A Cotton Bud Coated with Chitosan/N-Carbon Dot Composite as SPE Sorbent for Extraction of Steroid Hormones from Fish Farm Water

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Abstract. 17-β Estradiol (E2), estrone (E1), and testosterone (T) are steroid hormones formed naturally by humans and animals or derived from synthetic sources. These steroid hormones are frequently used in aquaculture farms to increase production and treat diseases. However, there are concerns about the presence of steroids in farm wastewater since steroid hormones are known to be potent endocrine disruptors even at their lowest concentration. Therefore, it necessitates the development of an analytical method for monitoring these hormones in fish farms. This work developed a cotton bud coated with N-doped carbon dot and chitosan composite in vortex-assisted solid-phase extraction to extract E2, E1, and T before quantitative analysis by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD). Under optimum HPLC-DAD condition, the linear range for E2, E1, and T were 0.1–1000 mg L^{-1}, 0.05–1000 mgL^{-1}, and 0.1–1000 mgL^{-1}, respectively, with a coefficient of determination (R^2) greater than 0.99. In terms of limits of detection (LOD), the values were 40.26 ± 0.11 µg L^{-1} for E2, 38.41 ± 0.10 µg L^{-1} for E1, and 34.26 ± 0.07 µg L^{-1} for T. And the LOQ was 134.2 ± 0.4 µg L^{-1} for E2, 128.1 ± 0.3 µg L^{-1} for E1 and 114.2 ± 0.2 µg L^{-1} for T. This study's developed method can extract spiked E2, E1, and T at a concentration of 75 µg L^{-1} with a satisfactory extraction efficiency of 75.8 ± 6.9%, 80.2 ± 5.5%, and 72.9 ± 4.6% for E2, E1, and T respectively. As per the result of the experiment, this simple and cheap sorbent can extract steroid hormones, and further optimization of the desorption and extraction parameters is expected to improve recovery.

Keywords: steroid hormones · N-carbon dot · chitosan · solid phase extraction

1 Introduction

According to the Food and Agriculture Organization (FAO) of the United Nations, global per capita fish consumption has increased to over 20 kg a year in mid-2010s to 25 kg per year in early 2020s [1]. Steroid hormones like 17-β estradiol (E2), estrone (E1), and

testosterone (T) are often used to increase fish production depending on either of the sexes to go bigger and faster [2, 3]. Studies have reported the presence of these hormones in aquaculture farms and their surrounding water bodies [3, 4]. And this is concerning because steroid hormones are known to be endocrine-disrupting compounds (EDCs) even at the lowest concentrations. Hence their presence in environmental matrices should be monitored.

The Food and Agriculture Organization of the United Nations defines maximum residue limits (MRLs) of E2 and T in cattle meat as unnecessary. And the acceptable daily intake (ADI) is set as 0.05 µg kg^{-1} bw per day and 2.0 µg kg^{-1} bw per day, respectively via meat consumption. Nevertheless, the maximum contaminant levels are yet to be set for steroid hormones or EDCs in water. As per the overview of the watch list of European Union (EU), E2 and E1 are included in the watch list. Thus, more studies must be conducted to determine the concentration of steroid hormones in water samples from various sources.

To obtain high recoveries and minimize matrix interferences, determining steroid hormones in any environmental sample requires extraction and clean-up steps prior to instrumental analysis [2]. Therefore, the sample preparation method is essential. Solid phase extraction (SPE) is the most extensively used technique due to its advantages, such as simplicity, flexibility, high selectivity, rapidity, and higher enrichment factors [5, 6]. However, sorbent preparations and extraction procedures still require improvement to enhance extraction efficiency and simplify development methods [7].

N-doped carbon dot (N-CDs) is a promising nanomaterial offering multiple benefits such as large surface area, low production cost, various functional groups, biocompatibility, and low toxicity [8]. The possible interaction of sorbent with target analytes includes the formation of H-bond, π–π interaction, and π-lone pair interaction. Therefore, it has potential uses in separation processes. Despite this, they are not suitable for use as free-standing sorbents because of inherent drawbacks such as the difficulty of separation, aggregation, and loss of activity [8, 9]. In pursuit to address the issue, chitosan (CS) was chosen as the polymer matrix to entrap and stabilize N-CDs mainly by functionalizing at its amine and carboxyl group [10]. This polymer exhibits several desirable properties, including biodegradability, film-forming ability, low cost, and nontoxic properties [7]. Moreover, it could also enhance extraction efficiency by forming H-bond and π-lone pair interaction. To simplify the development method, the cotton used as substrate was dipped in the composite to form the complete sorbent called CS/N-CDs@cotton bud.

