House Microwave-Assisted Solid Phase Extraction for Residual 17α-Methyltestosterone Determination in Nile Tilapia Tissues by High-Performance Liquid Chromatography

Kawin KHACHORNSAKKUL¹, Sudtida Pliankarom THANASUPSIM² and Wijitar DUNGCHA¹,*

¹Department of Chemistry, Faculty of Science, King Mongkut’s University of Technology Thonburi, Pracha-U-Tit, Thungkru, Bangkok, 10140, Thailand; ²Chemistry for Green Society and Healthy Living (ChGSH) Research Unit, Department of Chemistry, Faculty of Science, King Mongkut’s University of Technology Thonburi, Pracha-U-Tit, Thungkru, Bangkok, 10140, Thailand.

(Received December 23, 2020; Accepted February 1, 2021)

An effective sample preparation procedure using a house microwave-assisted extraction (hMAE) procedure, followed by cleaning with solid-phase extraction (SPE) by using HPLC with general UV-visible spectrometry was developed for residual 17α-methyltestosterone (MT) determination in Nile tilapia tissues. Our developed method showed a wide linear range from 25 to 800 ng g⁻¹ with $R^2$ as 0.9985 and presented a low detection limit of 1.53 ng g⁻¹ with good precision. Evaluation of accuracy for the MT determination in real samples showed a great recovery percentage ranging from 98.41 to 100.78%. In conclusion, it can be said that the developed method has the potential for sample preparation when compared with another method, and can be used for the determination of MT residues in fish tissue with simplicity, rapidity, cost-effectiveness, low solvent consumption, and minimum waste generation as well as having high accuracy and precision.

1. Introduction

Nile tilapia, known as the “economical fish”, is used to make various food owing to its low price, taste and high nutritional value. However, male tilapia is more favored than female because it can convert energy from feeding into muscle better than female tilapia, which provides higher economic productivity and profits, therefore, a synthetic anabolic-androgenic steroid was administered in order to increase the male hormone [1-3]. 17α-methyltestosterone (MT), a steroid hormone, is mixed with tilapia feed and is provided at an early stage to selectively attain a male population. Generally, fish food containing 60 mg kg⁻¹ of MT is given to Nile tilapia for 21 days to produce a male Nile tilapia population [4-6], but a small amount of MT can affect human health which is not approved for use in fish in the United States [7]. For the abovementioned, it is necessary to develop a high sensitivity technique for monitoring the residues of MT in Nile tilapia in order to evaluate the risk from their influence.

A sensitive assay for detecting MT at a small ng level in residues of fish tissue is desired because it is required by the Food and Drug Administration (FDA). However, fish samples are complex in terms of composition which causes more difficult analysis. Hence, the development of separation techniques for residues of MT detection in fish can be challenging to attain high reproducibility and recovery. Currently,
numerous chromatography method combined with sample preparation processes, i.e., subcritical fluid extraction (SFE), solid-phase extraction with salt (QuEChERS), solid-phase extraction (SPE), and solid-phase microextraction (SPME), for determining MT content in various samples have been developed to reduce the matrix effect and enhance measurable efficiency such as HPLC, liquid chromatography-mass spectrometry (LC-MS), gas chromatography (GC) and GC-MS, [8-17]. Although MS detector can be widely used to detect MT at ng level, it is expensive and needs experts for operation and analysis. Thus, our main strategy was to develop an effective sample preparation method for measuring MT residues in Nile tilapia tissues by using HPLC with a UV-Visible spectrometric detector (HPLC-UV), which is a general chromatographic instrument and detector.

