การใช้ฮอร์โมนเมสทาโนโลนเหนี่ยวนำเพศผู้ในลูกปลานิล

นางสาวนิอร วินารักษ์วงศ์

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MESTANOLONE INDUCED MALE SEX-REVERSED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FRY

Miss Nion Vinarukwong

A Dissertation Submitted in Partial Fulfillment of the Requirements

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นิอร วินารักษ์วงศ์ : การใช้ฮอร์โมนเมสทาโนโลนเหนี่ยวนำเพศผู้ในลูกปลานิล (MESTANOLONE INDUCED MALE SEX-REVERSED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ ดร. เจนนุช ว่องธวัชชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภก. ดร. พรชัย โรจน์สิทธิศักดิ์, 156 หน้า.

ปลานิลเป็นปลาน้ำจืดที่สำคัญชนิดหนึ่งของประเทศไทยโดยมีผลผลิตประมาณ 250,000 ตันต่อปี การเลี้ยงในระบบอุตสาหกรรมมีการนำฮอร์โมนเพศผู้มาใช้ระหว่างการเลี้ยงเพื่อผลิตปลานิลแบบเพศผู้เพศเดียว เพราะจะให้ผลผลิตที่ดีกว่า การเลี้ยงปลาเพศเดียวทำให้การจัดการเป็นไปได้อย่างมีประสิทธิภาพ เมสทาโนโลน หรือ 17 แอลฟาเมทิลไดไฮโดรเทสโทสเตอโรน เป็นฮอร์โมนเพศผู้สังเคราะห์ที่นำมาให้ลูกปลานิลที่เพิ่งฟักเป็นตัวเพื่อเหนี่ยวนำเพศ แต่ระดับการตกค้างของฮอร์โมนนี้ในปลายังไม่มีรายงานการศึกษา ผลของฮอร์โมนแอนโดรเจนจากภายนอกต่อพัฒนาการทางเพศถูกทำการทดสอบในลูกปลานิล การเหนี่ยวนำให้เป็นเพศผู้ทำโดยให้ปลากินเมสทาโนโลนขนาด 80 มิลลิกรัมต่อกิโลกรัมอาหาร เป็นระยะเวลา 5, 10, 15 และ 20 วัน การตรวจสอบทางจุลทรรศน์ของอวัยวะเพศปลาที่ติดสีย้อมอะซีโตคามีนเพื่อประเมินเพศผู้และเพศเมียของลูกปลาที่อายุ 60 วันหลังฟักเป็นตัว การให้เมสทาโนโลนทำให้ผลผลิตเป็นลูกปลาเพศผู้ 100% หลังจากให้ฮอร์โมนผสมอาหารเป็นระยะเวลา 15 วัน หรือ 20 วัน ในขณะที่ระยะเวลาการให้ 5 และ 10 วัน จะได้ปลาเพศผู้ 87% และ 90% ตามลำดับ ผลของเทคนิคทางจุลวิทยาพบว่าไม่มีความแตกต่างของเนื้อเยื่อระบบสืบพันธุ์ในปลากลุ่มที่ได้รับฮอร์โมนเมื่อเทียบกับปลาปกติ นอกจากนั้นจากการวิเคราะห์ทางสถิติพบว่าน้ำหนักปลาไม่มีความแตกต่างกันอย่างมีนัยสำคัญ ($P>0.05$)ในกลุ่มที่ทำการทดลองและกลุ่มควบคุม การศึกษานี้ทำการตรวจวิเคราะห์ปริมาณเมสทาโนโลนตกค้างหลังจากการผสมอาหารให้ลูกปลานิลกินในขนาด 80 มิลลิกรัมต่อกิโลกรัมอาหารติดต่อกันเป็นระยะเวลา 15 วัน (ระยะเวลาที่ลดลง) และ 23 วัน (ระยะเวลาที่ปฏิบัติจริงในฟาร์ม) การวิเคราะห์ทำในวันที่ 1, 2, 3, 5, 7, 14 และ 21 หลังจากการให้อาหารผสมฮอร์โมนครั้งสุดท้าย โดยใช้เทคนิคลิควิดโครมาโทกราฟีแทนแดมแมสสเปคโทรเมทรี ปริมาณเมสทาโนโลนที่พบในลูกปลาที่ได้รับฮอร์โมนเป็นระยะเวลา 15 และ 23 วัน จากการตรวจในวันที่ 1, 2, 3 และ 5 หลังหยุดการให้ฮอร์โมน อยู่ในช่วง 0.28-3.20 และ 0.29-3.22 นาโนกรัมต่อกรัมตามลำดับ ตรวจไม่พบเมสทาโนโลนในลูกปลาหลังหยุดการให้ฮอร์โมนเป็นระยะเวลา 7 วัน (ขีดจำกัดการวัดเชิงปริมาณ หรือ แอลโอคิว เท่ากับ 0.09 นาโนกรัมต่อกรัม) ดังนั้นจึงไม่ควรมีปริมาณเมสทาโนโลนตกค้างในปลานิลที่อายุ 6-8 เดือน ที่มีระยะหยุดยาที่เหมาะสมหลังจากได้รับฮอร์โมนในระยะปลาวัยอ่อน โดยสรุปคือการทดลองนี้ประสบความสำเร็จในการลดระยะเวลาการใช้เมสทาโนโลนในการเหนี่ยวนำลูกปลานิลเพศผู้ที่ระยะเวลาการให้ 15 วัน โดยยังคงให้ผลผลิตเป็นลูกปลาเพศผู้ 100% และไม่มีผลข้างเคียงเชิงลบต่อการเจริญเติบโตของปลา และการวิเคราะห์สารตกค้างในการศึกษานี้สำคัญในแง่ที่จะสร้างความไว้วางใจของผู้บริโภคเกี่ยวกับความปลอดภัยในการบริโภคปลานิลที่ได้รับฮอร์โมน

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....

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NION VINARUKWONG: MESTANOLONE INDUCED MALE SEX-REVERSED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FRY. ADVISOR: ASSOC. PROF. JANENUJ WONGTAVATCHAI, D.V.M., M.Sc., Ph.D., D.T.B.V.M., CO-ADVISOR: ASSOC. PROF. PORNCHAI ROJSITTHISAK, B.Sc.(Pharm), M.Sc., Ph.D., 156 pp.

Tilapia (*Oreochromis niloticus*) is an important freshwater fish in Thailand yielding approximately 250,000 tons production per year. Along with intensive farming, androgenic hormones are applied during the farming process to produce monosex-male tilapia because of its better yield. A monosex population also allows for more effective management of a single crop. A synthetic androgenic steroid, mestanolone or 17α-methyldihydrotestosterone, is used in newly hatched tilapia fry for sex reversal, but its residual level in these fry has not been examined. The effects of exogenous androgenic hormone on sex differentiation was examined in Nile tilapia fry. Male sex reversal was mediated using oral administration of mestanolone at a dose of 80 mg/kg diet for 5, 10, 15, and 20 days. The microscopic examination of fish gonad stained with aceto-carmine was used to determine male and female fish fry at 60 days post hatching. Treatment with mestanolone yielded 100% male after feeding with hormonal diet for either 15 or 20 days, while treatment for 5 and 10 days presented 87% and 90% male, respectively. The results of histological examination revealed no differences in gonadal tissues of hormonal treated fish compare with normal fish. In addition, statistical analysis of the total weight gain among the fish fry revealed that there was no significant difference ($P > 0.05$) between the treated groups and the control group. This study also investigated residual mestanolone after a course of oral administration to tilapia fry at a dose of 80 mg/kg feed for 15 (minimized dose) and 23 (practical dose) consecutive days. The analyses were performed at 1, 2, 3, 5, 7, 14 and 21 days after the last dose using liquid chromatography tandem mass spectrometry. The amounts of mestanolone detected in 15 and 23 days hormonal treated fry on days 1, 2, 3 and 5 after hormone withdrawal ranged from 0.28-3.20 ng/g and 0.29-3.22 ng/g, respectively. Mestanolone was not detectable in fry after hormonal withdrawal for 7 day (limit of quantitation, LOQ, 0.09 ng/g), which suggests that negligible levels of mestanolone will be present in tilapia during the growth stage of 6-8 months after an adequate withdrawal period following treatment of early-stage fry. In conclude, the present study successfully minimized the use of mestanolone in male sex-reversed tilapia to a 15-day period, while maintaining 100% masculinization and having no adverse effect on general fish growth. The residue analysis in this study is important for establishing consumer trust in food safety for hormonally treated tilapia.

Department:	Veterinary Medicine	Student's Signature	.....
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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BW	body weight
ºC	degree Celsius
cm	centimeter
CV	coefficient of variance
DEN	dienestrol
DES	diethylstilbestrol
DHT	dihydrotestosterone
Dpf	days post fertilization
Dph	days post hatching
E_2	17α-estradiol; estradiol
EE_2	17α-ethynylestradiol
ELISA	enzyme-linked-immunosorbent assay
g	gram
GAP	good aquaculture practice
GC-MS	gas chromatography mass spectrometry
H&E	hematoxylin and eosin
HEX	hexestrol
HPLC	high performance liquid chromatography
kg	kilogram
L	liter
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation

MDHT	17α-methyldihydrotestosterone; mestanolone
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
MRL	maximum residual limit
MT	17α-methyltestosterone
ND	no data available
ng	nanogram
PAR	peak area ratio
ppb	part per billion
QC	quality control
RIA	radioimmunoassay
SD	standard deviation
TBME	*tert*-butyl methyl ether
USD	united states dollar
µL	microliter
µm	micrometer

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CHAPTER I

INTRODUCTION

1.1. Importance and rationale

Aquaculture is one of the fastest growing sectors of world animal food production. Hundreds of fish species are cultured and become famous supplying the high quality food for consumer. Nile tilapia (*Oreochromis niloticus*) is a species of major interest aquaculture around the world because of its rapid growth, high yield potential and tolerance wide range of environmental conditions. The demand of tilapia continue raises worldwide as a lower priced freshwater fish and white-meat fish which is suitable for people in all of age even the patient. Many products that made from tilapia were also interested such as tilapia's leather-made bag and cosmetic industry. The climate in Thailand is suitable for bringing tilapia culture up. All-male tilapia is more prefer than rearing mix sex because monosex-male tilapia can grow faster with better feed conversion ratio and avoid facing with overpopulation problem. Various methods for inducing female incompatibility in tilapia such as physical methods and chemical methods are used but the most efficient and least expensive method is the administration of androgenic hormones. The result of this technique is satisfied because it can yield nearly 100 percentage of male. Nevertheless, there is no study reported about the residual level of mestanolone in tilapia fry after the course of oral administration for sex reversal.

Although hormone is given to tilapia by dipping or oral administration in a short period, the use of hormone has been under increasing public criticism due to their possible health and environmental impacts. Furthermore, the European Union has banned using androgenic hormone as the growth promoter in food animals through the Council Regulation 2377/90/EC. Nowadays, there is no any published law forbidding the use of mestanolone in tilapia but in the future, some imported countries may use these topics as the justification in order to deprive Thailand from international trade. Then every food products from Thai's tilapia will be declined and lead to tremendous economic loss of Thailand. To lend credence to consumer and the imported country in international market, this study will come up with a solution to these problems by determination the mestanolone residue in sex-reversed tilapia and finding out the minimum period for hormonal administration.

1.2. Keywords

เพศผู้ที่เปลี่ยนจากเพศเมีย ฮอร์โมนเมสทาโนโลน ปลานิล การวิเคราะห์ส่วนตกค้าง

male sex-reversed mestanolone hormone Nile tilapia residual analysis

1.3. Research objectives

1.3.1. To study effect of the current mestanolone application on sexual development of tilapia fry.

1.3.2. To determine mestanolone residue in tissue of male sex-reversed tilapia.

1.3.3. To minimize the use of mestanolone in male sex-reversed farmed tilapia

1.4. Research benefits

1.4.1 Database of mestanolone residue in tilapia meat observed in Thailand aquaculture which show the useful data to enhance customer confidence and strengthen Thailand's tilapia culture on the global market.

1.4.2 Alternative method to minimize the use of mestanolone administration in male sex-reversed farmed tilapia that can reduce the production cost for agriculturist and reduce the risk for customer.

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CHAPTER II

LITERATURE REVIWS

2.1 Nile tilapia

Taxonomic Hierarchy of Nile tilapia (ITIS, 2015)

Kingdom: Animalia

Subkingdom: Bilateria

Infrakingdom: Deuterostomia

Phylum: Chordata

Subphylum: Vertebrata

Infraphylum: Gnathostomata

Superclass: Osteichthyes

Class: Actinopterygii

Subclass: Neopterygii

Infraclass: Teleostei

Superorder: Acanthopterygii

Order: Perciformes

Suborder: Labroidei

Family: Cichlidae

Genus: *Oreochromis*

Species: *Oreochromis niloticus*

(Linnaeus, 1758)

The name “tilapia” is derived from the African Bushman word meaning fish (Trewavas, 1982) originating from Africa. Tilapia represent a large number of freshwater fish species within the family Cichlidae and the genus Tilapia have been classified into three genera; *Tilapia* (substrate spawners), *Sarotherodon* (parental mouthbrooders) and *Oreochromis* (maternal mouthbrooders) based on the parental incubation of eggs. Later Tilapia were introduced into many countries in the Americas, Europe, Australia and Asia (El-Sayed, 2006) of which the economically important species are Nile tilapia (*Oreochromis niloticus*), blue tilapia (*O. aureus*), Mozambique tilapia (*O. mossambicus*), Zanzibar tilapia (*O. hornorum*), and the red belly tilapia (*O. zilli*). Tilapia have a compressed body covered with cycloid scales. The dorsal and anal fins have hard spines and soft rays (the dorsal fin with 16 - 17 spines and 11 to 15 soft rays and the anal fin with 3 spines and 10-11 rays). The pectoral and pelvic fins are large and used for controlling swimming and locomotion (El-Sayed, 2006). Their fins color in the spawning season; pectoral, dorsal and caudal fins becoming reddish and caudal fin with numerous black bars (FAO, 2015). Tilapia have well-developed sense organs, represented by prominent nares, relatively large eyes and visible lateral lines (El-Sayed, 2006). Tilapia are herbivorous/ omnivorous. The feeding habits and dietary preferences depend on many factors; the tilapia species and size, photoperiod, temperature, water depth and geographical rearing location.

Tilapia was brought into Thailand in 1965 when Emperor Akihito, as His Royal Highness the Crown Prince of Japan, sent 50 Nile tilapias as the royal tribute to His Majesty King Bhumibol Adulyadej, (which were later called “Pla Nil” in Thai). At first, His Majesty the King allowed them to be fed in a pond in Chitlada garden, Dusit Palace. Because there seemed to be a lot of fry showing up, His Majesty intended to breed this

fish species for the sake of his population. Therefore, His Majesty sent 10,000 fish to the Department of Fisheries to feed and breed them at the experimental breeding plan and then distributed them to Thai agriculturists all over the country. Currently, the aquaculture of Nile tilapia has expanded throughout the country and they have become the No. 1 freshwater fish produce in Thailand.

2.2 Tilapia production

The Nile tilapia is a species of major interest to aquaculturists worldwide due to its excellent growth rate, high yield, disease resistance, and tolerance to a wide range of environments. Tilapia is thus on its way to become a major supplier of protein both in the developed and the developing world. This increased trend has come from consumers who care about the benefit to their health because of the white meat of the tilapia and its good taste. Tilapia tissue can be used for various types of cooking and its cost is lower than other fish so it is used in the manufacture of food production in order to increase profit. Tilapia exported products consist of whole frozen fish, frozen fillets and fresh fillets (FAO, 2015). Nowadays, tilapia processed products such as shoes and bags which are made from tilapia skin, which is more tensible and durable than those made from cowhide have become popular on the international market. Moreover, jelly and collagen derived from tilapia skin can be used in the cosmetics industry.

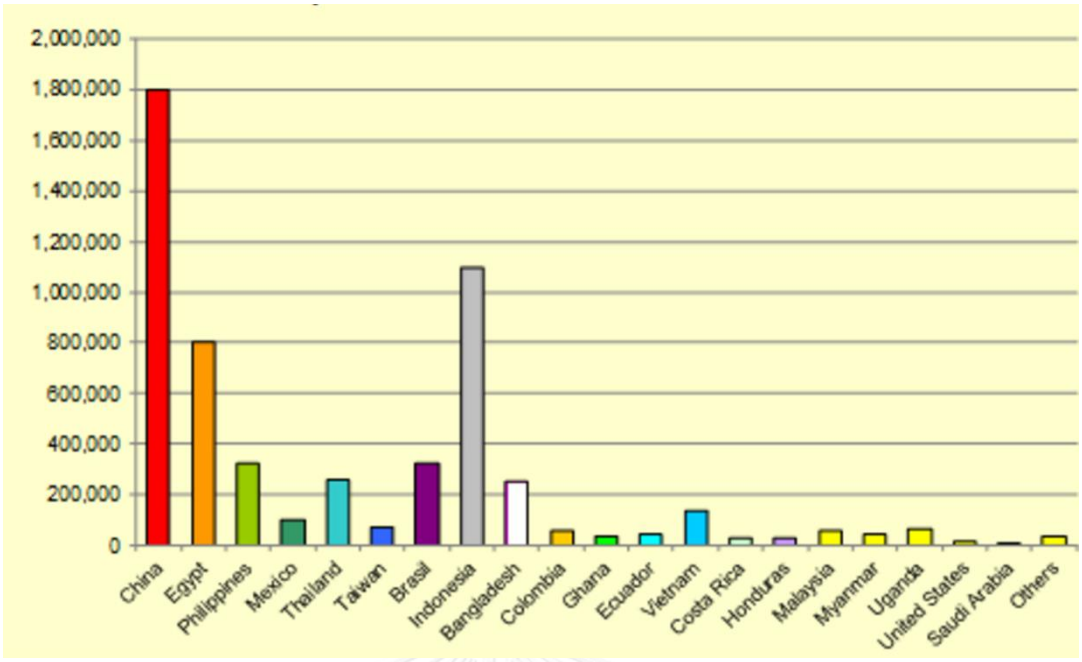

Figure 2.1 World tilapia production (Fitzsimmons, 2016)

The global production of farmed tilapia was 4,850,000 metric tons in 2014 and increased to 5,576,800 metric tons at the end of 2015 (Fitzsimmons, 2016). Over two-thirds of this volume was produced in Asia. China continued its position as the single largest producer (1,800,000 metric tons in 2015). Other countries in Southeast Asia are also major producers and consumers, such as Indonesia, Philippines and Thailand (Fitzsimmons, 2016). In 2014, Thailand was ranked fourth in world Nile tilapia production with an export quantity of 179,240 metric tons and an export value of about 306,571,357 USD (approximately 10,089,110,060 Baht) (FAO, 2016). The production of tilapia in Thailand is sufficient for Thai consumer's demand (around 10% of this production will go to the local market with the remaining 90% heading to export) (Tower, 2013) and this can be enhanced for export to the global market and such regions as the United States, Japan and Europe.

2.3 Intensive culture and problems

Intensive culture is one of the culture methods use to manage high stocking density in order to gain the highest production with the minimal use of supplies (El-Sayed, 2006). This method needs high technology and the cost of the inputs per unit of fish weight is higher than in extensive farming, especially because of the high cost of fish feed, which contain a higher level of protein. The other important factors are dissolved oxygen, fresh water and commercial feeds which must be adequately provided. However, increasing stocking density results in increasing ammonia concentration and decreasing dissolved oxygen levels which are needed for good manufacturing practices.

In addition, intensive culture can be the cause of stress in fish that disturbs fish homeostasis and leads to disease occurrence in tilapia culture. Normally, the severity of the illness will increase if fish have a secondary infection, such as, having a bacterial infection after a parasitic infestation. Bacterial diseases; edwardsiellosis, motile aeromonas septicaemia, vibriosis and streptococcosis, are among the most serious problems of tilapia culture which can cause high loss. The main bacterial disease is streptococcosis, a disease caused by the streptococcal bacteria, that has been reported in many countries and has economic consequences on mass mortality at all stages of tilapia farming (Evans et al., 2000; Shoemaker et al., 2000). The major pathogens of this disease in Thai tilapia are *Streptococcus iniae* and *Streptococcus agalactiae* (Wongtavatchai and Maisak, 2008). Diseased fish should be removed from the culture system and treated separately or discharged depending on the severity of the disease. The ability to detect disease at an early stage is important in reducing loss. The current regimen used to control bacteria in Thai tilapia farming is antimicrobial medication but

their residue in tilapia meat may have adverse effects on consumer. Good aquaculture practices which can reduce stress and disease incidence are preferable to using antibiotic treatments. A vaccination program; the current trend for bacterial prevention is recommended. Moreover, aquaculturists in intensive farming usually prefer rearing monosex fish because this culture permits a higher growth rate, a greater uniformity of size and better meat quality (Beardmore et al., 2001; El-Sayed, 2006).

2.4 Monosex culture

Monosex culture is desirable in many species of cultured fish since it permits a higher growth rate, greater uniformity of size and better meat quality due to prevention of unwanted or uncontrolled reproduction through undesirable sexual behavior and precocious sexual maturation. Moreover, monosex culture reduces the risk to the environment caused by escape of exotic species (Beardmore et al., 2001; El-Sayed, 2006). These advantages fulfill the needs of aquaculturists who have shifted from traditional systems to more intensive cultures.

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Monosex culture is based on production of a fish culture of all males or females, depending on which sex has a better food conversion ratio and growth rate. Male monosex is preferred in many species of fish, including Nile tilapia (*Oreochromis niloticus*) (Guerrero, 1975; Phelps et al., 1996; Cagauan, 2004; Mateen and Ahmed, 2007; Marjani et al., 2009), black crappie (*Pomoxis nigromaculatus*) (Arslan and Phelps, 2004), and channel catfish (*Ictalurus punctatus*) (Goudie et al., 1994; Galvez et al., 1995). The average weight of channel catfish harvested from monosex male ponds was found to be 8.5% higher than fish in mixed sex ponds and 15% higher than fish in monosex female

ponds (Goudie et al., 1994). Female monosex also has significant benefits by eliminating losses arising from early maturation of males and allowing for a doubling in roe production compare to mixed sex cultures (Refstie et al., 1982). Fish species that are commonly reared as female monosex cultures include chinook salmon (*Oncorhynchus tshawytscha*) (Hunter et al., 1983), coho salmon (*O. kisutch*) (Goetz et al., 1979), brook trout *(Salvelinus fontinalis*) (Haffray et al., 2009), rainbow trout (*O. mykiss*)((Arslan et al., 2010), and white amur (*Ctenopharyngodonidella Valenciennes*) (Stanley, 1976). Manual sorting of sex is the simplest method requiring the least technology. However, this technique is laborious, needs experience, causes the fish some stress and the size of fingerling must large enough to determine the sex (Cnaani and Levavi-Sivan, 2009). Several physical and chemical methods have been described for the production of monosex fish. Physical methods include the use of irradiated sperm and cold shock (Refstie et al., 1982). The results of many studies show that temperature has an effect on sex differentiation during the developmental stage of embryo; high temperature makes fry become female sex-incompatible (Baroiller et al., 1999; Devlin and Nagahama, 2002; Wessels and Hörstgen-Schwark, 2007), while low temperature induces male sex incompatibility (Haffray et al., 2009) (Table 2.1). High temperature make tilapia fry becoming female sex incompatibility in high rate but it is not completely 100% succeed due to the differentiation into male or female in fish which is a complex and labile mechanism under the control of genetic, physiological or/and environmental factors (Devlin and Nagahama, 2002).

Table 2.1 Temperature treatment during sex differentiation for male sex reversal in tilapia fry.

Fish	Sex of fry	Temperature (ºC)	Duration	Percentage of Male (%)	Reference
O. aureus	mixed sex	21ºC	40 day	0*	Desprez and
		27ºC	60 day	63.0	Mélard (1998)
		34 ºC	25 day	97.8	
O. niloticus	female	25.78±0.2 ºC	21-30 day	12.2	Abucay et al.
	monosex	27.87±1.4 ºC	21-30 day	20.2	(1999)
		36.54±0.4 ºC	21-30 day	37.3	
O. niloticus	mixed sex	28ºC	10 day	51.6	Wessels and
		36ºC	10 day	65.6	Horstgen-
					Schwark (2007)
O. niloticus	female	27ºC	10 day	0	Rougeot et al.
	monosex	34ºC	10 day	9.7	(2008b)
		35ºC	10 day	18.2	
		36ºC	10 day	17.5	
O. niloticus	mixed sex	27ºC	30 day	50.2	Bezault et al.
		36ºC	30 day	78.7	(2007)
O. niloticus	Mixed	19.0±0.9ºC	28 day	43.6	Azaza et al.
	sex	32.0±0.4ºC	28 day	46.9	(2008)
		34.0±0.3ºC	28 day	48.7	
		36.5±0.4ºC	28 day	64.2	

**undiffentiated*

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Chemical method; hormonal administration is the most commonly used chemical method for generation of sex incompatibility in farmed fish. The routes of administration include dietary feeding, immersion, injection, and implantation. However, hormone injection or implantation are not practical in fish farming due to the cost, labour and skills required (Pandian and Sheela, 1995). Hormonal feeding and immersion are preferable techniques because of their reliability and high success rates (Baroiller et al., 1999; Devlin and Nagahama, 2002). Moreover, these treatments can be handled easily and are cost effective for farming practice.

2.5 Hormonal treatment

Hormones play an important role in sexual differentiation in fish. Synthetic steroid hormones such as androgens and estrogens are commonly used for sex reversal at the early stage of fry (Piferrer, 2011). Hormones are administrated via dietary supplementation by dissolving them in alcohol prior to mix with the diet. The other techniques are immersion and injection applied successfully in some species (Cnaani and Levavi-Sivan, 2009). The differentiation occurs during a critical period (labile period) which is variable among species, it begins after hatching and lasts for about 10-40 days in cichlids (Pandian and Sheela, 1995). Hormonal administration permits production of a monosex population and can overcome the problems of mixed-sex culture such as over-reproduction, low growth rate, low feed utilization, and non-uniformity of size at harvest. Thus, many types of hormones have been used for induction of sex incompatibility in fish. Pandian and Sheela (1995) reviewed the use of natural and synthetic steroids in many species of fish (Cichlidae, Cyprinodondidae, Anabantidae, Poecilidae, Salmonidae and Cyprinidae) and suggested that 17α-methyltestosterone (MT, methyltestosterone) and

17β-estradiol (E_2, estradiol) are the preferred hormones for induction of masculinization and feminization, respectively. There are many previous studies reported about using androgen and estrogen hormone for sex reversal (Table 2.2 and Table 2.3).

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Table 2.2 Synthetic androgenic hormones used in medicated feed for male sex reversal of fish in dosage, kinds of fish and the effectiveness of hormones shown by percentage of males.

Hormone	Fish	Dose (mg/g)	Duration (day)	Feeding rate	Water temperature	Percentage of male (%)	Reference
1-dehydrotestosterone	*O. aureus*	0	18	4% BW	21°C	56.00	Guerrero
		15				69.00	(1975)
		30				59.00	
		60				44.00	
17α-ethynyltestosterone	*O. aureus*	15	18	4% BW	21°C	85.00	Guerrero
		30				98.00	(1975)
		60				100.00	
17α-methyltestosterone	*O. aureus*	15	18	4% BW	21°C	84.00	Guerrero
		30				98.00	(1975)
		60				85.00	
17α-methyltestosterone	*O. niloticus*	0	28	20% BW	ND	54.70	Phelps et
		60				97.80	al. (1992)
17α-methyltestosterone	*O. niloticus* (3-day old fry)	0	25	ND	ND	51.00	Mateen and
		50				80.00	Ahmed
		60				89.90	(2007)
		70				95.40	
		80				90.70	
17α-methyltestosterone	*O. mossambicus* (3 dph)	0	21	20% BW	28±1°C	51.80	Marjani et
		50				74.29	al. (2009)
		75				98.09	
		100				79.38	

ND, no data available.

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Table 2.2 (cont) Synthetic androgenic hormones used in medicated feed for male sex reversal of fish in dosage, kinds of fish and the effectiveness of hormones shown by percentage of males.

Hormone	Fish	Dose (mg/kg)	Duration (day)	Feeding rate	Water temperature	Percentage of male (%)	Reference
17α-methyltestosterone	*O. niloticus* (3-day old spawn)	0	28	ND	ND	48.60	Ferdous and Ali (2011)
		40				88.60	
		50				91.40	
		60				94.30	
		70				91.40	
17α-methyltestosterone	*O. niloticus* (<12 mm)	0	28	15% BW	27-32°C	44.50	Phelps and Okoko (2011)
		3.75				80.00	
		7.5				91.70	
		15				98.30	
		30				99.30	
		60				97.00	
		120				71.90	
		240				50.70	
		480				48.30	
		600				55.00	
		1200				52.00	
17α-methyltestosterone	*O. niloticus* (12-15 mm length)	0	28	15% BW	25°C	63.00	Amaraweera et al. (2012)
		40				92.00	
		60				98.00	
fluoxymesterone	*O. niloticus*	0	28	20% BW	ND	54.70	Phelps et al. (1992)
		0.2				87.30	
		1				100.00	
		5				100.00	
		25				100.00	

ND, no data available

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Table 2.3 Synthetic estrogenic hormones used for female sex reversal of fish in dosage, route, kinds of fish and the effectiveness of hormones shown by percentage of females.

Hormone	Fish	Dose	Duration	Route	Water temperature	Percentage of female (%)	Reference
17β-estradiol	*O. aureus*	30 mg/kg	35 day	feed	23±1°C	54.34	Jensen and Shelton (1979)
		60 mg/kg		10 - 12%		55.36	
		120 mg/kg		BW		50.00	
17β-estradiol	*Centropomus undecimalis*	0 mg/kg	45 day	feed	28±1°C	0.00	Carvalho et al. (2014)
		50 mg/kg				68.42	
		100 mg/kg				90.00	
17β-estradiol	*O. niloticus*	0 mg/kg	30 day	feed	28±1°C	40.40	Alcántar-Vázquez et al. (2015)
		60 mg/kg				60.20	
		120 mg/kg				69.80	
17α-ethynylestradiol	*O. niloticus* YY fry (4 dph)	0.01 µg/mL	3 day	immersion	26±1°C	0.00	Kobayashi et al. (2003)
		0.01 µg/mL				100.00	
		0.1 µg/mL				100.00	
17α-ethynylestradiol	*O. niloticus* XY < 12 hpf	0 mg/L	5 day	immersion	27ºC	0.00	Rougeot et al. (2008a)
		100 mg/L				55.50	
		500 mg/L				68.20	
17α-ethynylestradiol	*O. niloticus* XY fry (1 dpf)	0 µg/L	4 hr	immersion	27ºC	0.00	Gennotte et al. (2015)
		1000 µg/L				48.00	
		2000 µg/L				48.60	
	YY fry (1 dpf)	0 µg/L				00.00	
		2000 µg/L				00.00	
		6500 µg/kg				00.30	
β-estradiol 17-valerate	*Oryzias latipes* (4 dpf)	0 ng/kg	15 day	immersion	25±1°C	52.05	Lei et al. (2013)
		1 ng/L				58.57	
		10 ng/L				69.81	
		100 ng/L				70.73	
		1000 ng/L				76.19	

dph, days post hatching; dpf, days post fertilization; ND, no data available.

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Table 2.3 (cont) Synthetic steroid sex hormones used for female sex reversal of fish in dosage, kinds of fish and the effectiveness of hormones shown by percentage of females.

Hormone	Fish	Dose	Duration	Route	Water temperature	Percentage of female (%)	Reference
diethylstilbestrol	*O. niloticus* YY fry (10 dpf)	0 mg/kg	20 day	feed	ND	52.29	Abucay and Mair (1997)
		1000 mg/kg	10 day			100.00	
		1000 mg/kg	15 day			86.14	
		1000 mg/kg	20 day			93.50	
diethylstilbestrol	*O. niloticus* YY fry (10 dpf)	0 mg/kg	11 day	feed	28±1°C	0.00	Karayücel et al. (2003)
		1000 mg/kg				33.80	
diethylstilbestrol	*O. niloticus*	0 mg/kg	40 day	feed	22-29ºC	57.07	Hamdoon et al. (2013)
		50 mg/kg	25 day	10% BW		70.29	
		50 mg/kg	40 day			75.86	
		100 mg/kg	25 day			81.86	
		100 mg/kg	40 day			87.39	
diethylstilbestrol	*O. niloticus*	0 mg/kg	20 day	feed	28-30ºC	39.00	Ramírez et al. (2015)
		100 mg/kg		20% BW		62.00	
		200 mg/kg				67.00	
		300 mg/kg				64.00	
		400 mg/kg				91.00	
estriol	*O. aureus*	0 mg/kg	35 day	feed	23±1°C	50.74	Jensen and Shelton (1979)
		30 mg/kg		10 - 12%		50.98	
		60 mg/kg		BW		53.49	
		120 mg/kg				36.63	
estrone	*O. aureus*	30 mg/kg	35 day	feed	23±1°C	54.55	Jensen and Shelton (1979)
		60 mg/kg		10 - 12%		55.16	
		120 mg/kg		BW		50.00	
ethinylestradiol	*O. mossambicus*	0 mg/kg	20 day	feed	20±2°C	38.18	Nakamura and Takahashi (1973)
		50 mg/kg		4% BW		100.00	

dph, days post hatching; dpf, days post fertilization; ND, no data available.

Different testosterone derivatives have been administered to fish at the early stage of fry, including 17α-methyltestosterone (MT, methyltestosterone) by feeding and bathing in Nile tilapia (Phelps et al., 1996; Straus et al., 2013; Mateen and Ahmed, 2015) 17 α-ethynyltestosterone feeding in Blue tilapia (*O. aureus*) (Guerrero, 1975) 5α-dihydrotestosterone feeding in pejerrey (*Odontesthes bonariensis*) (González et al., 2015) and 17α-methyldihydrotestosterone (MDHT, mestanolone) bathing in Nile tilapia (Gale et al., 1999) (Figure 2.2).

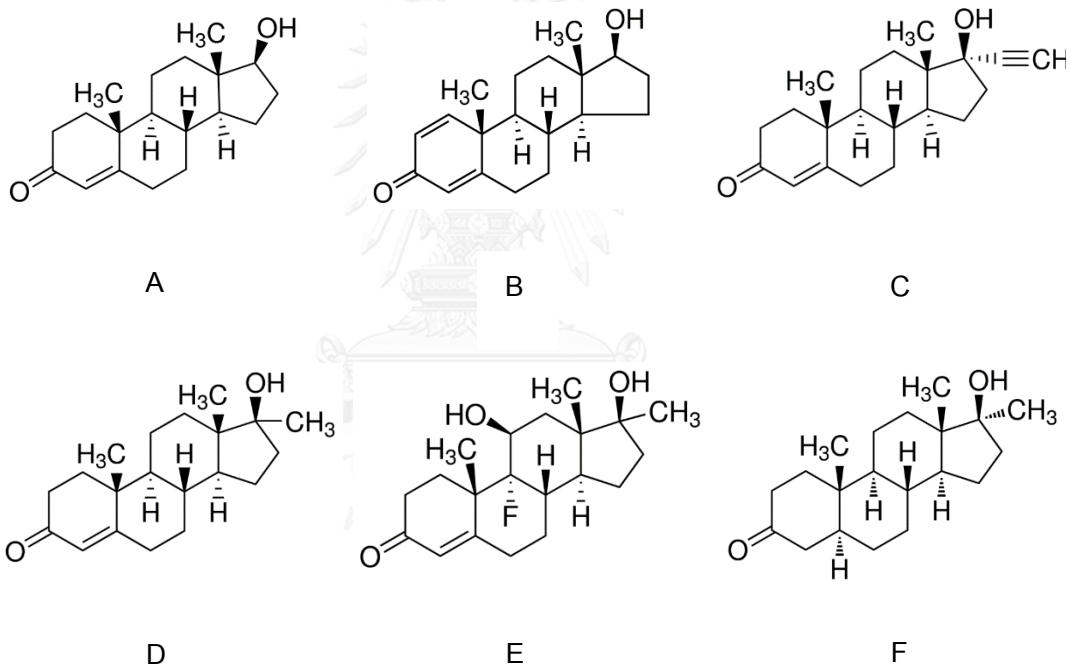

Figure 2.2 Chemical structures of widely used androgenic hormones for sex reversal in tilapia. (A) testosterone (B) 1-dehydrotestosterone (C) 17α- ethynyltestosterone (D) 17α-methyltestosterone(MT) (E) fluoxymesterone and (F) 17α-methyldihydrotestosterone (mestanolone).

However, these hormones may not be the best choice (Pandian and Sheela, 1995). Mestanolone (17α-methyldihydrotestosterone or (5α,17β)-17-hydroxy-17-methylandrostan-3-one) is the 17α-methylated version of dihydrotestosterone (DHT), which binds with greater affinity to the androgen receptor than testosterone (Kicman, 2008), may be more effective for male sex-reversal. Mestanolone is white powder, scentless, stable in the air, can be dissolved in alcohol and organic compounds but cannot be dissolved in water. Mestanolone was introduced to be used for prophylaxis and therapy of osteoporosis in postmenopausal woman which was issued in the United States Patent No. 5591735 (Mattern and Hacker, 1997). Because of its anabolic effect that can increase muscle mass and physical strength, it is abused for the purpose of enhancing physical performance in athletes (Franke and Berendonk, 1997) and racehorses (Yamada et al., 2008). Nowadays, mestanolone is classified as prohibited substances according to World Anti-Doping Agency (WADA, 2017). Currently, mestanolone is widely used in farmed tilapia in order to produce the male monosex population by giving hormone at an early stage to tilapia fry at a dose rate of 80 mg/kg feed for 23 consecutive days.

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2.6 Potential hazard of hormonal use

The adverse effects of androgenic hormone on various body systems were reported in many publication, such as hepatotoxic effects (Büttner and Thieme, 2010), cardiovascular effects (Fineschi et al., 2007; Basaria, 2010) and psychological effects (Kicman, 2008). In addition, MacIndoe et al. (1997) found the suppression of spermatogenesis because the high dose of testosterone suppress hypothalamic-pituitary-gonadal axis due to the negative feedback. Although there is still a little information about

mestanolone toxicity, one study said about the use of oral-mestanolone with contraceptive steroid preparations could increase the risk of liver dysfunctions and damages in human (Franke and Berendonk, 1997).

The use of animal drugs in livestock is generally antibiotics and growth promoters for the control of disease outbreaks and the efficiency of feed conversion. In the EU the use of antibacterial drugs is regulated by MRLs and the use of growth promoters is banned. Commission Decision 98/179/EC lays down the detailed rules on official sampling for the monitoring of certain substances and residues thereof in live animals and animal products. Therefore, Commission Decision 2002/657/EC establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. Although the use of mestanolone in tilapia is simple, reliable and cost effective, but may lead to residual problems in the fish and aquatic environment. A potential use of mestanolone as a growth promoter poses regulatory concerns therefore analytical methods are needed to monitor mestanolone residues in fish.

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2.7 Analytical methods for hormonal detection

Analytical methods for the determination of anabolic steroid hormones have been achieved by radioimmunoassay (RIA) (El-Neklawey et al., 2009), enzyme-linked immunosorbent assay (ELISA) (Hungerford et al., 2005), and chromatographic assays such as High performance liquid chromatography (HPLC), Gas chromatography mass spectrometry (GC-MS) and Liquid chromatography mass spectrometry (LC-MS). Many previous studies had reported about the analytical methods used for detection of

androgenic hormones in various kinds of sample as shown in Table 2.4. HPLC is an important analytical method for many hormones because of its high sensitivity and specificity. HPLC is currently used as the standard method for detection of hormonal residues in fish tissues and other matrices including, MT in carp muscle (Jiang et al., 2005). GC-MS has also been applied for detection of a wide range anabolic steroids in meat (Marchand et al., 2000; Stolker and Brinkman, 2005). Liquid chromatography tandem mass spectrometry (LC-MS/MS) is used for detecting MT in tilapia, rainbow trout and salmon tissues which is chosen as a marker for monitoring of MT residues in fish (Chu et al., 2006). Four anabolic steroids are trenbolone, methylboldenone, MT, and norethandrolone, which were determined in bovine muscle using LC-MS/MS (Kaklamanos et al., 2007). In addition, the LC-MS/MS method has also developed for detection of diethylstilbestrol (DES), dienestrol (DEN), and hexestrol (HEX) in muscle tissue of catfish, salmon, trout, and tilapia (Lohne et al., 2013). To the best of my knowledge, there is no report on the determination of mestanolone residues in Nile tilapia. As mentioned above, LC-MS/MS is the most accurate approach for measuring androgenic hormones (El-Neklawey et al., 2009) and was the chosen method in the current study in male sex-reversed Nile tilapia.

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The main purposes of this study are to define the proper dosage of mestanolone administration for production of monosex fry and to determine the mestanolone residues in male sex-reversed Nile tilapia. Studies in support of proposed withdrawal times and in support of minimize use of hormonal applications in tilapia might be expected to dilute the residues through growth of the fish to marketable size resulting in no human health hazard is left at the time of consumption.

Table 2.4 Analysis methods used for detection of androgenic hormones in various kinds of sample.

Hormone	Dose of treatment	Sample	Detection method	Level of hormone	LOD	LOQ	Reference
11-β hydroxyandrostenedione	No treatment	serum of goldfish	LC-MS	0.86-6.63 ng/mL	0.2 ng/mL	0.5 ng/mL	Blasco et al. (2009)
11- ketosterone	No treatment	serum of goldfish	LC-MS	11.7-19.38 ng/mL	0.1 ng/mL	0.3 ng/mL	Blasco et al. (2009)
17α-ethynyltestosterone	2 mg/kg feed; 14 day	muscle of tilapia	HPLC	4 hour: <LOD	50 ng/g	ND	Rothbard et al. (1990)
	60 mg/kg feed; 14 day	muscle of tilapia	HPLC	Day 3: <LOD	50 ng/g	ND	
17α-methyltestosterone	spiked hormone	muscle of carp	HPLC	Validation	0.05 mg/kg	ND	Jiang et al. (2005)
17α-methyltestosterone	unknown	fish feed	HPLC	15-120 mg/kg	3 mg/kg	10 mg/kg	Marwah et al. (2005)
17α-methyltestosterone	30 mg/kg feed; 4 day	muscle of tilapia	LC-MS	Day 21: <LOQ	0.04 ng/g	0.09 ng/g	Chu et al. (2006)
		muscle of rainbow trout	LC-MS	Day 21: =0.09 ng/g	0.04 ng/g	0.09 ng/g	
		muscle of salmon	LC-MS	Day 21: I<LOQ	0.04 ng/g	0.09 ng/g	
17α-methyltestosterone	spiked hormone	muscle of bovine	LC-MS/MS	Validation	0.3 ng/g	1.0 ng/g	Kaklamanos et al. (2007)

ND, no data available.

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Table 2.4 (cont) Analysis methods used for detection of androgenic hormones in various kinds of sample.