Overall, this work emphasizes the development of simple, fast, eco-friendly, and inexpensive vortex-assisted (VA) -SPE method to extract steroid hormones.

2   Materials and Methods

2.1   Chemicals and Reagents

Chitosan from shrimp shell (low viscosity, <200 mPas) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Urea (<99.5% purity) and citric acid monohydrate (99.5%-102% purity) was procured from Loba Chemie Pvt. Ltd (Colaba, Mumbai, India). Acetonitrile (>98.5% purity) and sodium hydroxide (97%) was purchased from RCI Labscan (Bangkok, Thailand) and JT Baker (Radnore, Pennsylvania, USA). 17-β

estradiol (98% purity), estrone (99% purity), and testosterone (98%) standards were purchased from Sigma- Aldrich (St. Louis, Missouri, USA). Ultrapure water with a resistivity of 18.2 MΩ.cm was obtained from a Milli Q system (Merck, Germany). All chemicals used in this study were of analytical grade. Cotton bud (United Medical Instruments Co., Ltd) in size small (S) was purchased from a local pharmaceutical store in Hat Yai, Songkhla, Thailand.

2.2 Instruments

Chromatographic separation was performed on a L-200 Series HPLC system (Hitachi HPLC, Japan). Data were analyzed using LaChrom Elite HPLC (Merck-Hitachi HPLC, Japan). Three steroids were separated on a reverse-phase VertisepTM UPS C18 column (5 μm particle size, 150 mm × 4.6 mm ID) (Vertical Chromatography Co., Ltd, Bangkok, Thailand). The mobile phase was a mixture of water and acetonitrile. An isocratic elution mode was performed with the mobile phase ratio of 32% water and 68% acetonitrile. The flow rate was set at 0.60 mL min^{-1}. The injection volume was 20 μL and the column temperature was maintained at 25 °C. A diode array detector detected the interested analytes at the detection wavelength of 225 nm.

2.3 Optimization of HPLC-DAD

Optimization of HPLC-DAD parameters were carried out by injecting three replicates of 1.0 mg L^{-1} of E2, E1, and T mixed and varying one parameter at a time. The optimal parameters were selected by focusing on the criteria such as short analysis time and reasonable resolution. Three parameters: mobile phase composition, flow rate, and detection wavelength, were optimized.

2.4 Preparation of Chitosan/N-Carbon Dot Composite Coated Cotton Bud

The summary of the sorbent preparation method is depicted in Fig. 1. N-carbon dot (N-CDs) was prepared by following the method developed by Qu and coworkers [11]. The chitosan (CS) and N-CDs composite were prepared as per the method reported by Jlassi and coworkers [12] with slight modification. Briefly, 1.0 g of CS was dissolved in 2.0% acetic acid by sonicating for 30 min. Then, carbon solution was added to it and stirred vigorously overnight. The composite was again sonicated for 1 h to ensure uniform dispersibility and enhanced interaction. CS contains several -OH and -NH$_2$, which can interact with the -COH group of N-CDs by forming H-bonding [8]. Moreover, N-CDs can also interact electrostatically with CS, ensuring strong adherence and uniform distribution in the composite formed [12].

This study used the exact brand and size of naked cotton buds throughout the experiment. Bare cotton bud was first washed with 1.0 M NaOH for 10 min to remove fats and alcohol. Then, it was dipped in 1.0 mL solution by a dip coating method and air dried for 60 min. It was dried in an electric oven at 65 °C for 45 min and stored in a desiccator until used. Each naked wooden support from coated cotton bud was cut off and weighed to ensure each sorbent's uniform weight (0.15 g). The final product was
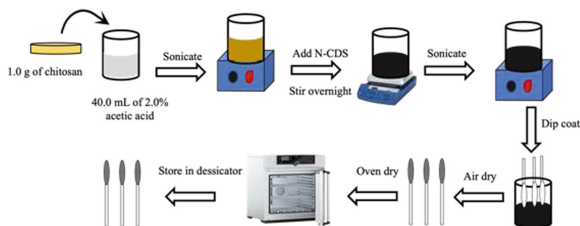
Fig. 1. Schematic presentation of preparation of sorbent
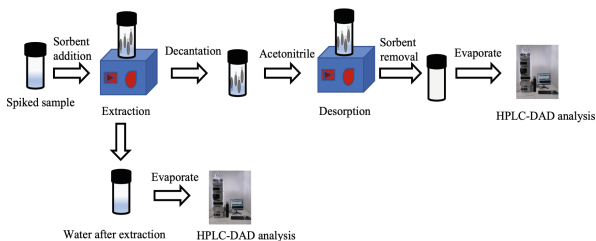
Fig. 2. Extraction and desorption procedure

