17α-METHYLTESTOSTERONE REMOVAL BY *Salvinia molesta* BASED REMEDIATION TREATMENT SYSTEM

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Abstract: The aim of this study was to explore the potential ability of *Salvinia molesta* for remediating 17α-methyltestosterone (MT) from wastewater. MT is a kind of Endocrine Disrupting Compounds (EDCs). Exposing to this compound may cause disturbance to natural organisms. The remediation treatment system was conducted in batch process which was containing synthetic wastewater. The potential of this plant for treating MT wastewater in a remediation system was observed. A high performance liquid chromatographic (HPLC) method using ultraviolet (UV) detection has been used to analyze the samples. During the pre-test, it was found that *Salvinia molesta* was able to remove MT to 0.03 mg.L^{-1} within 7 days. For kinetic studies, the MT sorption was fitted with Pseudo-second order with the rate constant (k) of 0.04 g.mg^{-1}.day^{-1}. Based on this result, it is likely to say that *Salvinia molesta* can be used to phyto-remediate MT from wastewater. Further investigations on factor affecting the removal efficiency involve several variables such as, weights of plant, concentrations of compound, and wavelengths of exposure-light.

1. Introduction

17α-methyltestosterone (MT) is an anabolic androgenic steroids hormone which has endocrine disrupting property at part per billion levels that can interfere with normal functions of the reproductive systems of humans and animals. [1] MT is commonly used to induce sex reversal of Tilapia *sp*. To obtain mono-sex cultures with economically viable mean, mixing fry feed with 60 mg of MT per one kilogram of feed was conducted. The tilapia fry will be fed with this feed for 21 days in the nursing pond. The un-eaten feed containing MT remains in the water and deposits as sediments at the bottom of the pond. Improper discharge of this wastewater may lead to surface water contamination, and cause deterioration on biodiversity and problems in human health. [2] Hornung et al., (2004) has studied in fathead minnow (*Pimephales promelas*), reported that MT in high dosage can be converted to 17α-methylestradiol (ME2) by aromatase enzyme in which producing estrogenic activity. [3] Another research has found that male sex organs such as penis and vas deferens in female freshwater ramshorn snail (*Marisa cornuarietis*) were developed when exposed to 100 – 1000 ng.L^{-1} of MT for six months. [4] Many studies have reported about the decrease in fertility of fish after having been exposed to certain amounts of MT. MT was reported to decrease feducity and fertility of Medaka fish when exposed to about 46 ng.L^{-1} of MT. [5]

It was also reported that exposure to MT at very low concentration (4.5 ng.L^{-1}) can affect vitellogenin synthesis and steroid levels in the reproductive system of male zebra fish. [6]

At present, Advanced Oxidation Processes (AOPs) is one of the effective technologies for treatment of recalcitrant and persistent organic contaminants. With Fenton's reagent, degradation of MT can be achieved through oxidation reaction. [7] It was also reported that MT was degraded by 48 – 62% when exposed to sunlight, partly degraded with UVA and UVB while UVC showed complete irradiation at the same power level. [8] [9] Although AOPs are effective to remove this group of contaminants, major drawback of this technology lie about the complicate reactions and specific operating conditions. Moreover, the intermediate substances produced during the AOP process become one of the major environmental concerns.

Phytoremediation has been developed rapidly as a technology for the remediation of organic contaminants in soil and water because it can achieve the goal of complete mineralizing the contaminants into relatively non-toxic substances (e.g. carbon dioxide, nitrate) and offers potential advantages in low cost and minimum landscape disruption. Phytoremediation used for removal of some organic contaminants has been reported in several studies. [10][11][12]

The aim of this study was to explore the potential ability of *Salvinia molesta* for remediating 17α-methyltestosterone (MT) from wastewater.

2. Materials and Methods

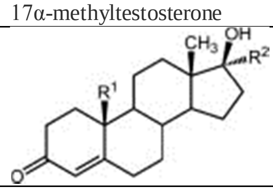
2.1 Chemicals and Materials

All reagents used during the extraction and analysis were analytical reagent grade. 17α-methyltestosterone (17β-Hydroxy-17α-methyl-4-androsten-3-one, CAS n: 58-18-4, purity 97.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were all HPLC-grade and purchased from Merck (Darmstadt, Germany). Deionized (DI) water was obtained in the laboratory. The cartridges used for, SPE were OASIS HLB C-18 3 mL, 60 mg (Waters, USA). Some physical and chemical properties of MT are given in Table 1.