Hormone	Dose of treatment	Sample	Detection method	Level of hormone	LOD	LOQ	Reference
17α-methyltestosterone	1.0 mg/kg bw; nasogastric tube	urine of horse	GC-MS/MS	5-50 ng/mL	ND	ND	Yamada et al. (2008)
17α-methyltestosterone	1.5 ml of 5 mg/ml injection	hair of bovine	LC-MS/MS	Validation	0.07 ng/g	0.12 ng/g	Regal et al. (2010)
17α-methyltestosterone	spiked hormone	muscle of tilapia	HPLC	Validation	19 µg/L	58.3 µg/L	Barbosa et al. (2013)
methyltestosterone-17-O-glucuronide	60 mg/kg bw; single dose	bile of tilapia	LC-MS	Day 7: =0.5 ng/mg	ND	ND	Amarasinghe et al. (2012)
testosterone	spiked hormone	serum of trout	GC-MS	Validation	0.1-0.4 ng/g	ND	Budzinski et al. (2006)
		bile of trout	GC-MS	Validation	1.6-14 ng/g	ND	
testosterone	No treatment	serum of goldfish	LC-MS	4.28-13.25. ng/mL	0.1 ng/ml	0.2 ng/mL	Blasco et al. (2009)
testosterone	unknown	muscle of tilapia	RIA	4.22 ng/g	ND	ND	El-Neklawey et al. (2009)
		muscle of carp	RIA	ND	ND	ND	

ND, no data available

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CHAPTER III

MATERIALS AND METHODS

Apparatus and equipment

1. High performance liquid chromatographic system:; SHIMADZU prominence®: CBM-20Alite communications bus module, DGU-20A5 Degasser, LC-20AD Liquid Chromatograph, SIL-20AC Auto Sampler, CTO-20A Column Oven, API4000 MS/MS Detector; LC solution® version 1.22 SP1 software (Shimadzu Corporation. Kyoto, Japan)
2. Analytical balance; Mettler Toledo® Model XS Dual range (Mettler - Toledo AG, Switzerland)
3. Ultrasonic cleanser ; Elma® D 78224 Model T490 DH (Elma GmBH & Co. KG, Denmark)
4. Vortex mixer; Vortex-Genie®2 Model G560E (Scientific Industries, Inc., USA)
5. Centrifuge; Jouan® Model BR4i (Jouan S.A., France)
6. SpeedVac Concentrator; Thermo® Model SC250EXP-230, Refrigerated Vapor Trap (RVT4104), VaporNet Controller (VN100DDA), ValuPump (VLP200) (Thermo Electron Corporation, USA)
7. Micropipette 10-100 µL; Eppendorf® Model Research 3111, single channel Serial No. 3554196 (Eppendorf, USA)

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8. Micropipette 100-1000 µL; Eppendorf® Model Research 3111, single channel Serial No. 4759656 (Eppendorf, USA)
9. Micropipette 100-1000 µL; Eppendorf® Model Research 3111, single channel Serial No. 4318198 (Eppendorf, USA)
10. Micropipette 500-5000 µL; Eppendorf® Model Research 3111, single channel Serial No. 4766876 (Eppendorf, USA)
11. Micropipette 1000 µL(Fix); Eppendorf® Model Research 3111, single channel Serial No. 1827594 (Eppendorf, USA)
12. Freezer; Jouan® Model VXS 490 (Jouan S.A., France)
13. Glassware
14. Microcentrifuge tube; Hycon® size 1.5 mL (Hycon plastic Inc., USA)
15. Pipette tip; Sorenson® size 1-200 µL and 100-1000 µL (Bioscience Inc., USA)
16. Column; ZORBAX Eclipse XDB-C8 4.6x100mm, 3.5µm (Agilent Technologies)
17. Water Purification; Milli-Q® gradient Model ZMQS5V001 (Millipore Corporation, France)

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The present study was divided into 2 phases as follows: phase 1, investigation of sex reversal efficacy of mestanolone in farmed Nile tilapia *Oreochromis niloticus*; and phase 2, detection of mestanolone residues in male sex-reversed Nile tilapia. The experimental outline is shown in Figure 3.1.

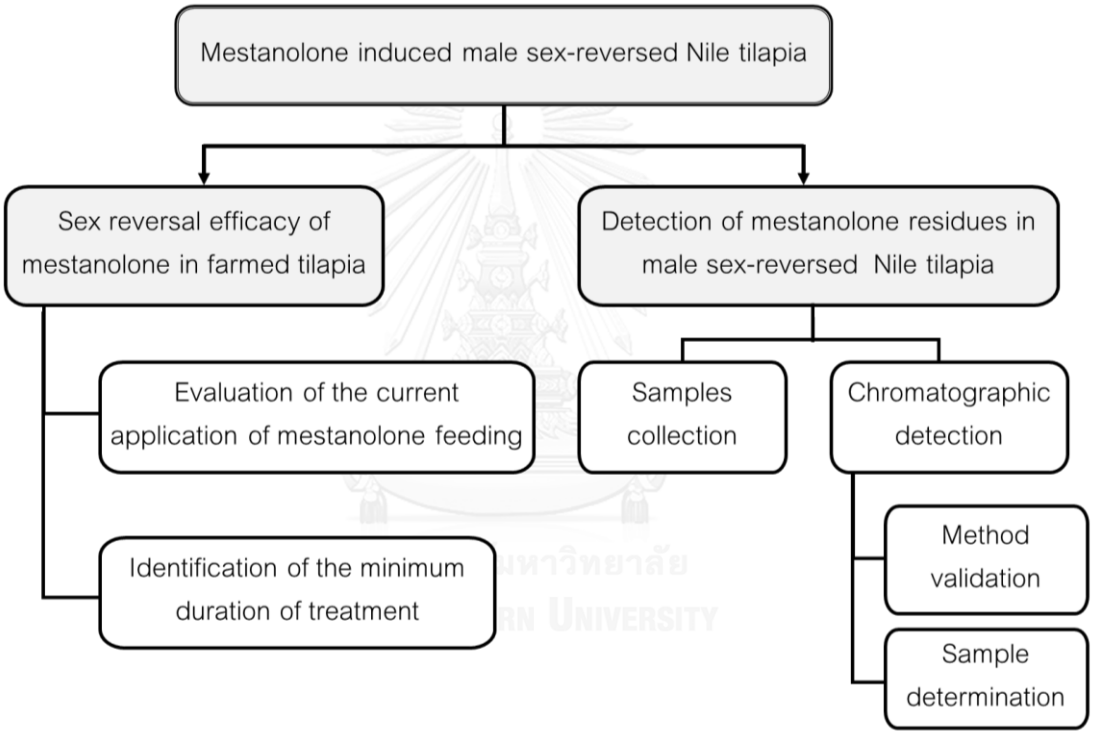

Figure 3.1 Schematic diagram showing the research plan.

3.1 Sex reversal efficacy of mestanolone in farmed tilapia

3.1.1 Hormonal preparation

17α-methyldihydrotestosterone (MDHT, mestanolone) and 17α-ethynylestradiol (EE_2) (purity ≥98%) (Sigma-Aldrich, St Louis, Missouri, USA) were dissolved in 95% ethanol. For immersion treatment, the solution of EE_2 was freshly diluted into the water at a dose of 100 mg/L, and the solution of mestanolone and EE_2 for dietary supplementation were sprayed onto commercial fish feed at a dose of 80 mg/kg and 100 mg/kg, respectively. The moistened hormonal feeds were air dried and kept at 4°C under dark and dry conditions. The preparation of mestanolone feed was shown in Figure 3.2.

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Figure 3.2 Mestanolone feed preparation. (A) mestanolone powder, (B) the tilapia feed, (C) the mixture (D) the alcohol was allowed to evaporate.

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3.1.2 Animals

Nile tilapia (*O. niloticus*) obtained from hatchery unit of commercial tilapia farm in Chachoengsoa province were used for mestanolone treatment (10 dph larvae) and EE_2 treatment (5 dph larvae). Five thousand larvae at the age of 5 dph were housed in incubation trays (8 × 24 × 30 cm^3) at the stocking density of 250 fry/tray and 6,000 larvae at the age of 10 dph were placed in hapas (1 x 1.25 x 0.8 m^3) at the stocking density of 250 fry/net. The water parameters were maintained as; mean temperature of 29±3 ºC, pH 7.0-8.0, dissolved oxygen 7.0-8.0 mg/L, ammonia (NH_3) 0.00-0.50 mg/L, and nitrite 0.00-0.25 µg/L.

3.1.3 Hormonal treatment

Animal management was approved by the ethics committee of Chulalongkorn University Animal Care and Use Committee (CU-ACUC; Approval No. 11310046). The sex differentiation responded to various dosages of mestanolone dietary supplementation was studied as the schematic diagram in Figure 3.3. Four groups were fed with 80 mg/kg mestanolone with different durations (5, 10, 15 and 20 days) and the remaining groups were fed with normal diet without mestanolone to serve as control, with 4 replications in all groups. EE_2 treatment effects were examined initially by long-term immersion in EE_2 and followed by dietary supplementation (Figure 3.4). At 5 dph, groups of fry were randomly divided into 6 treatments: F-K, with 4 replications (groups F and G were immersed in water without EE_2; groups H and I were immersed in 100 mg EE_2/L for 3 days, and groups J and K were immersed in 100 mg EE_2/L for 5 days). After immersion, fry were collected and placed in new hapas that contained fresh water. Oral treatments starting

at 10 dph of fry consisted of a feeding with 100 mg EE_2/kg diet for 35 days in groups G, I and K. Other groups were fed commercial powder feed without EE_2. Throughout the feeding experiment, fry were fed with 10-15% of body weight four times daily. In both experiments, the groups of fish were held in the hapas until the age of 60 dph. A sample of 25 fish was randomly selected from each replication. The body weight and length were measured and sexing was performed.

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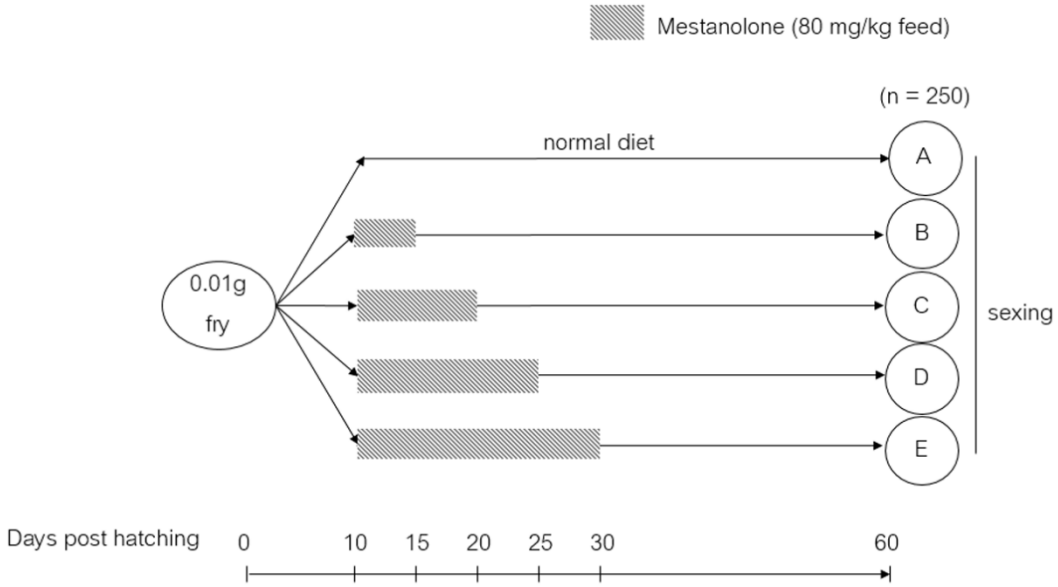

Figure 3.3 Schematic diagram showing mestanolone treatment. Groups A-E (n=250 fry for each group, 4 replicates). A, control group; B to E, treated groups, mestanolone feeding at 80 mg/kg 4 times a day for 5, 10, 15 and 20 days in groups B, C, D and E, respectively. After this treatment, fry were reared with a normal diet until reaching a size that could be sexed (60 dph).

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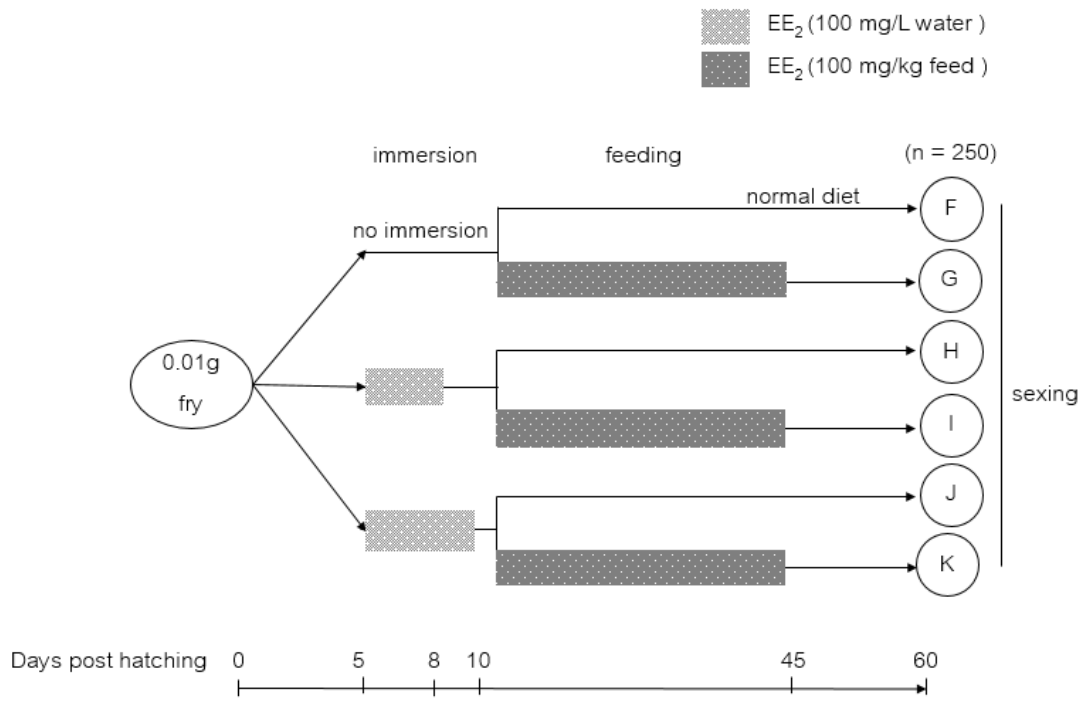

Figure 3.4 Schematic diagram showing EE_2 treatment. Groups F-K (n=250 fry in each group, 4 replicates). F, control group; H-K, immersed in EE_2 (100 mg/L water) for 3 days (H and I) or 5 days (J and K) then G, I and K fed EE_2 (100 mg/kg feed) for 35 days. After this treatment, fry were reared with a normal diet until reaching a size that could be sexed (60 dph).

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3.1.4 Sexing

Sex percentages of each treatment (male and female) were determined by examination of gonad squash mount technique (Wassermann and Afonso, 2002). Gonads of fry (25 fish per replication) were removed from the upper part in peritoneal cavity, mounted on a clean microscope slide, stained with few drops of aceto-carmine, squashed with a cover slip and examined with a light microscope (×40 and ×100 magnification) as shown in Figure 3.5. The gonads were characterized based on morphological and structural traits that were differential between males and females. For histological investigation, gonads (10 fish per replication) were collected at 60, 80 days and 10 months post-hatching. Gonads were fixed in 10% buffered formalin for overnight and then transferred to graded alcohol series until dehydration was completed. The fixation process was followed by dehydration, paraffinization and sectioning. After that, the gonad sections were stained with haematoxylin and eosin (H&E) and then examined under a light microscope (×10, ×20 and ×100 magnification).

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3.1.5 Data analysis

The effects of mestanolone and EE_2 treatment on sex ratio, body weight and total length of fry were reported as mean ± standard deviation (SD). In order to assess the stoutness of the fish, condition factor was calculated according to the formula as suggested by Mortuza and Al-Misned (2013).

$$\text{Condition factor (K)} = \frac{\text{W (g)}}{[\text{L (cm)}]^3} \times 100$$

where W is the weight and L is the total length of the fish.

Sex ratio was evaluated by chi-square test (x^2), whereas the body weight, total length and condition factor were evaluated by analysis of variance (ANOVA) and mean differences were found using Tukey post-hoc test. All the statistical analyses were carried out using SPSS statistical package (version 22; SPSS Inc., Chicago, Illinois, USA). Differences were considered statistically significant at $p < 0.05$.

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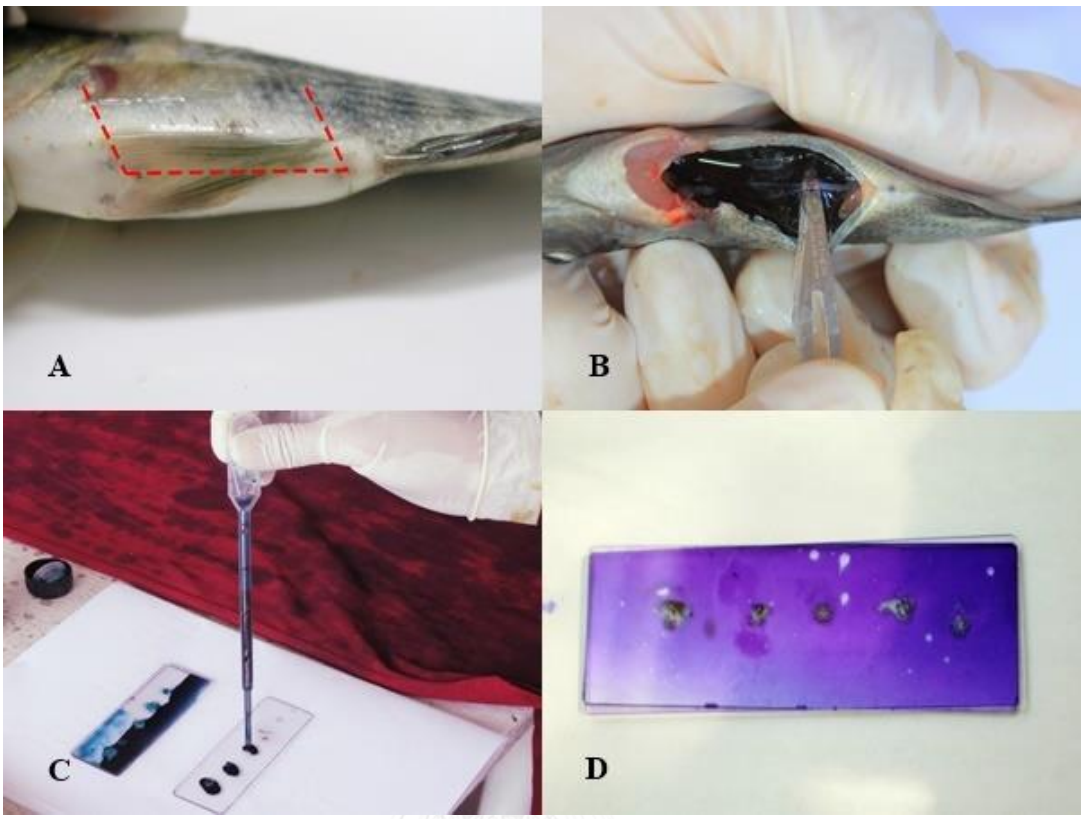

Figure 3.5 Fresh tissue squash. (A) the fish was euthanized and cut at the abdominal area, (B) the lateral wall of the fish was removed using a blade and the gonads were dissected and placed on a clear glass slide, (C) a few drops of aceto-carmine stain was topically applied to the gonads and (D) another clean glass slide placed over and pressed then they were examined by light microscope.

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3.2 Detection of mestanolone residues in male sex-reversed Nile tilapia

3.2.1 Chemicals and reagents

Acetonitrile (LEDA, Spain) and methanol (Scharlau, Spain) were HPLC grade. Formic acid (Carlo Erba, Germany), *tert*-butylmethylether (TBME) (Merck, Germany), ammonium formate (Carlo Erba, Germany) were reagent grade. Standard mestanolone (98.32% dry weight) and standard finasteride (99.50% dry weight) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used in preparing solutions was LC grade and purified with a Milli-Q water system (Millipore Corp., France). The mobile phase was prepared by dissolving 0.3153 g of ammonium formate in 1,000 mL of deionized water and adjusting to pH 3.5 with formic acid. Various mixtures of 5 mM ammonium formate and acetonitrile were used in the chromatographic system.

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3.2.2 Standard preparation

3.2.2.1 Mestanolone stock solution preparation

Mestanolone stock solution 1

Stock solution of mestanolone was prepared by accurately weighing 50 mg of mestanolone working standard, transferring to a 100-mL volumetric flask, dissolving and adjusting to volume with acetonitrile to obtain a nominal concentration of 0.5 mg/mL.

Mestanolone stock solution 2

Stock solution 2 was prepared by pipetting 0.5 mL of mestanolone stock solution 1 into a 100-mL volumetric flask, diluting to obtain a nominal concentration of 2,500 ng/mL with acetonitrile.

Mestanolone stock solution for calibration curve and accuracy and precision

Mestanolone stock solutions for calibration curve (10, 20, 40, 60, 100, 150 and 200 ng/mL) and accuracy and precision samples (20, 100, and 150 ng/mL) were prepared by diluting exactly measure volume of the standard mestanolone stock solution 2 (2500 ng/mL) to 10 mL in volumetric flask using acetonitrile as shown in Tables 3.1 and 3.2.

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Table 3.1 Mestanolone stock solutions for calibration curve preparation.

Mestanolone stock solution (ng/mL)	Spike volume (µL)	Diluent (µL)	Final volume (mL)	Final concentration (ng/mL)
2500	0.8		10	200
2500	0.6	Dilute to final	10	150
2500	0.4	volume with	10	100
200	3.0	acetonitrile	10	60
200	2.0		10	40
200	1.0		10	20
200	0.5		10	10

Table 3.2 Mestanolone stock solutions for accuracy and precision preparation.

Mestanolone stock solution (ng/mL)	Spike volume (µL)	Diluent (µL)	Final volume (mL)	Final concentration (ng/mL)
2500	0.6	Dilute to final	10	150
2500	0.4	volume with	10	100
200	1.0	acetonitrile	10	20

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3.2.2.2 Finasteride stock solution preparation (internal standard)

Finasteride stock solution 1

A stock solution of finasteride was prepared by accurately weighing 50 mg of finasteride working standard, transferring to a 100-mL volumetric flask, dissolving and adjusting to volume with acetonitrile to obtain a nominal concentration of 0.5 mg/mL.

Finasteride stock solution 2

A stock solution 2 was prepared by pipetting 0.1 mL of finasteride stock solution 1 into a 100-mL volumetric flask, and diluting to obtain a nominal concentration of 500 ng/mL with acetonitrile.

3.2.2.3 Mobile phase preparation

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Ammonium formate solution (5 mM) was prepared by weighing 0.3153 g of ammonium formate, transferring into a 1000-mL beaker, dissolving with deionized water, and adjusting to pH 3.5 with formic acid. Variable mixtures of 5 mM ammonium formate and acetronitrile were prepared as directed in the chromatographic system.

3.2.3 Method validation

3.2.3.1 System suitability test

Mestanolone stock solution (100 ng/mL) and finasteride stock solution 2 (500 ng/mL) were used for system suitability test. Fifty microliters of each solution were added into homogenized fish tissue (1 g) which was blended with dry ice and mixed in a 5-mL centrifuge tube, *tert*-butyl methyl ether (TBME) was added to achieve the concentrations of 5 ng/g and 25 ng/g, respectively. The extraction procedure was described in the section 3.2.6 and then it was analyzed using a LC-MS/MS system. Analysis was performed 5 times and the data of chromatogram; retention time, peak area, resolution and tailing factor, were recorded. The %CV of each parameters was calculated.

3.2.3.2 Linearity

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The calibration curve was prepared by spiking 50 µL of each mestanolone stock solution (10, 20, 40, 60, 100, 150 and 200 ng/mL) into homogenized fish tissue (1 g) which blended with dry ice in a 5-mL centrifuge tube. Fifty microliters of finasteride stock solution 2 and 950 µL of TBME were added to obtain sample fish spiked at 7 concentrations; 0.5, 1.0, 2.0, 3.0, 5.0, 7.5 and 10.0 ng/g. The extraction procedure was described in the section 3.2.6 and then it was analyzed using a LC-MS/MS system. Each concentration was analyzed for 3 times and the data were recorded. The data of concentrations and the peak area ratio of mestanolone and finasteride were analyzed together using the linear equation;

$$y = ax+b$$

where x is the concentration ratio, y is the peak area ratio, a is the slope and b is the y-intercept.

3.2.3.3 Accuracy and precision

The standard mestanolone solutions at the concentrations of 20, 100 and 150 ng/mL were used as the quality control samples (QC samples). In each concentration, five samples were arranged to evaluate intra-day accuracy and precision and three samples were arranged to evaluate inter-day accuracy and precision while the internal standard was the finasteride solution 2 at a concentration of 25 ng/mL. Fifty microliters of each mestanolone and finasteride stock solution were added into 1 g homogenized fish tissue which was blended with dry ice and mixed in a 5-mL centrifuge tube. TBME (950 µL) was added to each tube. Each tube was extracted as described in the section 3.2.6 and then it was analyzed using a LC-MS/MS system.

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The accuracy of the detection method was identified by %recovery of mestanolone while the precision of the detection method was identified by %CV of the quality control sample. They were calculated using the following equation:

$$\%\text{Recovery} = \frac{\text{observed concentration}}{\text{actual concentration}} \times 100$$

$$\%\text{Recovery} = \frac{\text{Peak area of extracted concentration}}{\text{Peak area of standard concentration}} \times 100$$

$$\%CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

3.2.3.4 Limit of detection (LOD) and limit of quantitation (LOQ)

The Guidance for Industry Q2B of US FDA (1996) was used for determination of the limit of detection (LOD) and the limit of quantitation (LOQ) for mestanolone. Each tilapia sample (1 g) was spiked with standard finasteride (25 ng/g) and standard mestanolone at three concentrations (1.0, 5.0 and 7.5 ng/g). While blank sample was not spiked with mestanolone. A total of 8 regression lines was obtained from least-square regression analyses of the residual peak areas versus the three concentrations of mestanolone (1.0, 5.0 and 7.5 ng/g). The peak area ratio was each peak area of mestanolone-fortified samples divided by the peak area of finasteride. The LOD and LOQ of the method was calculated using the equation;

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$$LOD = \frac{3\sigma}{S}$$

$$LOQ = \frac{10\sigma}{S}$$

where σ is the standard deviation of y-intercepts and s is the average slope of the 8 linear regression analyses.

3.2.3.5 Specificity

The chromatographic interferences from the fish tissue were investigated by comparing the chromatogram of blank sample with finasteride and the mestanolone spiked sample. The blank sample was made by adding 50 µL of acetonitrile and 50 µL of finasteride stock solution 2 into 1 g of homogenized fish tissue, while the mestanolone spiked sample was made by adding 50 µL of 100 ng/mL mestanolone stock solution and 50 µL of finasteride stock solution 2 into 1 g of homogenized fish tissue. The fish tissue was blended with dry ice, mixed in a 5-mL centrifuge tube and then added with TBME for extraction. The extraction procedure was described in the section 3.2.6 and then it was analyzed using a LC-MS/MS system.

3.2.4 Fish dosing

Mestanolone was dissolved in 95% ethyl alcohol to prepare a solution for moistening commercial fish feed. The hormonal feeds were air dried and kept at 4°C under dark and dry conditions. Nile tilapia fry from a Good Aquaculture Practice (GAP) farm in Chachoengsao province, Thailand, were used in the study. The fry were reared in concrete tanks with net pens, where they all received their first feeding. Mestanolone was given to the fry at 80 mg/kg feed, 4 times a day for 15 or 23 consecutive days. The conditions were temperature of 23-30°C, pH of 7.0-8.0, ammonia (NH_3) (0.00-0.50 ppm), and nitrite (0.00-0.25 ppm). All parameters were monitored daily.

3.2.5 Sample collection

Samples were taken at the nursery stage at 1, 2, 3, 5, 7, 14 and 21 days after hormone withdrawal. All fish were euthanized with an overdose of anesthetic agent Aquanes®(Better Pharma, Thailand) and ice knocking, and were then stored at -80°C until analysis.

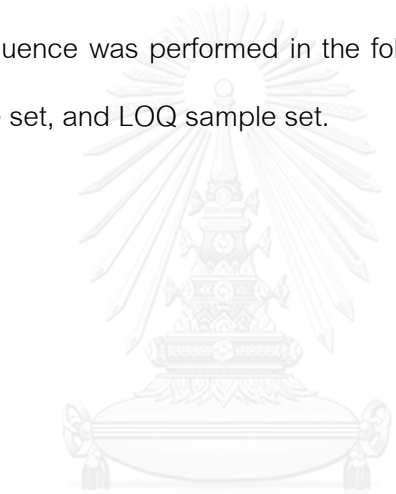
3.2.6 Extraction procedure

Sample fish tissue was ground to a fine powder with dry ice using a blender and about 1 g homogenized tissue was transferred to a 5-mL centrifuge tube. Finasteride (50 µL at 500 ng/mL) as an internal standard and 950 µl of TBME were added to each sample. The mixture was vortex mixed for 30 sec and centrifuged at 12,000 rpm for 10 min at 10°C. Only clear supernatant was transferred to a microcentrifuge tube and evaporated to dryness in a Speed vacuum concentrator at 50°C, 1.0 torr for 60 min. The residue was reconstituted with 500 µL of the acetonitrile, vortexed for 30 min, sonicated for 5 min, and centrifuged at 14,000 rpm at 10°C for 10 min. The clear supernatant was transferred to an autosampler vial and 10 µL was injected into the LC-MS/MS system.

3.2.7 LC-MS/MS analysis

The Shimadzu Prominence® HPLC system consisting of a binary gradient pump, a degasser, an autosampler, an API4000 MS/MS detector, and LC solution® v. 1.22 SP1 software (Shimadzu Corp., Kyoto, Japan) was used in the study. The ammonium formate-

acetonitrile gradient (5 mM ammonium formate pH 3.5: acetonitrile) was used as the mobile phase, with a linear gradient of 10-80% acetonitrile in 2 min, hold at 80% for 4 min, linear gradient of 80-100% acetonitrile in 0.5 min, hold at 100% for 1.5 min, linear gradient of 100-10% acetonitrile in 1 min, followed by equilibration at 10% for 1 min before the next injection. The flow rate was 0.8 mL/min. A C_8 column (100x4.6 mm, 5 µm; Agilent Technologies) was used and the injection volume was 10 µL. The column oven was held at 30ºC and the autosampler temperature was maintained at 20ºC. Detection was performed by MS at 305.3/269.3 m/z for mestanolone and 373.5/355.4 m/z for finasteride. A typical injection sequence was performed in the following order: blank of calibration, calibration set, sample set, and LOQ sample set.



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CHAPTER IV

RESULTS

4.1 Sex reversal efficacy of mestanolone in farmed tilapia

Using the fresh smear technique, male gonadal tissue showed a tubular conformation; female gonadal tissue showed a vesicle containing abundant round objects (Figure 4.1). Feeding of 10 dph tilapia fry with mestanolone at 80 mg/kg resulted in each mestanolone treated group gave a mean male/female ratio that deviate significantly from the normal 1:1 ratio, with the males significantly higher than females, while the control group (group A) showed normal 1:1 ratio. The percent of phenotypic males increased as the level of hormone increased from 5-day treatment (87%) to 15 and 20-day treatments (100%), and the percentage of females decreased obviously from 5-day treatment (13%) to 10-day treatment (10%) and 15, 20-day treatment (0%) (Table 4.1). In the female sex induction, the EE_2 feeding group (group G) showed no significant difference in female percentage (49%) compared to the control group (group F) (48%). Three and five days of EE_2 immersion only (groups H and J) gave the same result of 70 and 68% female, respectively. The combination of immersion and feeding yielded a significantly higher female percentage than either single method which presented 82 to 94% female populations (groups I and K) (Table 4.1). Furthermore, statistical analysis on gain in body weight, total length and condition factor showed no significantly difference among different sex reversal treatments ($P > 0.05$).

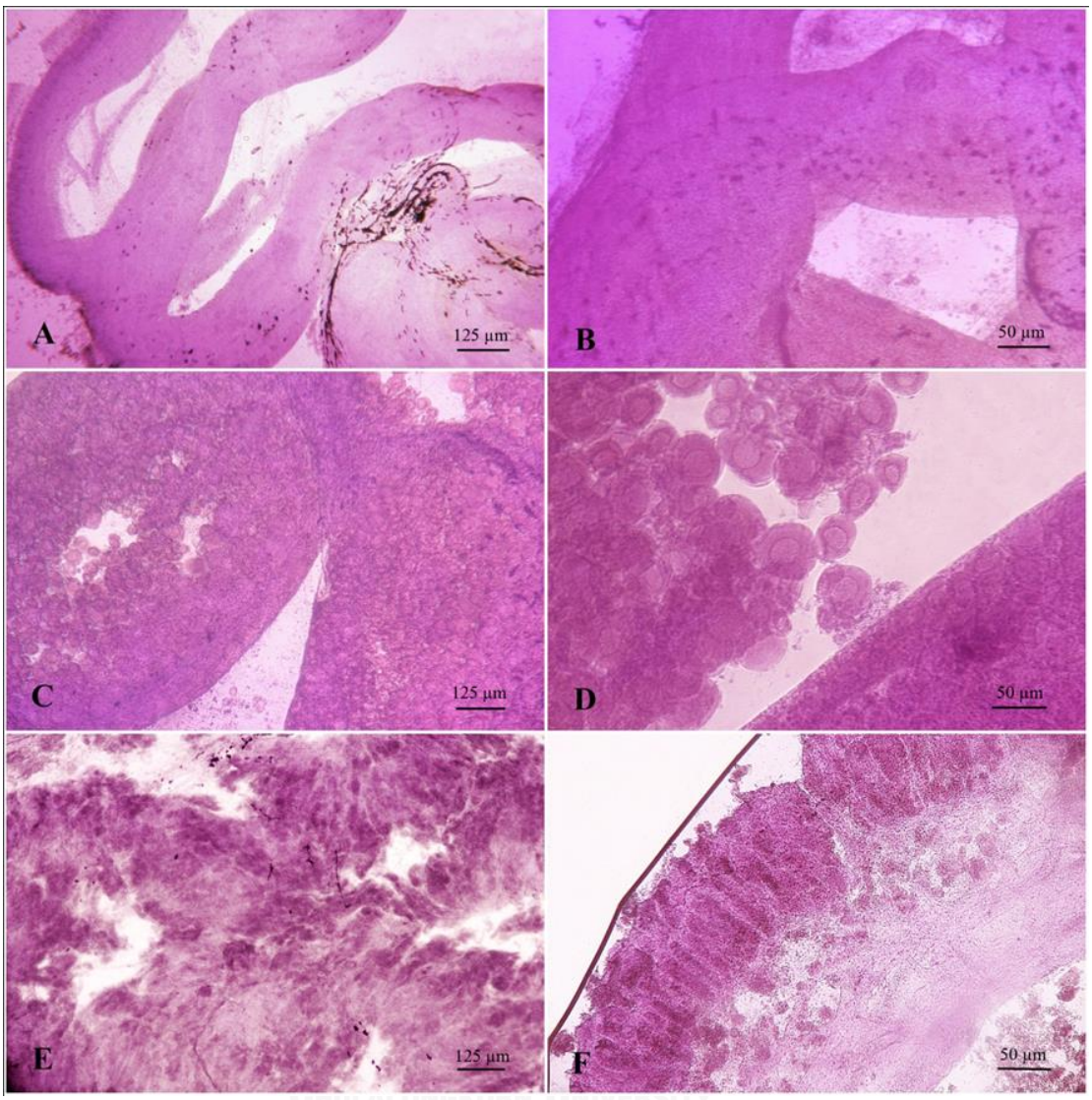

Figure 4.1 Gonadal tissue of 60-day old Nile tilapia stained with aceto-carmine. Male gonadal tissue showing a long smooth tube shape of the testis ×40 (A) and ×100 (B). Female gonadal tissue showing an ovary containing abundant round oocytes ×40 (C) and ×100 (D). Undifferentiated gonadal tissue showing a long non-smooth tube-shaped organ containing no oocytes in the tube ×40 (E) and ×100 (F).

Table 4.1 Effects of hormones on growth and sex ratio of Nile tilapia, *Oreochromis niloticus* (mean±SD).

Treatment	A	B	C	D	E
Route					
Immersion (day)	-	-	-	-	-
Feeding (day)	-	MDHT (5)	MDHT (10)	MDHT (15)	MDHT (20)
Parameter[*]					
Male (%)	53[a]	87[b]	90[bc]	100[c]	100[c]
Female (%)	47[a]	13[b]	10[b]	-	-
Intersex (%)	-	-	-	-	-
Weight (g)	12.82±0.66[a]	12.79±0.76[a]	12.85±0.70[a]	12.78±0.73[a]	12.75±0.84[a]
Total length (cm)	8.51±0.45[a]	8.49±0.39[a]	8.51±0.33[a]	8.45±0.46[a]	8.44±0.44[a]
Condition factor	2.12±0.35[a]	2.11±0.29[a]	2.10±0.26[a]	2.15±0.33[a]	2.15±0.32[a]

MDHT, mestanolone, 17α-methyldihydrotestosterone; fed with MDHT at 80 mg/kg feed.

EE_2, 17α-ethynylestradiol; immersed in EE_2 at 100 mg/L water, fed with EE_2 at 100 mg/kg feed.

A, control group; B, fed with MDHT for 5 days; C, fed with MDHT for 10 days; D, fed with MDHT for 15 days; E, fed with MDHT for 20 days; F, control group; G, fed with EE_2 for 35 days; H, immersed in EE_2 for 3 days; I, immersed in EE_2 for 3 days and fed with EE_2 for 35 days; J, immersed in EE_2 for 5 days; K, immersed in EE_2 for 5 days and fed with EE_2 for 35 days (n=1000).

*means in the row sharing the same letter are not significantly different at the $p > 0.05$ level.

Table 4.1 (cont) Effects of hormones on growth and sex ratio of Nile tilapia, *Oreochromis niloticus* (mean±SD).

Treatment	F	G	H	I	J	K
Route						
Immersion (day)	-	-	EE_2 (3)	EE_2 (3)	EE_2 (5)	EE_2 (5)
Feeding (day)	-	EE_2 (35)	-	EE_2 (35)	-	EE_2 (35)
Parameter[*]						
Male (%)	49[a]	39[d]	27[e]	13[f]	24[e]	6[g]
Female (%)	48[a]	49[a]	70[c]	82[d]	68[c]	94[e]
Intersex (%)	3[a]	12[b]	3[a]	5[ac]	8[bc]	-
Weight (g)	12.79±0.63[a]	12.81±0.64[a]	12.89±0.67[a]	12.90±0.83[a]	12.86±0.73[a]	12.88±0.76[a]
Total length (cm)	8.43±0.46[a]	8.53±0.51[a]	8.53±0.57[a]	8.43±0.49[a]	8.47±0.44[a]	8.49±0.46[a]
Condition factor	2.18±0.37[a]	2.11±0.36[a]	2.12±0.39[a]	2.20±0.42[a]	2.15±0.37[a]	2.14±0.36[a]

MDHT, mestanolone, 17α-methyldihydrotestosterone; fed with MDHT at 80 mg/kg feed.

EE_2, 17α-ethynylestradiol; immersed in EE_2 at 100 mg/L water, fed with EE_2 at 100 mg/kg feed.

A, control group; B, fed with MDHT for 5 days; C, fed with MDHT for 10 days; D, fed with MDHT for 15 days; E, fed with MDHT for 20 days; F, control group; G, fed with EE_2 for 35 days; H, immersed in EE_2 for 3 days; I, immersed in EE_2 for 3 days and fed with EE_2 for 35 days; J, immersed in EE_2 for 5 days; K, immersed in EE_2 for 5 days and fed with EE_2 for 35 days (n=1000).

*means in the row sharing the same letter are not significantly different at the $p > 0.05$ level.

Histological examinations of gonads from treated Nile tilapia with exogenous hormone at 60, 80 dph and 10 months were revealed in Figures 4.2 and 4.3. In male gonadal tissue, the seminiferous tubules was observed in male gonad containing spermatogonia and primary spermatocyte with meiotic division at 60 dph. Spermatogonia increased rapidly in number, and spermatocytes developed, suggesting active spermatogenesis took place in tilapia at the age of 80 dph. Mature testis showed testicular lobes with seminiferous tubules filling with germinal cells at different stages of development and surrounded by sertoli cells. The spermatogenic cells were spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa (Figure 4.2). Transverse and longitudinal sections of female gonadal tissue showed ovarian tissue containing round oocytes in various stages of oogenesis. Oogonia, and oocytes of perinucleolar stage were found during 60 dph. The perinucleolar stage was oocyte with several nucleoli at the periphery of the nucleus. The layer of simple squamous cells surrounding each oocyte were follicle cells. The cortical alveolar stage of oocytes containing germinal vesicle could be observed at 80 dph. At 10 months, oocytes of different developmental stages were found in mature ovary (Figure 4.3).

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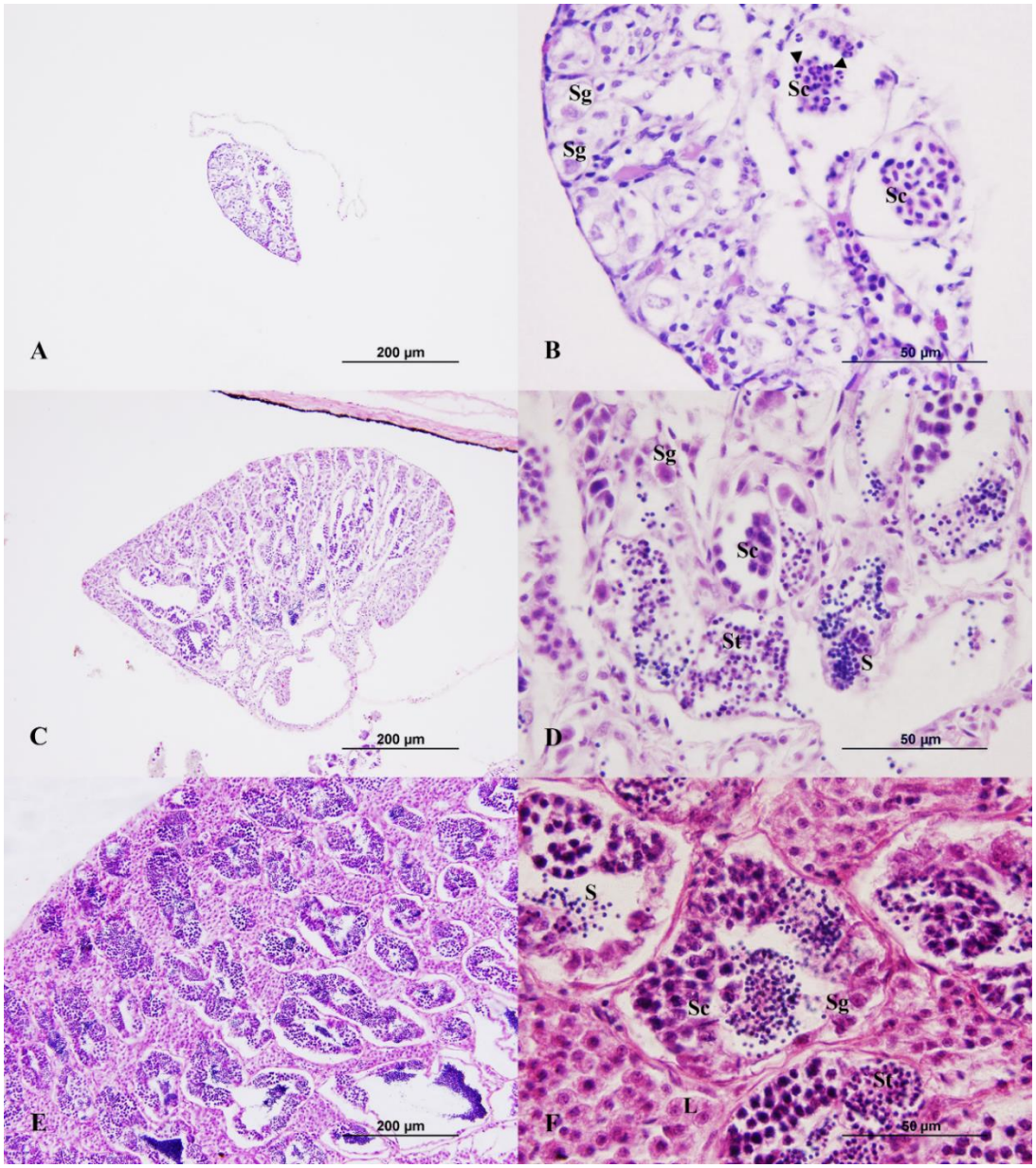

Figure 4.2 Histology of the testes development of Nile tilapia with H&E staining. Transverse section of male gonadal tissue showing a bean shaped testis and seminiferous tubules. Initiation of meiosis or spermatocytes (arrow head) was found at 60 days post-hatching (dph) ×20 (A) and ×100 (B). Active spermatogenesis in the testis at 80 dph. Many cysts containing spermatogenic germ cells at various stages were distributed throughout the testis ×20 (C) and ×100 (D). Mature testis at 10 month was shown ×20 (E) and ×100 (F). Sg, Spermatogonia; Sc, Spermatocytes, St, Spermatids; S, Sperm; L, Laydig cell.