called CS/N-CDs@cotton bud. The composite formed can interact with cotton through the formation of H-bonding [13]. In addition, a C = N bond formation between the aldehydic carbonyl group of cotton and the amino group of CS can lead to Schiff base formation [10]. Before every use, ten pieces of CS/N-CDs@cotton buds were washed at a time with acetonitrile for 30 min and DI water for one hour using a magnetic stirrer (200 rpm) to remove unreacted N-CDs and impurities.

2.5   Extraction and Desorption Procedures

The extraction and desorption procedures were carried out, as depicted in Fig. 2. The CS/N-CDs@cotton bud was immersed in 4.0 mL of 75 $\mu g\,L^{-1}$ mixed standard solution of E2, E1, and T. The extraction was carried out using a vortex speed of 2400 rpm. Then, the solution was decanted, and desorption solvent was added. The desorption was performed at 2400 rpm vortex speed using acetonitrile as the desorption solvent. Both the organic solvent and water after extraction was evaporated to dryness before redissolving with acetonitrile. After that, the solution was transferred to an autosampler vial before it was injected into the HPLC-DAD for analysis.

3   Results and Discussion

A.   *Optimization of HPLC-DAD system*

The optimum mobile phase composition was determined by varying the composition of acetonitrile from 50% to 70% in deionized water. A composition ratio of 68:32 (ACN:

Table 1. OPTIMUM CONDITIONS FOR HPLC-DAD FOR E2, E1 AND T.

HPLC-DAD parameter	Investigated condition	Optimum condition
Composition of mobile phase (ACN: H_2O, v/v)	50:50, 60:40, 68:32, 70:30	68:32
Flow rate (mL min^{-1})	0.2, 0.3, 0.4, 0.5, 0.6	0.6
Detection wavelength (nm)	190, 195, 200, 225, 230	225

H2O) provided good separation, the shortest analysis time, and a good peak shape. Therefore, it was chosen as the optimum condition. The flow rate of the mobile phase was investigated from 0.2 to 1.0 mL min^{-1}. The optimum flow rate of 0.6 mL min^{-1} provided the minimum plate height with a total analysis time of 8 min. Thus, this study selected 0.6 mL min^{-1} as the flow rate. Similarly, we optimized the detection wavelength from 225 nm to 230 nm. The highest response was recorded at 225 nm. Therefore, this wavelength was selected for further study. The optimum conditions of HPLC-DAD are summarized in Table 1. The linearity was in the range of 0.1–1000 mg L^{-1} for E2, 0.05–1000 mg L^{-1} for E1, and 0.1–1000 mg L^{-1} with a good coefficient of determination ($R^2 = 0.9999$). The LODs and LOQs were determined based on 3SB/m and 10SB/m, respectively (SB is the standard deviation of 20 blank responses, and m is the slope of the calibration curve). LODs for E2, E1 and T were 40.26 ± 0.11 µg L^{-1}, 38.41 ± 0.10 µg L^{-1}, and 34.26 ± 0.07 µg L^{-1}, respectively. And the LOQ was 134.2 ± 0.4 µg L^{-1} for E2, 128.1 ± 0.3 µg L^{-1} for E1 and 114.2 ± 0.2 µg L^{-1} for T.

B. *Optimization of VA-SPE conditions*

a) *Desorption solvent*

The desorption solvent should be able to elute the target analytes from the sorbent. Therefore, various desorption solvents with polarity closer to target analytes were studied. We tested acetone (polarity index 5.1), acetonitrile (polarity index 5.8), ethanol (polarity index 5.2), and methanol (5.1) as desorption solvents (Fig. 3a). As per the Fig. 3a, acetonitrile (ACN) provided the highest recovery. It could be possible as it is the most polar solvent. Moreover, it can provide three possible interactions with target analytes: H-bond, π–π interaction, and π-lone pair interaction. Therefore, ACN was chosen as the desorption solvent.

