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Method Validation for Determination of Methyltestosterone in Nile Tilapia by Supercritical Fluid with Carbon Dioxide as a Green Extraction Technique for HPLC Analysis

Sreynich Chhun[1*], Nisakorn Thongkon[1], Sudtida Pliankkarom Thanasupsin[1]

[1] Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, Thailand, 10140

*Corresponding Author: sreynich.chhun@mail.kmutt.ac.th

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Abstract: *Methyltestosterone (MT) is a synthetic anabolic steroid hormone. It is worldwide known for its use in fisheries and aquaculture to induce a mono-sex population, especially in Nile tilapia (Oreochromis niloticus) production. In this study, the quantitative analysis for residual methyltestosterone in Nile tilapia tissue was conducted using HPLC (high-performance liquid chromatography). A green extraction technique was carried out. Supercritical fluid with carbon dioxide was conducted at 31 ºC, 75 bars, and 10 minutes. The analysis was performed on RP-C18 (4.6 mm × 250 mm, 5 µm) using the mobile phase water-acetonitrile (30:700, v/v), 1.0 ml min-1 of flow rate, temperature at 25 ºC, and wavelength of 245 nm. A good linearity was obtained for methyltestosterone detection between concentrations of 25.0 – 600.0 µg L-1, R2 = 0.9991 and LOD = 8.0 µg L-1. Samples were spiked with methyltestosterone at levels of 10.0, 20.0, 30.0, and 40.0 ng g-1 and used to validate the method. The mean recovery value greater than 95% was achieved and RSD was less than 5%. These established results demonstrate the applicability of supercritical fluid carbon dioxide as a green extraction technique for residual methyltestosterone extraction in fish tissue.*

Keywords: Methyltestosterone, supercritical fluid, carbon dioxide, Nile tilapia, high-performance liquid chromatography

1. Introduction

Methyltestosterone (MT) is a 17α-alkylated synthetic anabolic steroid hormone (Figure 1). It is classified as endocrine-disrupting chemicals (EDCs) among more than 87,000 other compounds of EDCs due to its potential capability to interfere in the endocrine systems of numerous organisms (Komesli, Muz, Ak, Bakırdere, & Gokcay, 2015). Methyltestosterone is worldwide known for its use in fisheries and aquaculture to induce mono-sex production (Rivero-Wendt et al., 2016). Generally, oral administration of Methyltestosterone has been employed to tilapia fry at the prematurely life-phase of 7 – 12 day post-hatch (DPH) with the In a feeding trial, the duration of 21 – 28 days, concentration level at 30 to 60 mg MT/1 kg of fish feed is used(Hoga, Almeida, & Reyes, 2018). This procedure usually results in greater than 95% of sex-reversed male production. Moreover, in fisheries industrials, the residual from uneaten and unmetabolized Methyltestosterone impregnated food remains in the pond and, if discharged can infect the aquatic

environment (Liu et al., 2014). Methyltestosterone has the capacity to produce harmful biological effects on human and aquatic organisms even at part per billion levels (Gao et al., 2015).

Therefore, it is essential to analyze Methyltestosterone levels in numerous environmental samples such as water, sediments, and fish samples to beneficially understand its environmental chemical aspects and potential influences on the environment. Generally, for the detection of androgenic steroid hormones, chromatographic methods are conventionally used but the most challenging for scientists before the determination is the extraction of androgenic steroids from various types of samples including Methyltestosterone.

Recently, there are several extraction techniques have been reported for steroid hormones extraction such as microwave-assisted extraction (MAE) (Guedes-Alonso, Sosa-Ferrera, & Santana-Rodríguez, 2017), solid-liquid extraction (SLE) (Li et al., 2018), QuEChERS (Lopez-Garcia, Romero-Gonzalez, & Garrido Frenich, 2018), pressurized liquid extraction (PLE) (Wang et al., 2019), solid-phase extraction (Barbosa et al., 2013), dilute and shoot (Dahlin, Palte, LaMacchia, & Petrides, 2019), solvent ultrasonic extraction (X. Han & Liu, 2019), and subcritical fluid extraction (Y. Han, Ma, Lu, Xue, & Xue, 2012). The samples that were analyzed using these extraction techniques include water, sediment, fish tissues, animal meat, bovine tissues, human urine, commercial infant, and other fatty food, etc. All the above modern methods were validated and provided a good accuracy, precision, reproductivity, repeatability, and acceptable recovery percentage range to analyze steroid hormones. However, this technique required a number of processing steps, time-consuming, and used large quantities of chemicals.