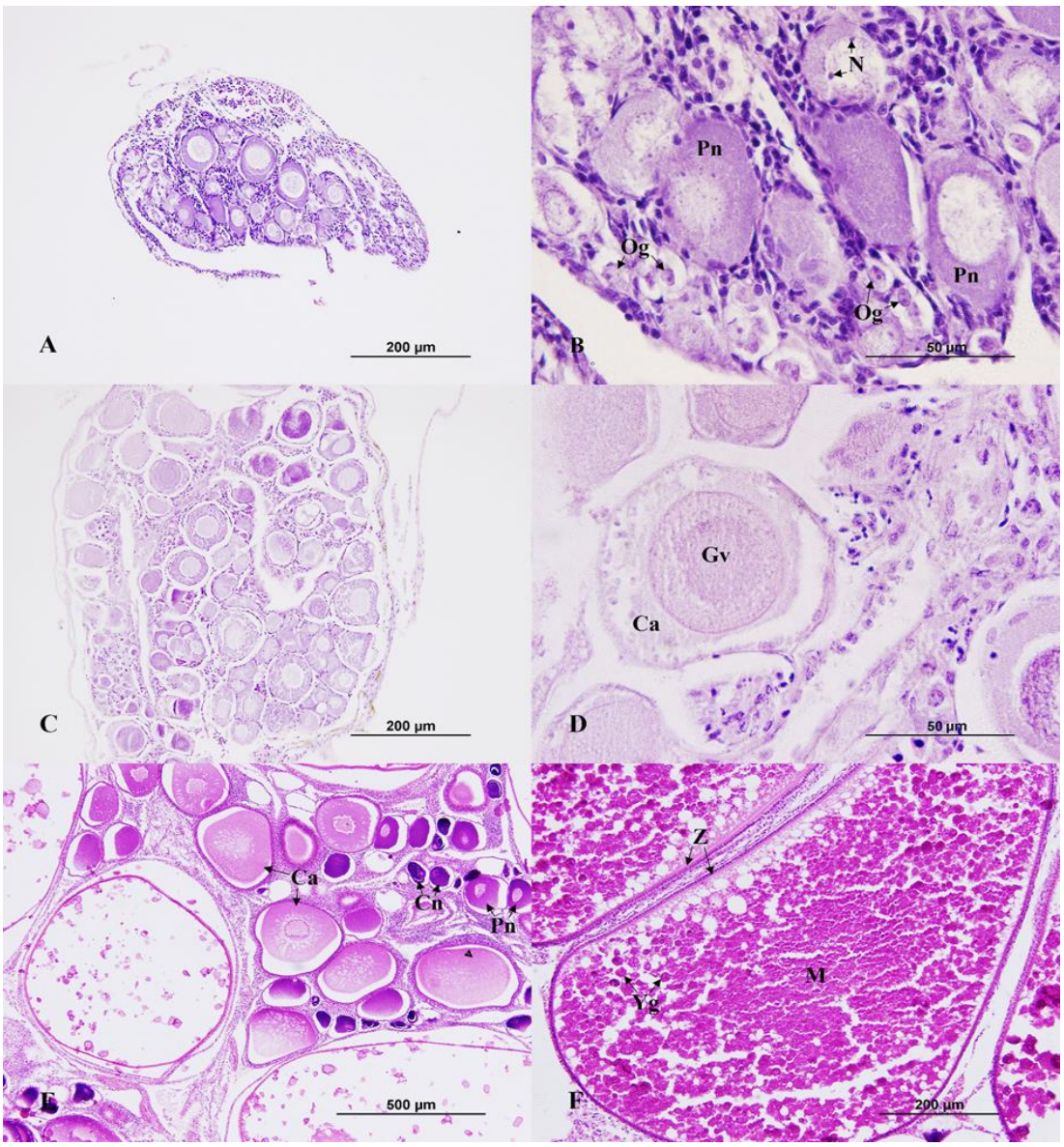

Figure 4.3 Histology of the ovaries development of Nile tilapia with H&E staining. Transverse section of 60 dph female gonadal tissue showing oogonia and perinucleolar stage of oocytes with several small nucleoli attached to nuclear membrane ×20 (A) and ×100 (B). The cortical alveolar stage of oocytes containing of germinal vesicle could be observed at 80 dph ×20 (C) and, ×100 (D). At 10 month, abundant oocytes in various stages embedded in ovarian interstitial tissue. Mature follicles contained cytoplasm which is full of large yolk globules and surrounded by trilayer consists of acidophilic zona pellucida, cuboidal follicular cell layer and stratified squamous thecal cell layer ×10 (E) and ×20 (F). Og, oogonia; Pn, perinucleolar stage of oocyte; N, nucleoli; Ca, cortical alveolar stage of oocyte; Cn, chromatin nucleus stage; Gv, germinal vesicle; M, mature follicle; Yg, yolk globule; Z, zona pellucida.

4.2 Detection of mestanolone residues in male sex-reversed Nile tilapia

4.2.1 Method validation

4.2.1.1 System suitability test

LC-MS/MS chromatograms of mestanolone residue in tilapia are shown in Figure 4.4. The average values of the retention time of mestanolone and finasteride were 5.67 and 5.04 min, consecutively. The %CV were 0.097 and 0.177, consecutively. The average of peak resolution of mestanolone and finasteride was 2.32 with %CV of 0.472. The tailing factor values of mestanolone and finasteride were 1.132 and 1.130, respectively, with %CV of 0.739 and 0.885, respectively. Detailed data are given in Table 4.2.

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Table 4.2 Method validation of mestanolone residues in Nile tilapia: system suitability test (n=5).

	Parameter (%CV)		
	Retention time (min)	Resolution	Tailing factor
Mestanolone	5.67 (0.097)	2.32 (0.472)	1.132 (0.739)
Finasteride	5.04 (0.177)	-	1.130 (0.885)

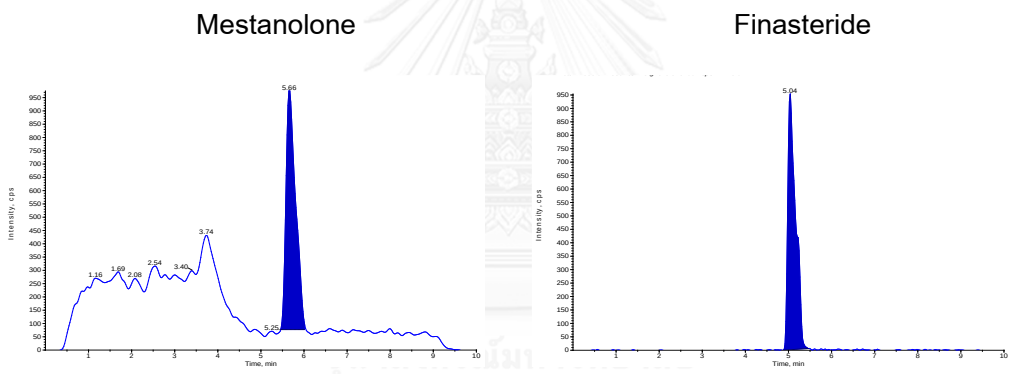

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Figure 4.4 LC-MS/MS chromatogram of 5 ng/g standard mestanolone (left) and 25 ng/g standard finasteride (right) for system suitability test.

4.2.1.2 Linearity

The linearity and range of the analytical assay were performed by analyzing the amount of mestanolone residues from fish spiked with mestanolone at 7 concentrations; 0.5, 1.0, 2.0, 3.0, 5.0, 7.5 and 10.0 ng/g. Each concentration was analyzed 3 times. The data of the concentrations and the peak area ratio between mestanolone and finasteride were in the linearity ($y = 4.3499x + 0.0069$) and $R^2 = 0.9997$ (Table 4.3, Figure 4.5 and Figure 4.6)

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Table 4.3 Method validation of mestanolone residues in Nile tilapia: linearity and ranges.

concentration range (ng/g)	parameters		
	a	b	R^2
0.0-10.0	4.3499	0.0069	0.9997

The linear relationship between spiked concentrations and analyzed values was found between 0.0-10.0 ng/g.

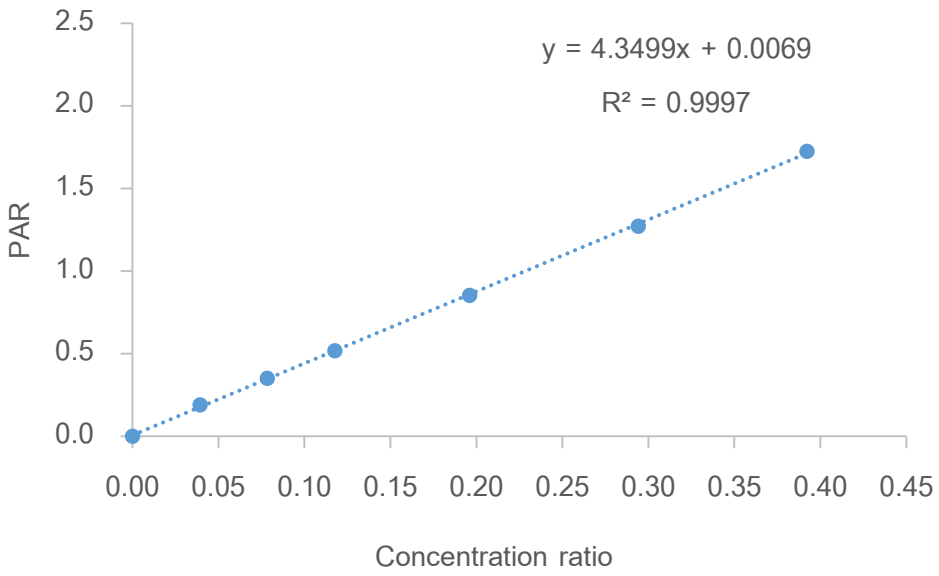

Figure 4.5 Linear regression analysis of the relation of concentration ratio and peak area ratio (PAR).

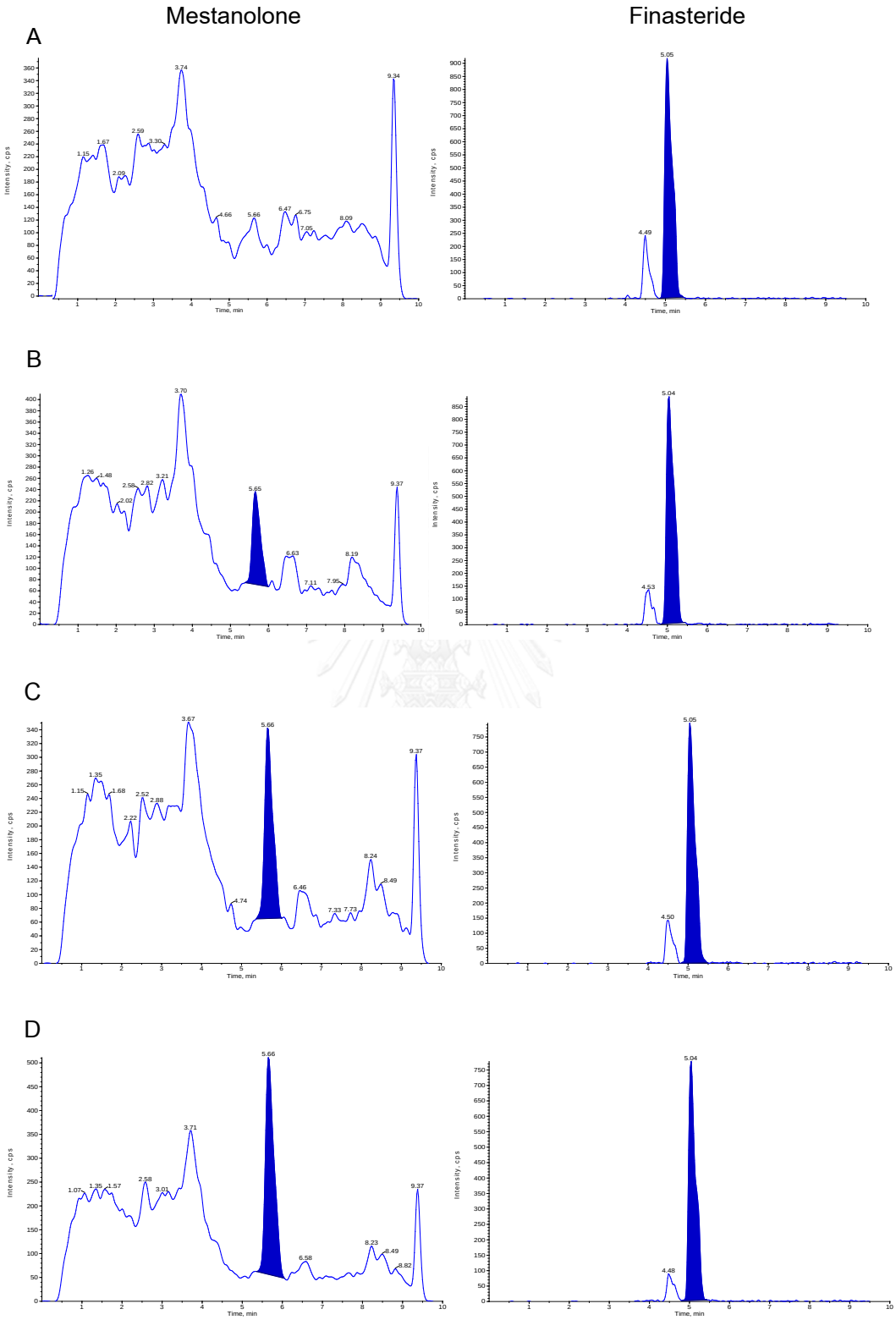

Figure 4.6 LC-MS/MS chromatogram of the standard mestanolone at a concentration of 0.0 ng/g (A), 1 ng/g (B), 2 ng/g (C), 3 ng/g (D), 5 ng/g (E), 7.5 ng/g (F) and 10 ng/g (G) (left) and the standard finasteride at a concentration of 25 ng/g (right) for linearity.

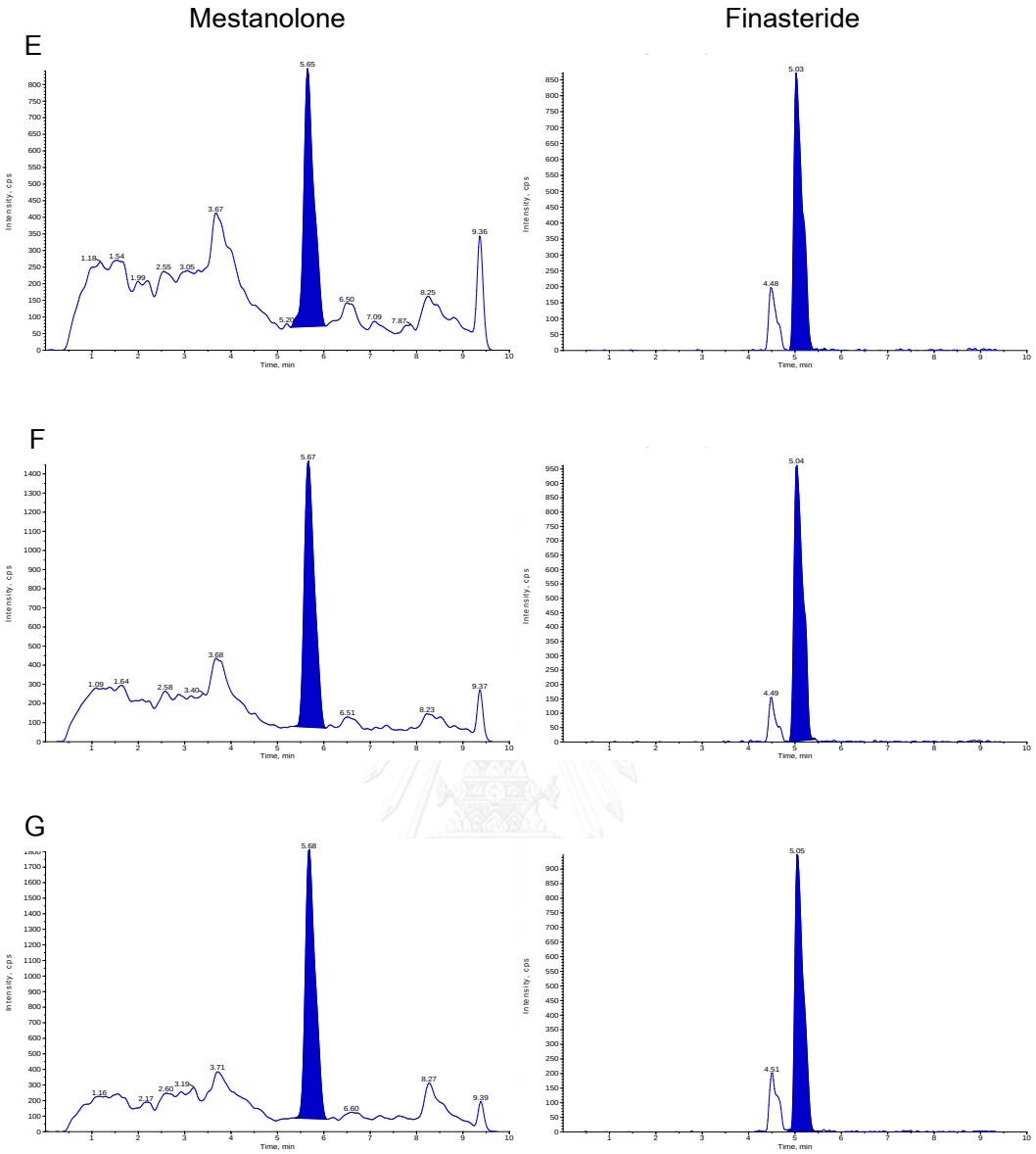

Figure 4.6 (cont) LC-MS/MS chromatogram of the standard mestanolone at a concentration of 0.0 ng/g (A), 1 ng/g (B), 2 ng/g (C), 3 ng/g (D), 5 ng/g (E), 7.5 ng/g (F) and 10 ng/g (G) (left) and the standard finasteride at a concentration of 25 ng/g (right) for linearity.

4.2.1.3 Accuracy and precision

The accuracy of the method, expressed as %recovery, ranged from 84.75 to 107.80, with an average value of 98.06. The precision of the method, expressed as %CV, ranged from 2.15 to 12.96, with an average value of 7.17. Table 4.4 summarizes the accuracy and precision of determination of mestanolone for tilapia sex reversal at three concentrations, with intra-day and inter-day analyses.

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Table 4.4 Method validation of mestanolone residues in Nile tilapia: accuracy and precision.

	amount added (ng/g)	amount found (ng/g)	%CV	%recovery
repeatability (intra-day) (n = 5)				
	1.007	1.04 ± 0.11	10.63	102.99
	5.035	4.88 ± 0.19	3.83	96.89
	7.522	7.65 ± 0.59	7.66	101.24
intermediate precision (inter-day) (3 days, n = 3)				
first day	0.983	1.06 ± 0.04	4.11	107.80
	4.915	4.17 ± 0.18	4.26	84.75
	7.373	7.43 ± 0.93	12.50	100.71
second day	1.007	0.99 ± 0.13	12.96	98.76
	5.035	4.93 ± 0.22	4.52	97.96
	7.522	7.56 ± 0.52	6.85	100.05
third day	1.007	0.95 ± 0.11	11.73	93.92
	5.035	4.79 ± 0.10	2.15	95.05
	7.552	7.29 ± 0.35	4.84	96.56

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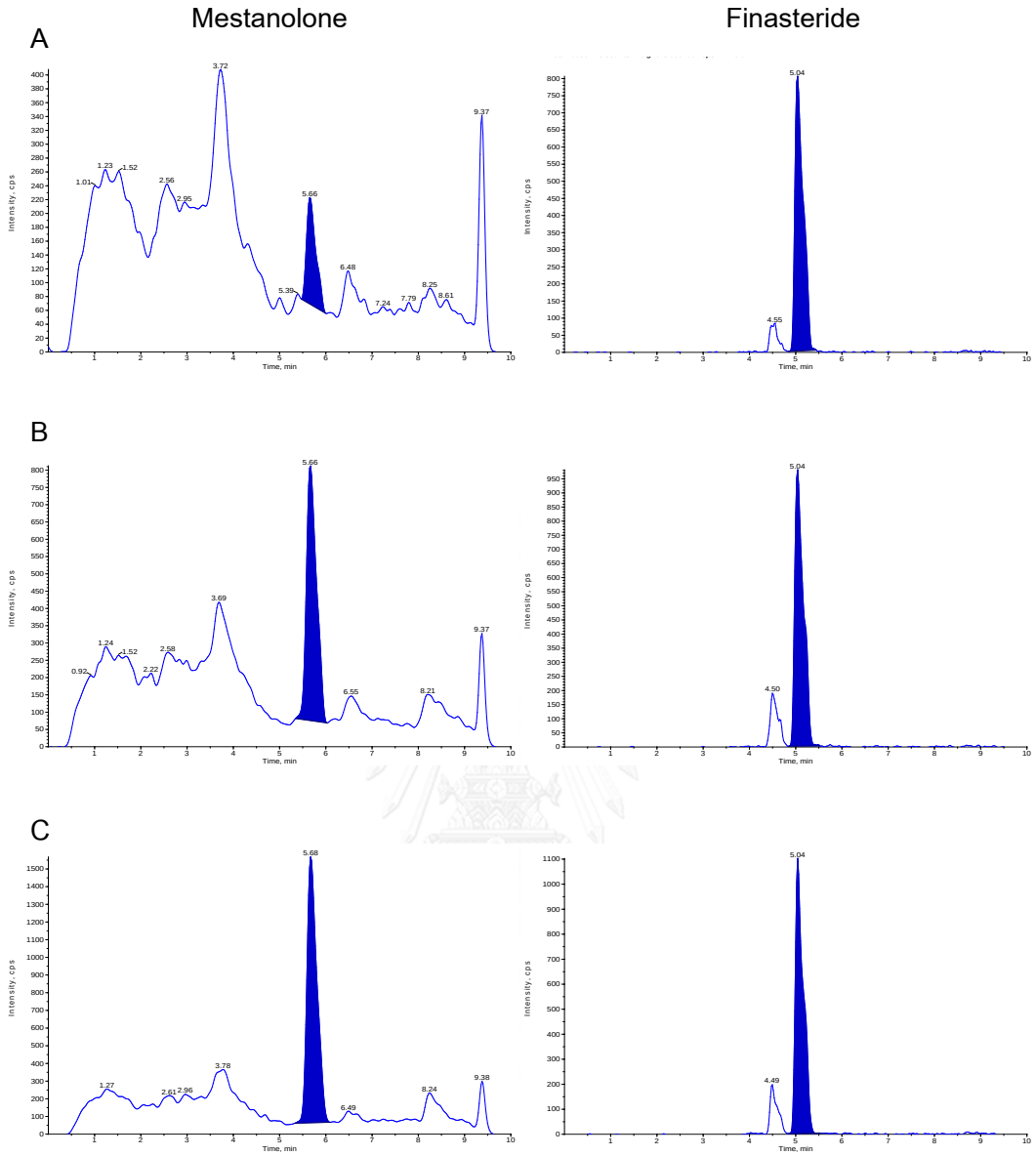

Figure 4.7 LC-MS/MS chromatogram of the standard mestanolone at a concentration of 1 ng/g (A), 5 ng/g (B), 7.5 ng/g (C) (left) and finasteride at a concentration of 25 ng/g (right) for accuracy and precision.

4.2.1.4 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection (LOD) can be calculated as following

$$LOD = \frac{3\sigma}{S}$$

$$= \frac{3(0.0535)}{5.9083}$$

$$= 0.027$$

$$= 0.03 \text{ ng/g}$$

$$LOQ = \frac{10\sigma}{S}$$

$$= \frac{10(0.0535)}{5.9083}$$

$$= 0.090$$

$$= 0.09 \text{ ng/g}$$

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The LOD was 0.03 ng/g (0.03 ppb) and LOQ was 0.09 ng/g (0.09 ppb).

Table 4.5 Slopes and y-intercepts of 8 regression lines used for determination of LOQ for mestanolone.

parameter	y-intercept	slope
	0.2171	5.3564
	0.2318	5.4337
	0.2106	5.9064
	0.2285	5.8597
	0.2078	6.3353
	0.1194	6.3532
	0.1006	6.1833
	0.1355	5.8381
mean	0.1814	5.9083 (S)
SD	0.0535 (**σ**)	0.3766

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4.2.1.5 Specificity

No interference was observed for mestanolone and finasteride at the retention times of 5.67 and 5.04 min, respectively.

4.2.2 Sample detection

From the analysis, the concentrations of 15-days mestanolone treated tilapia were 3.198, 2.065, 0.856 and 0.280 ng/g in tilapia tissue on day 1, 2, 3 and 5 after hormone withdrawal, respectively, the data were shown in Table 4.6 and Figures 4.8 to 4.11. The amounts of mestanolone residue in 23 days hormonal treated tilapia were 3.224, 2.029, 1.046 and 0.285 ng/g in tilapia tissue on day 1, 2, 3 and 5 after hormone withdrawal, respectively (Table 4.7 and Figures 4.12 to 4.15). Mestanolone in tilapia tissue was < 0.09 ng/g (LOQ) on the day 7 of the hormonal diet withdrawal of both two courses of experiments.

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Table 4.6 Mestanolone residues (ng/g) in Nile tilapia following oral administration of mestanolone at 80 mg/kg feed for 15 consecutive days.

Fish sample	Day after last dose						
	1	2	3	5	7	14	21
1	3.231	2.104	0.827	0.276	ND	ND	ND
2	3.139	2.016	0.857	0.273	ND	ND	ND
3	3.224	2.074	0.885	0.292	ND	ND	ND
Mean	3.198	2.065	0.856	0.280	ND	ND	ND
%CV	1.601	2.167	3.387	3.644	ND	ND	ND

ND, not detected; Limit of quantitation (LOQ): 0.09 ng/g

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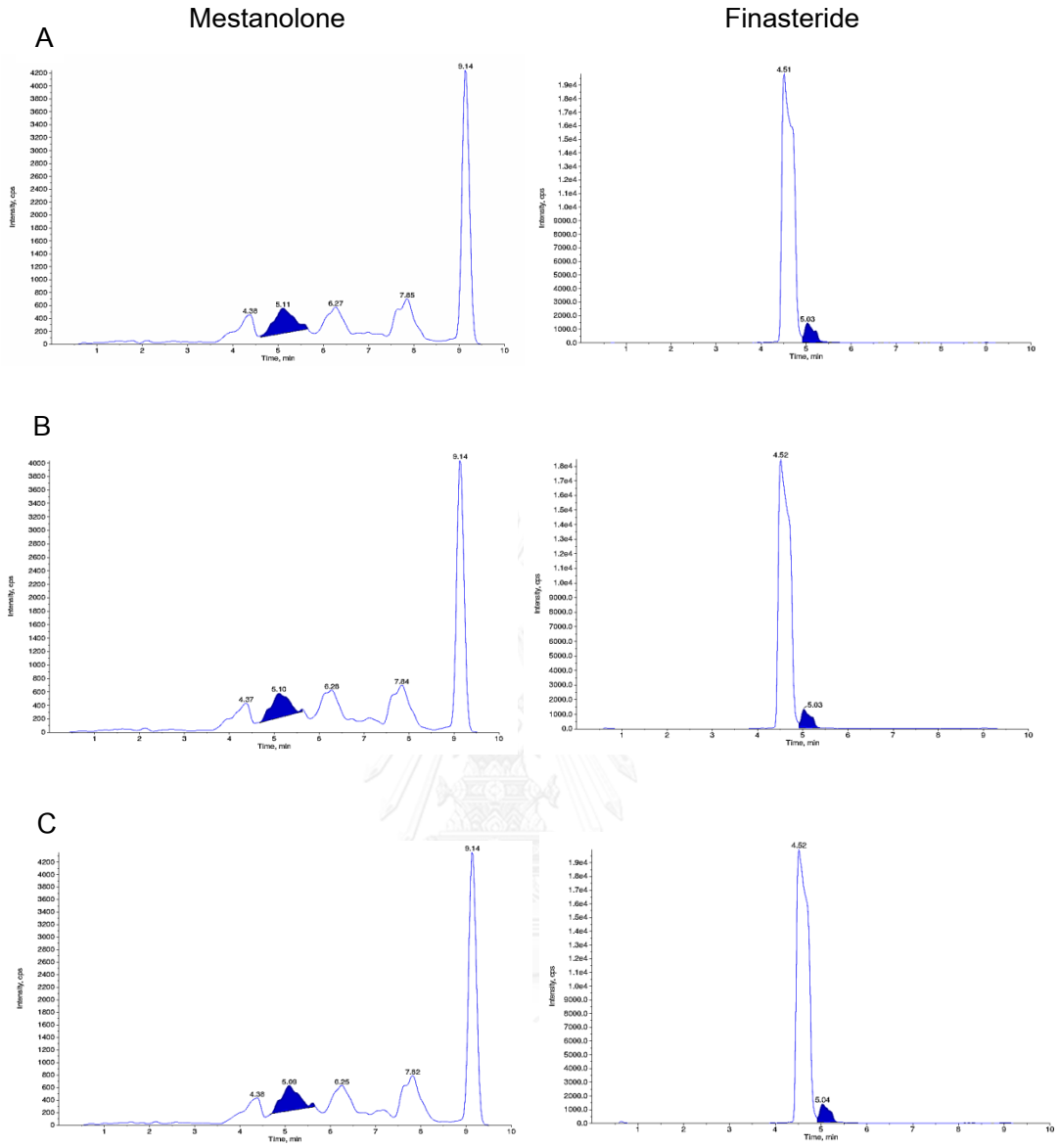

Figure 4.8 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 15 days-hormonal treated fry on the first day after hormone withdrawal.

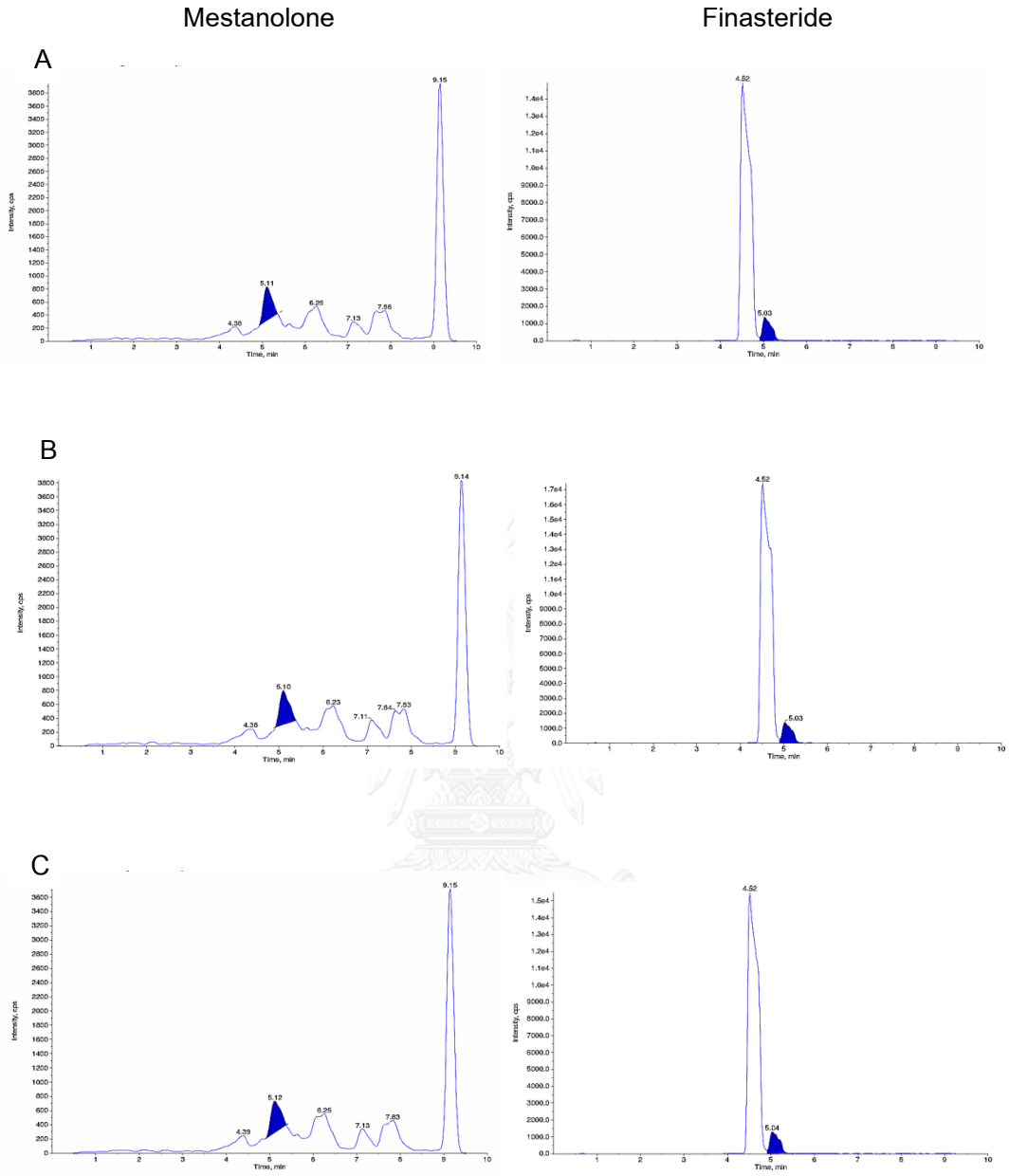

Figure 4.9 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 15 days-hormonal treated fry on the second day after hormone withdrawal.

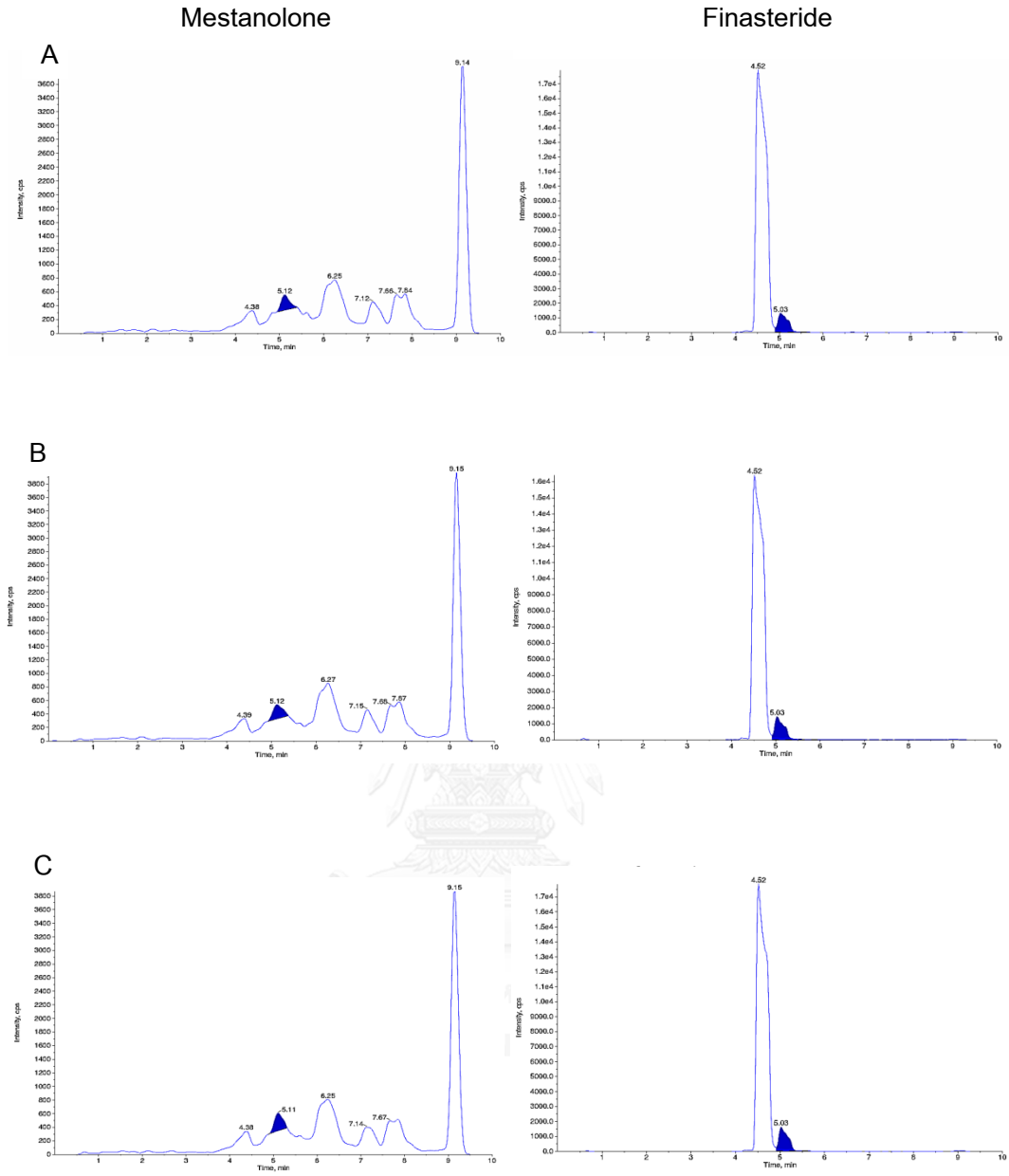

Figure 4.10 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 15 days-hormonal treated fry on the third day after hormone withdrawal.

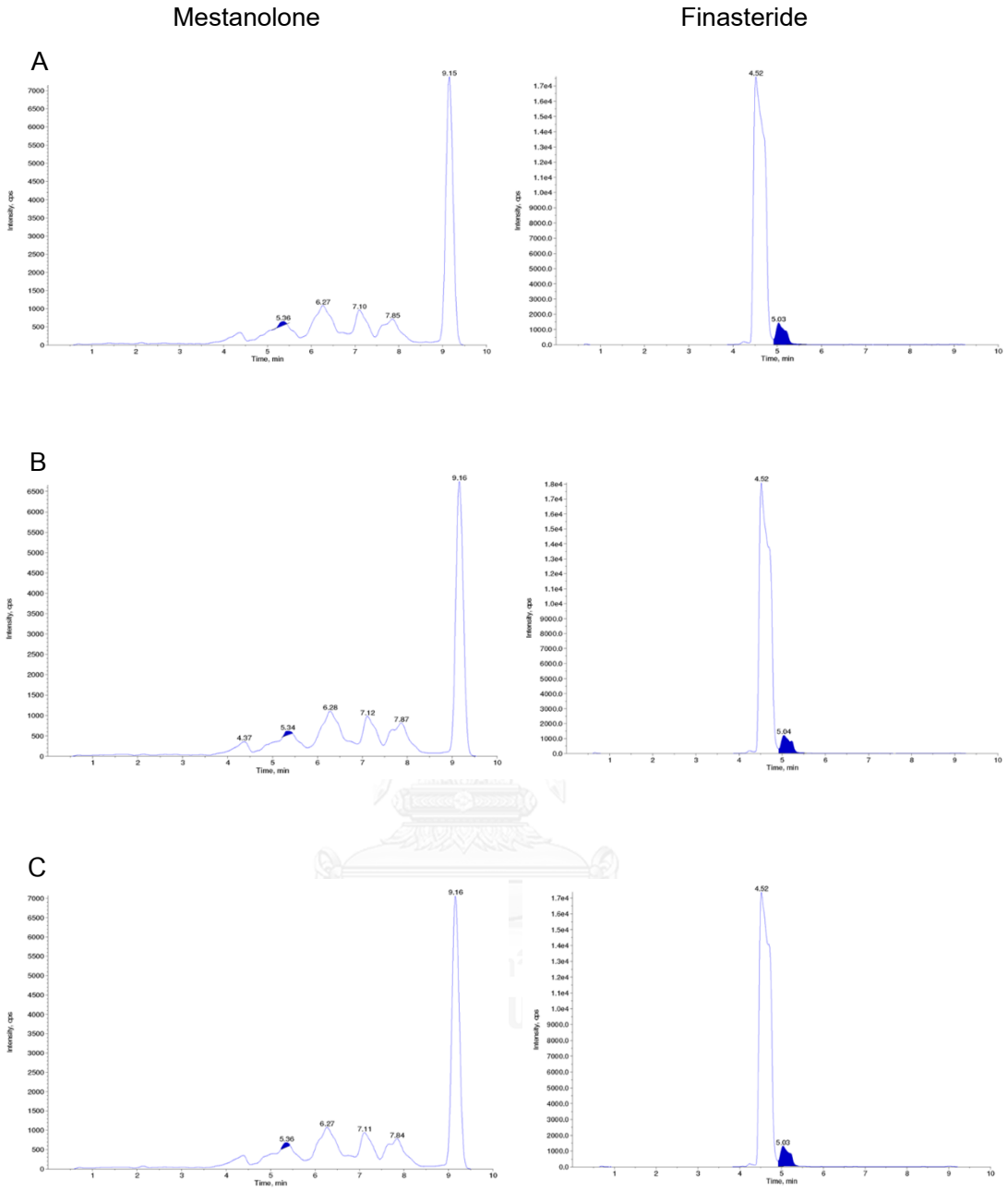

Figure 4.11 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 15 days-hormonal treated fry on the fifth day after hormone withdrawal.

Table 4.7 Mestanolone residues (ng/g) in Nile tilapia following oral administration of mestanolone at 80 mg/kg feed for 23 consecutive days.

	Day after last dose						
Fish sample	1	2	3	5	7	14	21
1	3.235	2.003	1.091	0.279	ND	ND	ND
2	3.230	2.028	1.033	0.277	ND	ND	ND
3	3.206	2.056	1.014	0.298	ND	ND	ND
Mean	3.224	2.029	1.046	0.285	ND	ND	ND
%CV	0.481	1.307	3.835	4.072	ND	ND	ND

ND, not detected; Limit of quantitation (LOQ): 0.09 ng/g

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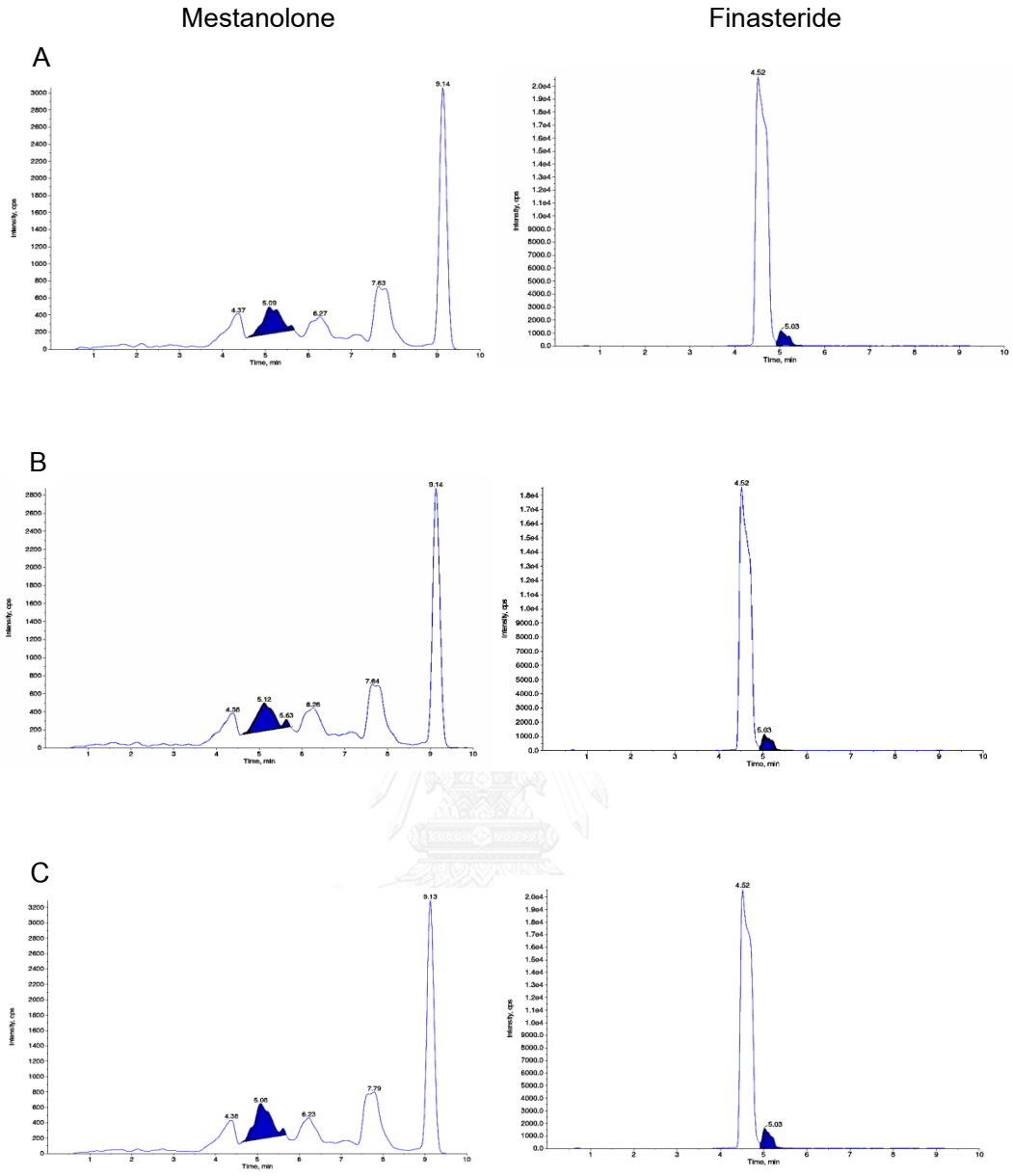

Figure 4.12 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 23 days-hormonal treated fry on the first day after hormone withdrawal.