2.2 Apparatus and Chromatographic Conditions

The method was developed on a Varian Pro Star HPLC system with quaternary pumps, autosampler, column oven, UV detector, degasser, and controlled by Varian Star software. The chromatographic separation was achieved with a C 18 reverse-phase (RP-C 18) column (ACE, 5 μm particle size, 250 × 4.6 mm) at 25℃. All injections were performed automatically using 20 μL loop on autosampler. Detection of analyte was carried out using UV detector at 245 nm. The analyte were separated by running a mobile phase consisted of acetonitrile and DI water (70:30, v/v) at a flow rate of 2 mL·min^{-1}.

Table 1: Properties of 17α-Methyltesterone (MT)

Name	17α-methyltestosterone
Structure	
R^1	$-CH_3$
R^2	$-CH_3$
R^3	-
Formula	$C_{20}H_{30}O_2$
Molecular Weight (MW)	302.45
CAS.No.	58-18-4
Melting point	162 – 168 °C
Water solubility	3.39 mg/lat 25 °C
pKa	15.13
log k_{ow} (log P)	3.559
Solubility	Soluble in methanol, ethanol, ether and other organic solvents

2.3 Plant culture conditions

Salvinia molesta was grown in a greenhouse at Faculty of Science, King Mongkut's University of Technology Thonburi (KMUTT), Bangmod campus. Plants at the same growth stage were selected and cultured containing Effective Microorganism (EM) solution to the start of the experiments.

2.4 Standard Solutions

All stock standard solutions of 100 mg.L^{-1} and 1000 mg.L^{-1}were prepared in acetonitrile. The working standard solutions of 17α-methyltestosterone (MT) for calibration curve were prepared through appropriated dilutions to obtain concentrations between 0.02 - 10 mg.L^{-1}. The stock standard solutions and the working standard solutions were stored at 4℃.

2.5 Synthetic wastewater

The synthetic MT solutions were used during experimental trials. Due to its low water solubility, synthetic solutions were prepared by dissolving MT in pure acetonitrile followed by the addition of DI water. The MT concentration in the stock solution is 1000 mg.L^{-1} in acetonitrile and then, was further dilute with DI water to obtain 10 mg.L^{-1} as working solution.

Table 2: Macronutrient and micronutrient composition of the synthetic water

Macronutrient	**C (mg/l)**	**Micronutrient**	**C (mg/l)**
CH_3COOH	93.75	EDTA	10.0
NH_4Cl	87.70	$FeCl_3.6H_2O$	1.50
$Na_2H_2PO_4.H_2O$	26.70	H_3BO_3	0.15
$MgSO_4.7H_2O$	9.00	$CuSO_4.2H_2O$	0.03
$CaCl_2.2H_2O$	4.72	KI	0.18
KCl	36.00	$MnCl_2.4H_2O$	0.12
Micronutrient	0.6 (ml/l)	$Na_2MoO_4.2H_2O$	0.06
Solution		$ZnSO_4.7H_2O$	0.12
		$CoCl.6H_2O$	0.15

The composition of the stock solution with respect to solvents was 1 % Acetonitrile and 99 % DI water (v/v). Synthetic wastewater was prepared to simulate the characteristics of effluent from fish pond, which is normally used as the pre-treatment of an integrated remediation system. The synthetic wastewater was prepared in DI water with some nutrients. Macronutrients and micronutrients were added as indicated in Table 2. [13]

2.5 Method and Sample Preparation

2.5.1 Sample Collection and Preparation

All water samples were collected in 60 mL PET bottles from different aquariums (batch system). The water samples were stored at 4℃. The freshwater samples of synthetic wastewater from aquariums were previously filtered through 0.2 μm membrane filter (Schleicher & Schuell) and then extracted by solid phase extraction (SPE).

2.5.2 Extraction and Clean-Up

The cartridge was preconditioned sequentially with 6 mL of methanol followed by 3 mL of ethanol and 6 mL of DI water. After that, the water samples of 10 mL were loaded on the preconditioned cartridge under vacuum. The washing step was performed with 10 mL of DI water. Then the cartridge was eluted with 4 ml of acetonitrile at a flow rate of 2 mL.min^{-1}. The elution was diluted in acetonitrile to obtain 5 mL of volume. The volume of elution injected was 20 μL.