Supercritical fluids, like carbon dioxide ($ScCO_2$) is the simplest used solvent for three main reasons; (1) it is harmless to human health and to the environment which is considered as “green solvent”; (2) its moderate critical temperature (31.1 ºC) and pressure (7.38 MPa) are the key issues for the preservation of extracts; and (3) it serves as a solvent for “difficult” chemical conversions, such as the direct reaction of forming hydrogen peroxide or various selective free-radical reactions (Budisa & Schulze-Makuch, 2014). This work aims to evaluate and validate the method of a green extraction technique; supercritical carbon dioxide extraction for residual analysis of Methyltestosterone in Nile Tilapia samples.

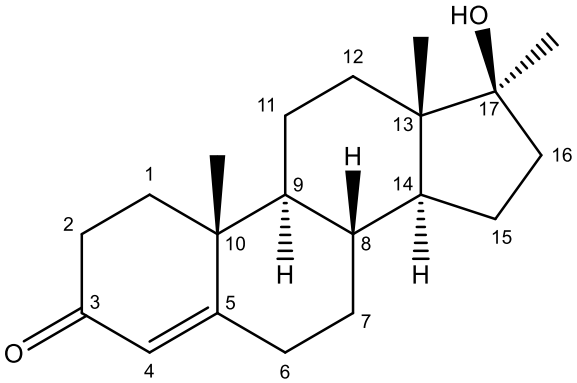

Figure 1: Methyltestosterone (MT) Structure (Math, Dungchai, & Thanasupsin, 2019)

2. Experimental

2.1 Materials

Nile tilapia (*Oreochromis niloticus*) was used as samples in this study which were obtained from local markets located in Bangkok, Thailand. Samples were cleaned with running water, followed by filleted to remove skin and bone then cut and blended with laboratory blender. The samples were sealed in polyethylene bags and stored in a −20 ℃ freezer for further extraction process.

2.2 Methyltestosterone Extraction Using Supercritical Fluid Carbon Dioxide

Supercritical fluid carbon dioxide was used as a solvent and operated by a high-pressure auto-clave (Figure 2). The extraction was conducted in a pressure vessel controlled at 30 ℃ and a pressure of 75 bars for the extraction for 10 minutes. The weight of the sample was fixed at 1:20 (w/v) solid-liquid ratio, i.e., 25 grams of sample in 500 mL distilled water. The pre-treated sample was centrifuged, and the supernatant was cleaned with SPE.

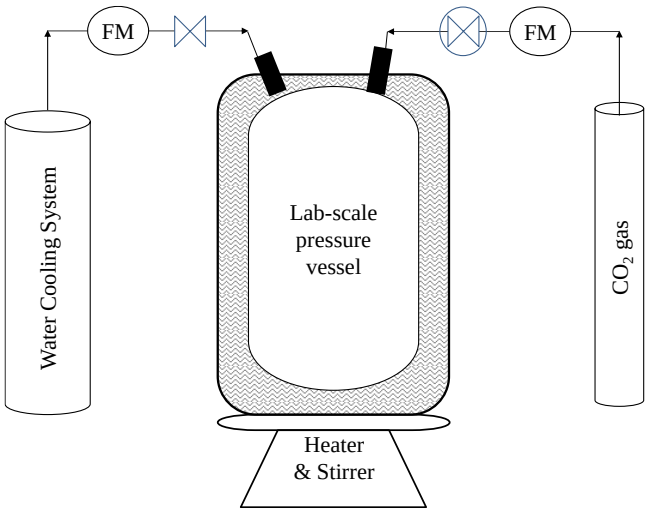

Figure 2: Schematic diagram of experimental; set-up of high-pressure autoclave (Amar model P2313)