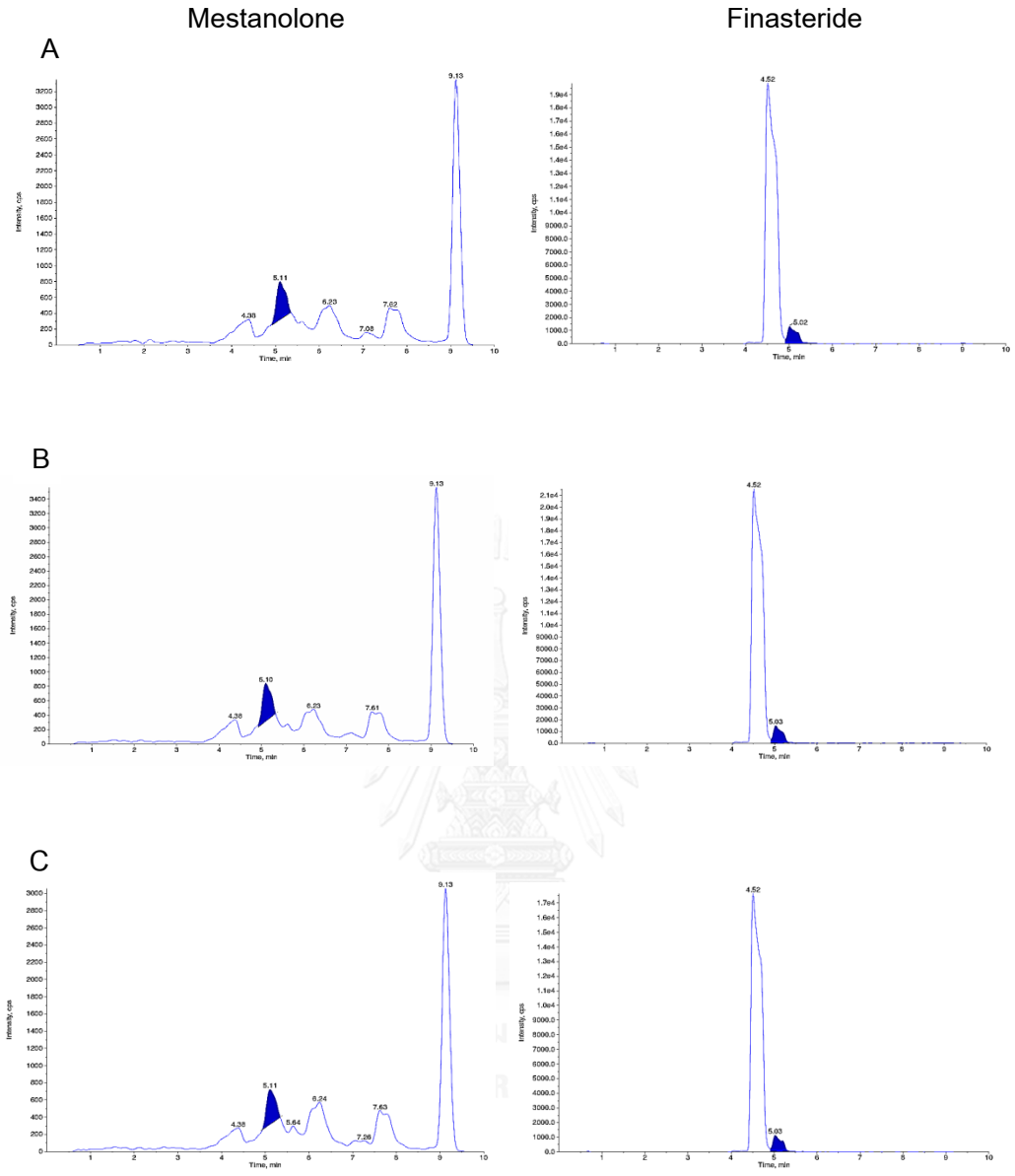

Figure 4.13 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 23 days-hormonal treated fry on the second day after hormone withdrawal.

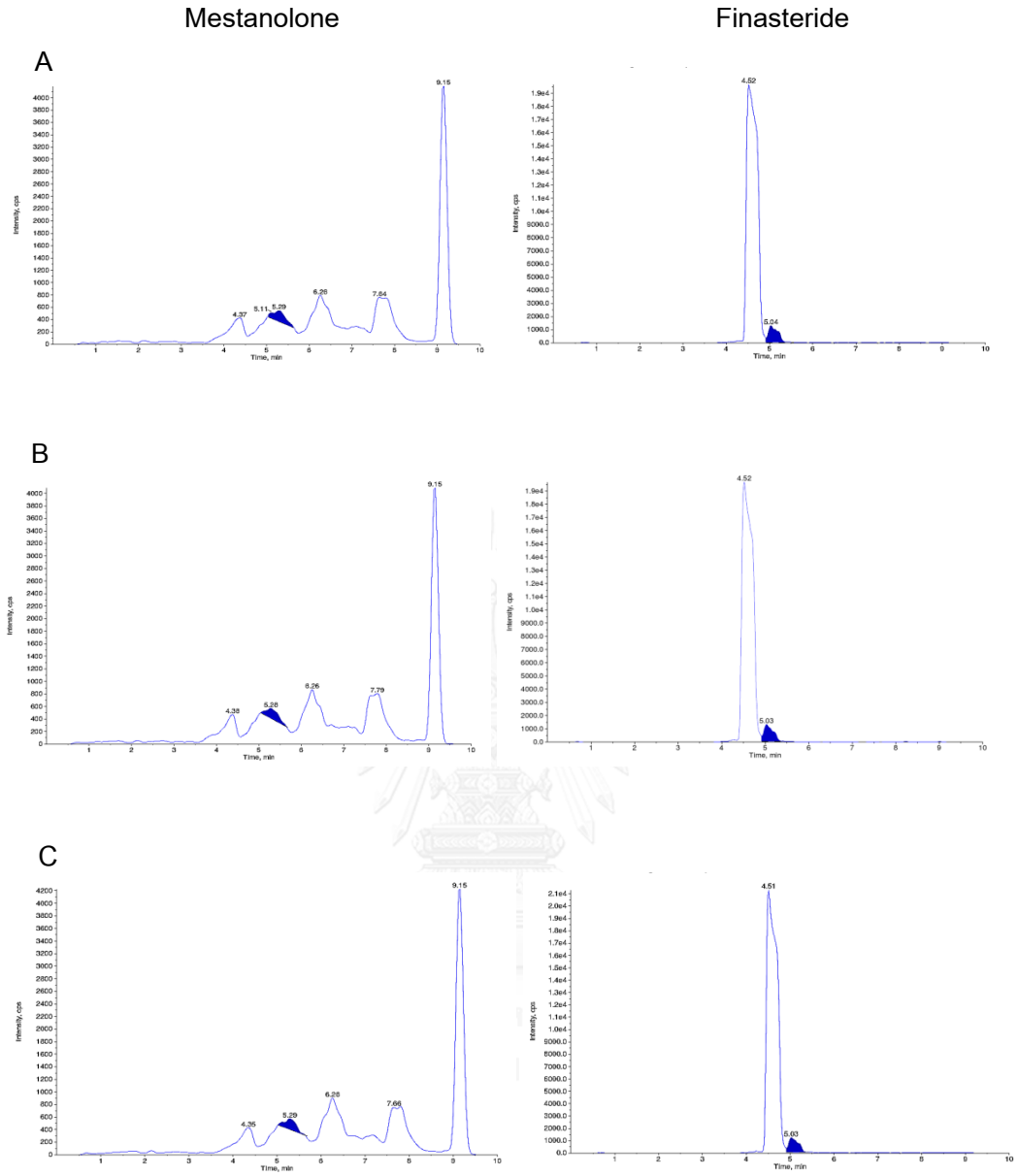

Figure 4.14 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 23 days-hormonal treated fry on the third day after hormone withdrawal.

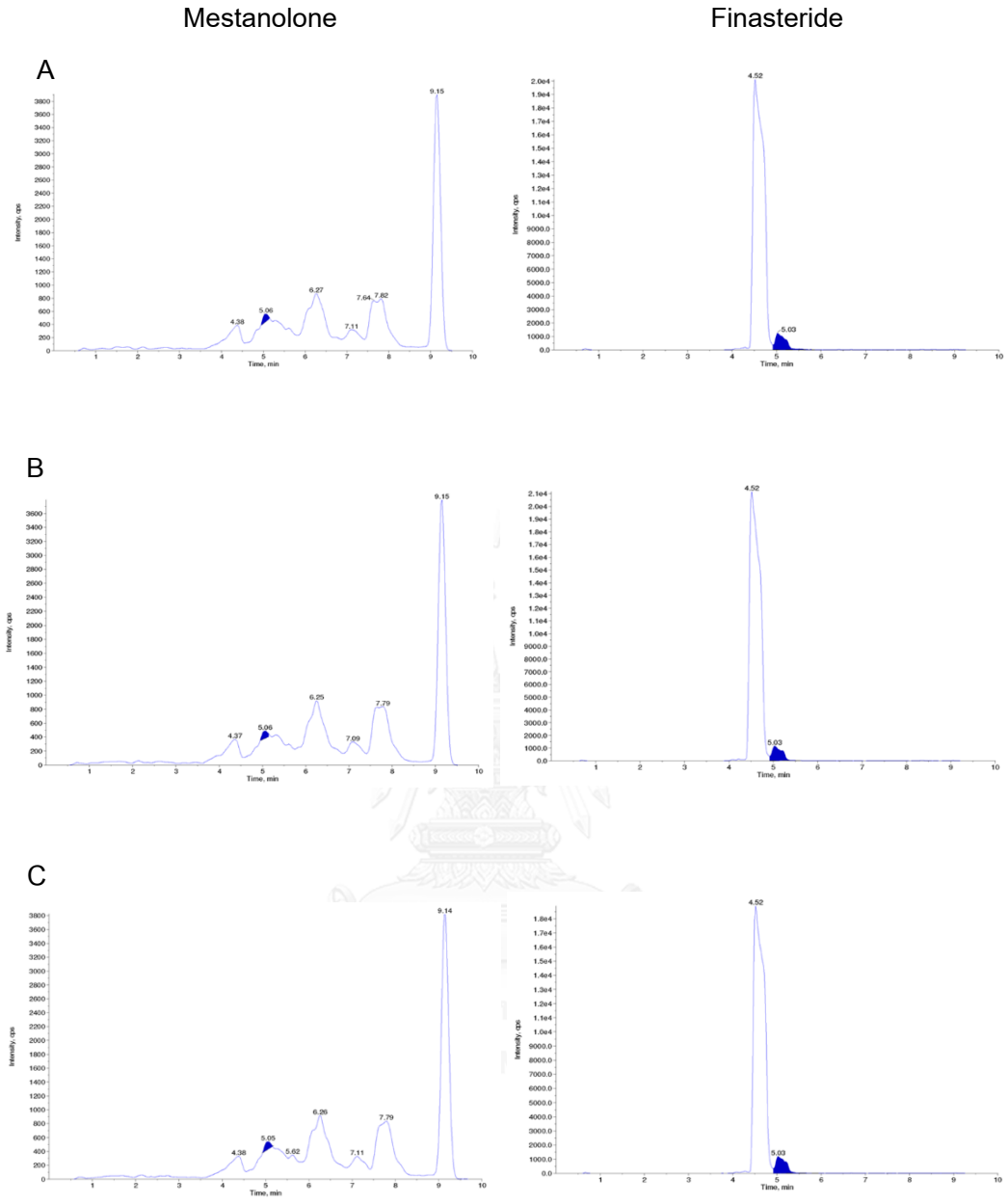

Figure 4.15 LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection (A), second injection (B), and third injection (C) from 23 days-hormonal treated fry on the fifth day after hormone withdrawal.

CHAPTER V

DISCUSSIONS

This study successfully minimized the use of mestanolone in male sex-reversed tilapia to a 15-day period, while maintaining 100% masculinization. This finding agrees with previous studies (Abucay and Mair, 1997; Mateen and Ahmed, 2007; Marjani et al., 2009) showing that sex reversal in tilapia species can be achieved by administration of exogenous androgens. The results exhibited that the estrogen hormone, EE_2, has a positive effect on feminizing in Nile tilapia. This agrees with the studies showing that administration of EE_2 to rainbow trout yielded a maximum female percentage of 94.5% without decreasing growth (Razmi et al., 2011) and that feeding blue tilapia with an EE_2 diet achieved a population that was 90% female (Hopkins et al., 1979). Hormonal treatments performed during the critical period require minimum steroid dosage to successfully reverse gonadal sex (Budd et al., 2015). It was observed that when the duration of hormonal administration was increased, the male percentage also increased considerably. This result indicated that the longer duration of mestanolone administration increases the number of male in Nile tilapia. Although EE_2 treatments in this study were performed during the critical period, fish could not be successfully feminized. This may be caused by using improper dosage or from environmental factors. For further study, it is recommended that EE_2 should be administrated in higher amount of hormone or longer treatment to yield 100% female.

Many researches said clearly that final body size of female tilapia is comparatively smaller than male tilapia due to utilizing their energy for oocytes development (Beardmore et al., 2001; Bhatta et al., 2012). Furthermore, female spawn frequently, or approximately every 2-3 weeks and during these spawning periods, they have markedly reduced feed intake or stop feed intake completely (Kadri et al., 1996; Bhatta et al., 2012). In this study, the body weight and total length of fry were recorded only one time at the day of sexing (60 dph) and found no significant difference between the treated groups and control group. This implies that treating fish with hormone does not negatively affect the growth rate. These results agree with the study of Bhatta et al. (2012), in which female and male fry aged 15-75 dph had the same growth rate.

The findings of this study on gonadal development by histological examination are in agreement with the results of Afonso et al. (2001), showing that histological examination reveals no differences in gonadal tissues treated with exogenous hormone and non-treated fish. Overall, the timing of each step of gonadal development observed in this study closely matches an earlier description for Nile tilapia (Esterhuyse et al., 2008; El-Sakhawy et al., 2011; El-Saba et al., 2013).

Many authors reported various rearing conditions for sex reversal experiment. In the earlier study, Mozambique tilapia (*Oreochromis mossambicus*) was treated by administration of MT at a dose of 75 mg/kg diet for 21 days at 27-29°C which could produce 98.1% male (Marjani et al., 2009). Phelps and Okoko (2011) also used MT to induce masculinization of Nile tilapia at 27-32°C which yielded 97.0% male at a dose of 60 mg/kg diet for 28 days, while in the study of Amaraweera et al. (2012), the experiment was done quite similar except the temperature condition was at 25°C, inducing 98.0%

male. Phelps and Popma (2000) suggested that there are many factors affecting sex reversal of tilapia such as size and age of fish, treatment duration, environment and dose rate of hormone. Therefore, these factors should be considered when we compare our masculinization percentage with the others.

In terms of size and age, the proper size and age to begin the hormonal administration are the smallest and youngest fry which its length should be lesser than 14 mm (Phelps and Popma, 2000). Hiott and Phelps (1993), feeding MT for 28 days obtained 95.7% males treating ≤11mm tilapia fry but obtained only 62.1% males when they treated 14-16 mm fry. Many studies started the hormonal treatment at the close age of tilapia. Fry at the age of 3 dph were used in the experiment of Marjani et al. (2009), Ferdous and Ali (2011) and Mateen and Ahmed (2015) resulting in 98.1%, 94.3% and 98.0% male, respectively. El-Greisy and El-Gamal (2012) obtained 97.0% male when they treated 7 dph fry and in this study, we obtained 100% male when we treated 10 dph fry. In term of treatment duration, the period of treatment might be associated with initial size and growth conditions. In general, fry less than 14 mm long should obtain hormone at least 14 days before reaching 18 mm but the duration can be extended to 28 days in case of slower growth (Hiott and Phelps, 1993). Therefore, treatment duration must be sufficient to allow all fish to complete gonadal differentiation during the treatment period (Phelps and Popma, 2000). In terms of environment, environmental factors such as temperature can influence growth and skew sex ratios. Phelps and Popma (2000) reported that the optimum temperature for sex reversal treatment was between 26-28°C and temperatures below 24°C significantly decrease growth and some fish may have uncompleted sex differentiation. Guerrero (1975) induced sex reversal of blue tilapia using

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1-dehydrotestosterone at 21°C the result showed only 44.0% male at the dosage of 60 mg/kg diet for 18 consecutive days.

In term of dose rate, excessive amount of hormone intake can reduce the efficacy of masculinization as shown in the study of El-Greisy and El-Gamal (2012). They fed MT to Nile tilapia for 21 days at 60 mg /kg diet and obtained 97.0% male, whereas, feeding at the higher dose, 80 mg/kg diet gave 93.0% male. Marjani et al. (2009) found the similar result in Mozambique tilapia which obtained 98.1% male after feeding 75 mg MT/kg diet, but at 100 mg MT/kg diet the percentage of male was only 79.4. In addition, overdoses and sub-doses could result in intersexes of tilapia fry which was shown in the study of Phelps and Okoko (2011). The optimum dose for sex reversal is hardly defined because the sufficient dose for sex reversal depends on the amount of feed eaten by fish. In the present study, the high dose of mestanolone (80 mg/kg) was given to fry when feeding at 13% body weight for 15 and 20 days. The total amounts of calculated hormone which treated fish intake were 0.156 and 0.208 mg mestanolone/g fish, respectively. These amounts came close to the results from previous studies. Mateen and Ahmed (2015) fed 0.175 mg MT/g fish at a dose rate of 70 mg MT/kg diet for 25 days (feed at 10% body weight) while El-Greisy and El-Gamal (2012) fed 0.189 mg MT/g fish at a dose rate of 60 mg MT/kg diet for 21 days (feed at 15% body weight).

Recently, many studies have revealed the mechanism of exogenous hormones on sex differentiation in Nile tilapia by dealing with genetic factors. For a natural pathway, *dmrt1* (a gene involved in testicular differentiation) displayed a male-specific expression from 6 dph in genotypic male gonads whereas, *cyp19a1* (a gene producing aromatase enzyme responsible for ovarian differentiation and the conversion of androgens into 17β-

estradiol) was expressed from 5 dph in genotypic female gonads. These results indicate that the differential expression of genes occurring in genotypic female gonads and genotypic male gonads during the period of 5-6 dph is critical for undifferentiated gonads to differentiate into either the ovary or testis in the Nile tilapia (Ijiri et al., 2008). In addition, Tao et al. (2013) explained why exogenous steroid treatment could induce sex reversal in genotypic female by using hormone receptors' information. The presence of androgen receptors (*ar1* and *ar2*) in genotypic female gonads at 5 dph causes genotypic female fry sensitive to exogenous androgen in masculinization. As described previously, the timing of gonadal development and sensitive period (5-6 dph) knowledge lead to the most precisely timed and thus effective treatments in Nile tilapia (Budd et al., 2015).

Aceto-carmine staining was suitable for sexing Nile tilapia above 0.5 g (approximate 45-47 dph) when fish were large enough for their genital organs to be distinguished (Wassermann and Afonso, 2002). Interestingly, knowledge of gene expression patterns in tilapia might suggest a molecular method for earlier detection of sexing in tilapia which is better than aceto-carmine technique. For example, the researcher could observe gene expression before the appearance of sex differences in histogenesis and purposed that *dmrt1* is a superior testicular differentiation marker in tilapia (Kobayashi et al., 2008). Further study on the biomarker for early detection of tilapia sexing will be critical to identify and develop.

Our results prove the possibility of skewing Nile tilapia into phenotypic females using estrogenic hormonal administration. This is a preliminary step towards the developing of breeding program to produce supermales (YY) through the mating of sex-reversed females (XY) with normal males (XY). The ultimate goal is gaining male monosex

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tilapia which will be hormone-free fish. Another way for producing hormone-free tilapia is triploidy induction which generated sterile tilapia (Hussain et al., 1991; Pradeep et al., 2014). However, this method has been studied in lab scale, the commercial application might be infeasible because of time and man power consumed (Pradeep et al., 2012; Pradeep et al., 2014). Hence, the hormone application for producing monosex tilapia is still the practical method.

The analytical method was validated in accordance to the criteria of VICH GL 49 (2011) which the accuracy, expressed as %recovery, should meet the acceptable range between 60-120%. The intra-day and inter-day precision, expressed as %CV, should meet the acceptable value 25% and 32%, respectively. The linearity has R^2 and r values which were greater than 0.995. All parameters had passed the criteria. Therefore, these results indicated that the method was suitable for the quantitation and confirmation of mestanolone in Nile tilapia due to its good precision and recovery.

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Many analytical methods have been developed to quantify androgenic hormones in muscles of fish species. LC-MS determination of MT was performed using a method validated at levels from 0.40 to 1.6 ng/g, with a limit of detection of 0.04 ng/g, an accuracy from 100% to 110% and CV values <10%. Residual amounts of MT were detected for 14 days after hormonal withdrawal before dropping below LOQ (<0.09 ng/g) on day 21 in tilapia, rainbow trout, and salmon (Chu et al., 2006). In a preliminary validation using HPLC for detection of MT in carp muscle tissue, recoveries were >80%, CV values were <10%, and the limit of detection was 0.05 μg/g (Jiang et al., 2005). LC-MS/MS system was developed for determination and confirmation of the anabolic steroids 17β-trenbolone, norethandrolone, methylboldenone, and MT in bovine muscle. The method

has verified to be highly specific and sensitive. Data obtained showed a satisfactory precision and accuracy with the recoveries ranging from 83 to 104% and the %CV not greater than the value of 7% (Kaklamanos et al., 2007). An analytical method to determine the use of three stilbene residues (DES, DEN, and HEX) in edible tissues of finfish; catfish, salmon, trout, and tilapia, was developed using LC-MS/MS with negative electrospray ionization. The overall average residue recoveries were 119, 99, and 104% with %CV of 18, 11, and 15% for DEN, DES, and HEX, respectively. LOD of DEN, DES, and HEX in each matrix were found to be at or below 0.21 ng/g, and LOQ averaged 0.3 ng/g (ranged from 0.18 to 0.65 ng/g) for all analytes in all matrices (Lohne et al., 2013).

Among other androgenic hormones, MT and fluoxymesterone have short half-lives, whereas testosterone enanthate and testosterone undecanoate have longer half-lives. The half-lives of MT are 57 hours in rainbow trout (Vick and Hayton, 2001) and 1 day in Nile tilapia (Curtis et al., 1991). Fluoxymesterone has a half-life of 9.2 hours in human (Modlinski and Fields, 2006). The half-life of testosterone is 1.99 days in Albino rats (*Rattus albus*) (James et al., 1969). Testosterone enanthate and testosterone undecanoate have the half-lives of 10.3 and 25.7 days, respectively, in cynomolgus monkeys (*Macaca fascicularis*) (Partsch et al., 1995). The exact half-life of mestanolone in tilapia has not been reported but the results of this study suggest rapid clearance since residual mestanolone was not detected on day 7 after hormonal diet withdrawal. Mestanolone half-life was calculated using the equation, $T_{1/2} = 0.693/k_e$, when k_e is the slope in semilogarithmic plot. The calculated half-life of mestanolone is 1.1 day.

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CHAPTER VI

CONCLUSIONS AND SUGGESTIONS

This study successfully minimized the use of mestanolone in male sex-reversed farmed tilapia at the practical dose of 80 mg mestanolone/kg feed from the 23 days present use to a 15 day period and resulted in 100% masculinization. This study has been developed to quantify mestanolone in muscles of Nile tilapia by using LC-MS/MS. The determination method was validated with the recovery (accuracy) ranging from 84.75 to 107.80%, inter-day and intra-day CV values (coefficient of variation) ranging from 2.15 to 12.85%, limit of detection (LOD) at 0.03 ng/g and limit of quantitation (LOQ) at 0.09 ng/g. We investigated residual mestanolone after a course of oral administration to tilapia fry at a dose of 80 mg/kg feed for 23 and for 15 consecutive days. The analysis was performed at 1, 2, 3, 5, 7, 14 and 21 days after the last dose. Mestanolone was not detectable in fry after hormonal withdrawal for 7 days.

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The minimal use and long interval between hormonal administration and harvesting of fish allows time for hormonal elimination, which reduces the risk of a health hazard in consumption and the cost of production for the agriculturist. The current study shows that mestanolone used for sex-reversal of tilapia is undetectable (LOQ 0.09 ng/g) in fry at 7 days after withdrawal of the hormonal diet and is likely to be similarly undetectable when the fish reach a marketable size at the age of 6-8 months. Despite its disadvantages, hormonal treatment is important for tilapia production in many countries due to economic pressure on the food supply. These data provide crucial information for

food scientists, knowledge for the aquaculturist, and confidence in food safety for consumers. Therefore, we report the current analysis in this context and as evidence that hormones can be used in this manner without endangering human health. Moreover, the less hormonal use in aquaculture, the less hormonal residue in fish products and environment. Nowadays, only a few drugs have been approved by Food and Drug Administration (FDA) for using in aquaculture such as MT, whereas other androgen hormones used for sex reversal have no approval. The concern about food safety from livestock husbandry is obligation of the veterinarian who has to insure the hygiene, quality of food and inspect to lay down the regulations of drug and hormonal use for serving public health. Further studies are necessary to establish the most effective hormonal treatment procedure for production of male monosex Nile tilpaia populations through both direct and indirect hormonal sex reversal.

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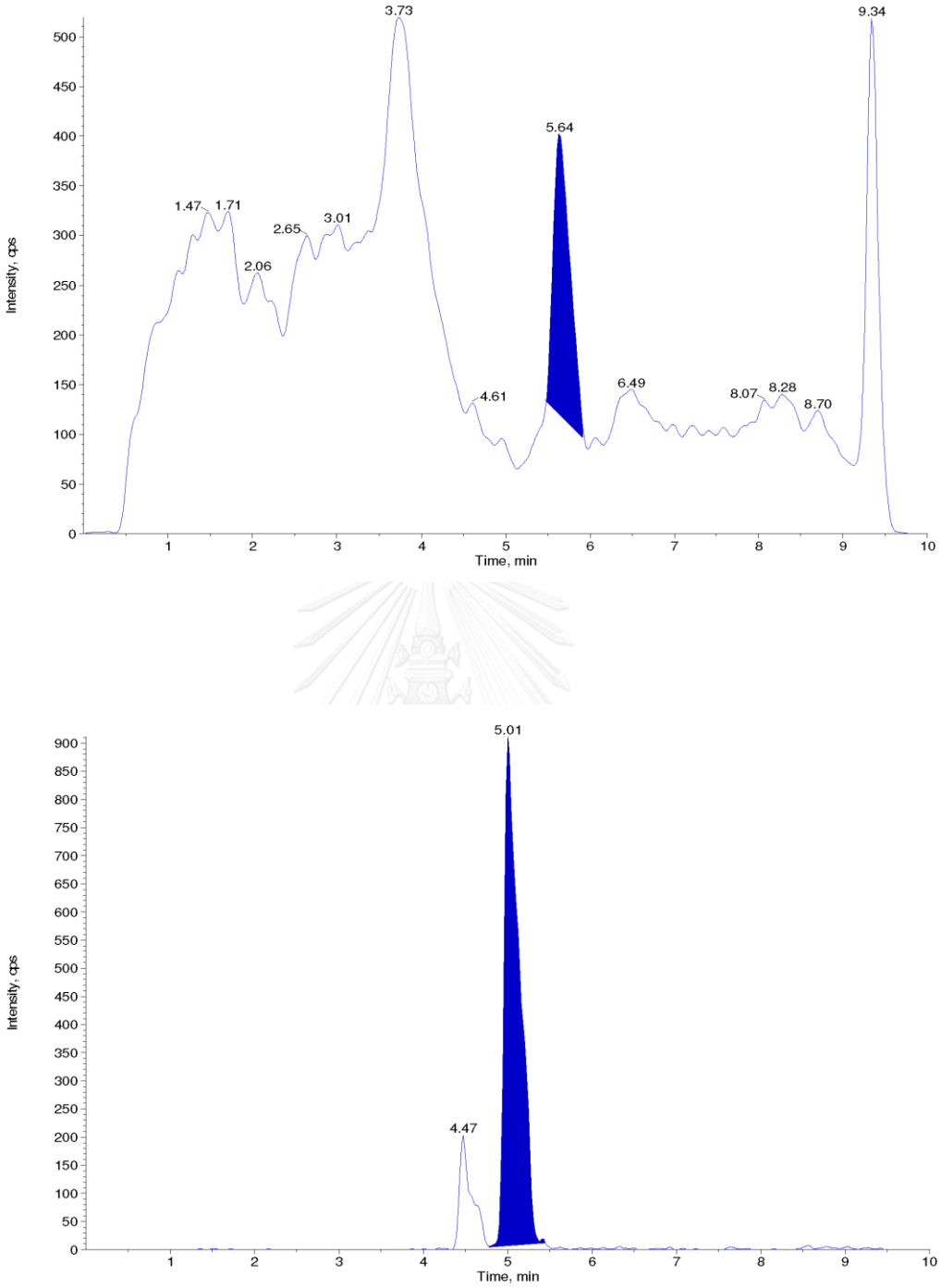
APPENDICES

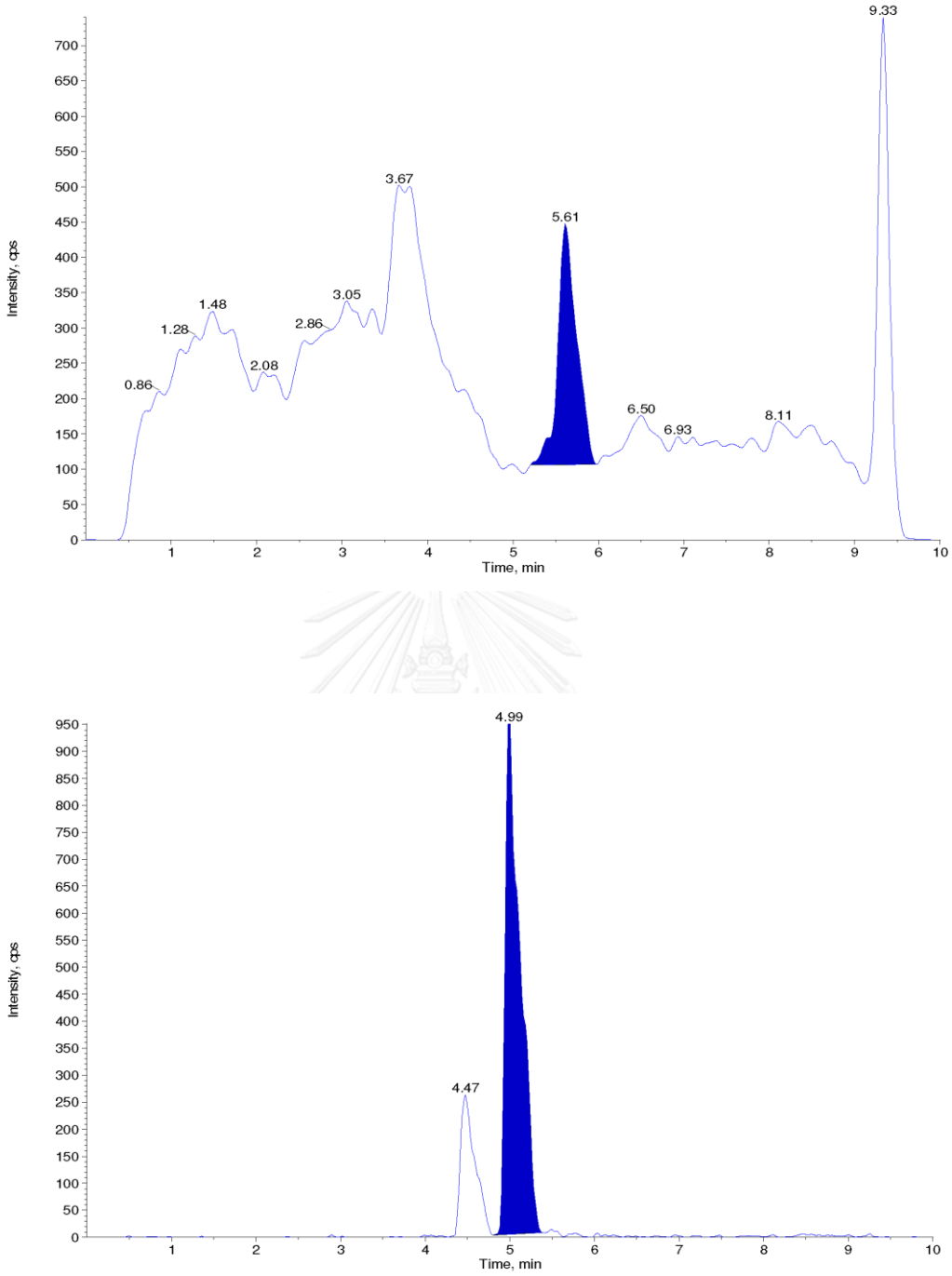
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX A LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 0.5, 1, 2, 3, 5, 7.5 and 10 ng/g for standard curve

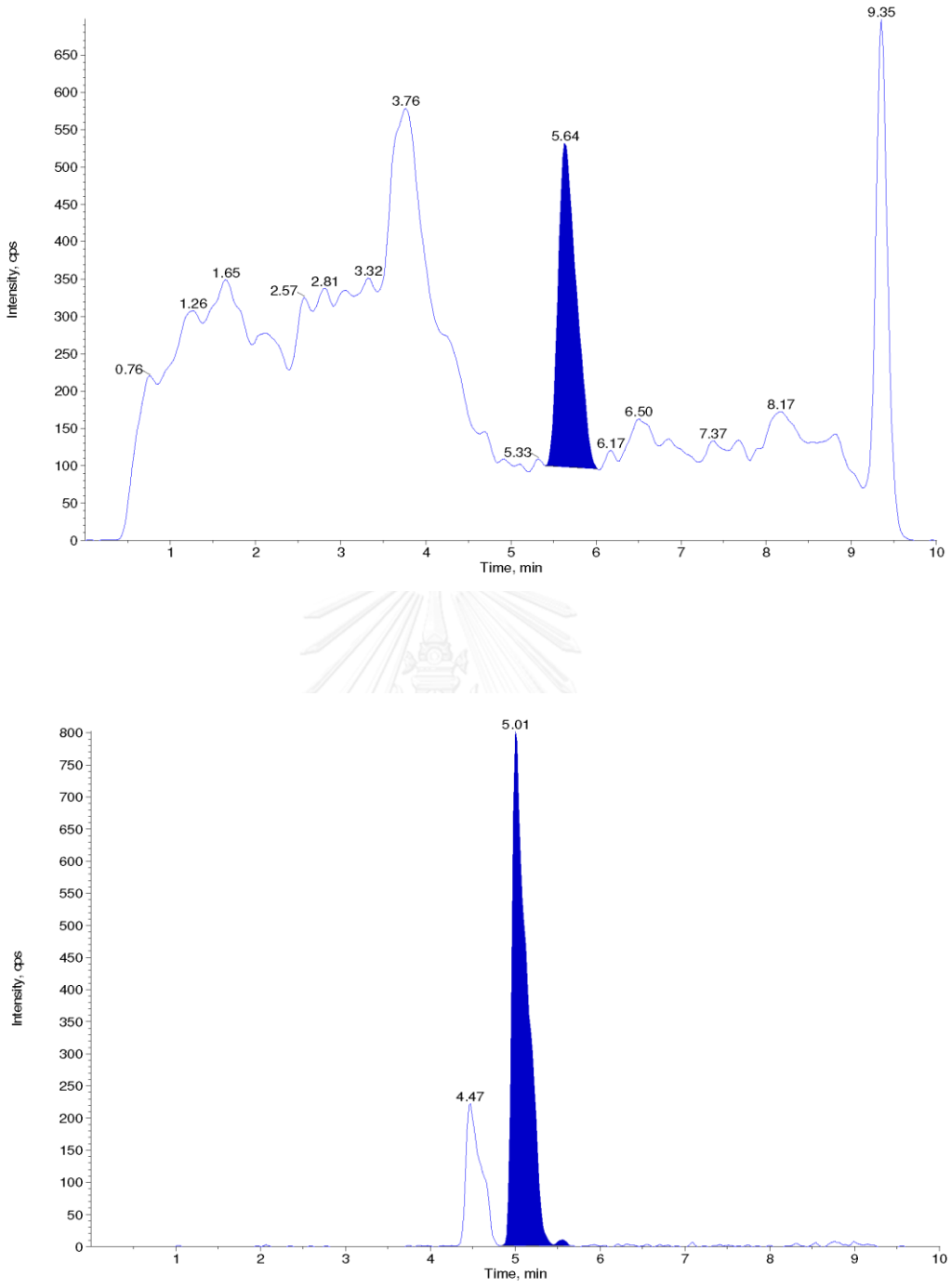
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University



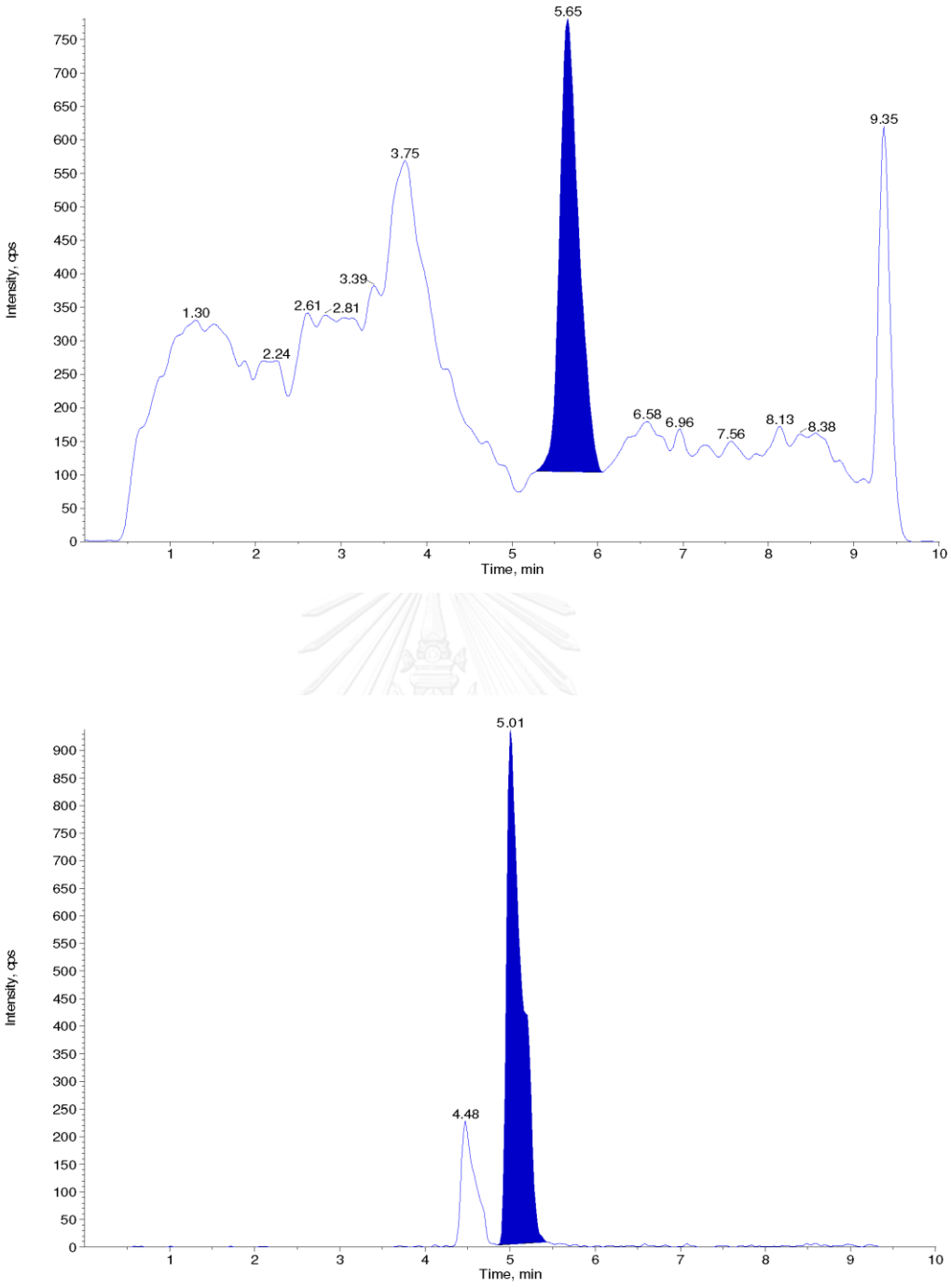
LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 0.5 ng/g.



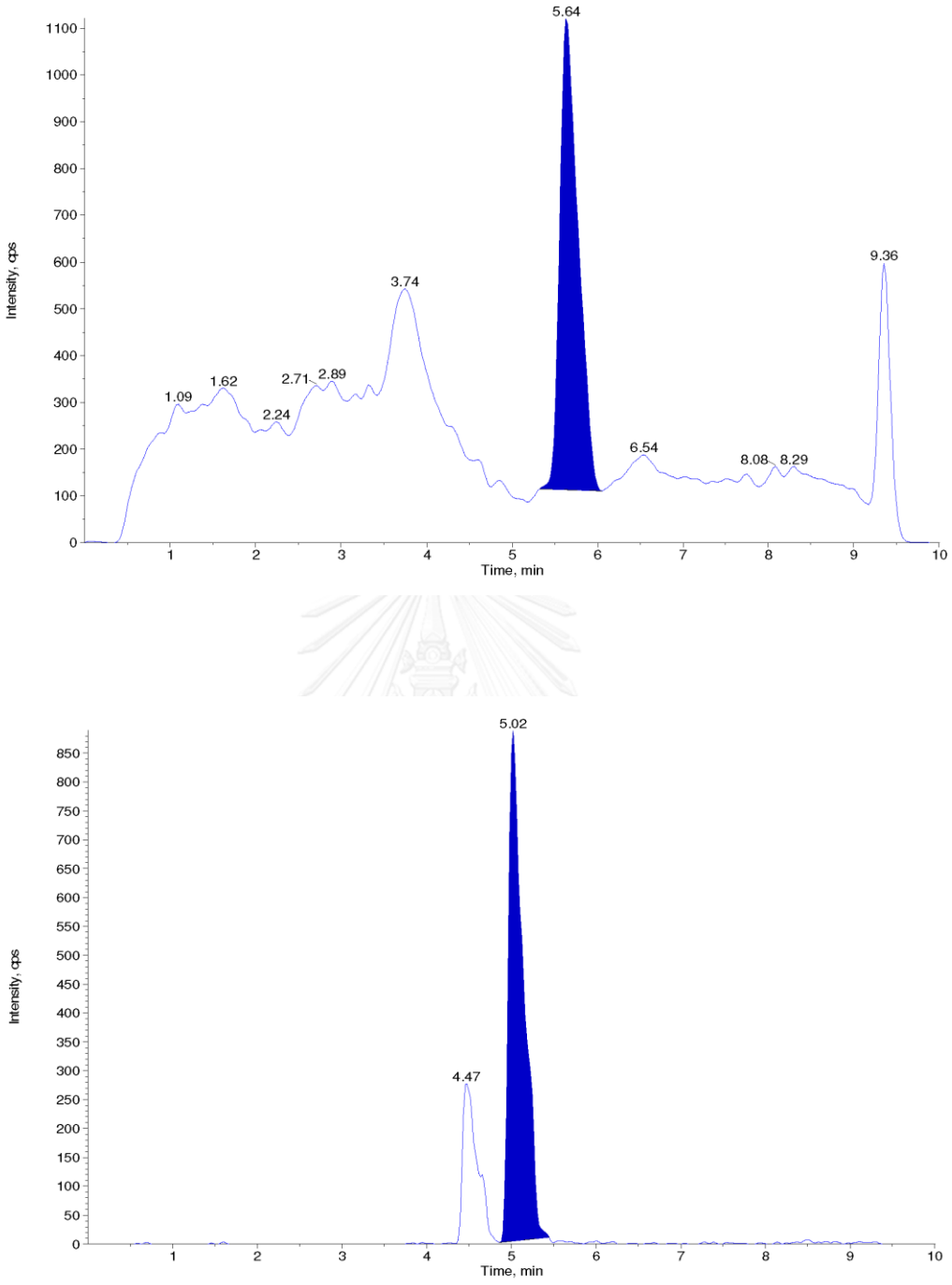
LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 1 ng/g.



