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Graphene oxide adsorbent based dispersive solid phase extraction coupled with multi-pretreatment clean-up for analysis of trace aflatoxins in traditional proprietary Chinese medicines

Congcong Ran[a], Dan Chen[b], Haiyan Ma[a], Ye Jiang[a,*]

[a] Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, 361 East Zhongshan Road, Shijiazhuang, Hebei, PR China
[b] Department of Pharmacy, Cangzhou Central Hospital, Cangzhou, Hebei, PR China

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ABSTRACT

Graphene oxide (GO)-based dispersive solid phase extraction (D-SPE) method combined with multi-step preparation has been proposed for the evaluation of trace aflatoxins in proprietary Chinese medicines (PCM). After being extracted by methanol, the sample was purified based on multi-step preparation, including dehydration with $MgSO_4$/NaCl and cleanup with neutral alumina. Then GO was used as an adsorbent in D-SPE method for further preconcentration of aflatoxins prior to high performance liquid chromatography-fluorescence detection. The selected conditions were investigated. The Box-Behnken design (BBD) was used to optimize factors affecting adsorption procedure. Under the optimized conditions, good linear relationships had been achieved with the correlation coefficient (R^2) varying from 0.9904 to 0.9990. The LODs and LOQs were ranging from 0.020 to 0.041 ng/mL and 0.061 to 0.125 ng/mL, respectively. The results of the recoveries were 74.0–102.7% for the four aflatoxins, while the precisions from 1.8% to 7.2% were obtained, which indicated that the method was suitable for the analysis of aflatoxins in PCM.

1. Introduction

Aflatoxins(AFs) are a class of toxic secondary metabolites, which are produced by certain strains of *Aspergillus parasitics* and *Aspergillus flavus*. The exposure of aflatoxins to human beings may result in genetic mutation and a variety of diseases such as cancer, malformation, etc. AFs widely exist in moldy food, agricultural products [1–3]. When the drying is delayed, being kept too moist and insect or rodent infestations during storage, raw material is susceptible to mould growth and thus to AFs contamination. Moreover, it is generally difficult to eliminate AFs because of the high temperature resistance and storage stability. Different from foods, agricultural products or medicinal herbs, AFs in PCM would be easily ignored due to the dark appearance or special taste. Once the raw material of PCM is polluted, which directly influences the quality of drug and has a threat to human health. PCM are manufactured according to a series of processes, which are derived completely from plants, animals or minerals or a combination of any one or more of them [4]. A common process for purification of PCM is the so-called "water extraction and alcohol precipitation". The raw materials of PCM were obtained from the water-alcohol extracts after preconcentration. However, the purification process might play a negative role in removing AFs or aggravate the potential risk of AFs contamination. Therefore, AFs in PCM should be detected more than others.

The extraction of trace AFs in complex samples is critical for the pretreatment step before instrumental analysis. The separation and enrichment of AFs are usually performed using methanol/water or chloroform extraction, followed by column chromatography with silica gel or Florisil, solid-phase extraction (SPE) and solid-phase microextraction (SPME). Generally, SPE with different adsorbents materials is widely used owing to its better performances in terms of yield and quantification limit [5,6]. Among these SPE methods, immunoaffinity columns (IACs) have been applied more frequently in foods, agricultural products or medicinal herbs [3,8,9]. Although IAC, as an authority method, allows a highly selective separation of AFs, it limits the sensitive fluorescence detection when facing the complex components in PCM such as additives, protein and some furocoumarin compounds of which polarity and structure are similar to AFs. Furthermore, IAC is also susceptible to ambient temperature, humidity and the value of pH, which results in low recoveries for some mycotoxins and a decrease of selectivity,

* Corresponding author.
E-mail addresses: jiangye@hebmu.edu.cn, jiangye1@126.com (Y. Jiang).

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even causes the poisoning of IAC [10]. Thus, the limitation of SPE with much interference is the main drawback for the collection of AFs. Thereby, in order to match the requirement of AFs analysis in PCM, great efforts are being paid to develop a novel, rugged and selective method.

Nowadays, featuring large surface area, chemical stability and unique adsorption properties, graphene oxide (GO) has been applied as a sorbent material in separation, purification and preconcentration [11–13]. GO, a new type of carbon material, is the oxidized derivative of graphene. GO possesses large quantities of oxygen atoms on the surface such as epoxy, hydroxyl, and carboxyl groups, which makes for formation of hydrogen bonding or electrostatic interaction between functional groups and organic compounds or metal ions [11,14,15]. In this case, the π-π stacking between the benzene rings of AFs and GO as well as the hydrogen bonds between the oxygen containing groups contained in AFs and GO might be responsible for the adsorption of AFs on GO absorbent. With the increase of oxygen functional groups, GO provides more active properties (such as solubility and dispersibility) than that of graphene in some solvents particularly in water. Owing to these superior characteristics, the application of GO in extraction of trace components in different matrices is greatly promising [16–18]. Simultaneously there is no study to apply GO extracting trace AFs from PCM and to quantify them. So, in this paper we applied GO to extract aflatoxins in PCM for further preconcentration procedure. However, a single pretreatment method can not meet the requirement of sensitive detection especially for the complex matrices. GO, as a sorbent material, applied in extraction of trace components in complex matrice has similar disadvantages to IAC. It follows that a new multi-step preparation method has been investigated and developed for the purification before the GO preconcentration step, aiming to remove interference as much as possible. Taking advantage of the highly dispersed GO solution, dispersive solid phase extraction (D-SPE) method is used during the GO preconcentration step.