2.3 Solid-phase Extraction

To clean up the impurities, interferents, and preconcentration, solid-phase extraction was used. The SPE was pre-conditioned using 5 mL of acetonitrile followed by 5 mL of water. After pre-condition, the supernatant was loaded through the SPE column. Then 5 mL of DI water was used. At last, 5 mL of ethanol was used to elute the Methyltestosterone. The eluting solvent was evaporated to dryness by using air-dry. The residue was dissolved in pure methanol and adjust to 5 mL. After that sample was filtered with a nylon syringe filter and transferred to a vial bottle (Barbosa et al., 2013).

2.4 Chromatography Analysis – High-performance Liquid Chromatography (HPLC)

HPLC analysis was done to characterize and determine the amount of Methyltestosterone in samples. 20.0 µL of the sample was successfully injected into a Prostar (230 No. 01488) HPLC system equipped with a UV/Vis photodiode array detector (SPD-M20A). The separation was conducted using RP-C_{18} (4.6 mm × 250 mm, 5 µm). The temperature of column was kept at 25

ºC. The mobile phase water-acetonitrile (30:70, v/v), with the flowrate of 1.0 ml min^{-1}. Methyltestosterone was detected at a wavelength of 245 nm (Barbosa et al., 2013; Y. Han et al., 2012).

2.5 Method Validation

The European Commission Decision 2002/657/EC (2002) (Commission, 2002) was used as the criteria to performed the method validation. Nile Tilapia samples were spiked at different levels (25.0 – 600.0 µg/L) then extracted. The linearity and sensitivity of the method were tested by injecting the analytes at various concentration levels into the HPLC system. To develop the calibration curves of the spiked blank fish samples for Methyltestosterone, a least-squares linear regression analysis was used by plotting the peak area of Methyltestosterone versus the Methyltestosterone concentration. The limit of detection (LOD) was built by depending on the 3s principle using a series of 10 solutions containing the lowest concentration (25 µg/L) of Methyltestosterone. Lastly, the lowest concentration used in the calibration curve was chosen as limit of quantification (LOQ) (FDA, 2018). Moreover, the accuracy and precision were examined. Nile Tilapia samples that were spiked with Methyltestosterone at levels of 10.0, 20.0, 30.0, and 40.0 ng/g were extracted and analysed on 3 different days which each group was duplicated three times.

3. Results and Discussion

3.1 Optimization of Supercritical Fluid Carbon Dioxide Extraction

For the Methyltestosterone extraction process using supercritical fluid carbon dioxide, the pressure of 75 bars and a temperature at 31 ºC were chosen since this condition allowed carbon dioxide to reach its critical point (Budisa & Schulze-Makuch, 2014). The density of carbon dioxide as a solvent will increase (and thus, its solvating power) due to the increase of pressure around the supercritical state. Also, as the density of solvent increases, the inter-molecular interactions of solvent increase, and it resulted in high solvent dissolution (Y. Han et al., 2012) for Methyltestosterone extraction. The extraction time became the main parameter that could affect the operating process as well as the extraction yield. To optimize the extraction time, the recovery percentage from varied extraction time from 10 to 60 minutes (i.e., 10, 20, 40, and 60 minutes) was studied. As a result, the recovery percentage was range from 60.9% to 106.2%. An extraction time of 10 minutes was selected because the highest recovery percentage of 106.2% was obtained as presented in Figure 3. Hence, the experiment chooses minimum time which provides the higher recoveries to reduce waste of carbon dioxide consumption.