Nowadays, commercial home-microwave device benefits our life in many ways. It can also be used in the science research application, especially for enhancing extraction techniques, called “Microwave-assisted extraction; MAE” which is a popular method for solid-liquid extraction due to its rapidity, simplicity, cost-effectiveness, and high performance. For example, Ying et al. presented MAE assisted SPE extraction for alkaloid analysis in Stephania cepharantha sample, which demonstrated that the combination of MAE and SPE enhanced the extraction and detection ability better than obtained the SPE method alone [18]. It can thus be said that MAE system can assist the increasing of separation and measurement performance for analysis. Likewise, organic compounds can be digested and extracted from solid matrix samples, such as soil, sludge and tissue samples, by electric power and electrothermal heating from a home microwave. With respect to green chemistry concepts, MAE is considered to be an efficiently useful tool because it can enhance extraction performance and measurability while incorporating low solvent consumption and pre-concentration at the same time [19-25].

Herein, we proposed the application of home microwave-assisted solid-phase extraction (hMAE-SPE) for the determination of MT residue in Nile tilapia tissues at ng level. This developed technique performed hMAE to increase the extractability and measurability in order to determine the trace of MT in the fish sample. Similarly, SPE was used to clean up the interference matrix such as phospholipids or proteins and pre-concentration of MT content. To our knowledge, this method is the first time to combine hMAE and SPE for the determination of MT in fish samples. Furthermore, the developed approach offers great analytical performance when compared with other methods, i.e., SPE or QuEChERS, as well as much benefit involved in less solvent consumption and cost-effectiveness.

2. Experimental

2.1 Chemical and materials

All solvents used in extraction and analysis steps were HPLC grade. Methanol, ethanol, acetonitrile (ACN), and dichloromethane (DCM) were purchased from Fisher Chemical. 17α-Methyltestosterone (MT) 97% HPLC grade and testosterone (T-II) 99% HPLC grade were purchased from Sigma-Aldrich. Preparation of 5.0 mg L⁻¹ of MT and T-II stock solution was performed by dissolving 0.50 mg of MT and T-II in methanol and adjusting to 100 mL in a volumetric flask. A house microwave (Samsung, ME81KS-1ST) was used in this study. Bond Elute C18 solid-phase extraction (SPE) cartridges (sorbent mass 1.0 g, volume 6.0 mL, particles size 120 µm, and frit pore size 20 µm) were purchased from Agilent and 0.25 µm syringe polyethylene terephthalate (PET) was purchased from Fisher Chemical.
2.2 Chromatographic conditions

HPLC-UV detector (Varian Pro Star, THAI UNIQUE CO., LTD, UV detector 310 ProStar and pump 230 ProStar) was performed with eclipse plus C18 column containing 4.6 mm × 150 mm and particles size as 5 µm by the surface area of 160 m²/g and controlled pore size of 95Å (Agilent, Zorbax). Mobile phase as methanol-ultrapure water (70:30 ratio) with isocratic eluent processing was conducted at a constant flow rate (1.0 mL min⁻¹) and detected at 245 nm.

2.3 Sample preparation procedures

Samples of tilapia were purchased from local markets. Fish tissue samples were mixed and homogenized. First, 0.10 g of samples were filled into the centrifuge tube and spiked with 0.20 mL of MT standard stock. Next, vortex was done for 2 min after that 4.0 mL of methanol was added into the mixture of samples. Then, the extraction was conducted using the home microwave at 600 W for 3 min. Later, the centrifuge tubes containing the mixture of samples were left at room temperature to cool down. Next, the mixture was filtered with a 0.25 µm syringe filter. After that, the filtered solution was added to 0.10 mL of T-II solution (which is used as an internal standard). Then, the total volume was adjusted to 5.0 mL by methanol. In order to clean up impurities and pre-concentration, the solution was passed through the SPE cartridge. Milli-Q water and ethanol were used as washing and eluting solvent, respectively (constant flow rate at 1.0 mL min⁻¹). Finally, the eluting solvent was evaporated to dryness, and the residue was dissolved in 0.10 mL of methanol. 0.02 mL of this successful solution was injected into HPLC for analysis.