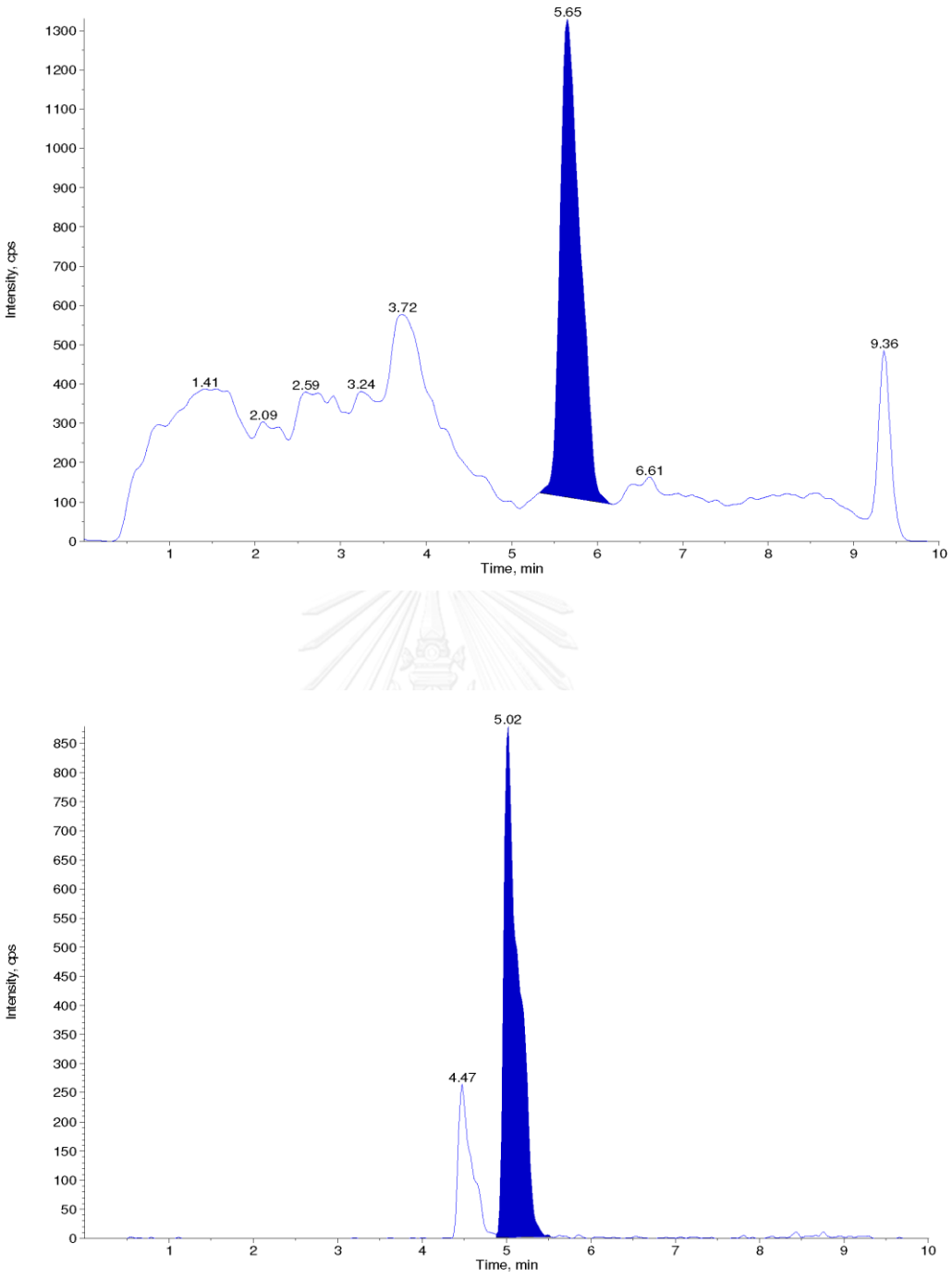
LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 2 ng/g.



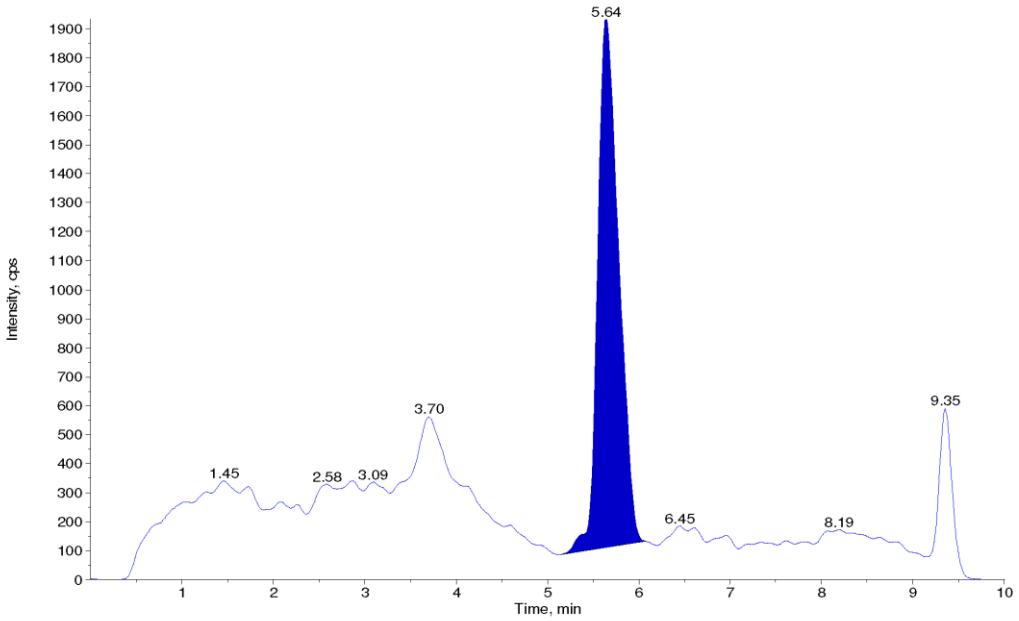
LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 3 ng/g.

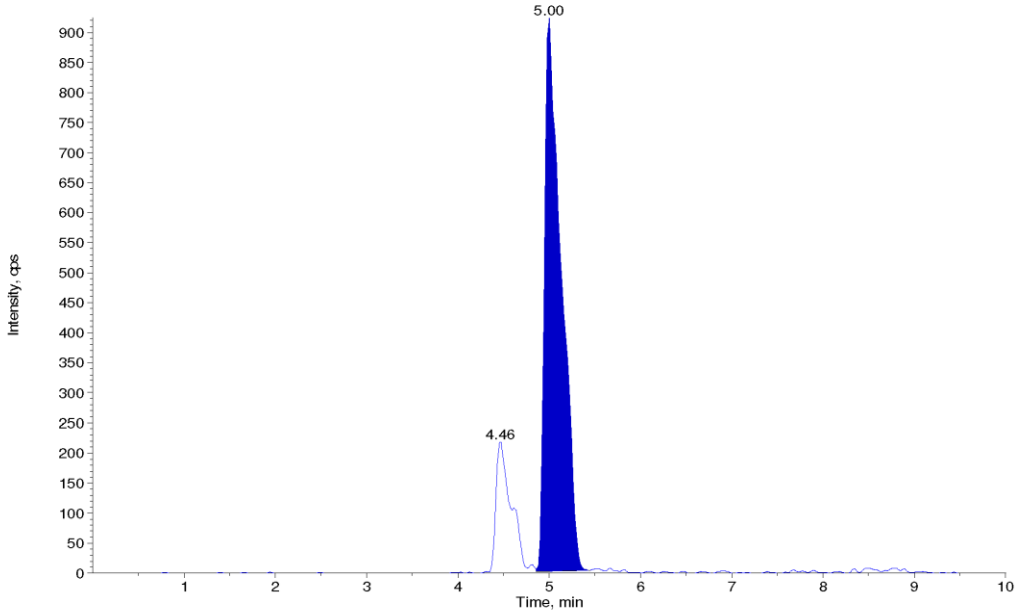


LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 5 ng/g.



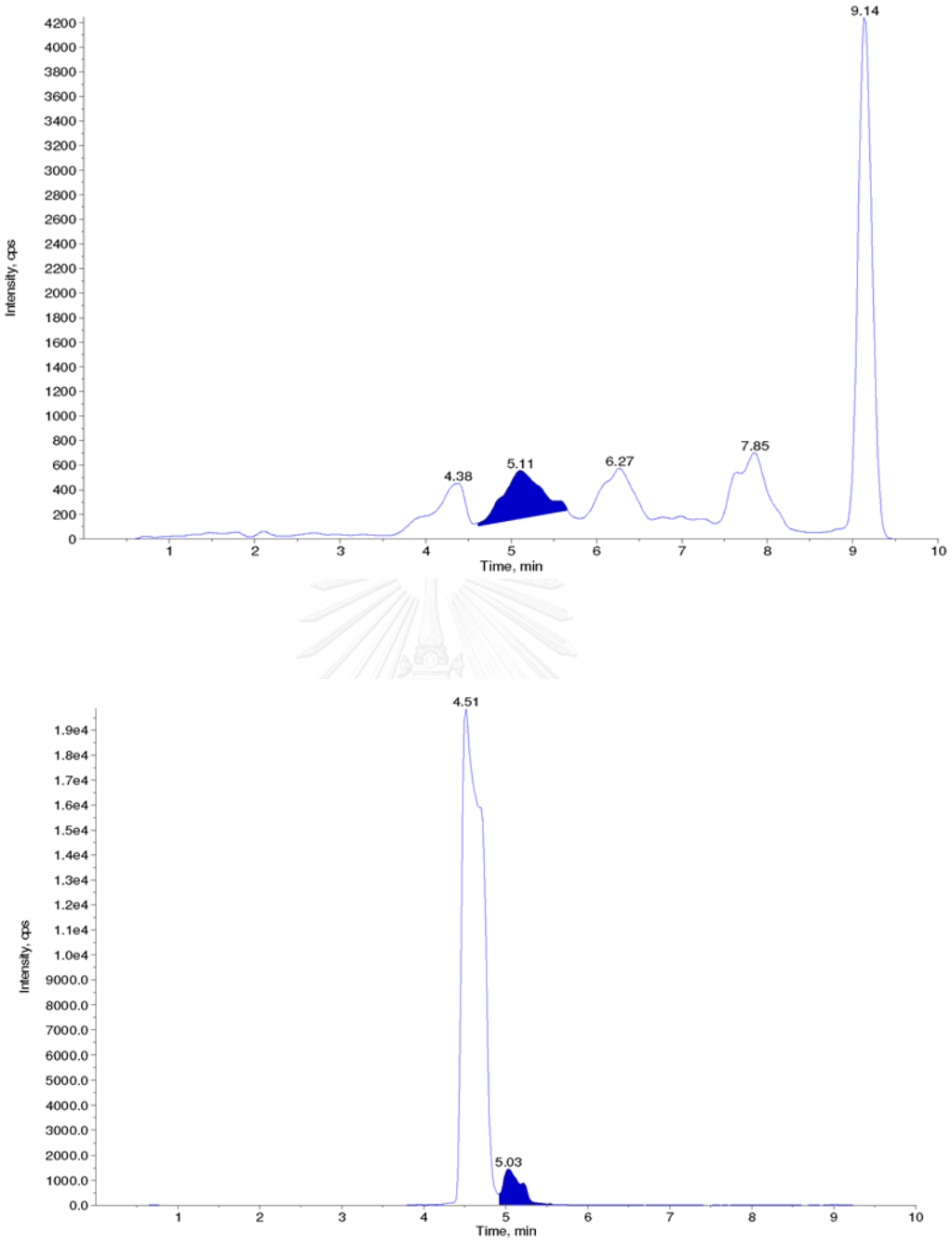
LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 7.5 ng/g.



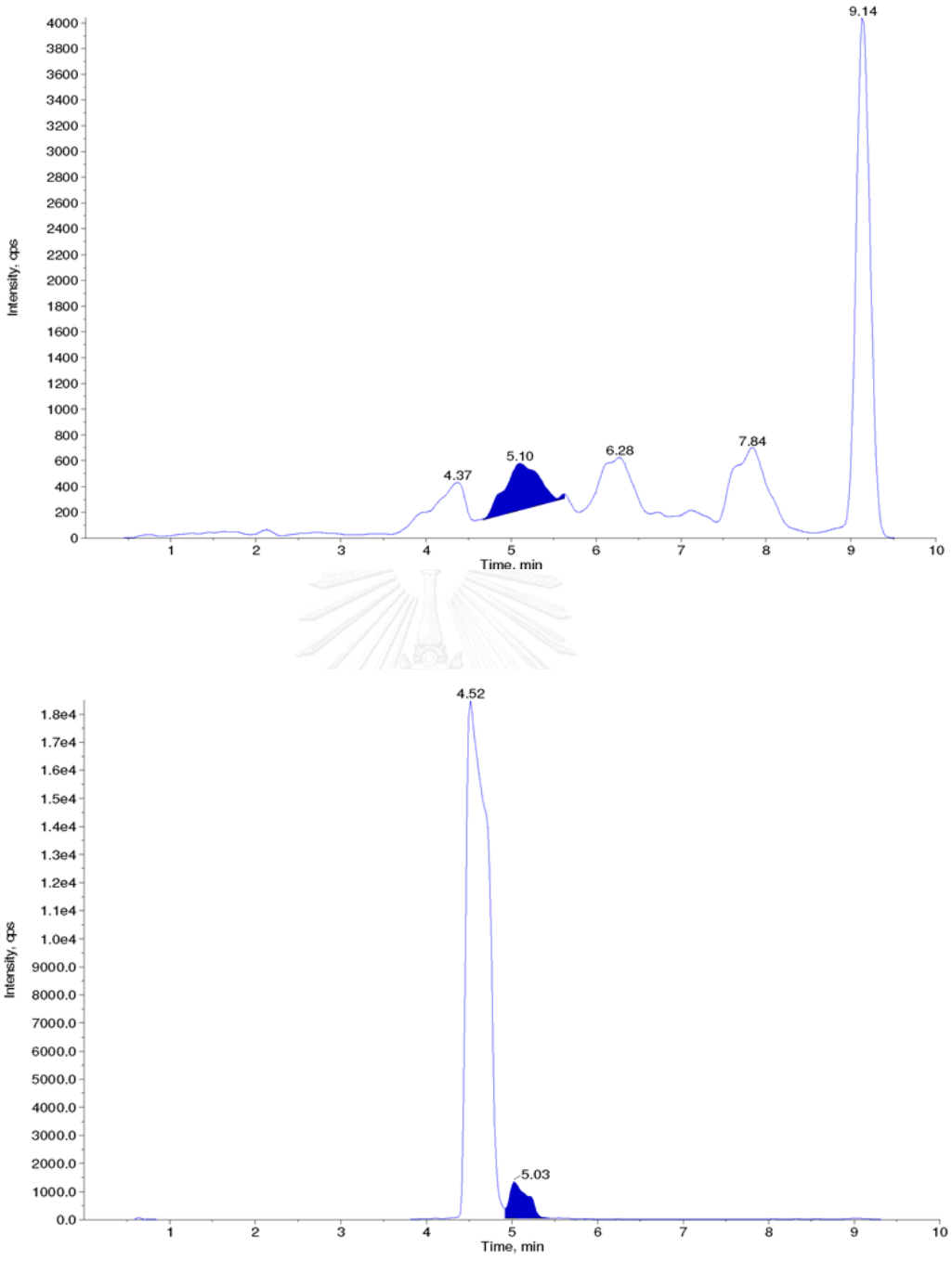


LC-MS/MS chromatogram of the standard mestanolone and finasteride at a concentration of 10 ng/g.

APPENDIX B LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride from 15 days-hormonal treated fry



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the first day after hormone withdrawal.



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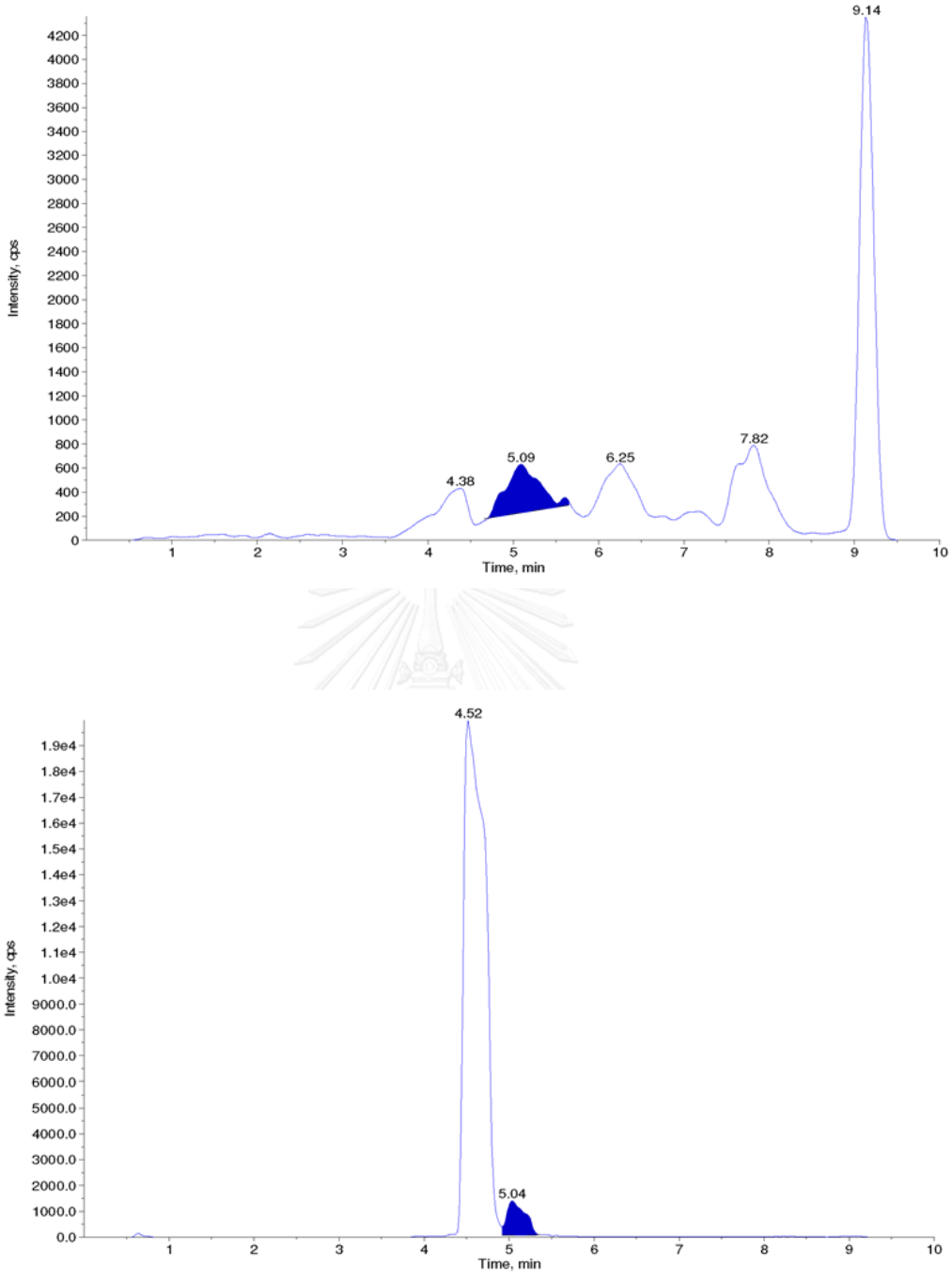
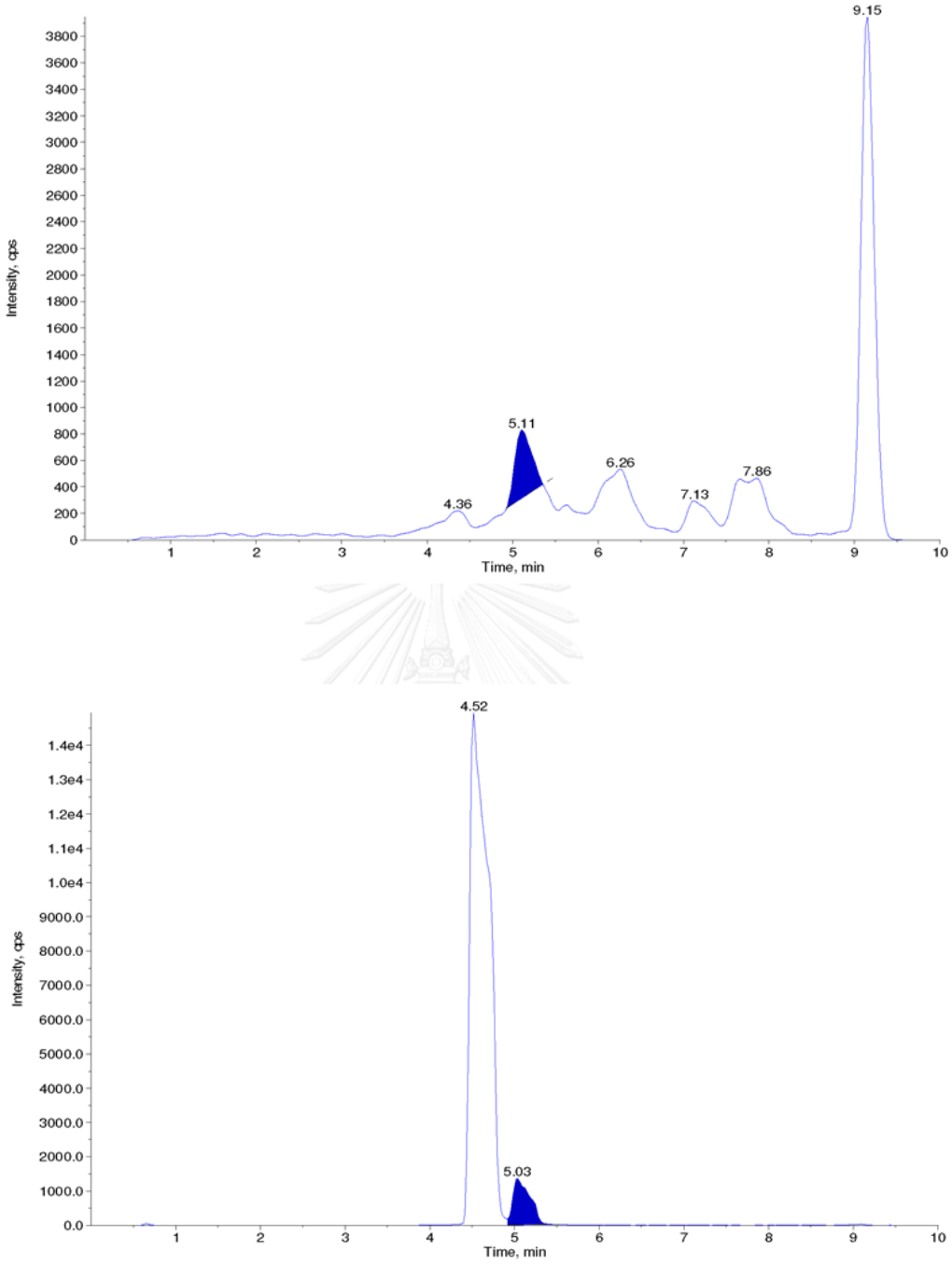
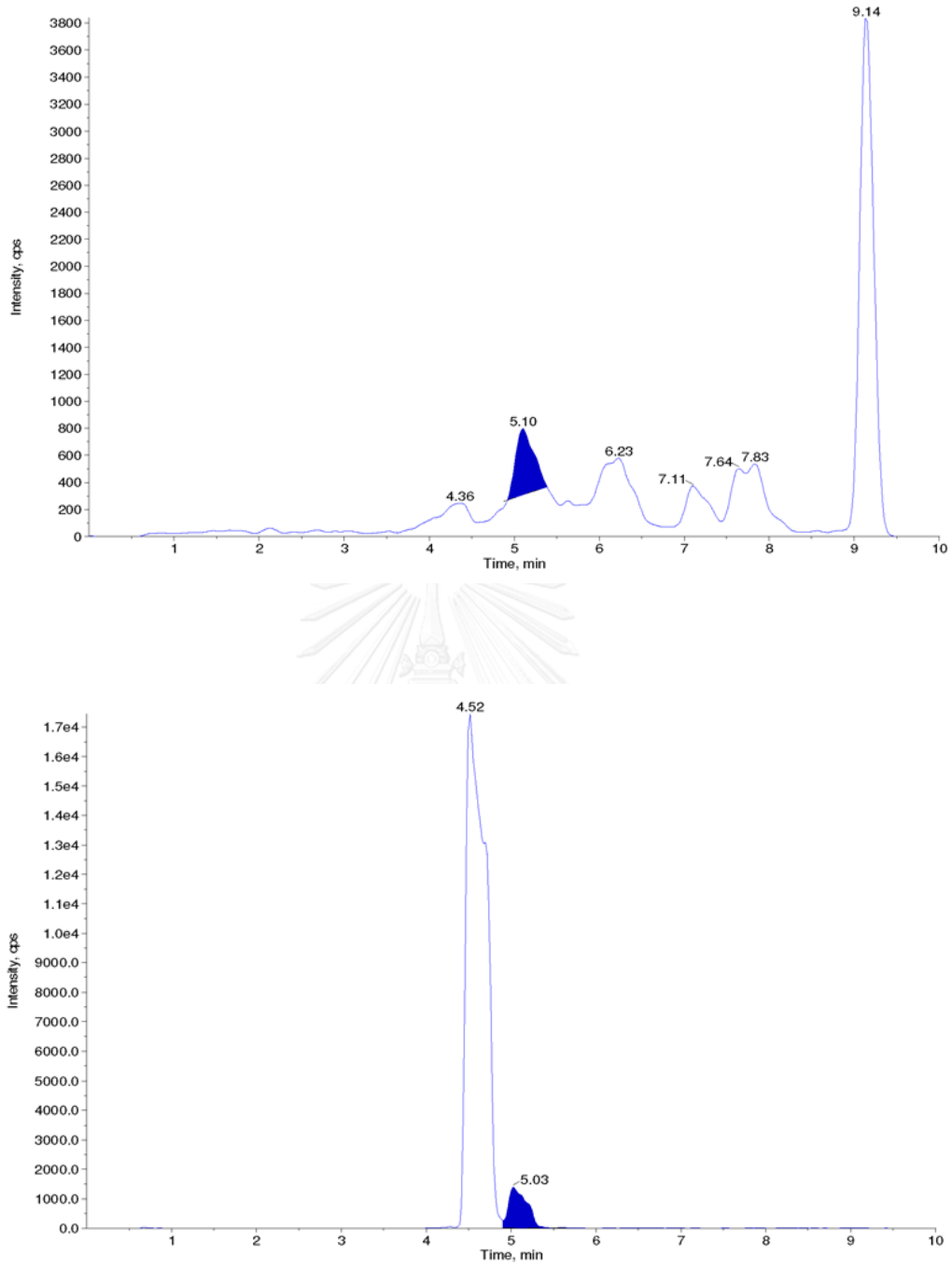


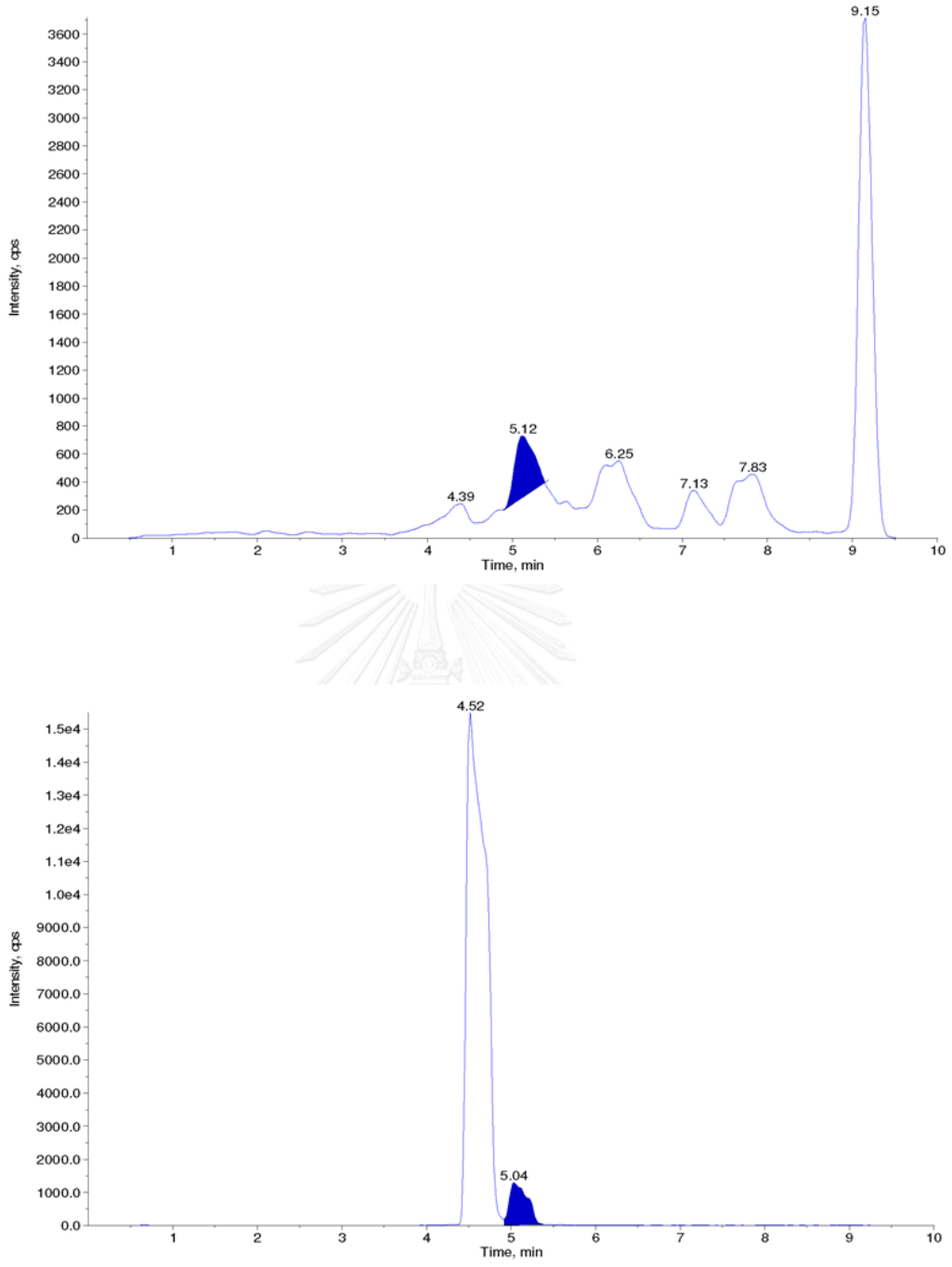
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 15 days-hormonal treated fry on the first day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the second day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 15 days-hormonal treated fry on the second day after hormone withdrawal.



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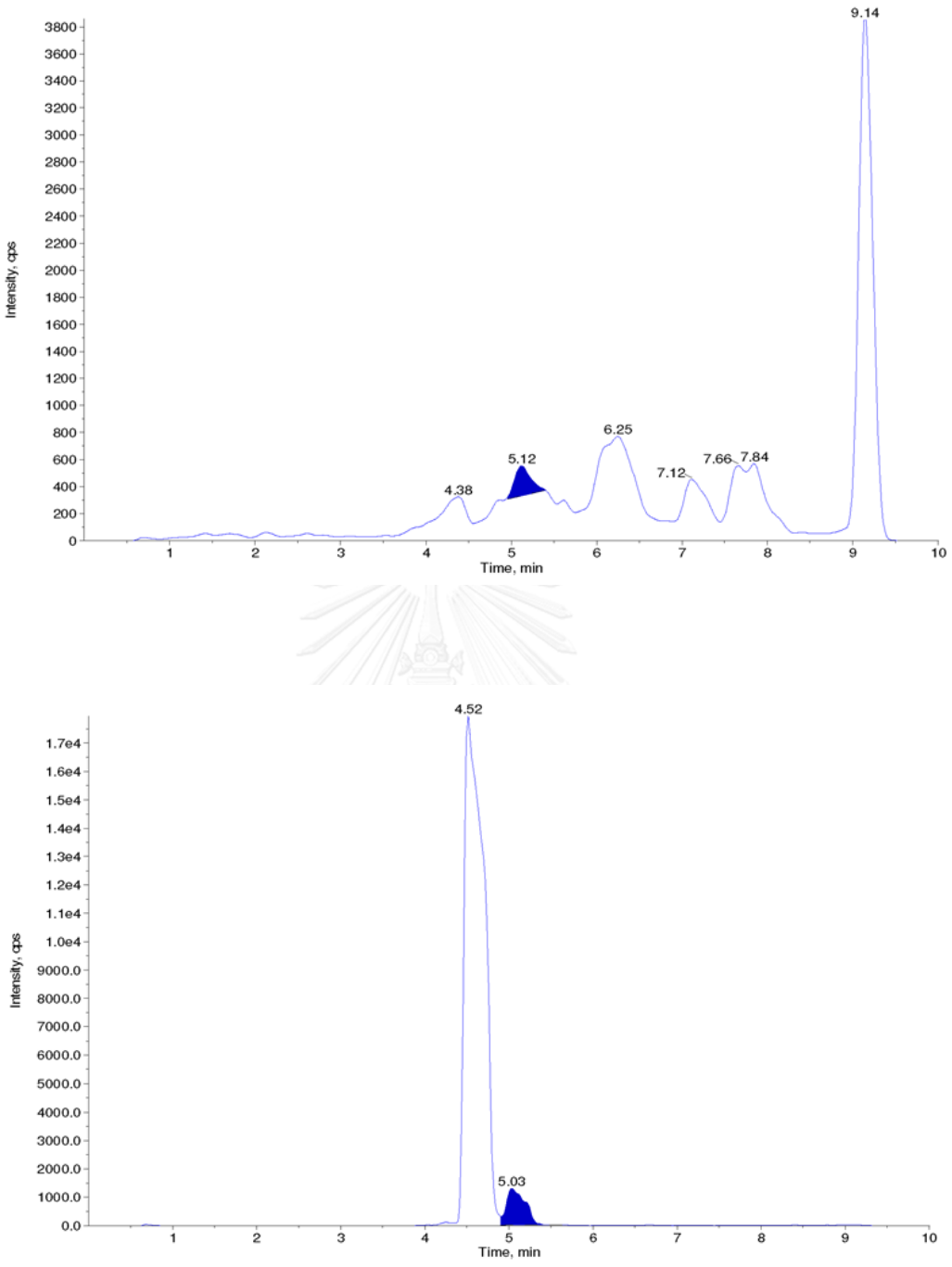
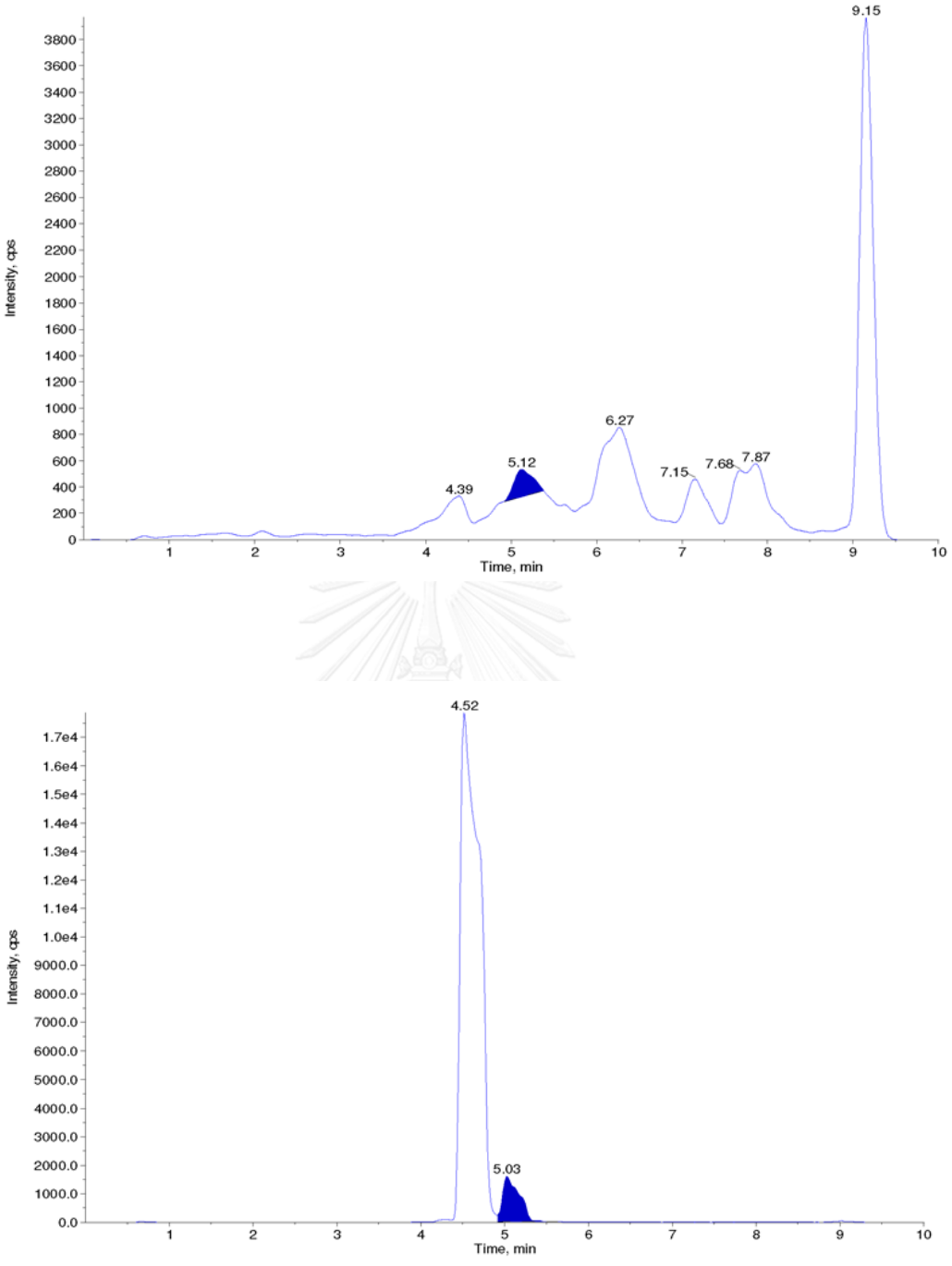


LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the third day after hormone withdrawal.



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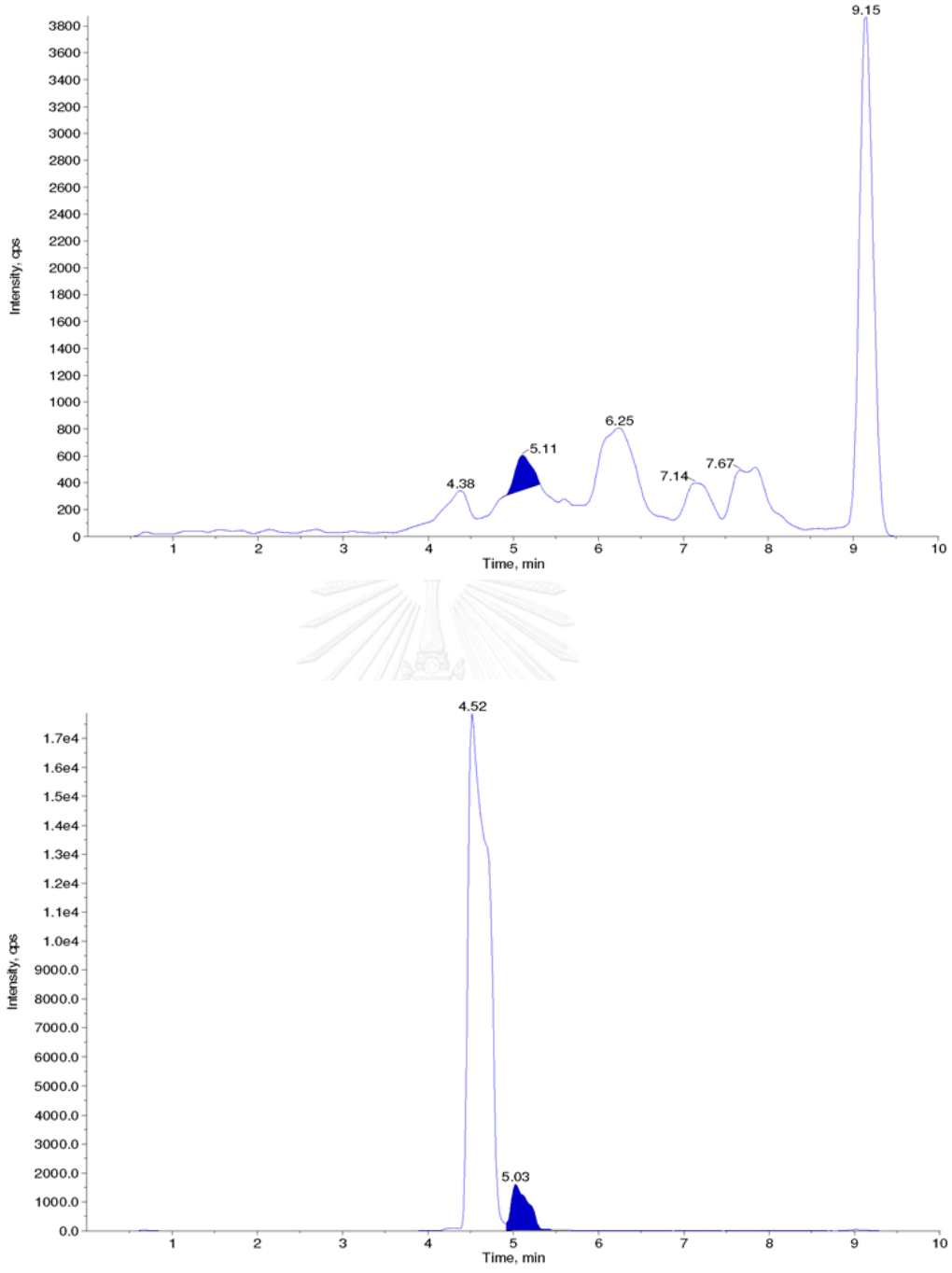