2.6 Batch Tests

The batch tests were carried out to study the degradation of MT with higher concentrations (about 10 mg.L^{-1}) in synthetic wastewater with *Salvinia molesta*. DI water was used as control. *Salvinia molesta* (10 g.L^{-1} fresh weight) was added to aquarium containing 5 L wastewater. All of aquariums were incubated at room temperature (30 °C). There was no agitation or shaking during the 7-days batch tests. Around 25 ml water samples were taken from each aquarium after 5 min, 10 min, 15 min, 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, and 7 days contact time to measure the MT concentration in the water phase.

3. Results and Discussion

3.1 Method of Validation

In the validation procedure of the analytical method for water samples, the following criteria were considered: sensitivity; linearity; recovery; precision and evaluation of the matrix effect.

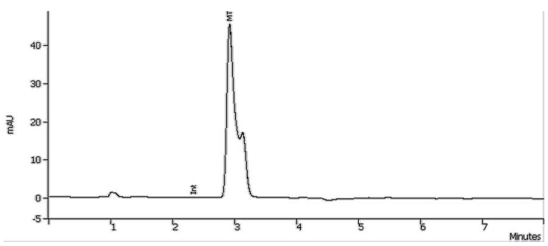
The calibration curves were prepared using linear regression analysis and gave good fits over the range 0.5-10 mg.L^{-1}. The mean regression coefficients (R^2) were 0.999 for 17*α*-methyltestosterone.

The limit of quantification, calculated according to the lower concentration that was 0.42 mg.L^{-1} for 17*α*-methyltestosterone. The limit of detection was 0.14 mg.L^{-1}, determined by half-dilution technique. In order to verify the absence of potential interfering substances around the retention time of 17α-methyltestosterone, water blank samples (n = 3) were analyzed in order to assess the specificity of the method. No interferences were observed in the region of interest where the 17α-methyltestosterone was eluted.

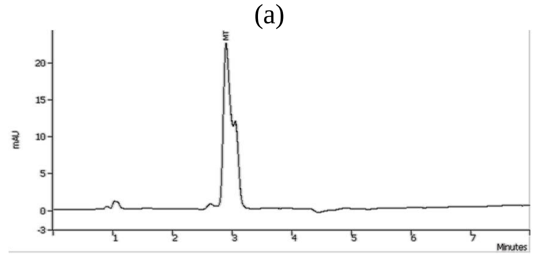
Table 3: Recovery (n=3)

Concentration level ($mg.L^{-1}$)	Recovery	
	Mean (%)	CV (%)
1	96 ± 2.56	1.64
2	97 ± 1.77	1.25
10	99 ± 0.13	0.098

The results has demonstrated that matrices in real samples had no effect on the performance of the analyzing method, which is therefore suitable for analysis of trace levels of 17α-methyltestosterone in water samples or in the surrounding aquatic channels. Within-day accuracy and precision data were determined by analyzing, on the same day, three replicates of samples, and one blank (to check the interferences). The recovery accuracy is shown in Table 3. The method was studied by spiking water samples at three MT concentration levels (1, 2 and 10 $mg.L^{-1}$).



(a)



(b)

Figure 1. Chromatograms of (a) Standard solution with MT 10 $mg.L^{-1}$; (b) Freshwater sample of aquarium after 1 day treatment containing 4.93 $mg.L^{-1}$ of MT

3.2 Equilibrium Time

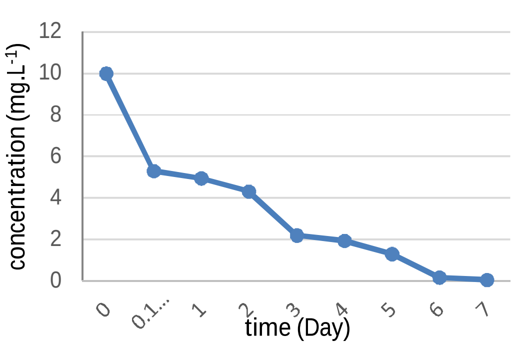


Figure 2. Concentration of MT against time

In this part describes the degradation of MT after treatment by Salvinia molesta after following day. From Figure 2, determination of equilibrium time is 7 days and removal efficiency of MT is 99.61%.