2.4 General procedure of analytical characteristics

The linearity range was obtained by various concentrations of MT in fish tissues using a spiked method from 25.0 to 800.0 ng g⁻¹ (n = 3), after that samples were prepared according to the proposed method, and detection was conducted with HPLC-UV. Repeatability and reproducibility of our proposed method were determined by analysis of MT at 50.0, 100.0, and 200.0 ng g⁻¹ (n = 10). The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the detection of the blank signal. The blank sample was performed without being spiked and followed the same procedure as for the analytes.

3. Results and Discussion

3.1 Optimization of sample preparation

Sample preparation is one of the crucial steps of good analytical practices. Therefore, it is necessary to study factors affecting the extraction performance of samples from the matrix. In this study, several factors were investigated, such as types of extractant solvent, solvent volume, microwave energy, extraction time, and types of washing solvent, washing volume, types of eluting solvent, and eluting volume. The optimized sample preparation factors were evaluated based on recovery percentage obtained from a spiked method of MT standard at 200.0 ng g⁻¹ and testosterone (T-II), which acts as an internal standard (at 100.0 ng g⁻¹) to reduce the tolerance in the analysis. The conditions were tested three times (n = 3). For detection, HPLC was used with fixed conditions for separation. According to the chromatogram of the proposed method, as shown in Figure 1, it gave a higher resolution (Rₜ) than 1.5. In the extraction of organic compounds in fish tissues, the influential parameters of sample preparation were evaluated. Firstly, organic solvents for microwave extraction are very important because they affect extracted performance of solid samples. Therefore, the initial study was on the type of extractant solvent in order to obtain the highest extraction efficiency.
Methanol and water were chosen as extractant solvents due to the high dielectric constant in which they were transformed into heat through ionic conduction and dipole rotation ability. The analysis was performed with various methanol-water ratio ranging from 0:100 to 100:0. As can be seen in Figure 2(a), increasing the methanol ratio caused an increase in recovery percentage. As expected, pure methanol showed the highest percentage recovery. Thus, pure methanol was selected as a suitable extractant solvent. The volume of extractant solvent used for extraction of a fixed amount of sample, known volume of solvent, plays an important part in the extraction step. Various solvent volumes (ranging from 2.0 to 10.0 mL) were studied. As shown in Figure 2(b). Four milliliters of methanol were selected because the methanol volume tended to be constant of extracted performance after 4.0 mL of methanol. With respect to one of the green chemistry principles which are ‘reducing solvent’, the minimum volume of methanol 4.0 mL was chosen as a sufficient volume of solvent for achieving the highest recovery percentage. The microwave energy level and extraction time were powerful parameters involved in the analysis because methanol can evaporate when it consumes excess energy and extraction time. However, low extraction efficiency may occur with insufficient energy and time. For this reason, suitable microwave energy levels and extraction time were examined. We found that the highest recovery for the MT determination in fish tissues was found at 600 W and 3 min for MAE while increasing the energy level and extraction time related to decreasing recoveries of analysis as the result indicated in Figure 2(c-d). We found that using too high of both conditions caused solvent evaporation because there was no liquid left in the sample container. Therefore, 4.0 mL of pure methanol was used as extractant solvent at 600 W for 3 min in the MAE process.

In order to clean up and pre-concentration the analyte after MAE step, SPE was performed to enhance a potential extraction of MT from complex matrices such as phospholipids and proteins. Optimization of washing and eluting process in SPE cartridge can reduce the matrix effect and improve the detecting ability of analysis. Therefore, the types and volume of solvents used for washing and eluting steps of MT extraction were studied. For this method, the consequence of reversed-phase sorbent showed no interference in the
analysis after washing with Milli-Q water. Figure 3(a) illustrated that the highest recovery percentage was obtained when using Milli-Q water as washing solvent because the target analyte cannot dissolve in the high polar solvent [12]. Figure 3(b) revealed that when adding more than 3.0 mL of Milli-Q water, the recovery percentage remained unchanged. Therefore, 3.0 mL of pure Milli-Q water was used as washing solvent to clean up in SPE. For the eluting step, various types of eluting solvents were studied. In comparison with other solvents, it was found that ethanol showed the highest recovery percentage because MT molecules can be greatly dissolved in ethanol due to their polarity [12]. Also, 3.0 mL of ethanol was selected as great eluent and sufficient to elute the analyte as shown in Figure 3(c-d).