The aim of our study is to develop a GO based D-SPE method combined with multi-step preparation for evaluation of trace AFs in PCM with an oral preparation as model matrix. To the best of our knowledge, this is the first time describing a D-SPE using GO as a sorbent combined with multi-step pretreatment method for the extraction of trace AFs in complex commercial drugs PCM, which refers to the real situation.

2. Materials and methods

2.1. Materials

Graphite flakes (~150 μm flakes) were purchased from Yongda Chemical Reagent Co., Ltd (Tianjin, China). All the reagents, including potassium permanganate ($KMnO_4$), hydrochloric acid (HCl), sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2, 30%), phosphorus pentoxide (P_2O_5), potassium persulfate ($K_2S_2O_8$), neutral alumina (Al_2O_3) and hydroxyapatite were all of analytical grade, which were from Sinopharm Chemical Reagent Co., Ltd (Tianjin, China). Acetonitrile(ACN) and methanol(MeOH) (HPLC grade) were purchased from Kangside Scientific (Tianjin, China). Double-distilled water (HPLC grade) was prepared using Milli-Q50 water purification system (Millipore, Bedford, MA). Xiao'er Qingre Zhike oral liquid was purchased from market. Standard solutions of AFs containing 2.00 μg/mL (B_1 and G_1) and 0.50 μg/mL (B_2 and G_2) were obtained from Beijing Tai Leqi (China). The working standard solutions of different concentrations were obtained by diluting the stock solution with MeOH/water (1:1, v/v). All solutions were kept at 4 °C in the dark. A 0.22 μm cellulose membrane filter was used for filtration of the stock standard solutions and PCM samples.

2.2. Apparatus and HPLC conditions

A Hitachi HPLC system equipped a L-6200A pump (Japan) and a Waters 2475 fluorimetric detector (USA) was used. Elution and separation were conducted on a Phenomenex® C18 column (250 mm × 4.6 mm, 5 μm). MeOH was used as mobile phase A, ACN was mobile phase B, and phosphoric acid aqueous solution (pH = 3.0) was used as mobile phase C. HPLC separation was achieved by an isocratic elution with a mobile phase consisting of A,B,C (3:3:5,v/v/v) at 0.8 mL/min. The injection volume was 20 μL.

A post-column derivatization was used to enhance the AFB_1 and AFG_1 responses. The post- column derivatization reagent was prepared by dissolving 500 mg of iodine in 100 mL of methanol and diluted to 1 L with ultrapure water, and delivered at a flow rate of 0.3 mL/min. The reaction coil was maintained at 70 °C. The eluate was monitored by a fluorescence detector set at excitation wavelength of 360 nm and emission wavelength of 450 nm.

2.3. Preparation and characterization of graphene oxide

GO was synthesized by Hummers and Offeman methods [19]. Briefly, graphite flakes (2 g) was added into an solution of concentrated H_2SO_4 (12 mL) containing 5 g of $K_2S_2O_8$ and 5 g of P_2O_5, and slowly heated to 80 °C for 4 h. Then, the mixture was washed to neutral with ultrapure water. The product was dried under 50 °C. 1 g of this pre-oxidized graphite was added into 36 mL of concentrated H_2SO_4 in an ice-bath and stirred for 30 min, then; 5 g of $KMnO_4$ was gradually added into the mixture under stirring for 30 min. Note that the rate of addition must be carefully controlled to prevent the temperature from exceeding 10 °C. Subsequently, the mixture was stirred at 35 °C for 8 h and then slowly diluted with 360 mL of ultrapure water. Shortly after the addition of water, 5 mL of H_2O_2 (30%,v/v) was added, causing the color turning to yellow along with bubbling, The solution was centrifuged at 4000 rpm for 20 min, and the supernatant was decanted away. The remaining solid material was successively washed with HCl (5%,v/v) three times and then to neutral with ultrapure water. The obtained solid was dried under ambient condition. The dispersion of GO was ultra-sonicated for 2 h to exfoliate graphite oxide to GO with 200 W. Then, the GO solution was centrifuged at 1000 rpm for 10 min to remove any unexfoliated GO. Thus the GO solution (5 mg/9 mL) was obtained.

UV-vis spectrum was collected by a T1901 double beam UV–vis spectrophotometer (Beijing Purkinje General Instrument., China) over the range of 200–400 nm. Fourier transform infrared spectra (FT-IR) were obtained with a FT-IR 8400 system (Shimadzu) by using the KBr pellet method.

2.4. Extraction and desorption procedure

The overall experimental procedures were performed as follows: (*i*) Extraction procedure: 2 mL of PCM was extracted with methanol (18 mL) in an ultrasonic cleaner for 20 min. The extracts were centrifugated for 5 min at 4000 rpm. (*ii*) Clean-up procedure: a $MgSO_4$/NaCl salt mixture (2/1,w/w)(1.5 g) was added to 10 mL extracts which was immediately and vigorously hand-shaken for 30 s before centrifugation at room temperature (4000 rpm, 3 min). For all samples, the resulting MeOH-based supernatant (5 mL) was transferred into a 10-mL Falcon tube and further cleaning with neutral alumina (0.5 g) under rotary agitation for 10 min. After centrifugation (4000 rpm for 5 min), the upper MeOH phase was collected. (*iii*) Preconcentration procedure: at this stage, the GO solution (4.5 mL, 2.5 mg) was placed in a centrifuge tube (polypropylene, 10 mL), then, the 1 mL sample extract was added, and the mixture was diluted with ultrapure water for decreasing the methanol concentration to 10.0%, and under agitation with magnetic stirring for 15 min. Subsequently, 0.1 g NaCl was added