b) *Number sorbents*

The number of sorbents was varied from one to five pieces of CS/N-CDs@cotton buds to assess its effect on extraction efficiency. From Fig. 3b, it is evident that the recovery increased from $25.6 \pm 3.4\%$ to $51.4 \pm 7.9\%$ for E2, $35.1 \pm 3.7\%$ to $72.4 \pm 4.5\%$ for E1, and $23.8 \pm 1.0\%$ to $31.8 \pm 4.6\%$ for T as the number increased from one to four. It is possibly due to increased surface area as the number of sorbents increased. No further improvement was observed when five pieces were used for extraction. This may
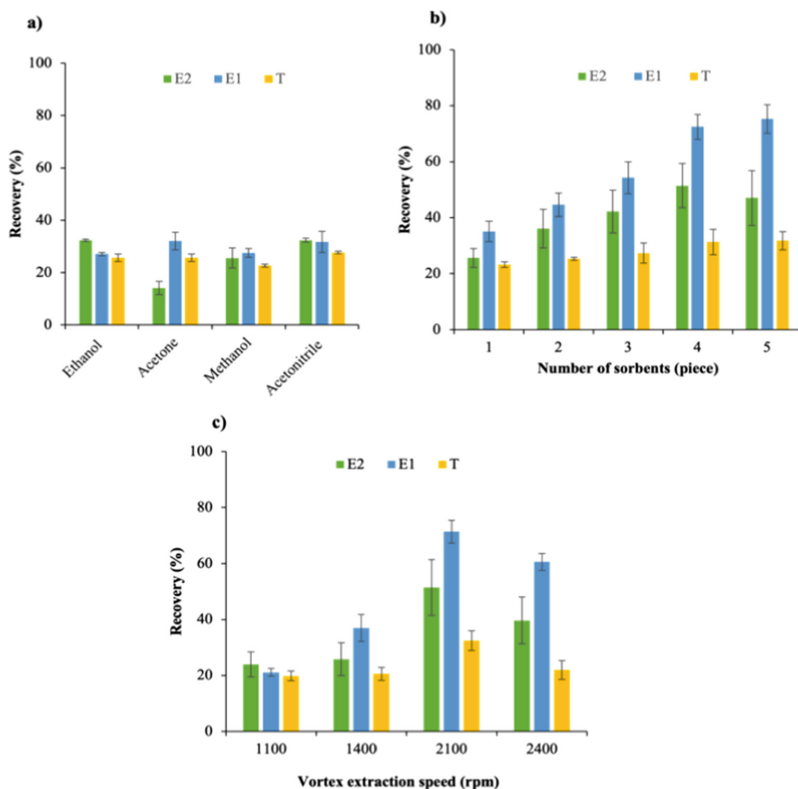
Fig. 3. The effects of (a) type of desorption solvents (b) number of sorbents, and (d) vortex extraction speed on the recovery of 75 µg L^{-1} E2, E1, and T.

be due to inhibited movement of sorbents in the glass vial and higher mass requiring a greater volume of solvent for desorption. Therefore, four pieces of CS/N-CDs@cotton buds were chosen as an optimum number to ensure the highest adsorptions for all three analytes.

c) *Vortex extraction speed*

The extraction speed plays a vital role in enhancing the diffusion of the analyte to the adsorbing site, thereby reducing the equilibration time. For evaluation of the effect of this parameter, vortex speed in the range of 1100 rpm to 2400 rpm was studied (Fig. 3c). The recovery of analytes increased with speed up to 2100 rpm and decreased beyond the speed of 2100 rpm. The increase in recovery could be because of the possibility of breaking off the molecules from the adsorbed site due to increased force. Furthermore, the decrease could result from a decrease in the contact time. Thus, the optimal speed used was 2100 rpm.

4   Conclusion

The developed CS/N-CDs@cotton bud shows the feasibility of its use as a VA-SPE sorbent for the determination of E2, E1, and T. It provided excellent adsorption efficiency of more than 75% for all three analytes, with a recovery of 70% for E1. The possible interaction between CS/N-CDs@cotton and target analytes include H-bond, π–π interaction, and π-lone pair interaction. However, further characterization is required to conclude the interaction involved. Further optimization of extraction and desorption conditions is required to obtain acceptable values before using them for analysis of actual samples.

Author Contribution. Deki: investigation, writing-original draft. Proespichaya Kanatharana: Conceptualization, methodology. Panote Thavarungkul: Conceptualization, methodology. Chongdee Thammakhet-Buranachai: Conceptualization, methodology, writing-review-editing, supervision.

Conflict of Interest. The authors declare that there is no conflict of interest.

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