3.2 Analytical characteristics

The potential analysis of the developed approach was validated for the determination of residual MT in Nile tilapia tissues by this appropriate sample preparation technique. Analytical performances were determined under the optimum parameters for the preparation of fish tissue samples. Figure 4 indicated that linearity was found in the range from 25.0 to 800.0 ng g\(^{-1}\) with a regression coefficient (\(R^2\)) equaled to 0.9985. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the following equations: LOD = 3 s/m and LOQ = 10 s/m, where ‘s’ was the deviation of blank signals (\(n = 10\)) and ‘m’ was the slope of the calibration curve. The values of LOD and LOQ were 1.53 and 5.08 ng g\(^{-1}\), respectively. The precision of the developed method was evaluated by conducting repeatability and reproducibility tests for the determination of MT standard at 50.0, 100.0, 200.0, and 400.0 ng g\(^{-1}\) concentration (\(n = 10\)) and found the highest relative standard deviation (RSD) as 1.5% as shown in Table 1.

A chromatogram was observed and no interference compound indicated a close position of the target compound. By comparing the analytical performances of the proposed method with other sample preparation methods for detecting MT using HPLC, it was found that the proposed method gave a lower detection limit and a better percentage recovery as the result indicated in Table 2. Concisely, it can be Figure 4. Linearity range for determination of MT using proposed preparation method (\(n = 3\)).
observed that the SPE process alone can detect MT in Carassius tissues with adequate recovery levels, nevertheless, a detection limit of this approach is still too high for monitoring of MT in fish tissues, i.e., 300 ng g⁻¹. Our technique offers the recoveries values and detection limit greater than that method.

Table 1. Results for repeatability and reproducibility for developed method (n = 10).

| Concentration of MT (ng g⁻¹) | Repeatability (%RSD) | Reproducibility (%RSD) |
|-----------------------------|----------------------|------------------------|
|                             | intra-day            | inter-day              |
| 50.0                        | 1.22                 | 1.33                   |
| 100.0                       | 1.32                 | 1.39                   |
| 200.0                       | 1.27                 | 1.40                   |
| 400.0                       | 1.32                 | 1.36                   |

Table 2. Analytical performance of the proposed method compared with other sample preparation methods for monitoring MT using HPLC.

| Separation methods | Linearity range (µg g⁻¹) | LOD (ng g⁻¹) | % Recoveries | Sample | Reference |
|--------------------|--------------------------|--------------|--------------|--------|----------|
| SFE                | 0.05 – 0.25              | 10.0         | 90.0         | Tilapia| 8        |
| QuEChERS           | 0.5 – 10.0               | 33.0         | 91.6 – 107.2 | Shrimp | 9        |
| SPE                | 1.0 – 100.0              | 300.0        | 82.9 – 93.6  | Carassius| 10       |
| LLE-SPE            | 15.0 – 120.0             | 1300.0       | 93.78 – 99.43| Fish feed| 11       |
| SPE                | 0.05 – 2.0               | 19.0         | 93.1 – 103.9 | Freshwater| 12       |
| MAE-SPE            | 0.025 – 0.80             | 1.53         | 98.41 – 100.78| Nile tilapia| This work|

SFE = Supercritical fluid extraction
QuEChERS = Solid phase extraction with salt
LLE = Liquid-liquid extraction

3.3 Application for real samples

This proposed method was used to determine the amount of residual MT content in the presence of Nile tilapia tissue samples. Six samples were collected at different areas in Thailand such as local markets and natural ponds. Samples were prepared by our proposed method and calculate the amounts of residual MT. Table 3 shows of amounts of MT concentration found in fish samples based on the developed method. The different samples (A, B, C, D, E and F) found the concentration of MT equal to 202.0, 454.3, 406.2, 597.9, 100.9 and 34.9 ng g⁻¹ respectively. Furthermore, the spiked method was implemented to validate the measurements with the proposed sample preparation method. Fish tissue samples were spiked with three different concentrations of MT standard solution (50.0, 100.0 and 150.0 ng g⁻¹).