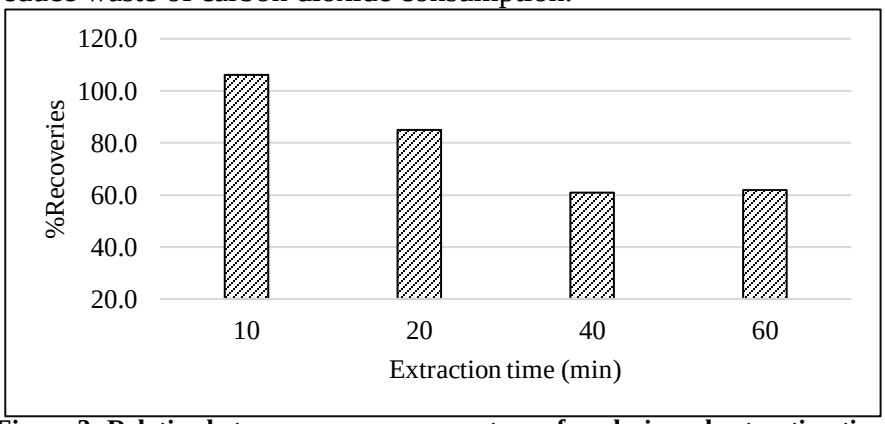

Figure 3: Relative between recovery percentage of analysis and extraction time

3.2 Result of Method Validation

In the method validation procedures, the experiment was conducted using the developed conditions (pressure, 75 bars; temperature, 31 ºC; extraction time, 10 minutes). By using the linear regression analysis, a good fit of the calibration curve was obtained over the range of 25 – 600 µg/L and the mean regression coefficient (R^2) was 0.9991 for Methyltestosterone. As shown in Figure 4, there was no presence of the potential interfering were noticed in interest where the Methyltestosterone was eluted, and it showed the specificity of the method. The limit of detection (LOD) for the method, as determined from the lowest standard concentration on the calibration curve (25 µg/L), was 8.0 µg/L (S/N=10).

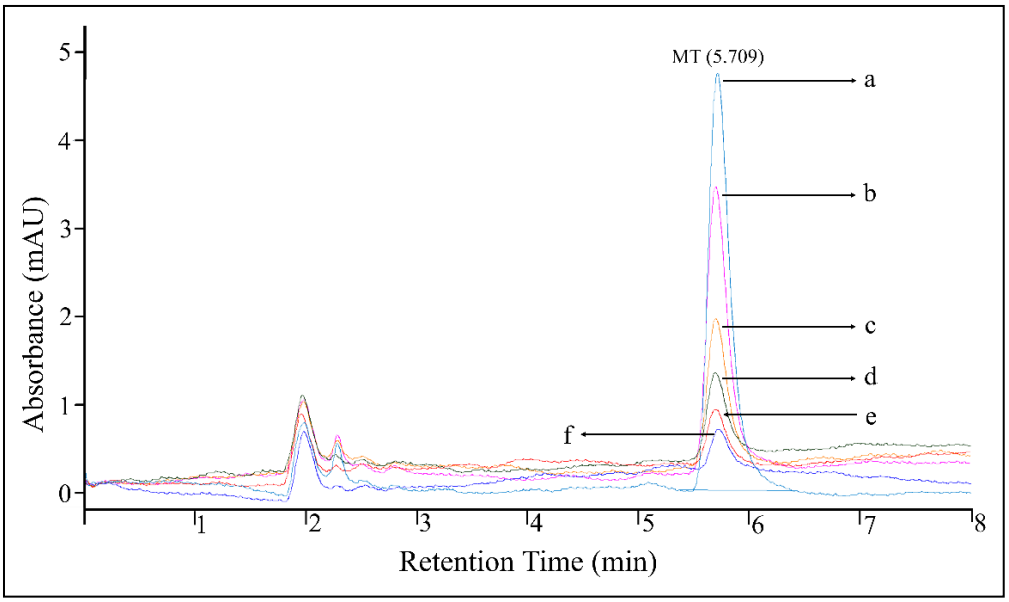

Figure 4: Standard curve chromatograms of Methyltestosterone; (a) 600 µg/L, (b) 400 µg/L, (c) 200 µg/L, (d) 100 µg/L, (e) 50 µg/L and (f) 25 µg/L.