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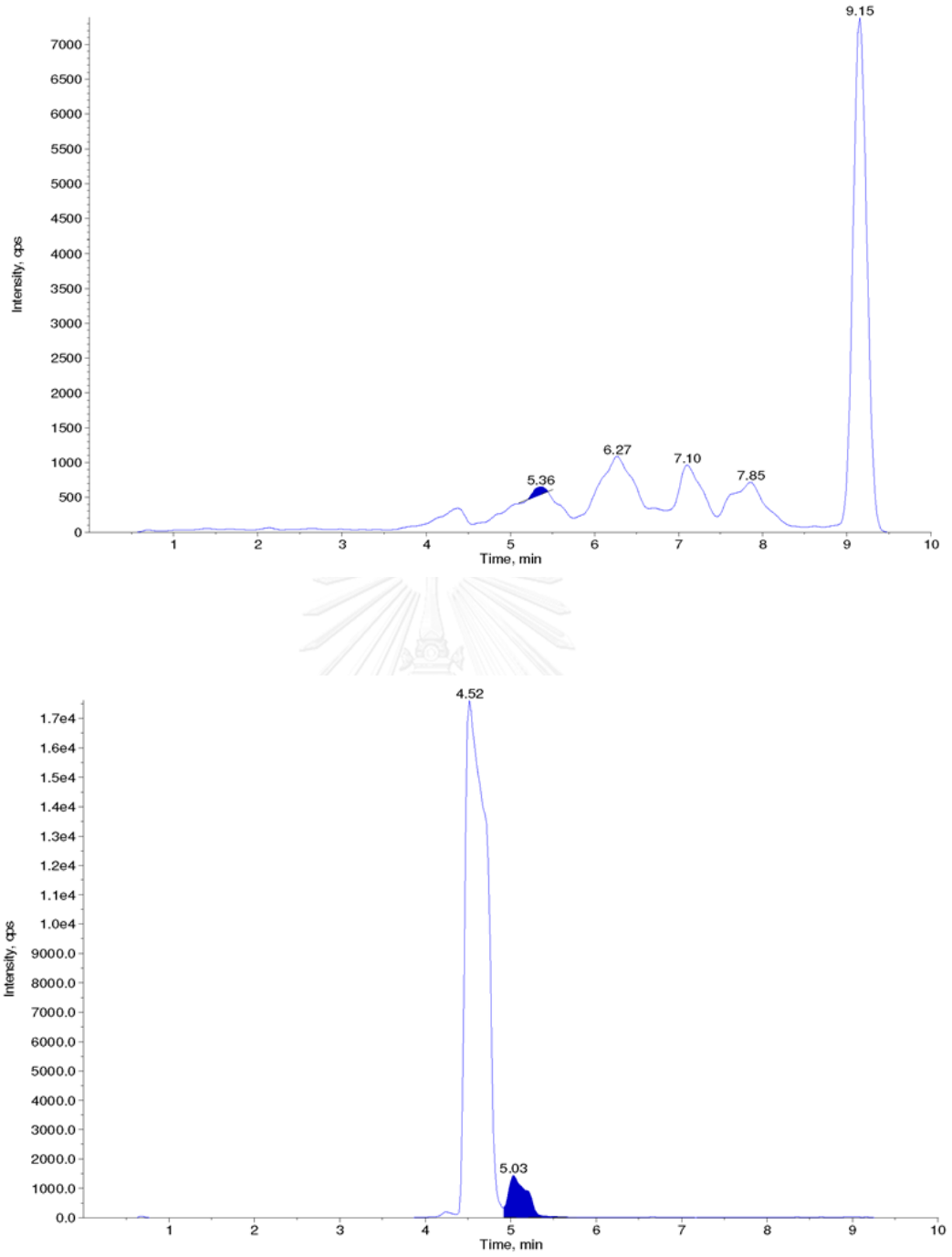
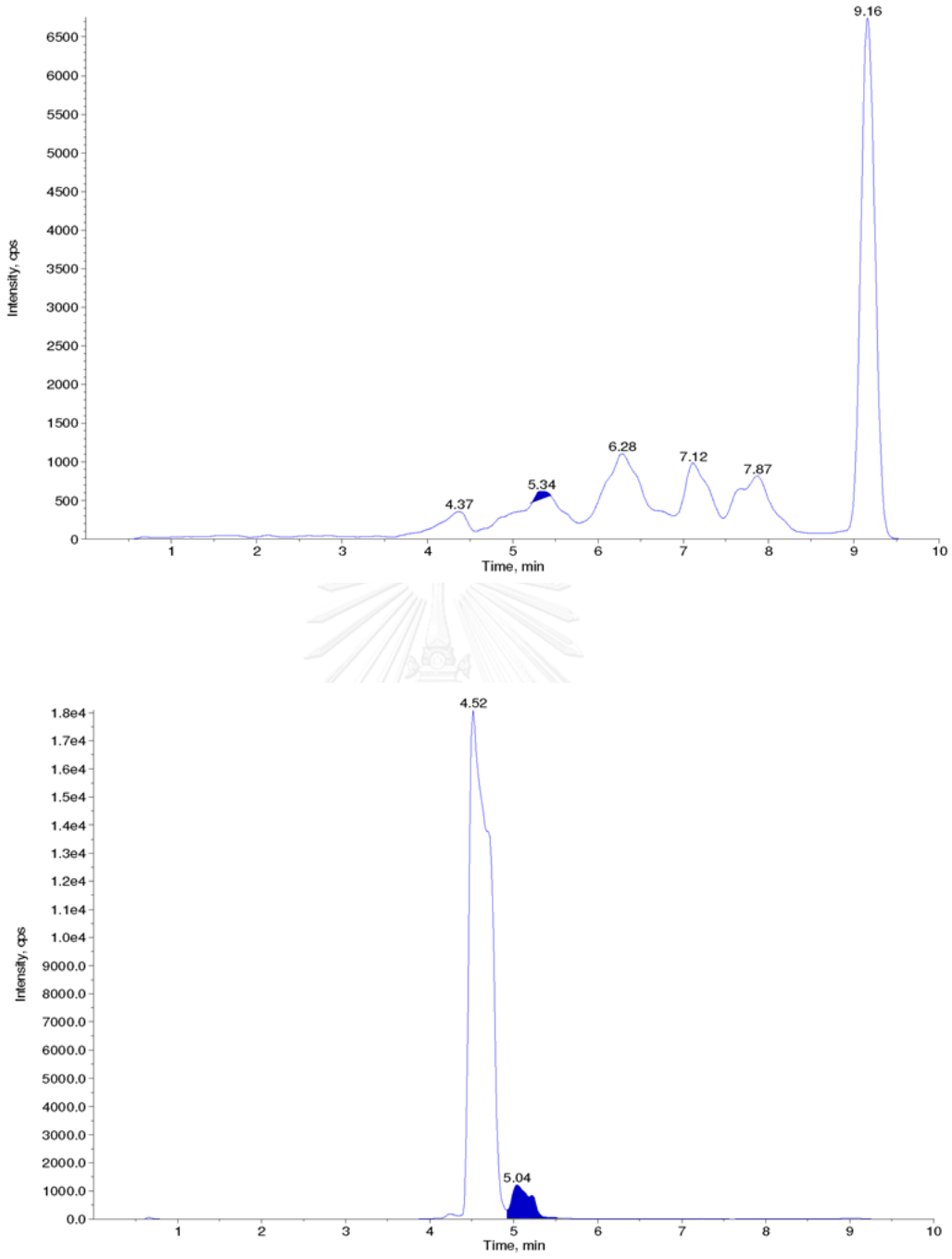
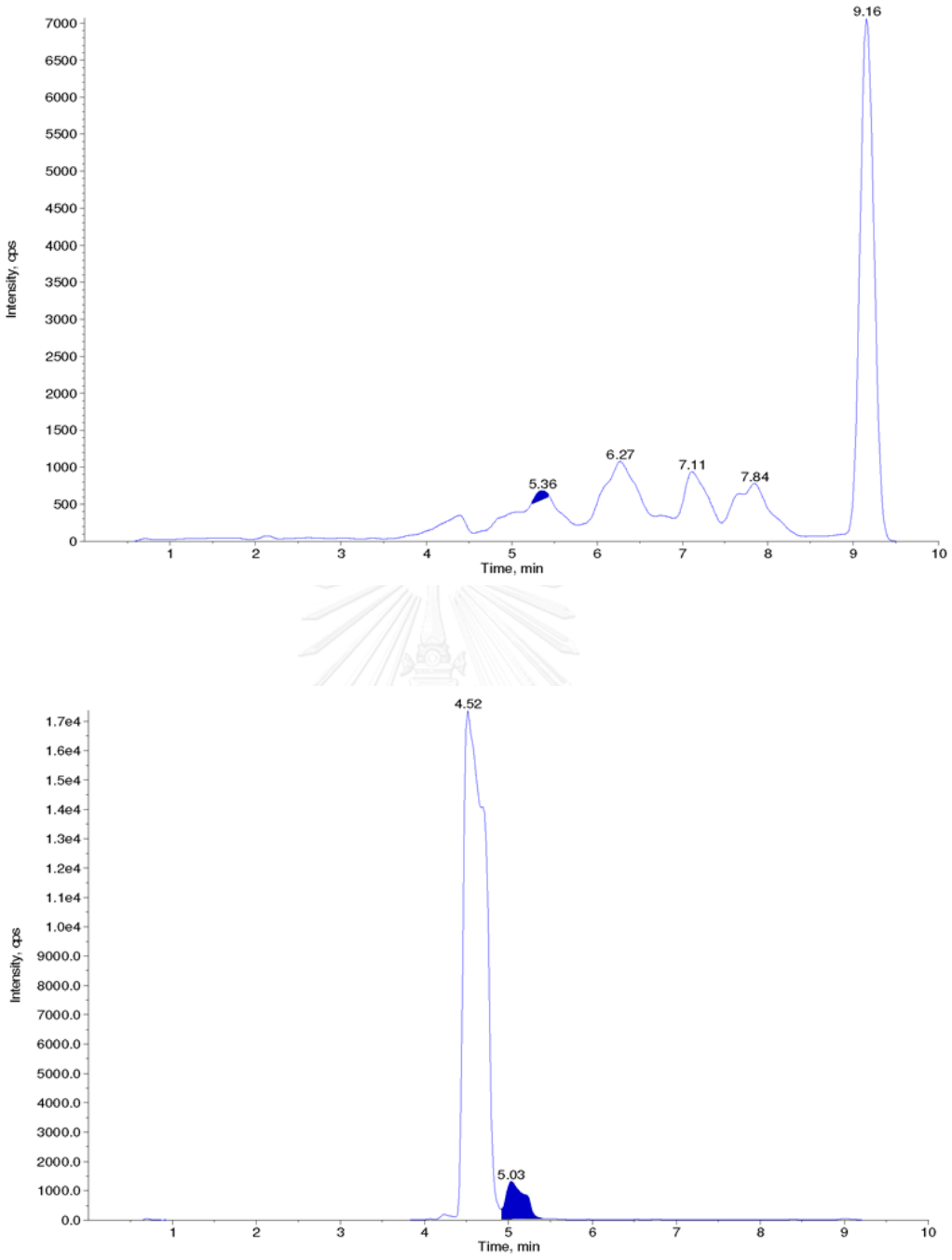


LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the fifth day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 15 days-hormonal treated fry on the fifth day after hormone withdrawal.



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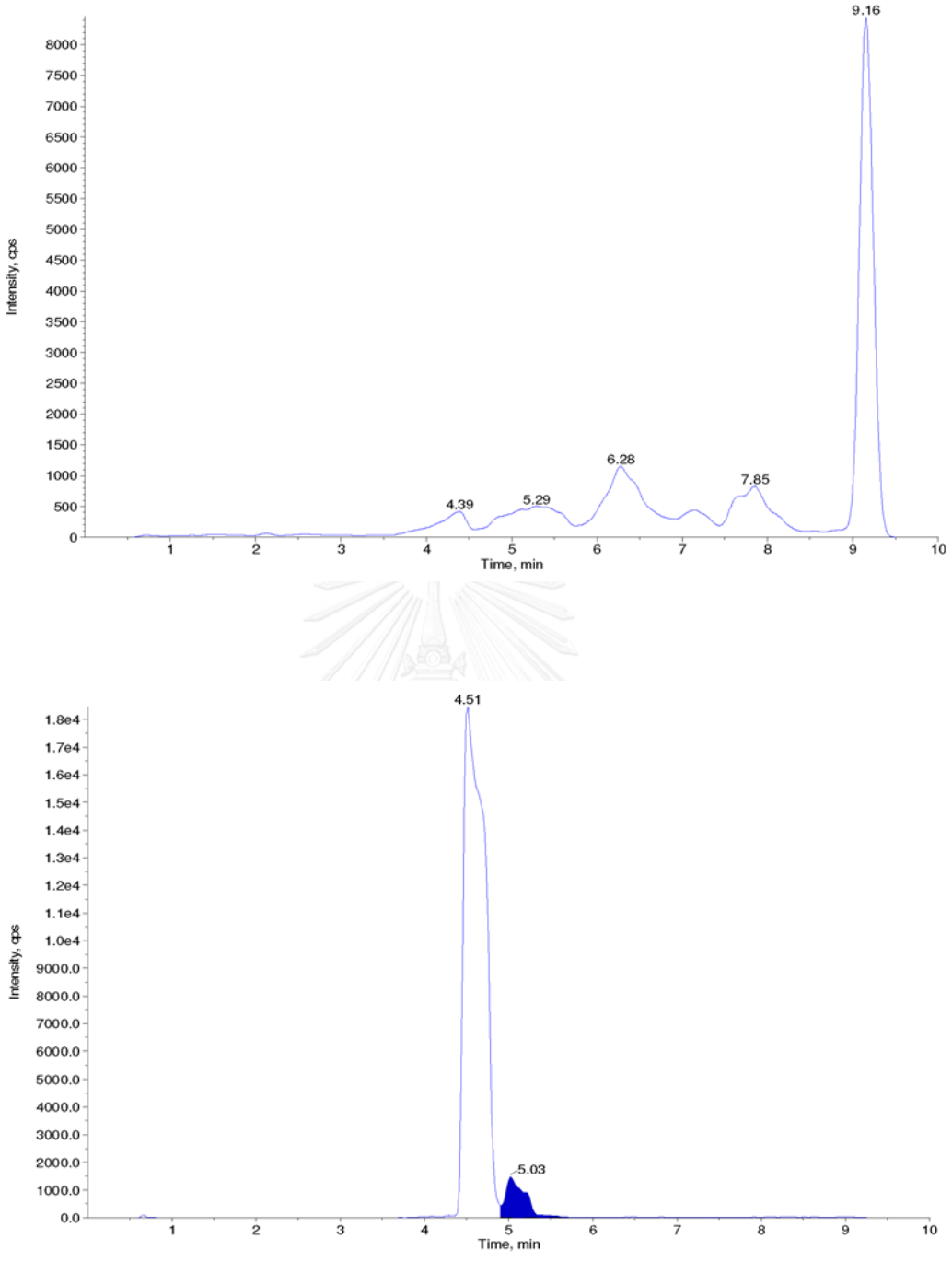
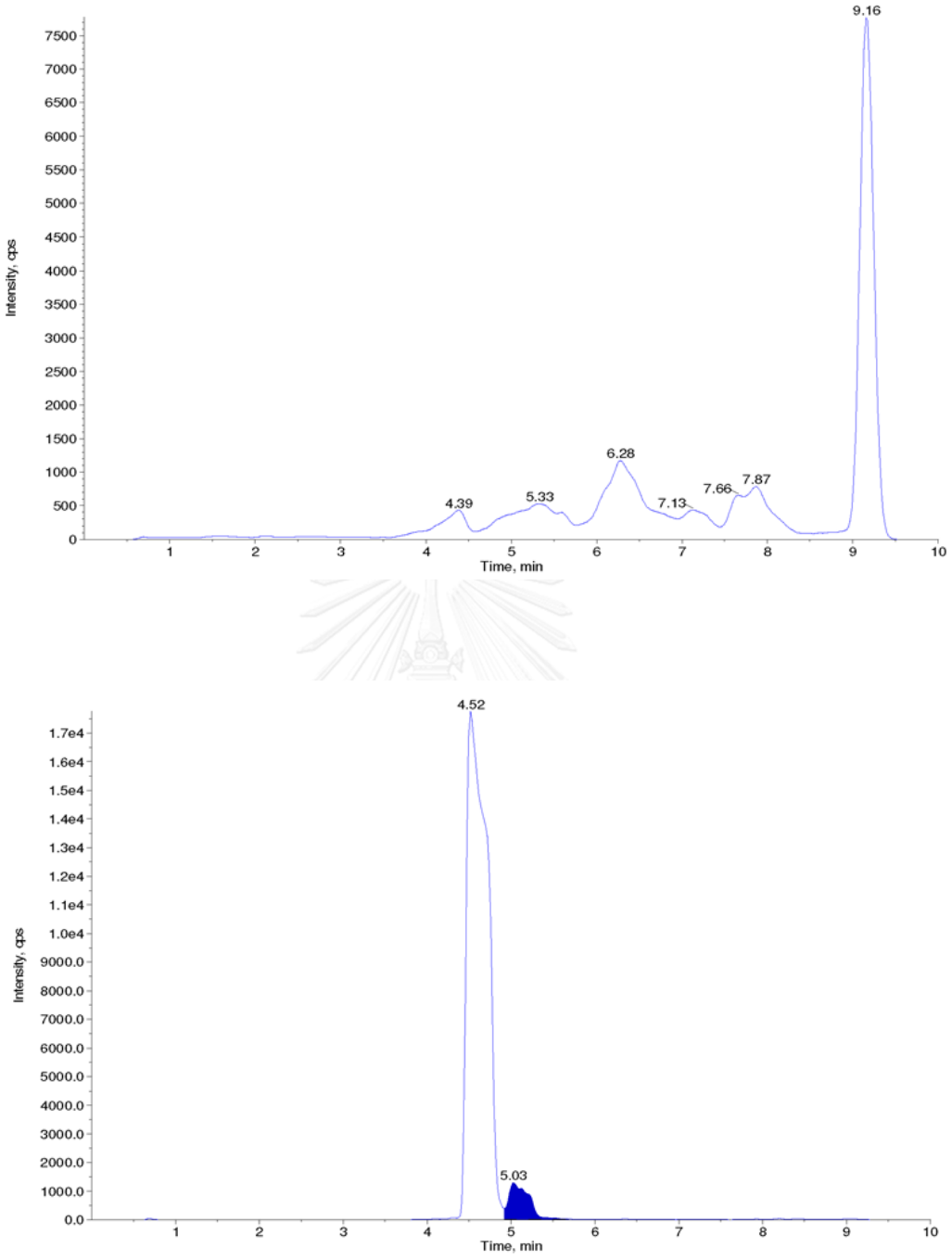


LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the seventh day after hormone withdrawal.



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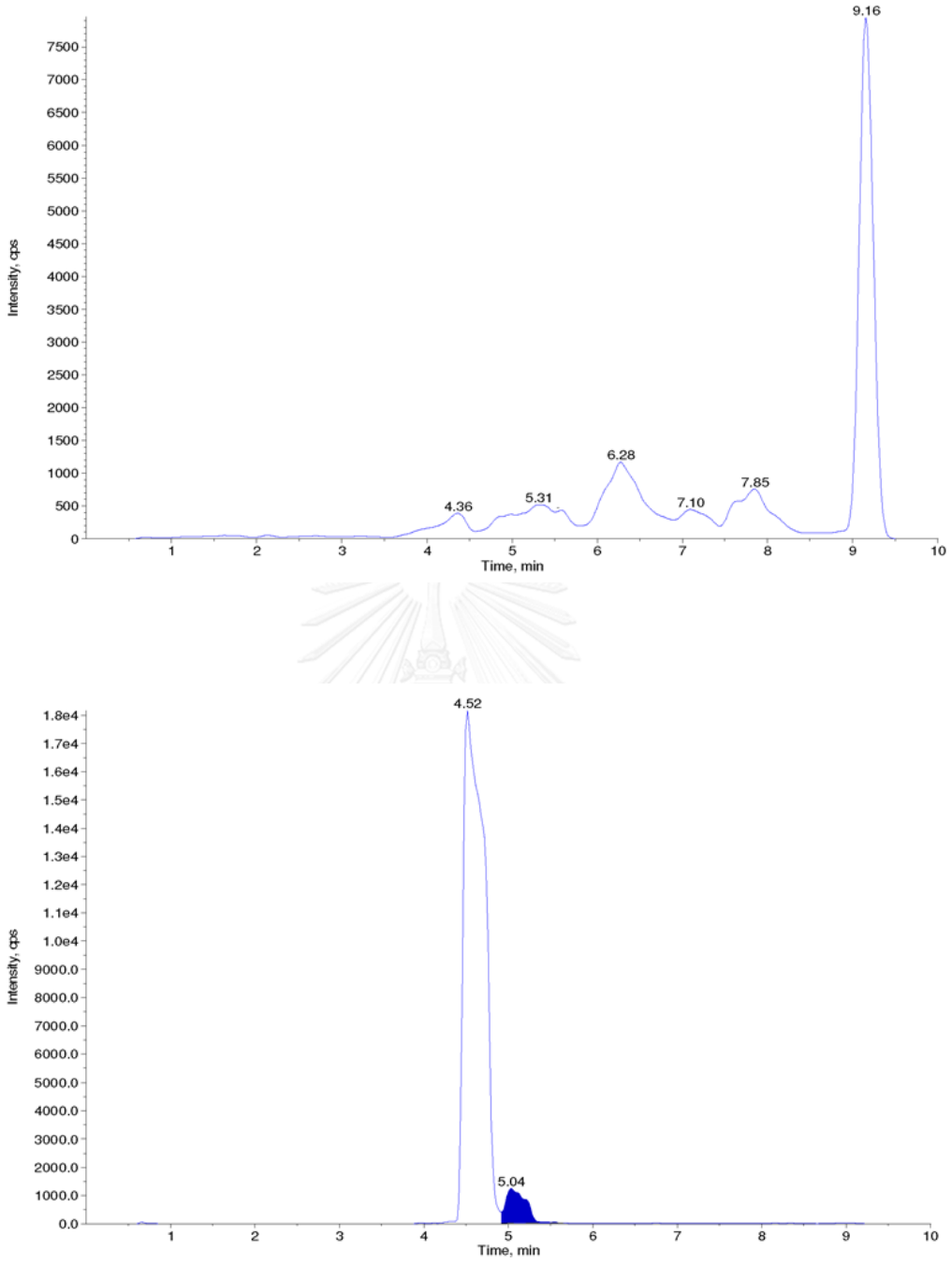

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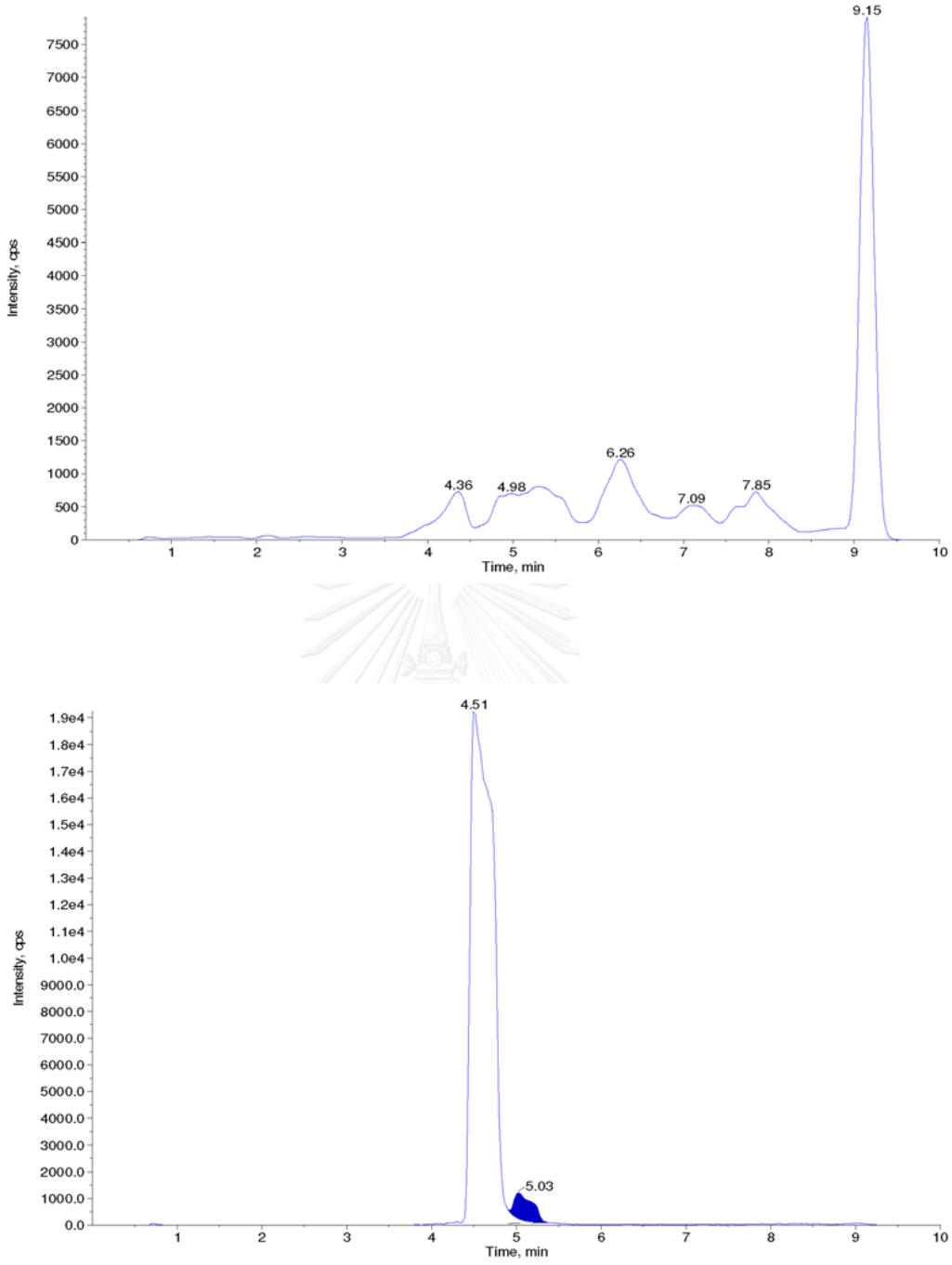

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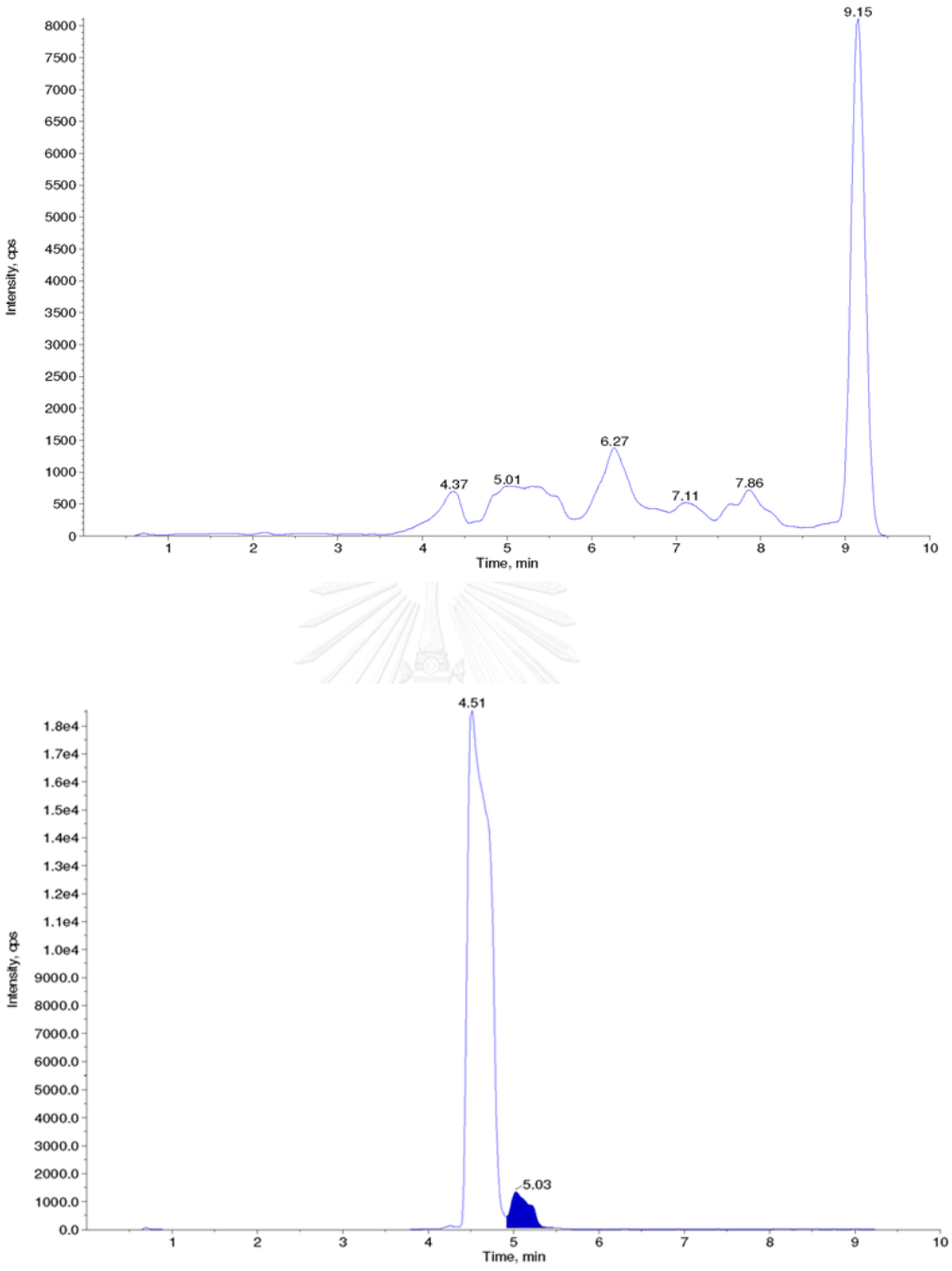

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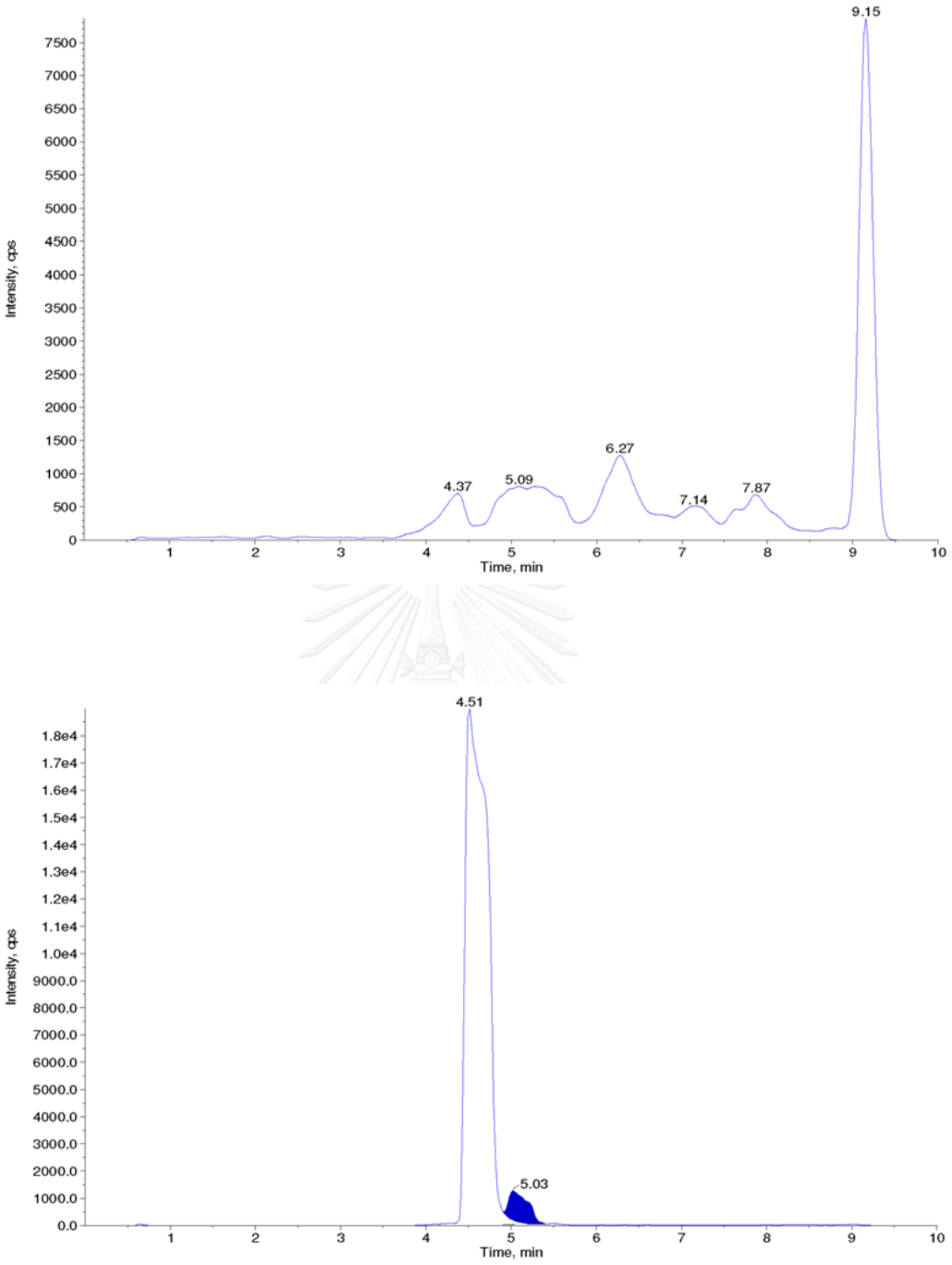
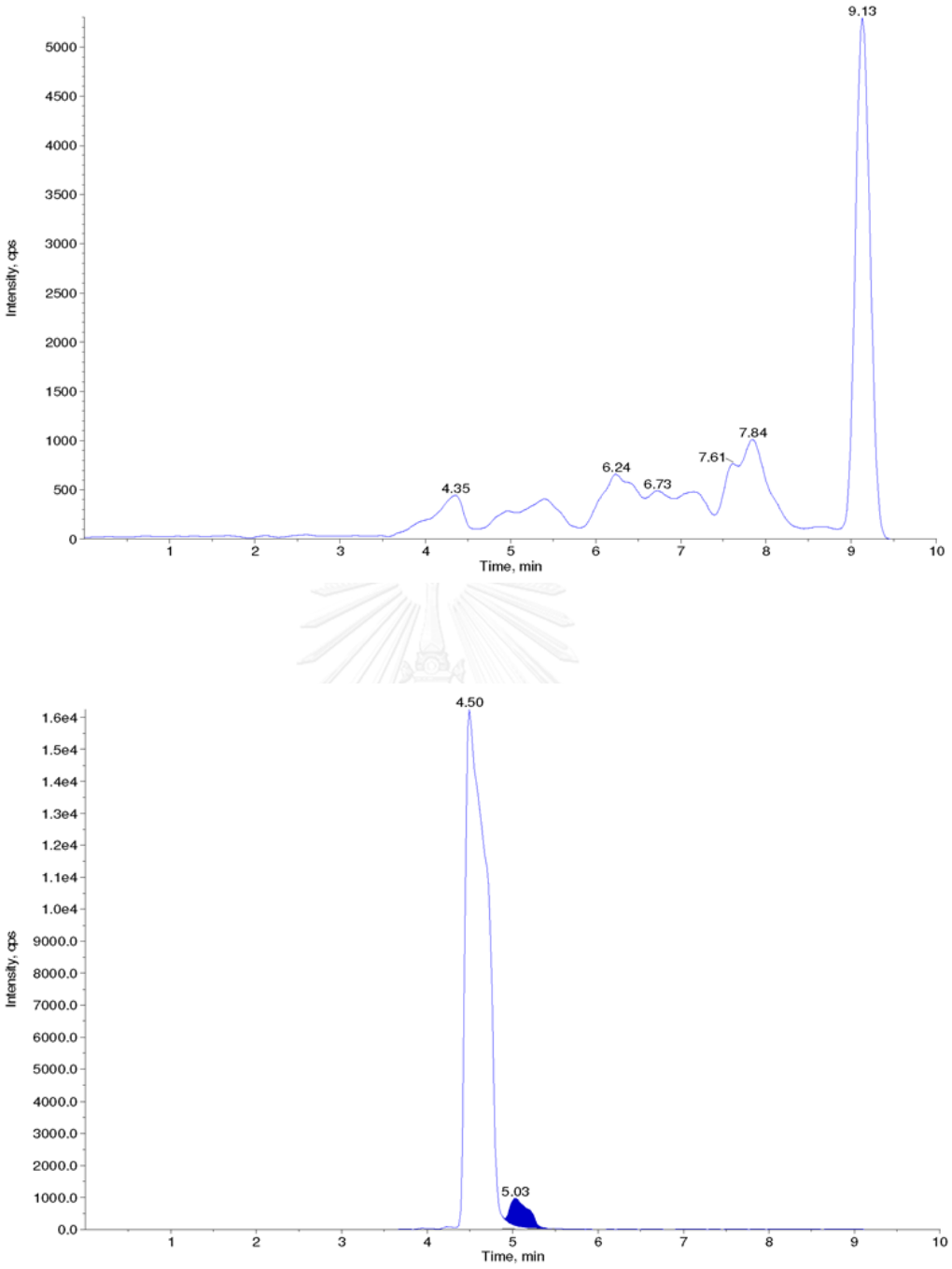
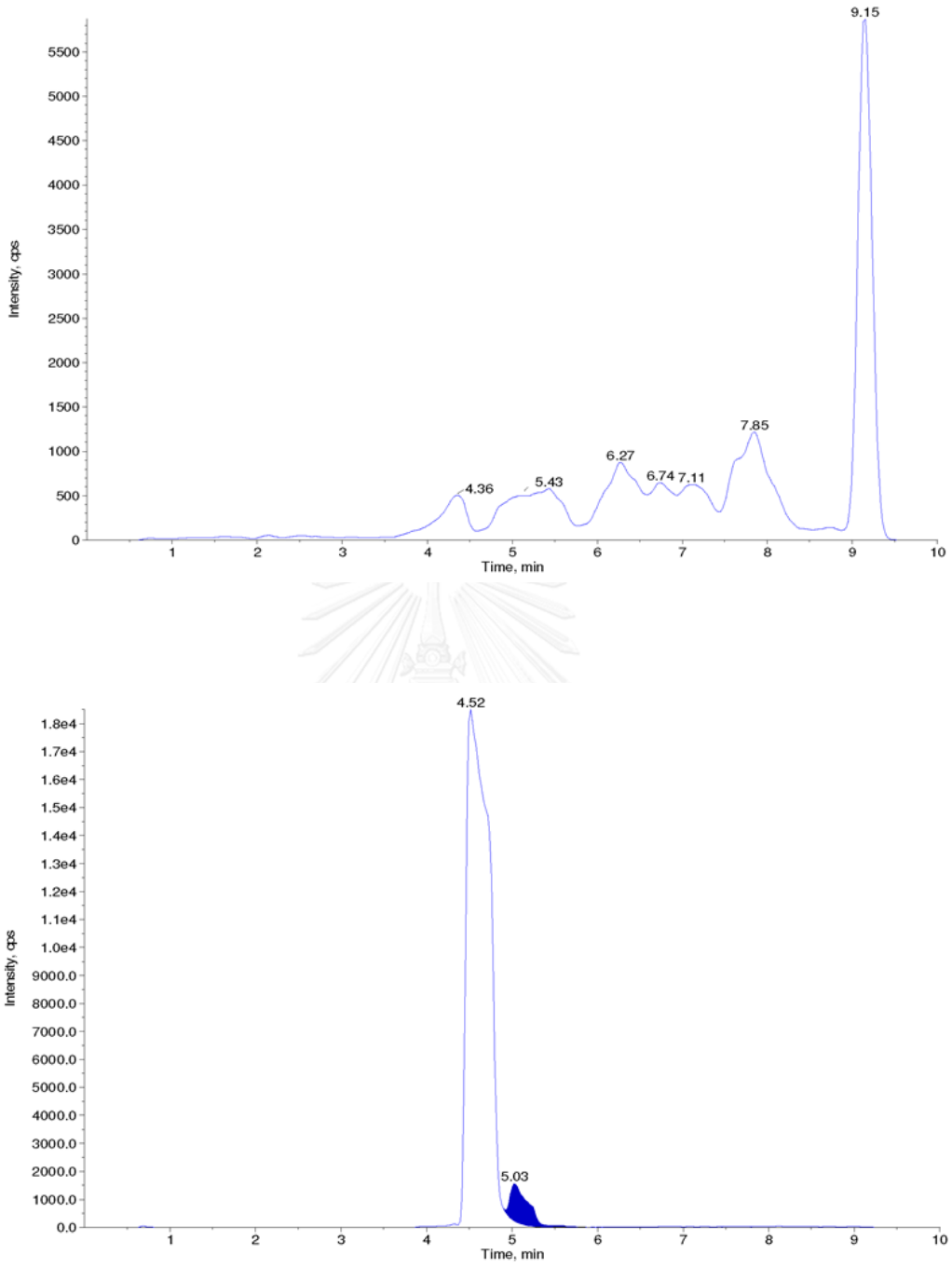


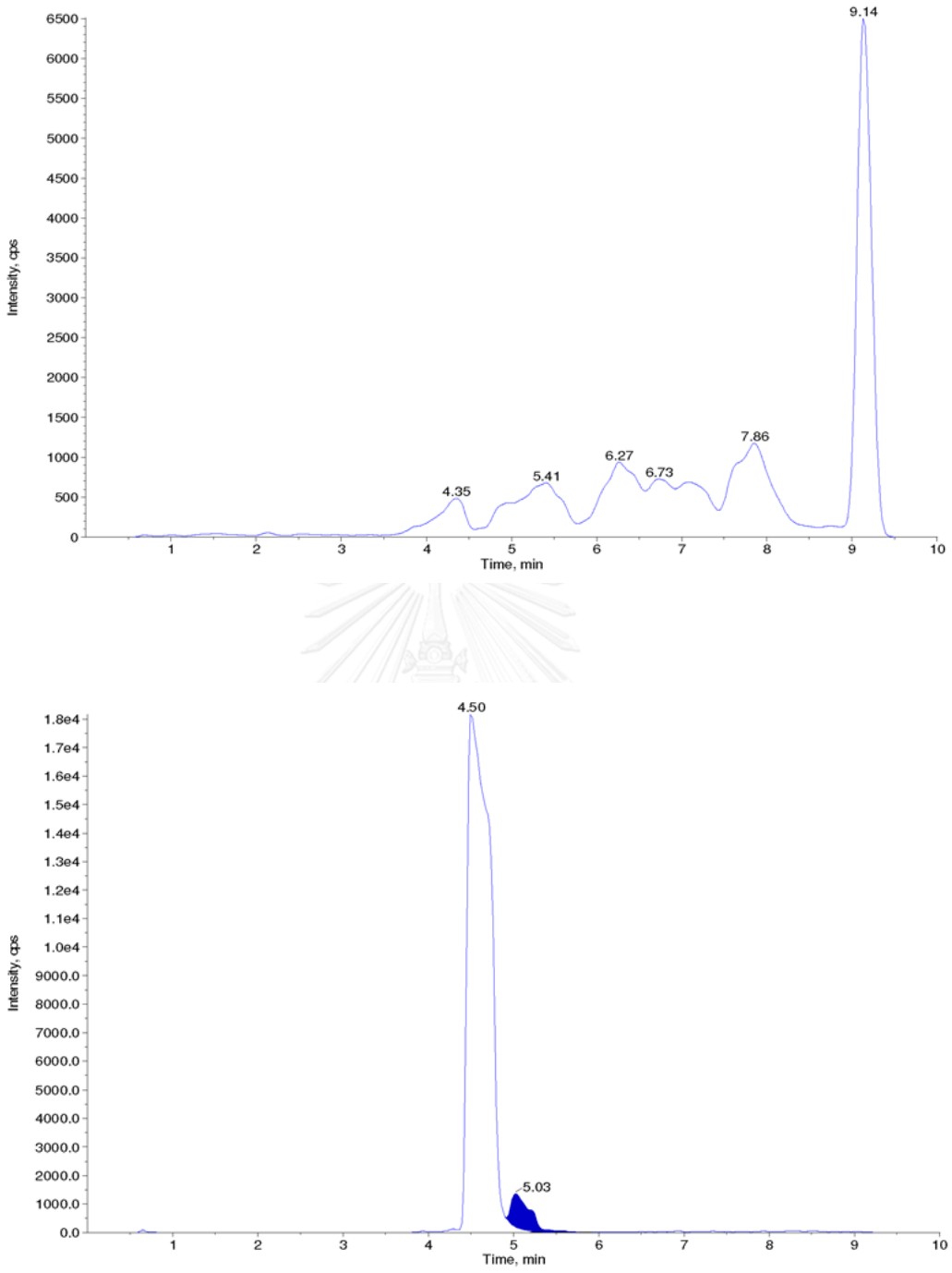
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 15 days-hormonal treated fry on the fourteenth day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 15 days-hormonal treated fry on the twenty first day after hormone withdrawal.

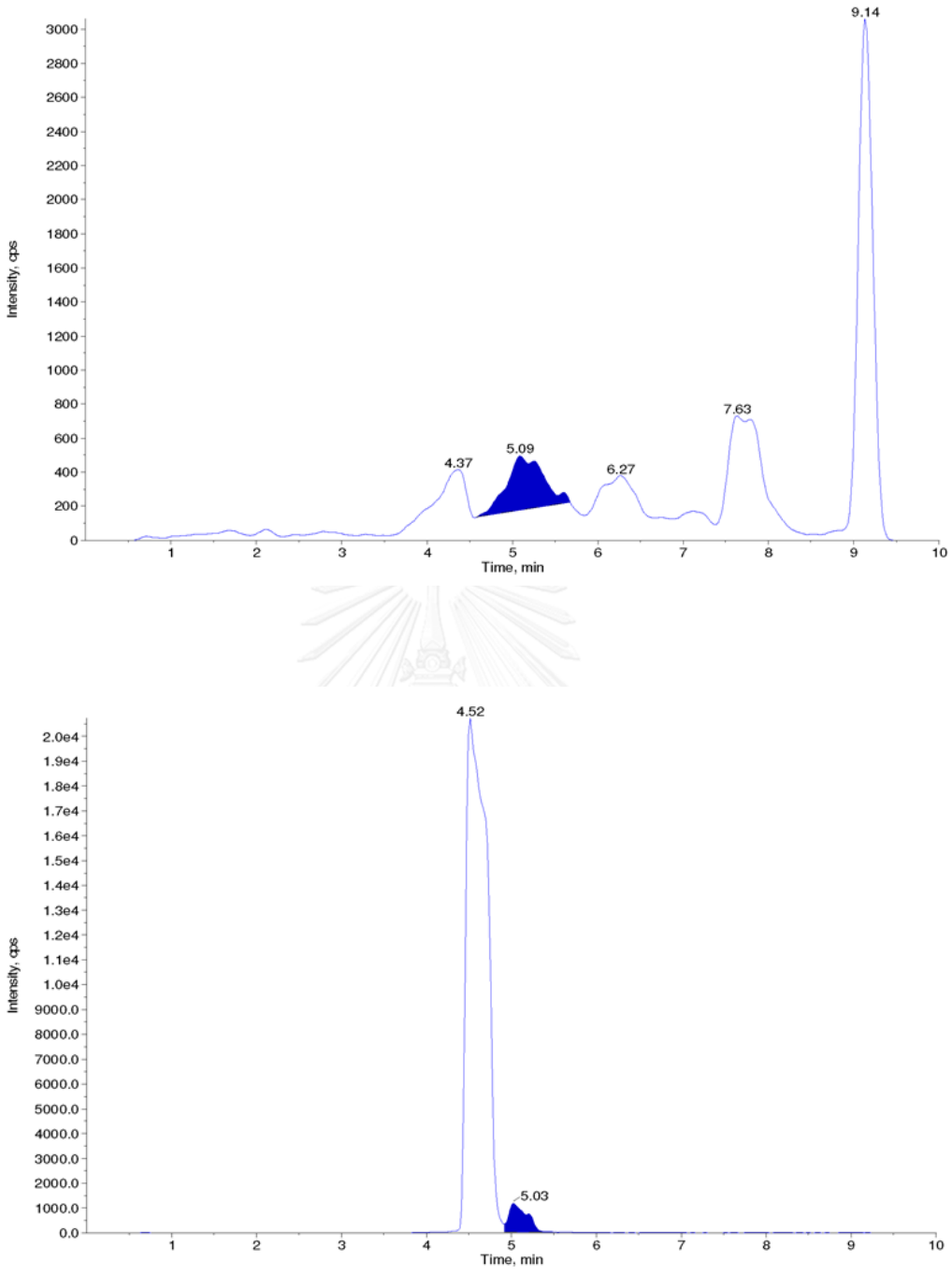


LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 15 days-hormonal treated fry on the twenty first day after hormone withdrawal.