Table 3. Results of MT level in fish tissues based on the developed method (n = 3).

| Samples | Total found (ng g⁻¹) |
|---------|---------------------|
| A       | 202.0 ± 0.10        |
| B       | 454.3 ± 0.60        |
| C       | 406.2 ± 0.20        |
| D       | 597.9 ± 0.20        |
| E       | 100.9 ± 0.15        |
| F       | 34.9 ± 0.18         |
Table 4 demonstrates the recovery percentages range from 98.4 to 100.8% with the highest RSD as 1.25%. These results indicated that the developed sample preparation method can be efficiently applied for the determination of MT in fish samples.

Table 4. Recovery testing in fish tissue samples in the proposed method ($n = 3$).

| Samples | Found (ng g$^{-1}$) | Added standard MT (ng g$^{-1}$) | Total found (ng g$^{-1}$) | % Recoveries | % RSD |
|---------|---------------------|-------------------------------|--------------------------|--------------|-------|
| A       | 202.0               | 50.0                          | 248.8                    | 98.7         | 0.40  |
|         |                     | 100.0                         | 297.2                    | 98.4         | 0.24  |
|         |                     | 150.0                         | 349.7                    | 99.4         | 0.31  |
| B       | 454.3               | 50.0                          | 508.2                    | 100.8        | 1.05  |
|         |                     | 100.0                         | 545.7                    | 98.4         | 0.45  |
|         |                     | 150.0                         | 598.4                    | 99.0         | 0.36  |
| C       | 406.2               | 50.0                          | 452.2                    | 99.1         | 0.62  |
|         |                     | 100.0                         | 506.6                    | 100.1        | 0.97  |
|         |                     | 150.0                         | 554.8                    | 99.7         | 0.62  |
| D       | 597.9               | 50.0                          | 616.6                    | 99.8         | 0.53  |
|         |                     | 100.0                         | 658.3                    | 98.6         | 1.12  |
|         |                     | 150.0                         | 718.4                    | 100.1        | 0.66  |
| E       | 100.9               | 50.0                          | 149.1                    | 98.8         | 0.69  |
|         |                     | 100.0                         | 199.8                    | 99.4         | 0.63  |
|         |                     | 150.0                         | 250.1                    | 99.7         | 1.25  |
| F       | 34.9                | 50.0                          | 83.6                     | 98.5         | 0.58  |
|         |                     | 100.0                         | 134.4                    | 99.7         | 0.76  |
|         |                     | 150.0                         | 184.8                    | 99.9         | 0.92  |

4. Conclusion

We can conclude that the determination of organic compounds in a sample containing a complex matrix requires an appropriate sample preparation technique to reduce the loss of the analyte and to increase the measurement efficiency. This proposed technique was developed for the detection of residual steroid hormone content, MT, in Nile tilapia tissue samples by HPLC-UV. With respect to its efficient and easy-to-use performance, a house microwave was chosen to assist solid-phase extraction for fish sample preparation. This technique can enhance detectability with a satisfactory recovery percentage and limit of detection. As well, it also exhibits many benefits for analysis such as cost-efficiency, rapidity, simplicity, high sample throughput, and low solvent consumption. More importantly, the analytical characterization of our proposed technique can be used to screen the residual MT at ng level in fish tissues with extreme accuracy and precision. This appropriate sample preparation can be applied as alternative sample preparation techniques to determine MT residues in fish tissues for both routine quality control and analytical research in various applications such as industry, agriculture, and medicine.