3.3 Kinetic Study

Several models were used to explain the mechanism of MT (solute) sorption onto *Salvinia molesta* (a sorbent). In order to investigate the mechanism of sorption, characteristic constants of sorption were determined using a pseudo-first order equation of Lagergren and a pseudo-second order equation based on solid phase sorption respectively.

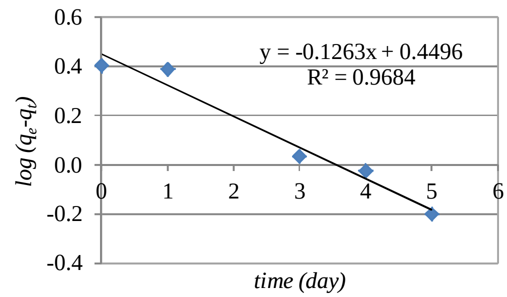


Figure 3.Pseudo-first order sorption kinetic of MT on *Salvinia molesta*

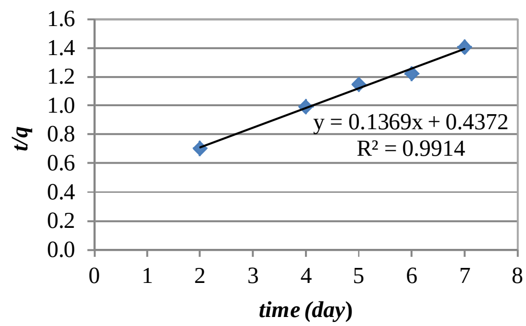


Figure 4. Pseudo-second order sorption kinetic of MT on *Salvinia molesta*

A kinetic model for sorption analysis is the pseudofirst order rate expression of Lagergren in the form:

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \quad (1)$$

Integrating this for the boundary conditions $t = 0$ to $t = t$ and $q_t = 0$ to $q_t = q_t$, Eq. (1) may be rearranged for linearized data plotting as shown by Eq. (2):

$$log q_e - log q_t = log q_e - \frac{k_1}{2.303}(t) \quad (2)$$

Where;

k_1 is the rate constant of first order sorption ($min.^{-1}$)

q_e is the amount of solute sorbed at equilibrium ($mg.g^{-1}$)

q_t is amount of solute sorbed on the surface of the sorbent at any time t ($mg.g^{-1}$).

The sorption kinetics may be described by the pseudo–second order model is expressed in Eq.(3) to (5):

$$\frac{1}{(q_e - q_t)} = \frac{1}{q_e} + kt \quad (3)$$

Eq. (3) can be rearranged to a linear form:

$$\frac{t}{q_t} = \frac{1}{kq_e^2} + \frac{1}{q_e}t \quad (4)$$

And, $h = k_2 q_e^2$ (5)

The data in straight-line plots of t/q_t against t have also been tested to obtain rate parameters.

Where;

k is pseudo–second order rate constant,

h is an initial sorption rate,

q_e is an equilibrium sorption capacity and

R^2 is the correlation coefficients for the MT under variable conditions were calculated from the data represented in these plots.

Table 4: Comparison the rate of sorption constant of MT sorption on *Salvinia molesta*

Data	Experimental Result
C_o (mg.L^{-1})	10
Pseudo-first order	
q_e (mg.g^{-1})	-
k_1 (mg.g^{-1}.day^{-1})	0.29
R^2	0.968
Pseudo-second order	
q_e (mg.g^{-1})	7.30
k_2 (g.mg^{-1}.day^{-1})	0.04
R^2	0.991
Sorbent	*Salvinia molesta*
Sorbate	MT

Based on Figure 3, 4, and Table 4, this MT sorption process followed Pseudo-second Order equation of Lagergren model. It has the rate constant 0.04 g/mg.day.

4. Conclusions

In this research, the proposed HPLC method to analyze MT was proven to be effective and convenient method for analyzing in aqueous matrices samples. In addition, the limit of quantification was found in amount of 0.623 mg.L^{-1} and the limit of detection was found in amount of 0.206 mg.L^{-1}. After 7 days treatment by *Salvinia molesta*, the initial concentration of MT decreased until 99.61 percentages in amount 0.03 mg.L^{-1}. Furthermore, Pseudo-second order was fitted to describe the sorption phenomenon. The kinetic rate sorption found in amount 0.04 g.$mg^{-1}$$day^{-1}$. In the future, to make sure the treatment result can discharge to environment, the next potential research can emphasize to describe the correlation of concentration of MT and the androgenic activity especially for human.

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