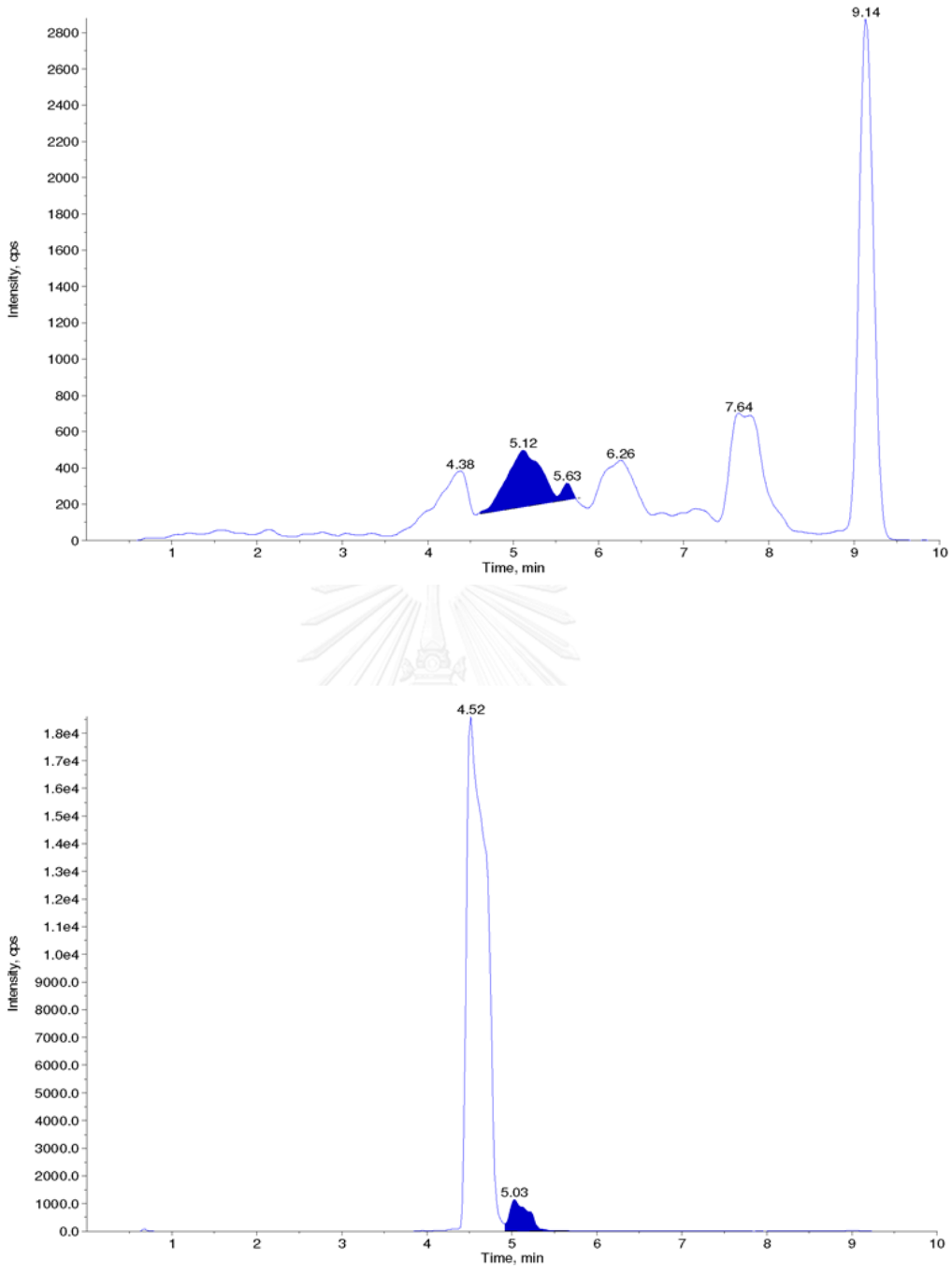


LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 15 days-hormonal treated fry on the twenty first day after hormone withdrawal.

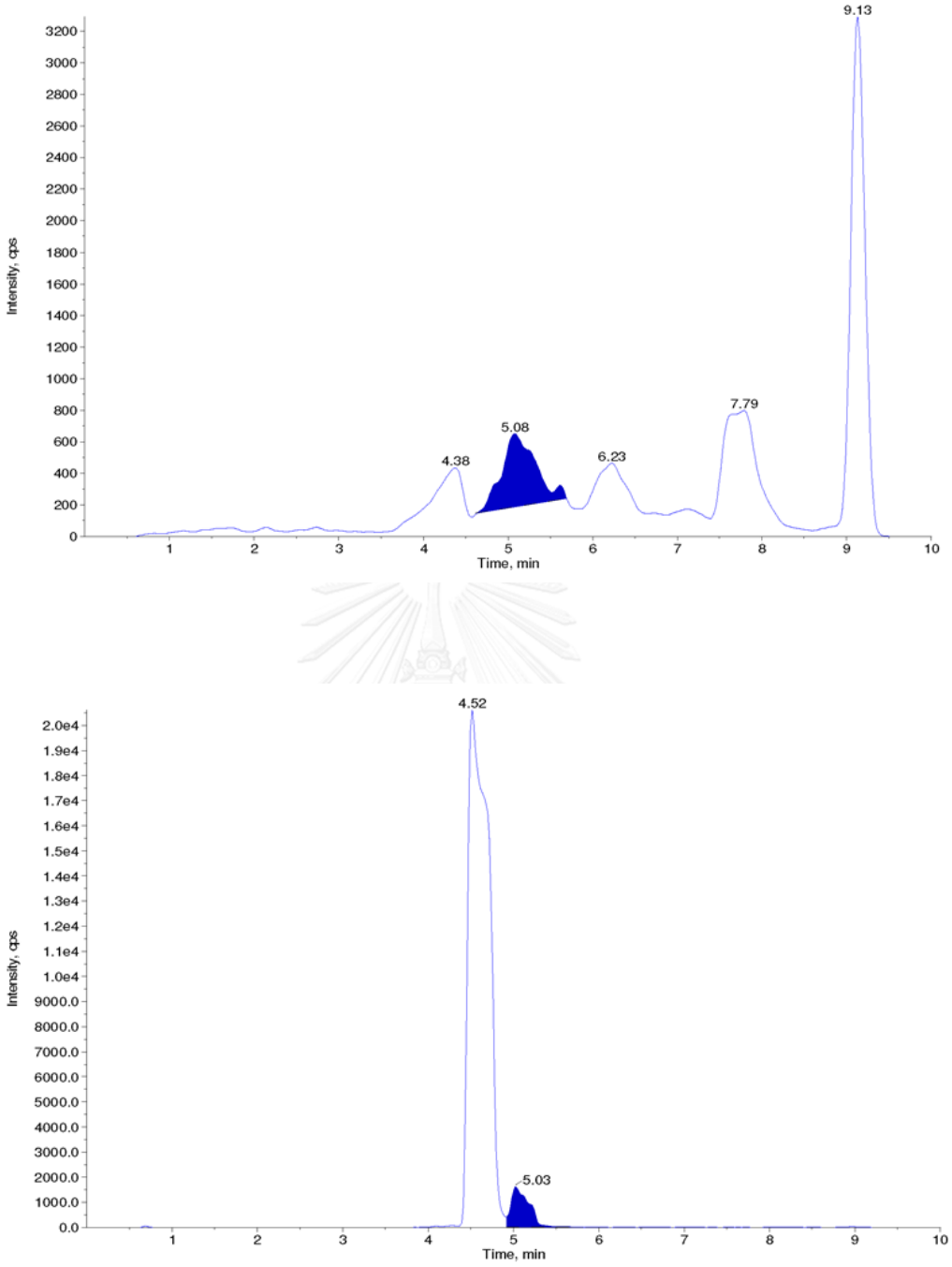
APPENDIX C LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride from 23 days-hormonal treated fry



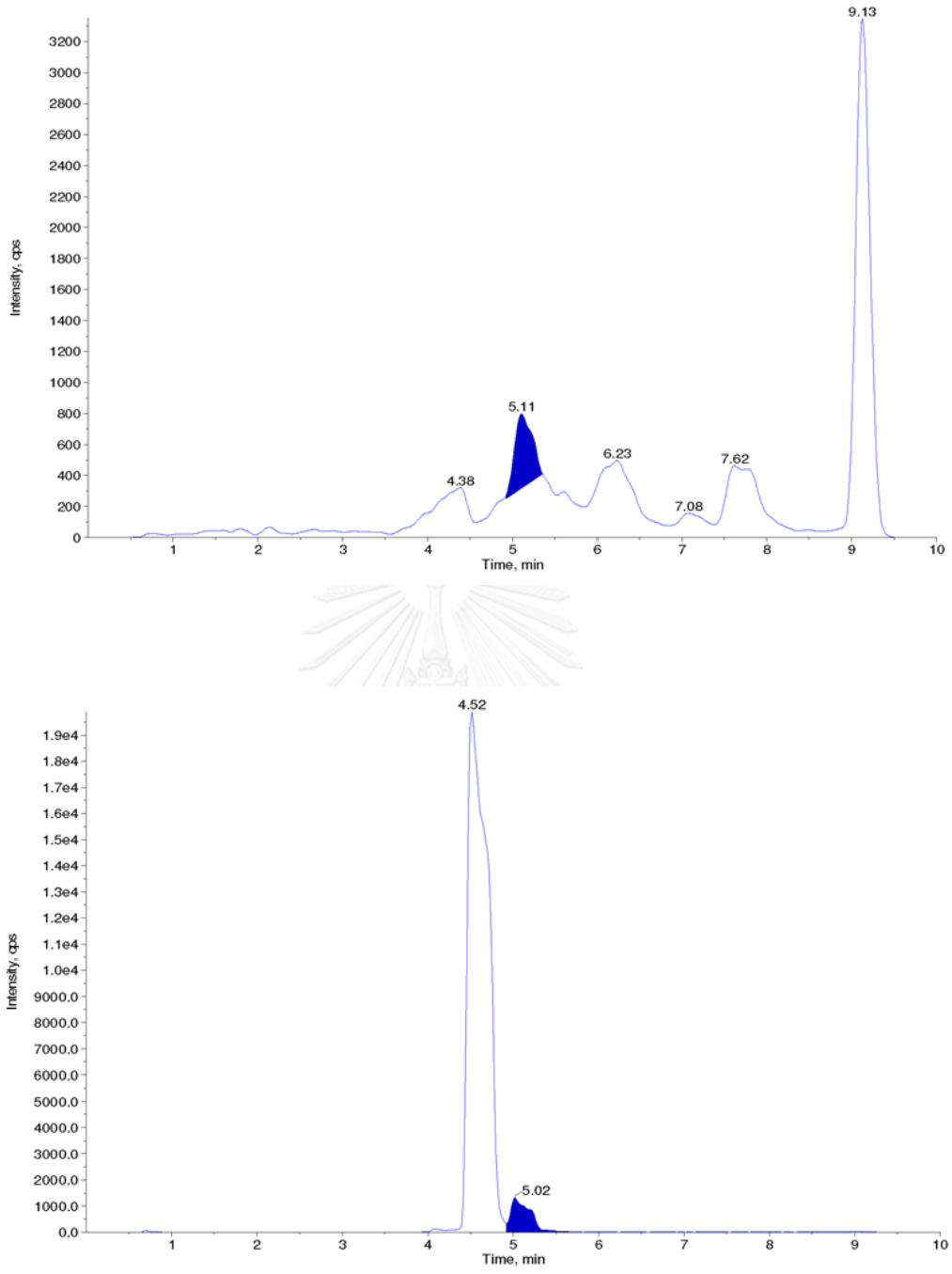
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the first day after hormone withdrawal.



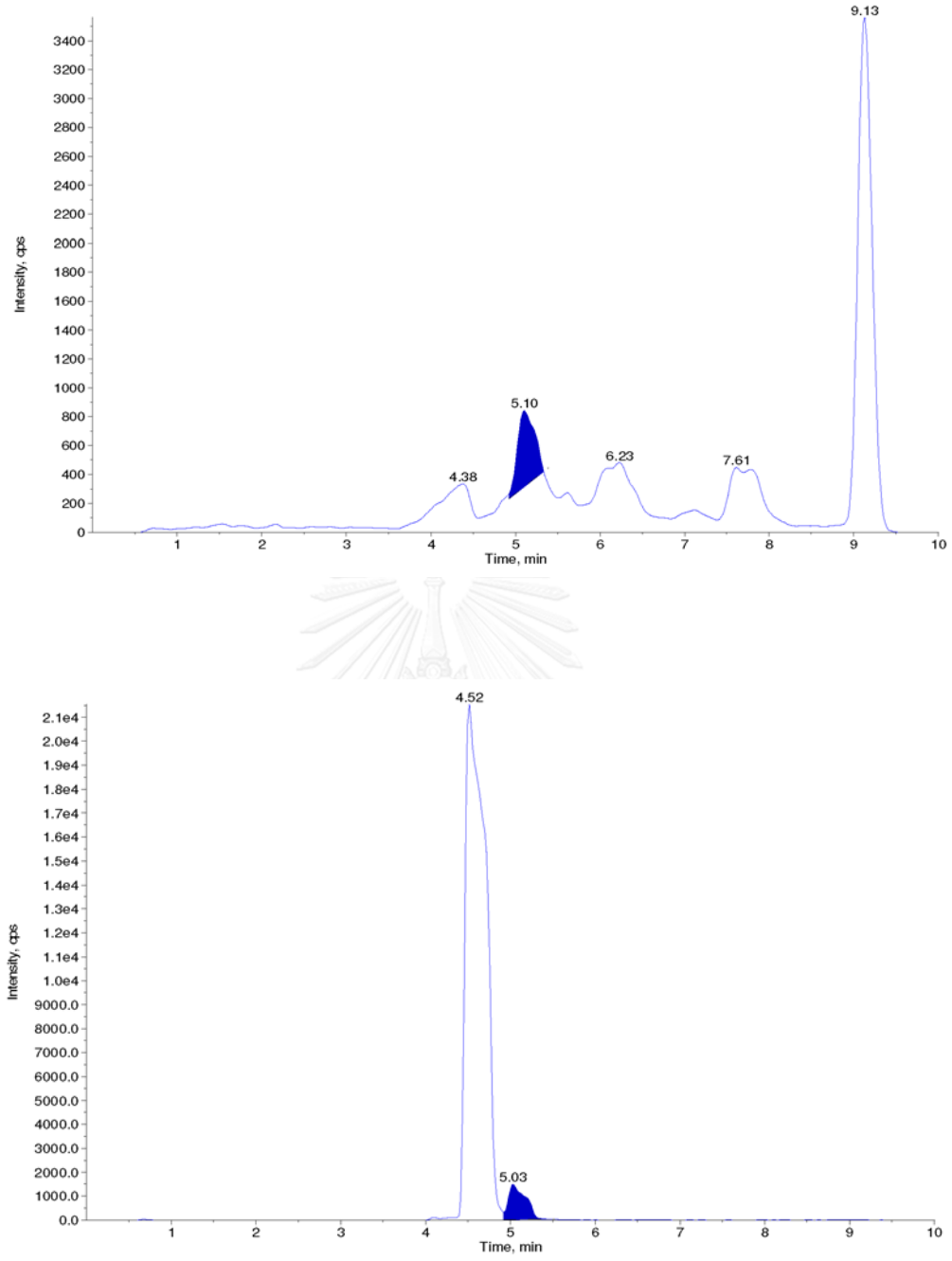
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the first day after hormone withdrawal.



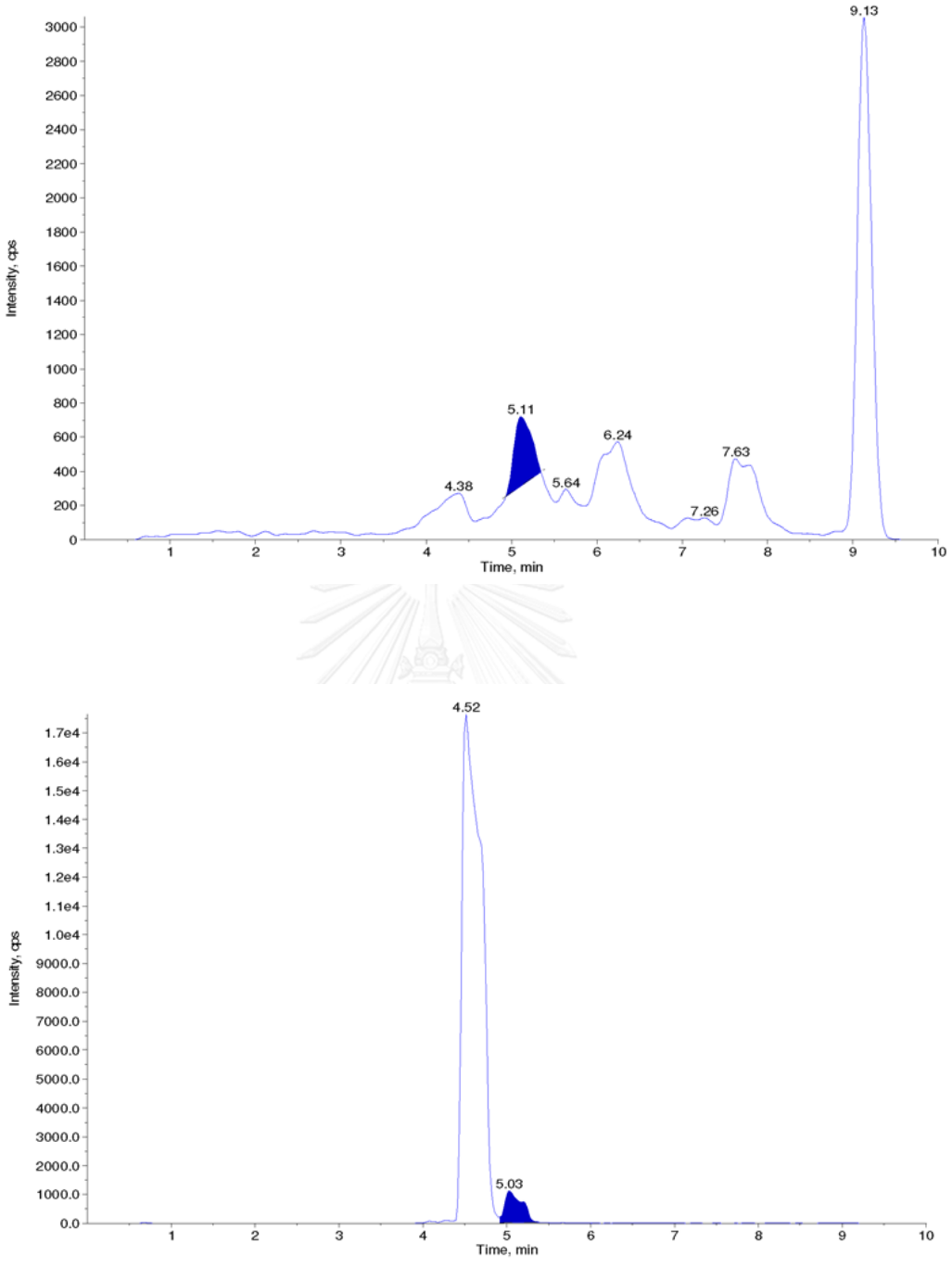
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the first day after hormone withdrawal.



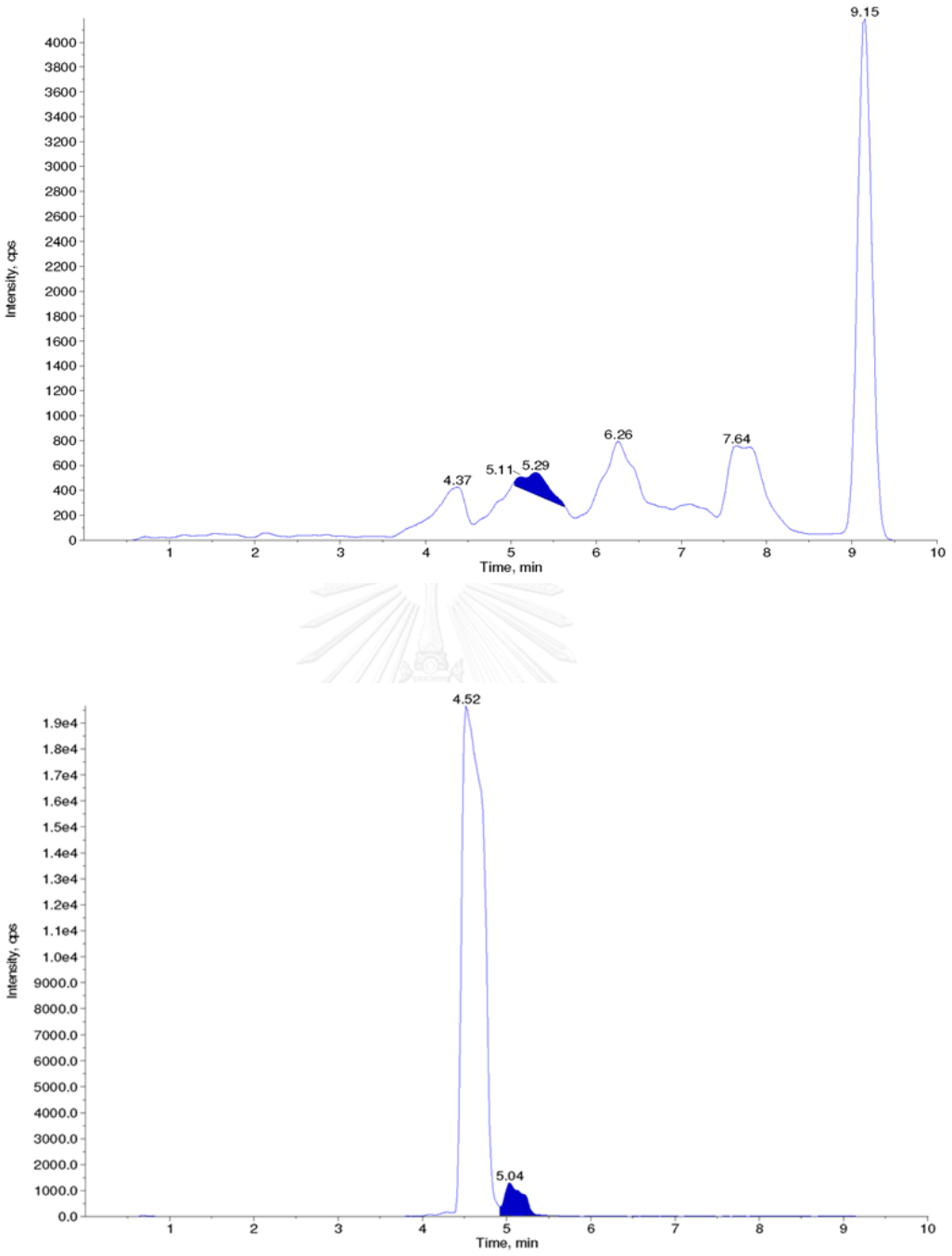
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the second day after hormone withdrawal.



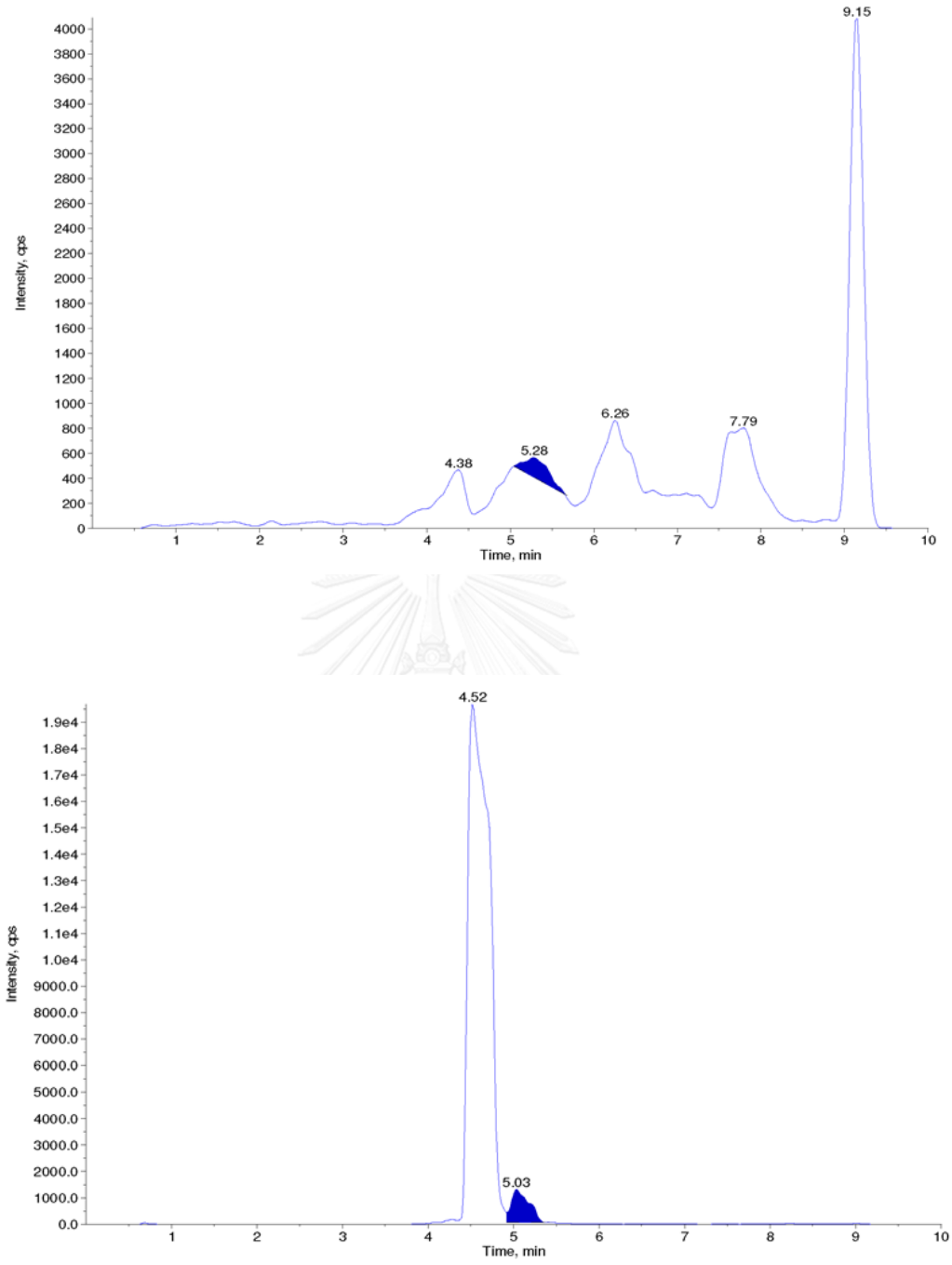
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the second day after hormone withdrawal.



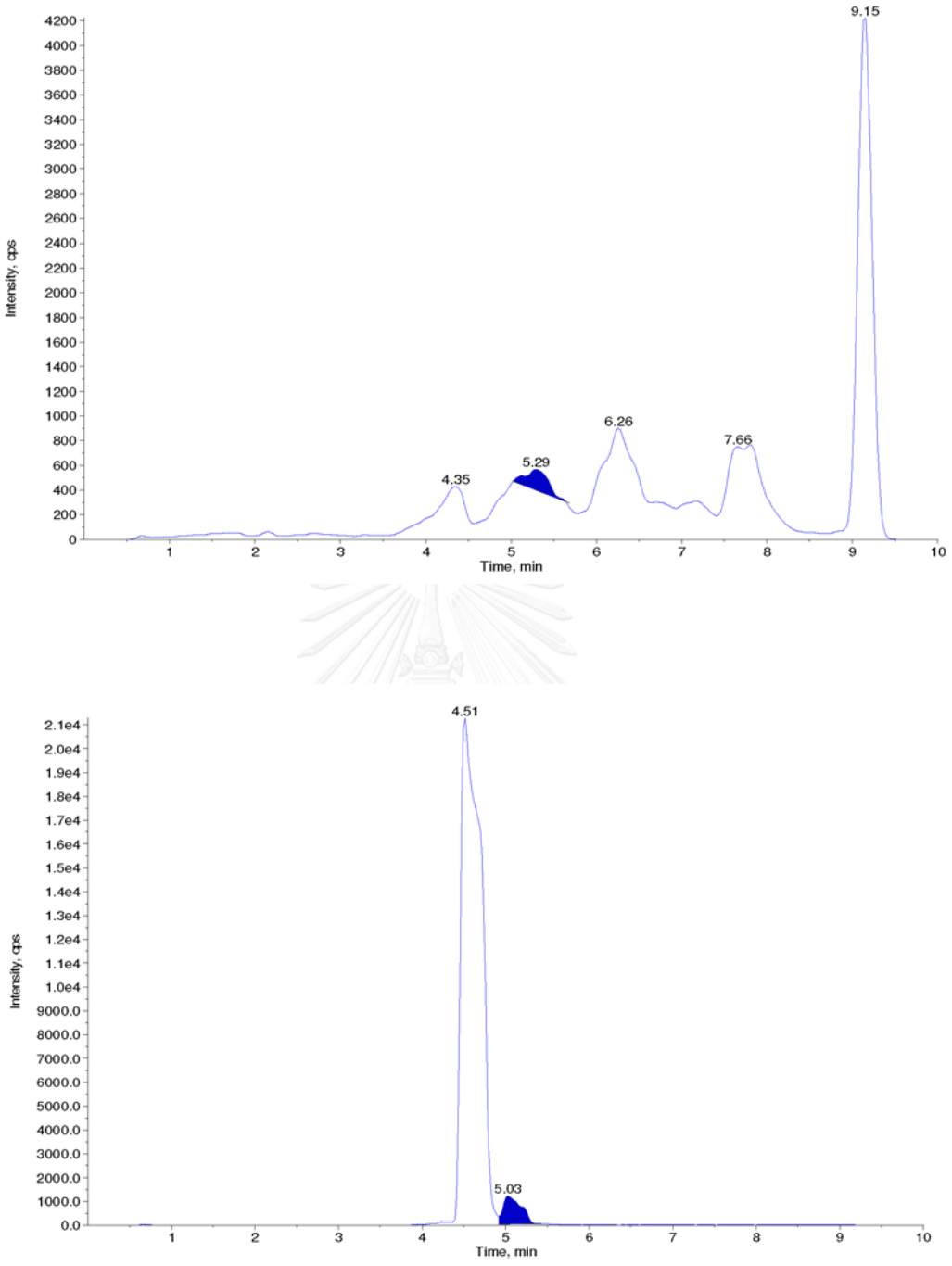
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the second day after hormone withdrawal.



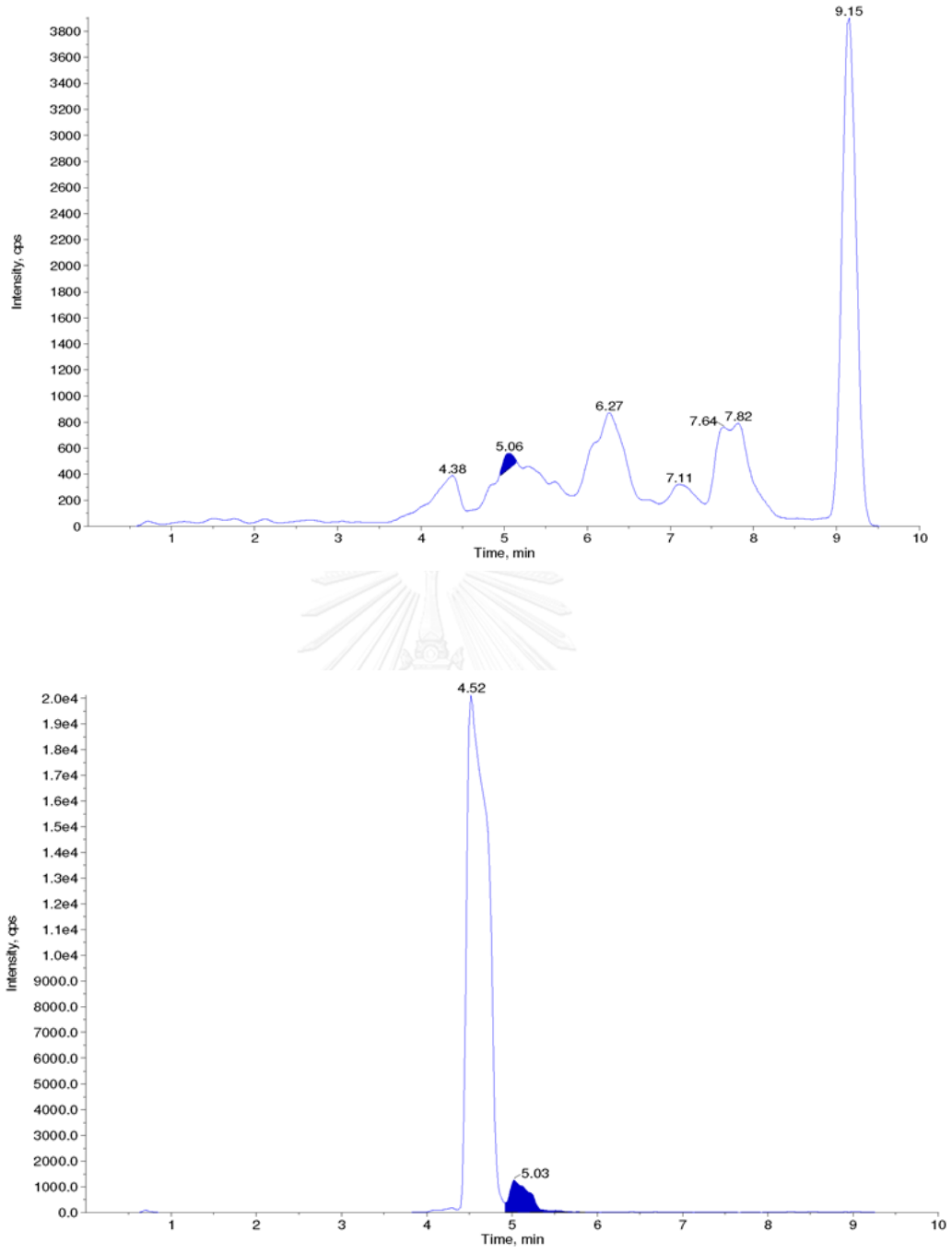
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the third day after hormone withdrawal.



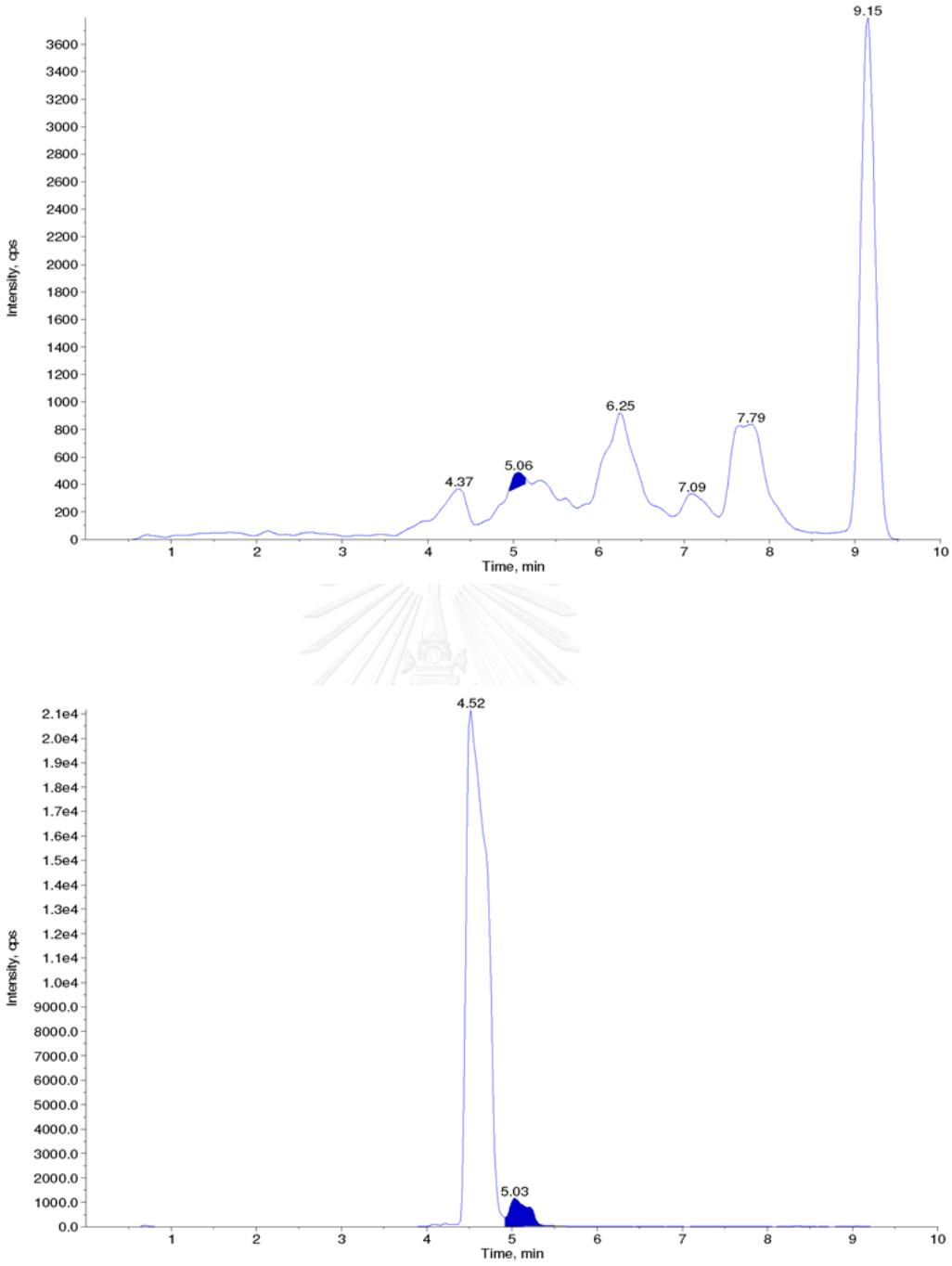
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the third day after hormone withdrawal.



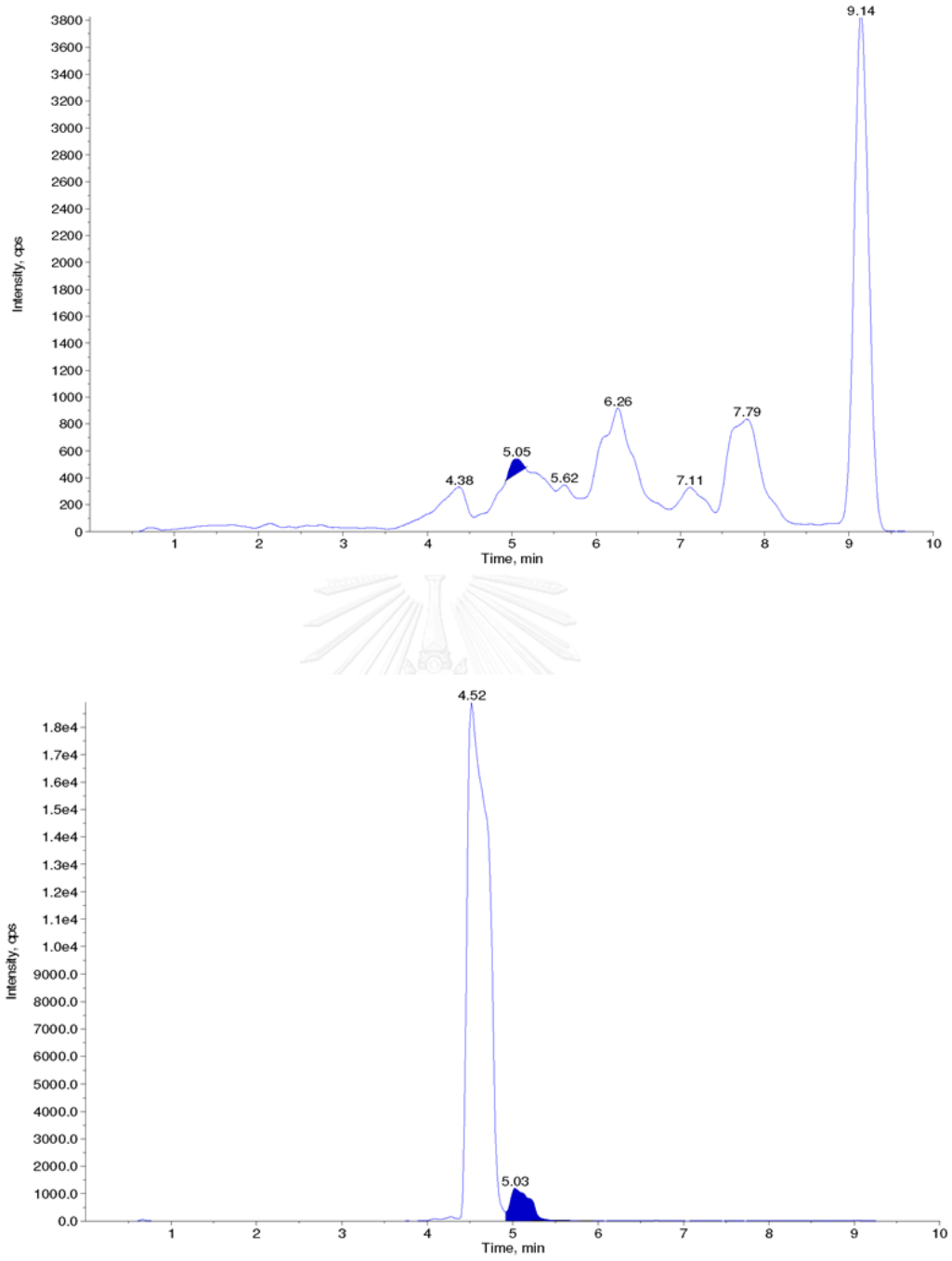
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the third day after hormone withdrawal.