Acknowledgement

The authors are thankful for the financial support from Petchra Pra Jom Klao Ph.D. Research Scholarship, King Mongkut’s University of Technology Thonburi.
References

1) A. Cnaani, B. Levavi-Sivan, *Sex. Dev.*, **3**, 164-175 (2009).
2) W. L. Gale, M. S. Fitzpatrick, M. Lucero, W. M. Contreras-Sánchez, C. B. Schreck, *Aquaculture*, **178**, 349-357 (1999).
3) J. Beardmore, G. Mair, R. Lewis, *Aquaculture*, **197**, 283-301 (2001).
4) T. J. Pandian, S. G. Sheela, *Aquaculture*, **138**, 1-22 (1995).
5) J. S. Abucay, G. C. Mair, *Aquacult. Res.*, **28**, 841-845 (1997).
6) L. R. Curtis, F. T. Diren, M. D. Hurley, W. K. Seim, R. A. Tubb, *Aquaculture*, **99**, 193-201 (1991).
7) P. S. Chu, M. Lopez, S. Serfling, C. Gieseker, R. Reimschuessel, *J. Agric. Food Chem.*, **54**, 3193-3198 (2006).
8) Y. Han, Q. Ma, J. Lu, Y. Xue, C. Xue, *Food Chem.*, **135**, 2988-2993 (2012).
9) R. Fu, A. Zhai, Agilent Technologies Co. Ltd., China, 5990-6589EN (2012).
10) C.H. Zhai, Y. Zou, C. Rou, N. Jin, Agilent Technologies Co. Ltd., China, 5990-3845EN (2009).
11) A. Marwah, P. Marwah, H. Lardy, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **824**, 107-115 (2005).
12) I. R. Barbosa, S. Lopes, R. Oliveira, I. Domingues, A. M. V. M. Soares, A. J. A. Nogueira, *Am. J. Anal. Chem.*, **4**, 207-211 (2013).
13) J. M. Storey, S. B. Clark, A. S. Johnson, W. C. Andersen, S. B. Turnipseed, J. J. Lohne, R. J. Burger, P. R. Ayres, J. R. Carr, M. R. Madson, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, **972**, 38-47 (2014).
14) D. Zeng, C. Lin, Z. Zeng, X. Huang, L. He, *Agric. Sci. China*, **9**, 306-312 (2010).
15) P. Regal, C. Nebot, B. I. Vázquez, A. Cepeda, C. A. Fente, *Meat Sci.*, **84**, 196-201 (2010).
16) Z. Zhang, H. Duan, L. Zhang, X. Chen, W. Liu, G. Chen, *Talanta*, **78**, 1083-1089 (2009).
17) P. Li, Z. Yan, X. Sun, S. Chen, Y. Chen, X. Zhang, *J. Ocean Univ. China*, **17**, 1171-1177 (2018).
18) Y. Liu, D. Xie, Y. Kang, Y. Wang, P. Yang, J. Guo, J. Huang, *J. Chromatogr. Sci.*, **54**, 670-676 (2016).
19) R. Guedes-Alonso, Z. Sosa-Ferrera, J. J. Santana-Rodríguez, *Food Chem.*, **237**, 1012-1020 (2017).
20) D.-L. Su, P.-J. Li, S. Y. Quek, Z.-Q. Huang, Y.-J. Yuan, G.-Y. Li, Y. Shan, *Food Chem.*, **286**, 1-7 (2019).
21) M. Magnusson, A. K. L. Yuen, R. Zhang, J. T. Wright, R. B. Taylor, T. Maschmeyer, R. Nys, *Algal Res.*, **23**, 28-36 (2017).
22) H. Wang, J. Ding, N. Ren, *TrAC, Trends Anal. Chem.*, **75**, 197-208 (2016).
23) A. Azouz, E. Ballesteros, *Sci. Total Environ.*, **419**, 208-215 (2012).
24) L. Sanchez-Prado, C. Garcia-Jares, T. Dagnac, M. Llompart, *TrAC, Trends Anal. Chem.*, **71**, 119-143 (2015).
25) Y. Fan, Z. Niu, C. Xu, L. Yang, F. Chen, H. Zhang, *Ind. Crops Prod.*, **141**, 111809 (2019).