To study the precision and accuracy of the method, Nile Tilapia samples were spiked with Methyltestosterone at four levels (10.0, 20.0, 30.0, and 40.0 ng/g). The experiments were done with triplicates. The average recovery percentage of Methyltestosterone was exceeding 95.0% which was between 80 – 120% presented a good accuracy and the value of relative standard deviation (RSD) was below 5.0% shown the appropriate method precision (Table 1).

Table 1: The Percentage Recovery of Methyltestosterone Extraction from Nile Tilapia

Analyte	Spiked Concentration (ng/g)	Recovery (%)	RSD (%, n = 3)
Methyltestosterone	10.0	107.0	3.1
	20.0	94.0	4.6
	30.0	105.1	3.9
	40.0	104.8	3.1

3.3 Analysis of Real Sample

The validity of this method was evaluated using real Nile tilapia samples. The Nile tilapia samples were purchased from the four local markets around campus located in Bangkok, Thailand. A total of twelve samples were analysed under the supercritical fluid carbon dioxide conditions described.

The experiments were repeated with triplicates. As result, it indicated that the Nile tilapia tissues had a level of residual Methyltestosterone of 5.2 – 17.5 µg/kg. Figure 5 represents the chromatogram found with one of the Nile tilapias fish samples analysed.

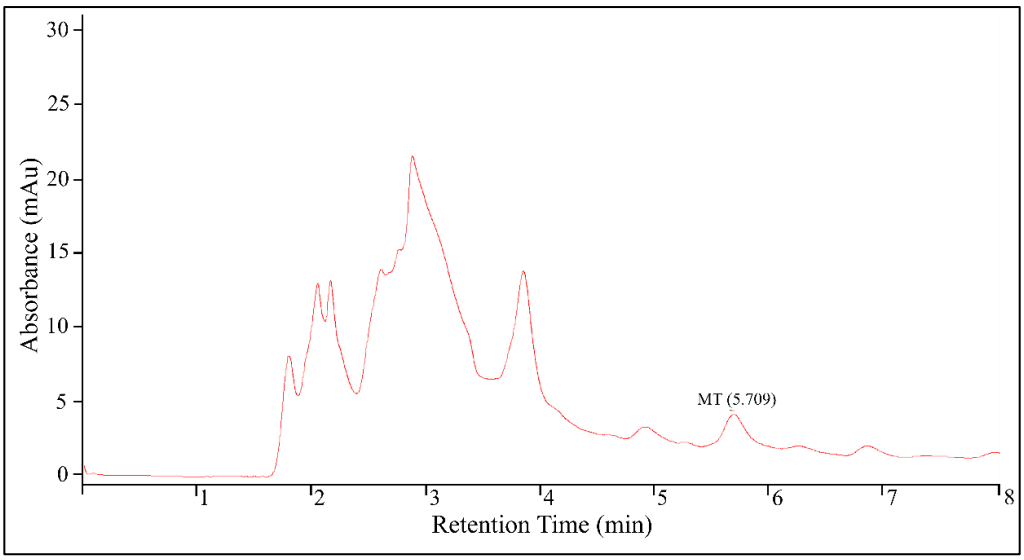

Figure 5: Nile tilapia sample containing 5.2 µg/kg of Methyltestosterone.

4. Conclusion

The supercritical fluid method as a green extraction technique for Methyltestosterone residue analysis in Nile tilapia has been validated, giving acceptable recovery, accuracy, and precision. In this study, the HPLC method is used for identification and quantification of trace levels of Methyltestosterone and the use of RP-C18 column coupled with UV detection allows the fast and precise analysis of Methyltestosterone in Nile tilapia. Additionally, the supercritical fluid method uses only 10 minutes of extraction time. Under the conditions, a temperature at 31 ºC, and pressure of 75 bar, the average recovery of Methyltestosterone from Nile tilapia is greater than 95%, and the RSD was less than 5%, demonstrating that the supercritical fluid carbon dioxide extraction is a practicable sample pre-treatment technology for the analysis of Methyltestosterone.

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