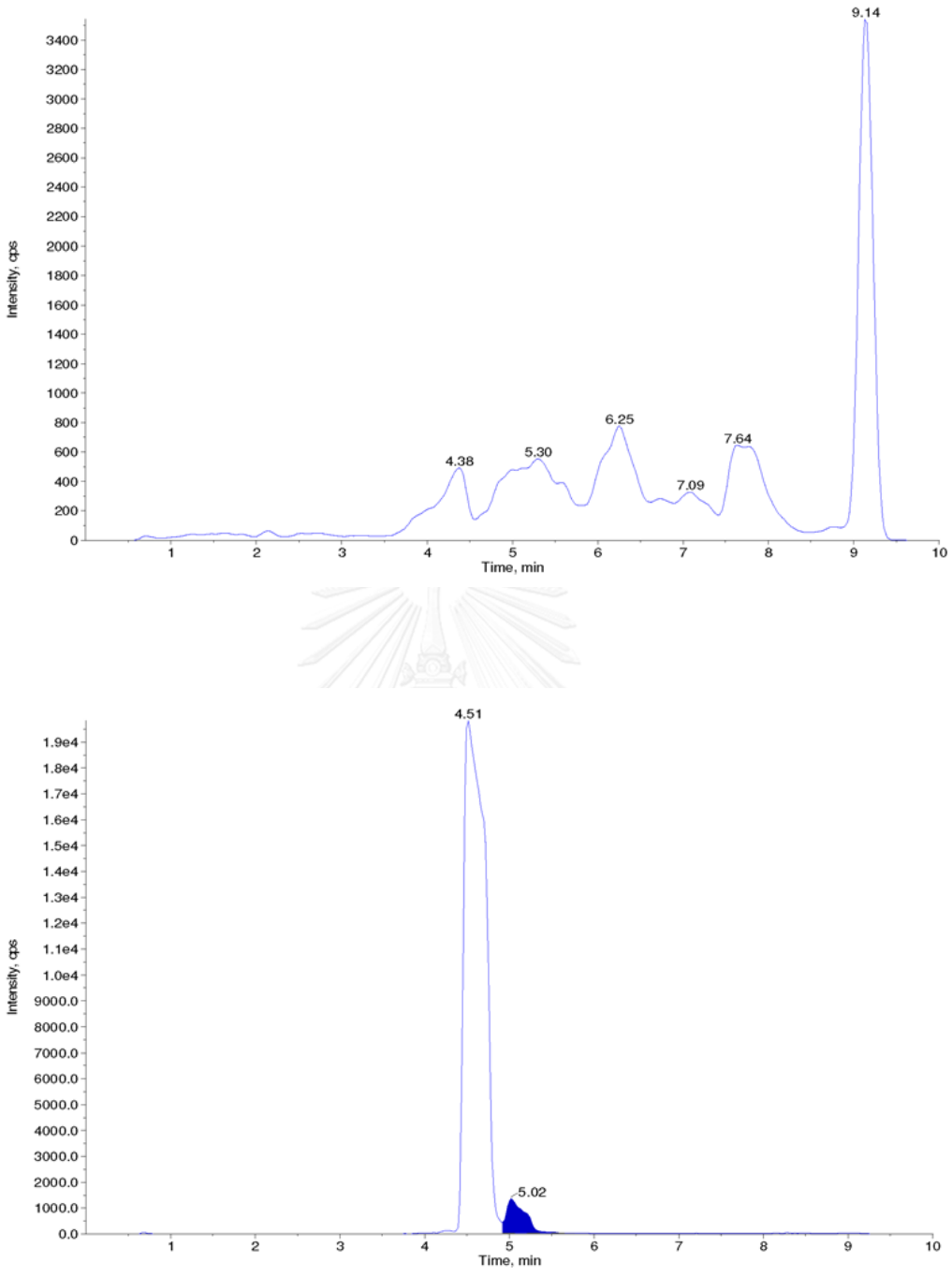
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the fifth day after hormone withdrawal.



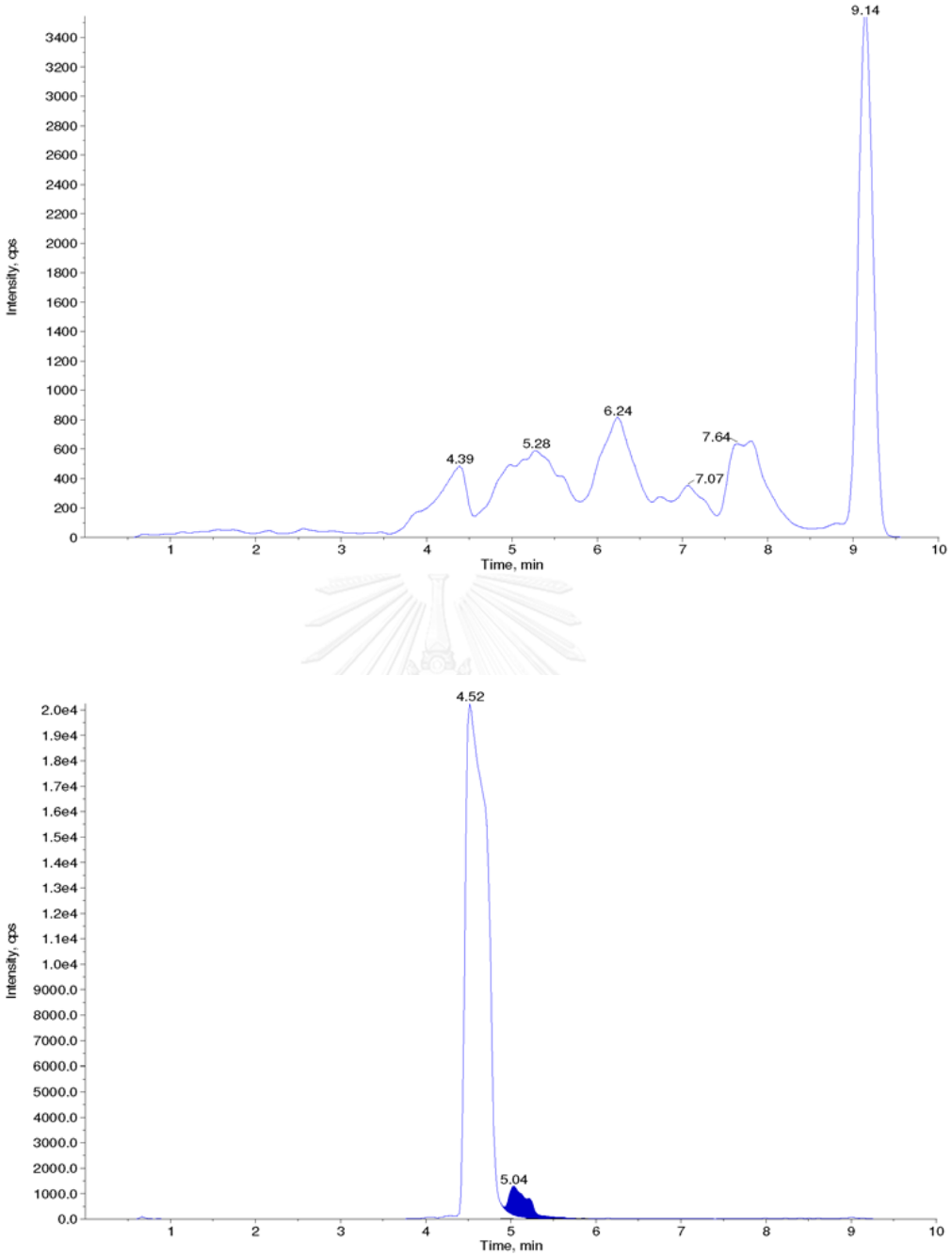
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the fifth day after hormone withdrawal.



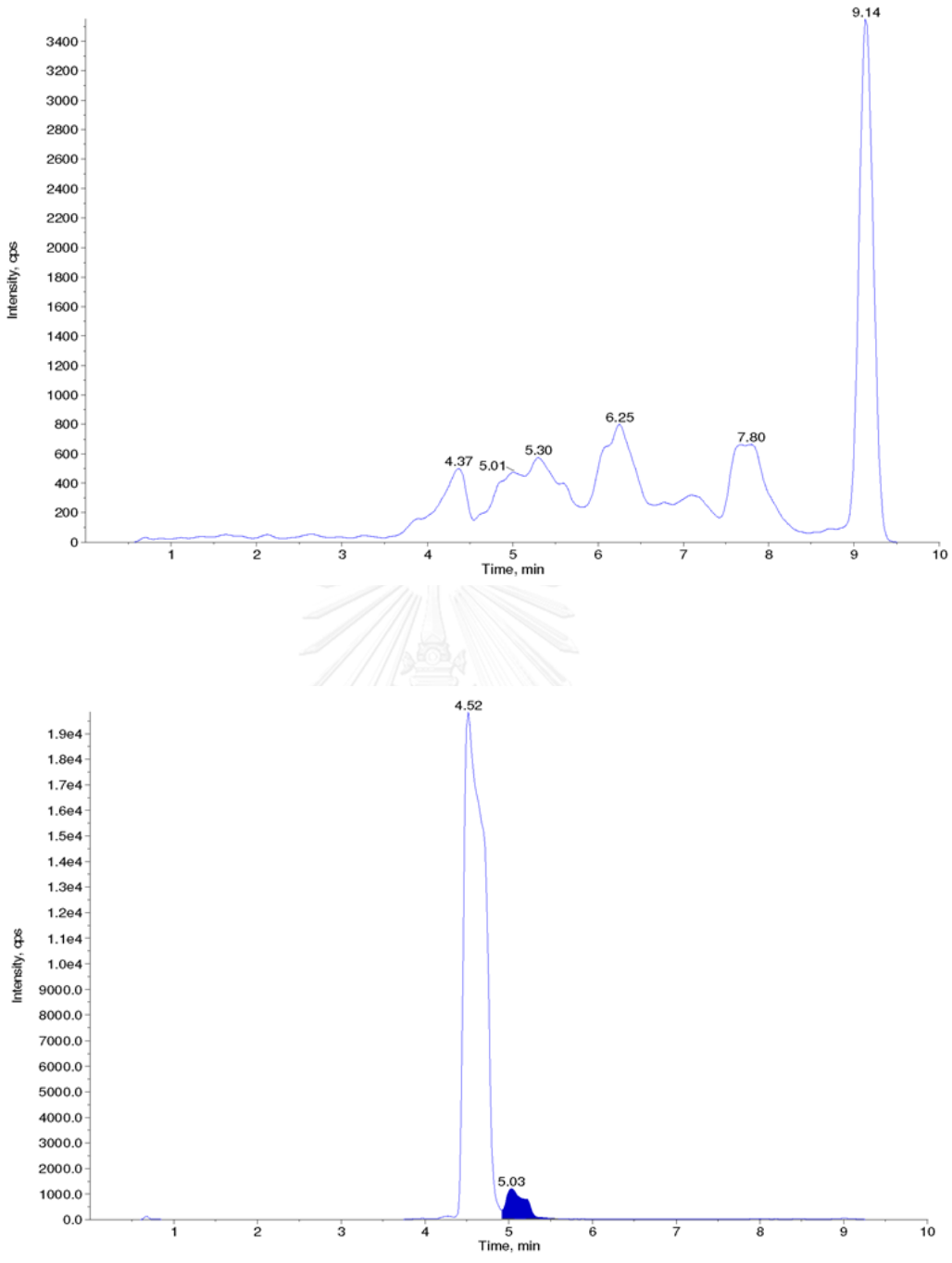
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the fifth day after hormone withdrawal.



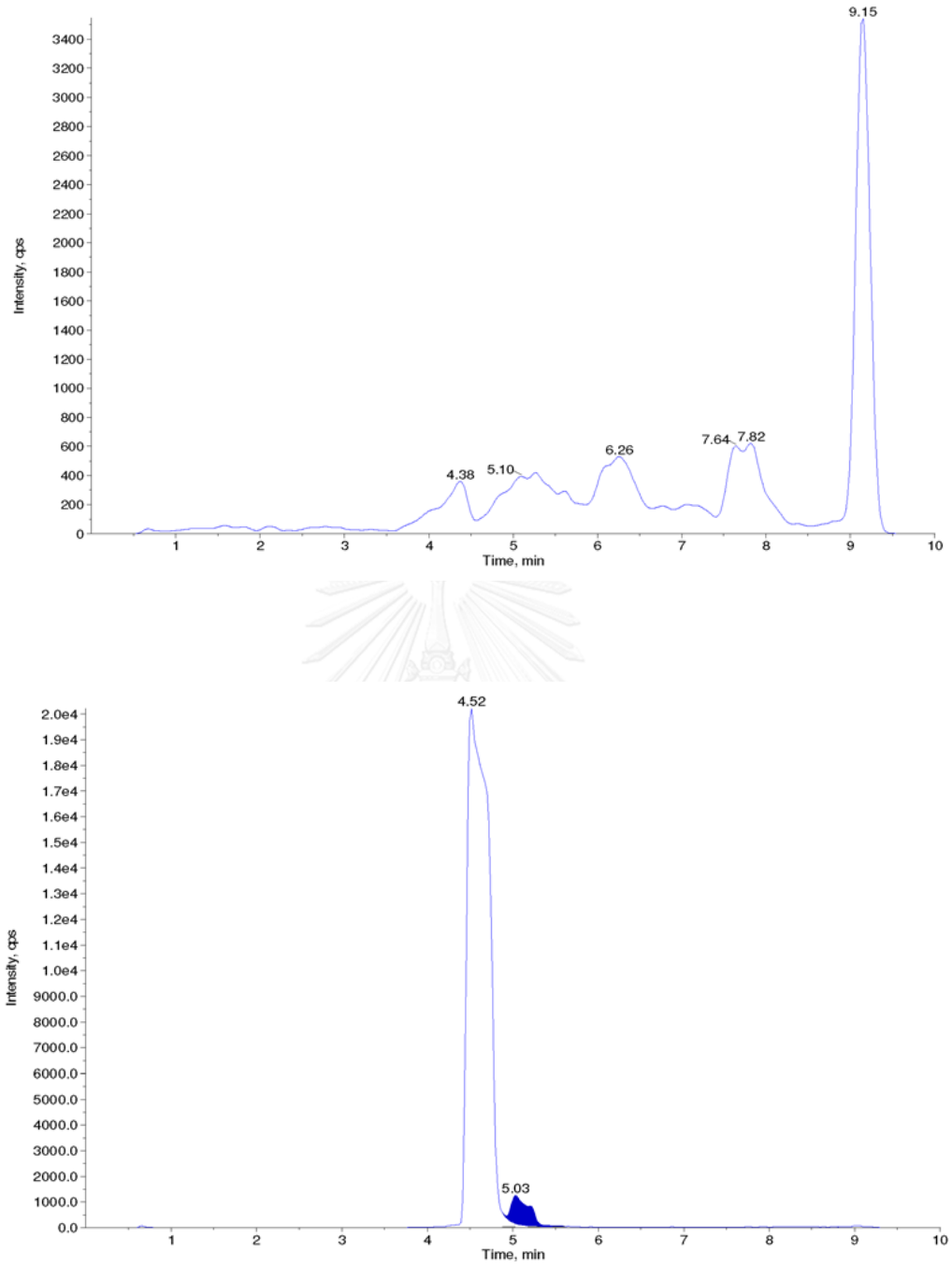
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the seventh day after hormone withdrawal.



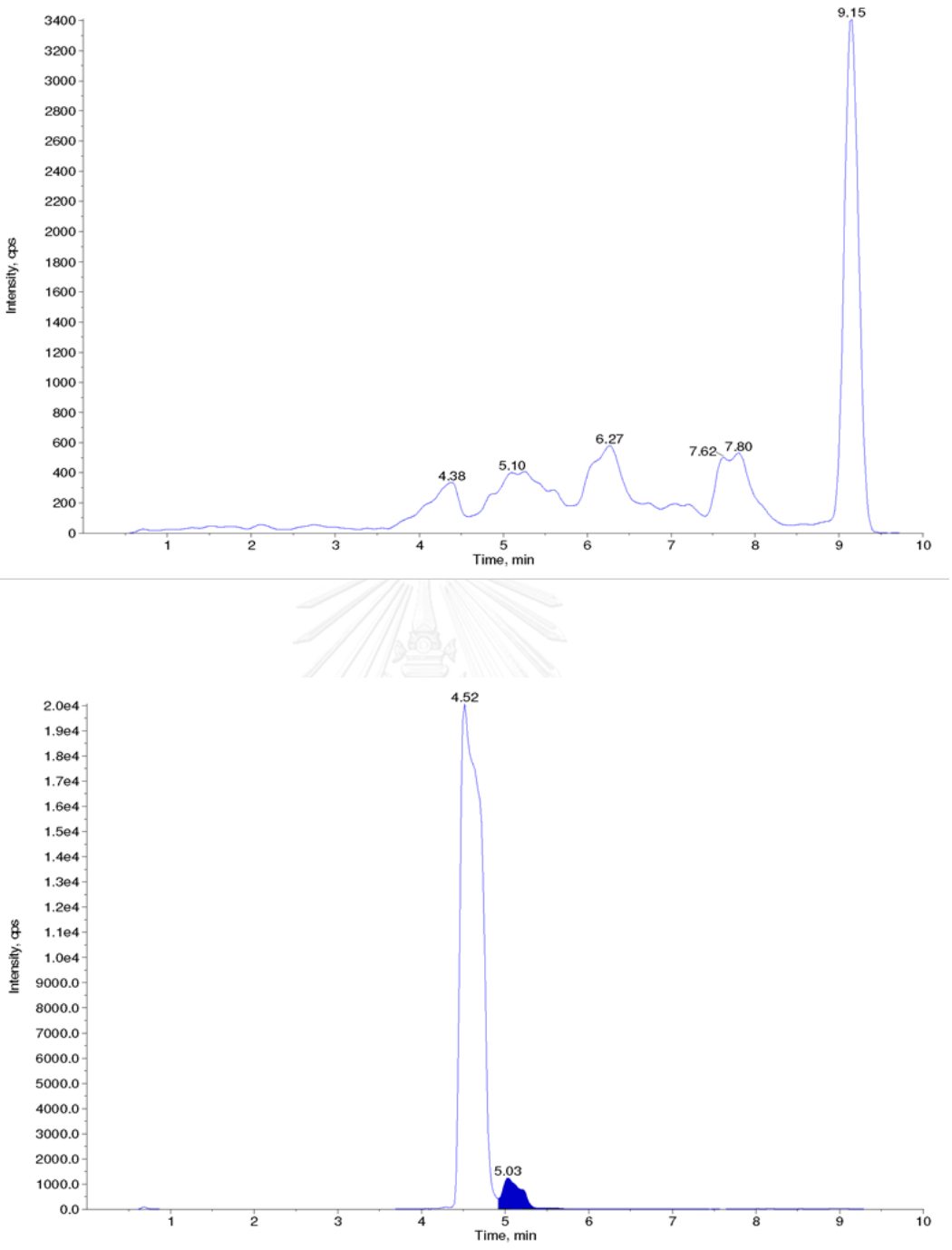
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the seventh day after hormone withdrawal.



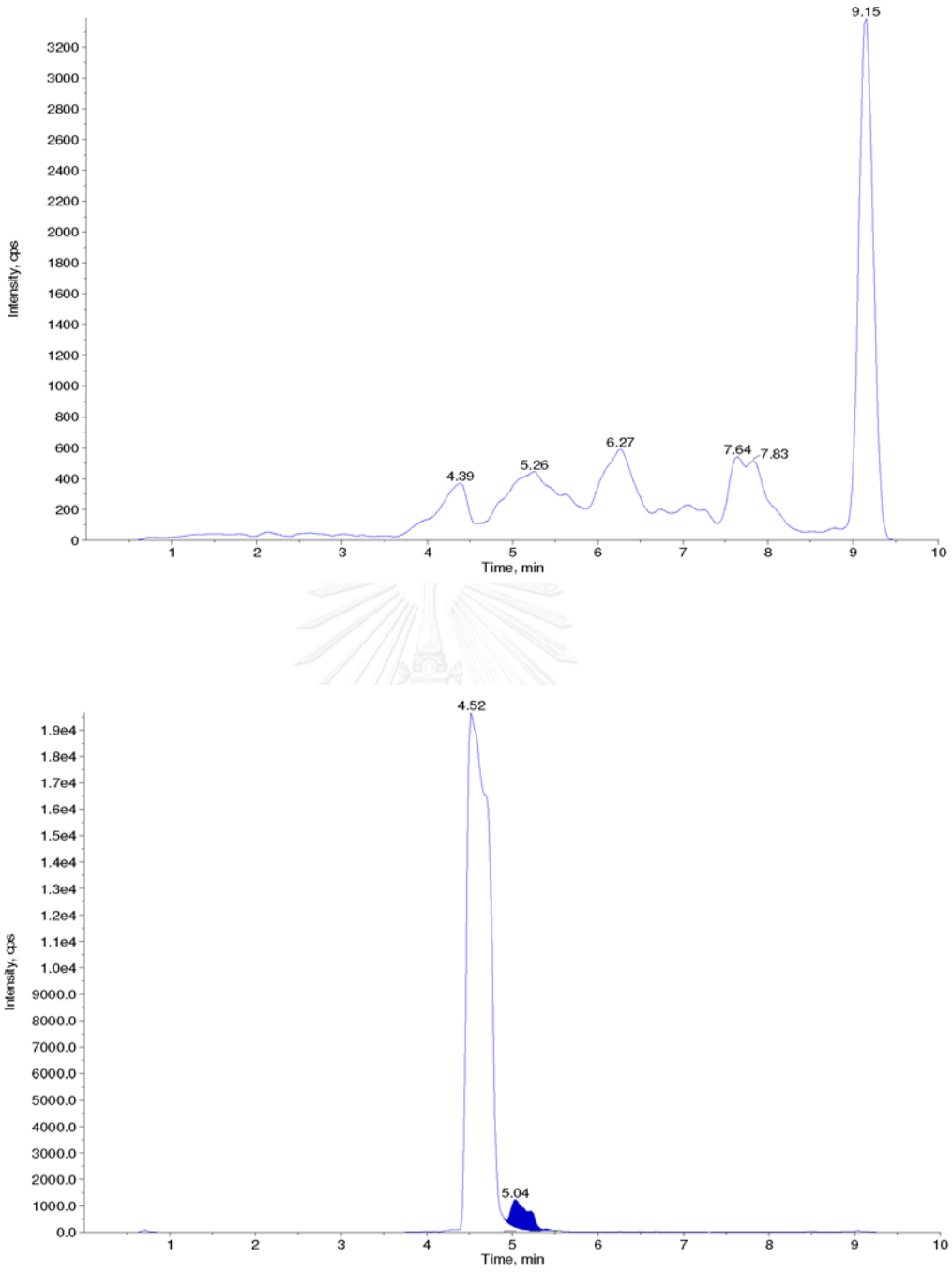
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the seventh day after hormone withdrawal.



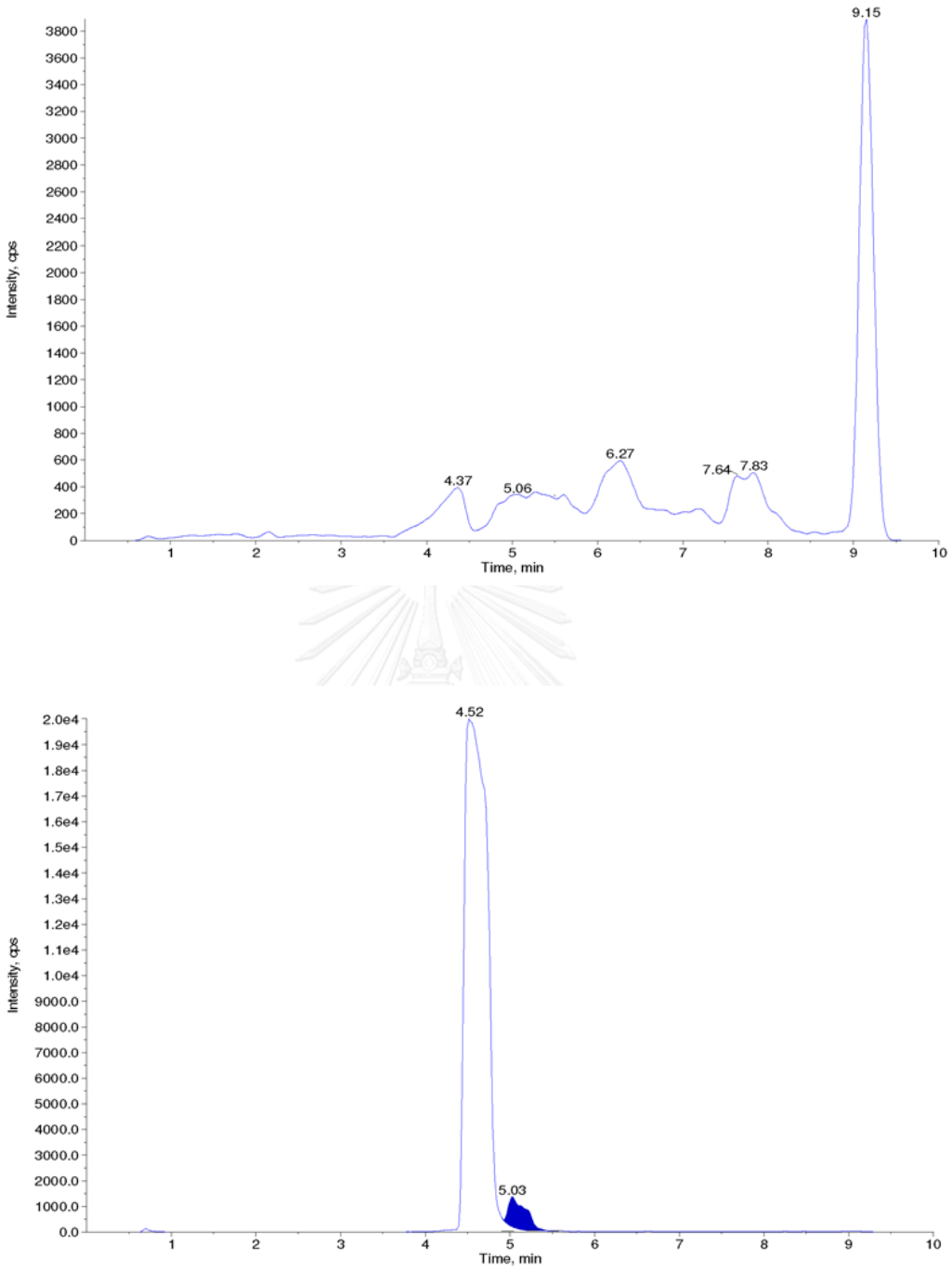
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the fourteenth day after hormone withdrawal.



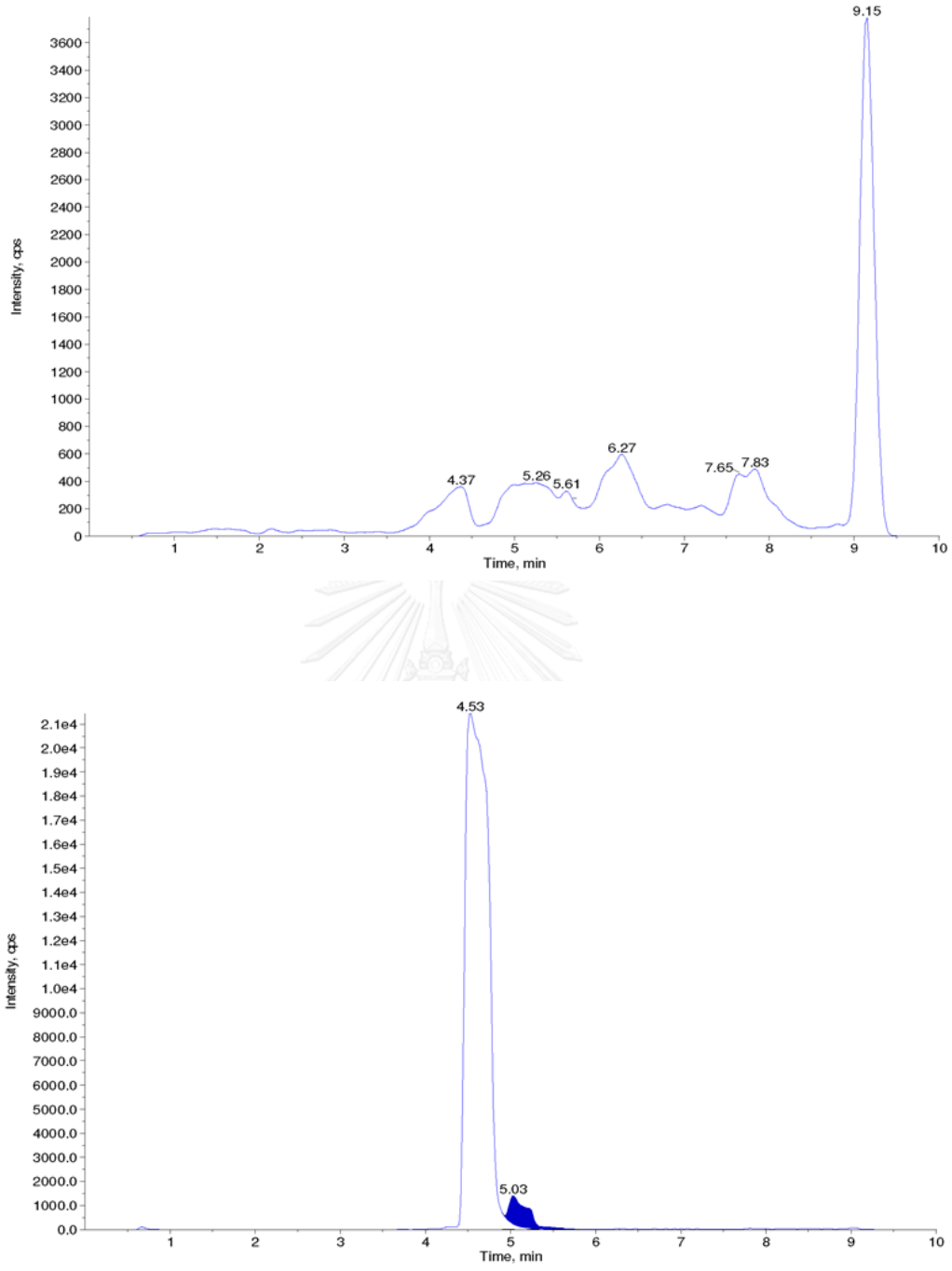
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the fourteenth day after hormone withdrawal.



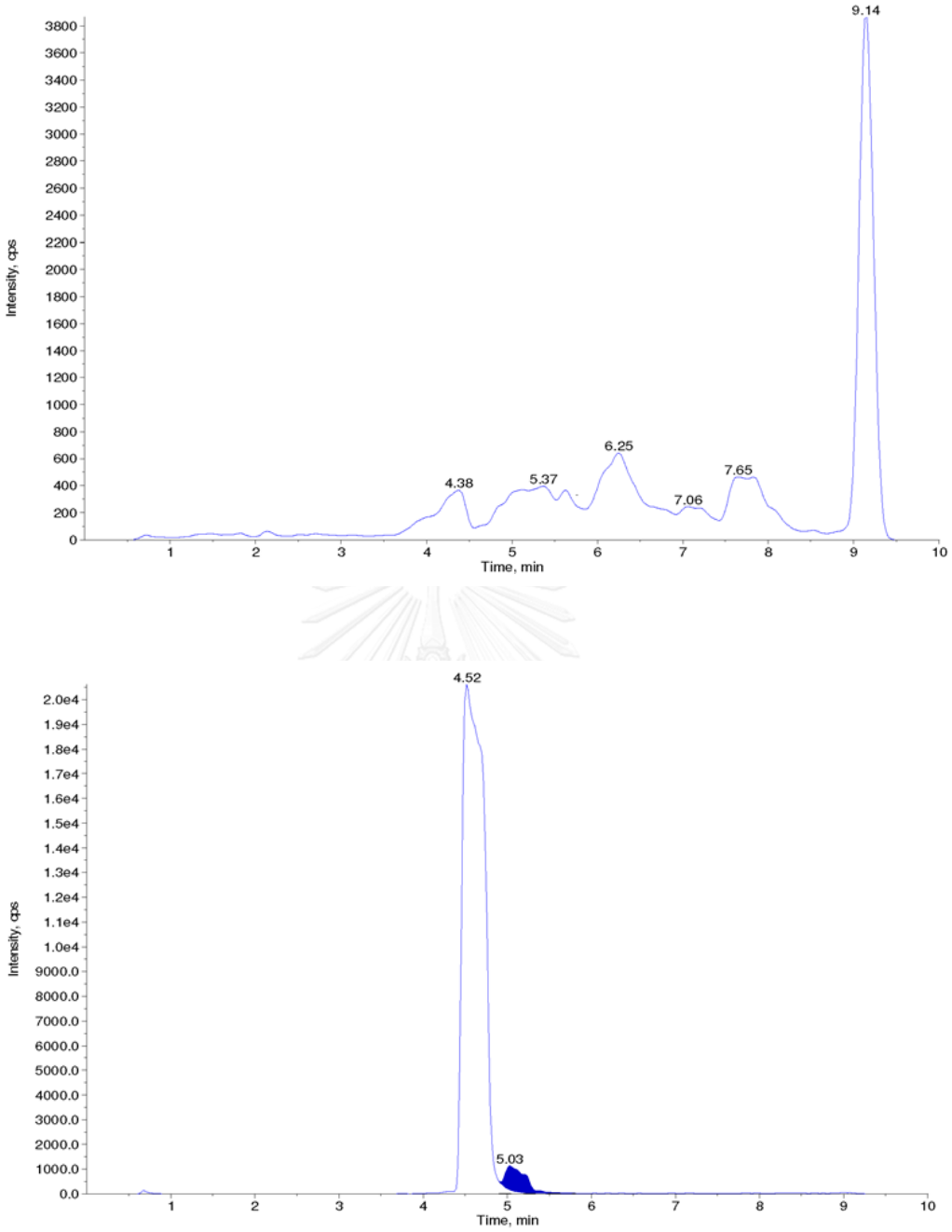
LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the fourteenth day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, first injection from 23 days-hormonal treated fry on the twenty first day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, second injection from 23 days-hormonal treated fry on the twenty first day after hormone withdrawal.



LC-MS/MS chromatogram of the extracted mestanolone and extracted finasteride, third injection from 23 days-hormonal treated fry on the twenty first day after hormone withdrawal.

APPENDIX D Linear regression analysis for determination of LOQ

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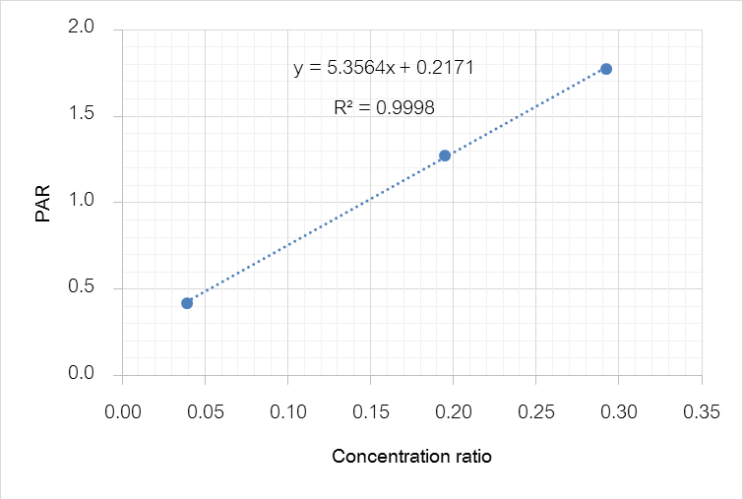
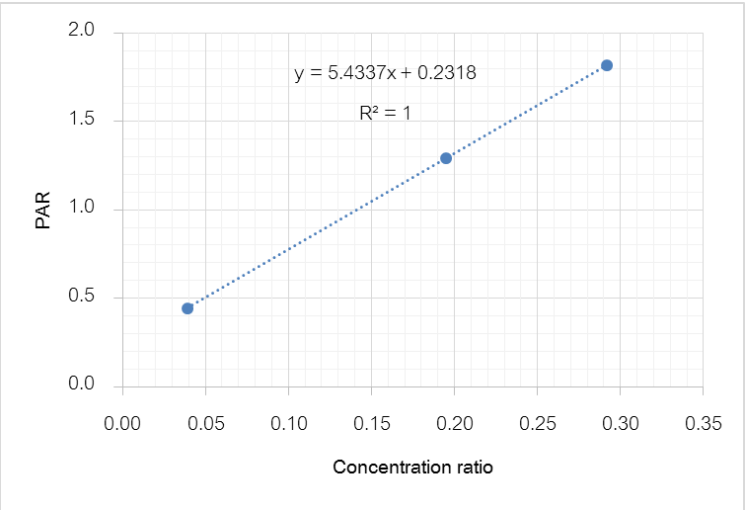
First regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	5022.027	11927.267	25.86	0.03894045	0.421054	0.8763742	87.028226
5.035	15622.909	12283.301	25.86	0.19470224	1.271882	4.7010832	93.368088
7.552	22759.902	12829.828	25.86	0.29203403	1.773983	6.9581701	92.136786



Second regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	5668.105	12793.364	25.86	0.03894045	0.44305	0.9752529	96.847357
5.035	15145.918	11736.561	25.86	0.19470224	1.29049	4.7847329	95.029452
7.552	22189.464	12204.746	25.86	0.29203403	1.818101	7.1564922	94.762873



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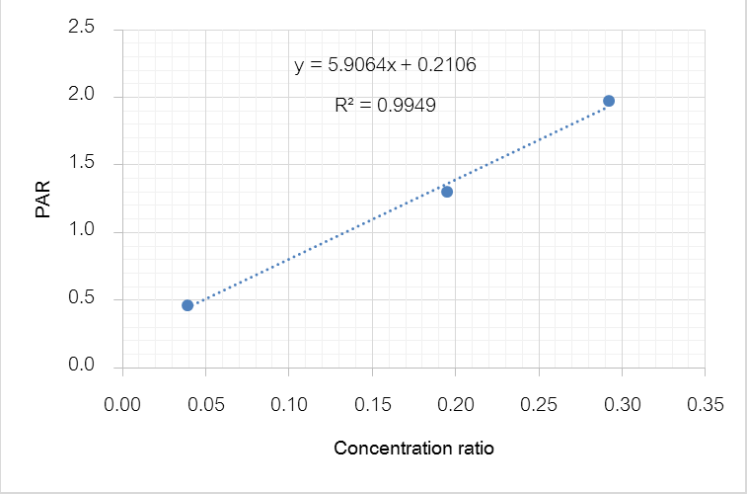
Chulalongkorn University

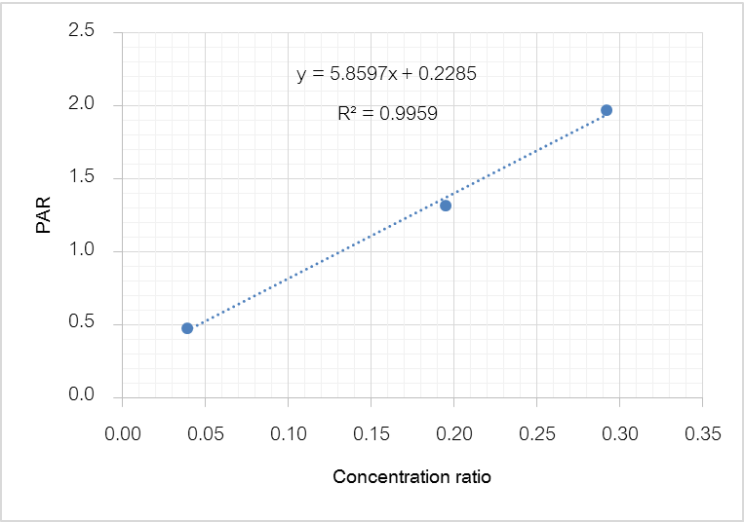
Third regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	6133.494	13209.407	25.86	0.03894045	0.464328	1.0709005	106.34563
5.035	14289.853	11000.743	25.86	0.19470224	1.29899	4.8229406	95.788294
7.552	22562.308	11433.125	25.86	0.29203403	1.973416	7.854674	104.00787



Fourth regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	5604.111	11730.341	25.86	0.03894045	0.477745	1.1312146	112.33511
5.035	14237.164	10828.748	25.86	0.19470224	1.314756	4.8938151	97.195931
7.552	21802.698	11048.165	25.86	0.29203403	1.973423	7.854705	104.00828



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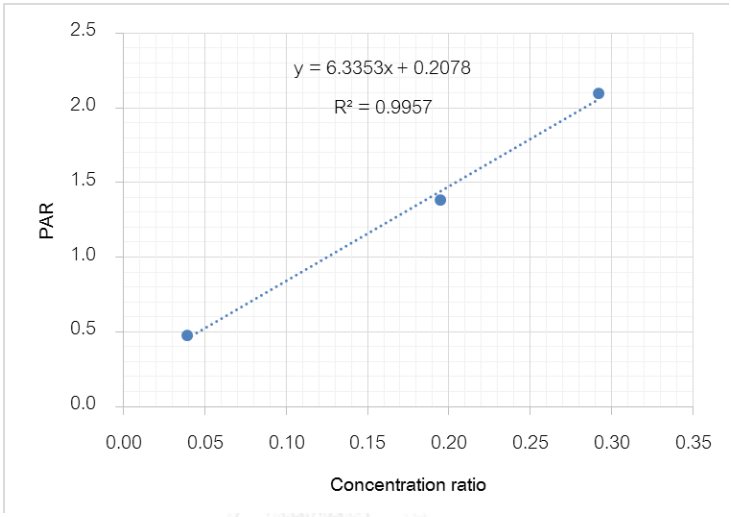
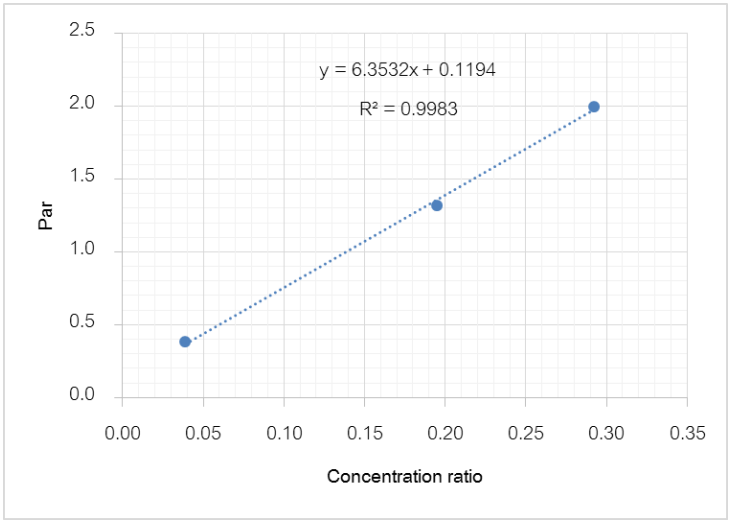
Fifth regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	4714.731	9865.608	25.86	0.03894045	0.477896	1.131892	112.40238
5.035	15917.484	11531.647	25.86	0.19470224	1.38033	5.1885898	103.05044
7.552	19675.033	9389.649	25.86	0.29203403	2.095396	8.4030098	111.26867



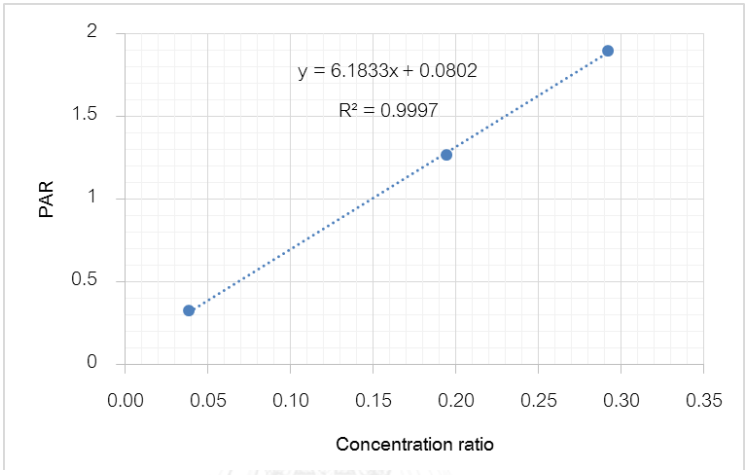
Sixth regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	4520.173	11848.994	25.86	0.03894	0.38148	1.057	104.96
5.035	13832.775	10493.023	25.86	0.19470	1.31828	4.886	97.04
7.552	19251.045	9633.98	25.86	0.29203	1.99824	7.665	101.50



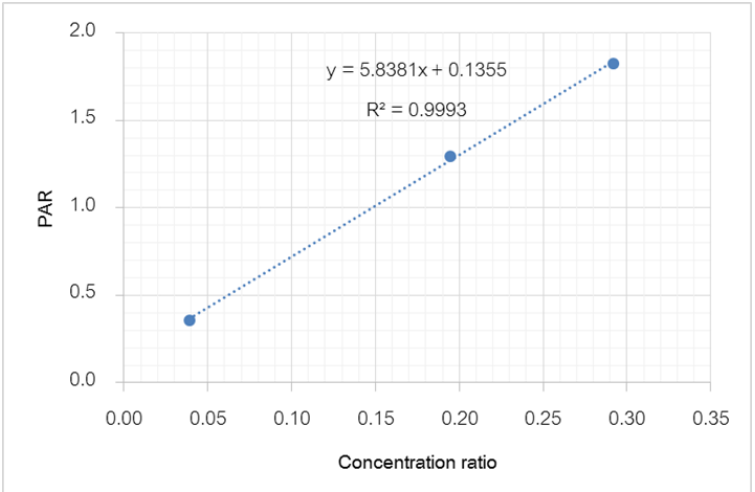
Seventh regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	4193.177	11838.227	25.86	0.03894045	0.32719	0.9454363	93.886422
5.035	12587.744	9926.706	25.86	0.19470224	1.268069	4.6807327	92.963906
7.552	19446.495	10646.352	25.86	0.29203403	1.89584	6.9636092	92.208808



Eighth regression line

Mes Conc.	Mes peak area	IS peak area	IS Conc.	Conc.Ratio	PAR	Cal.Conc.	Recovery
1.007	4008.383	12250.935	25.86	0.03894	0.35421	0.945	93.89
5.035	13827.648	10679.413	25.86	0.19470	1.29479	4.790	95.13
7.552	17984.300	9486.171	25.86	0.29203	1.82659	7.247	95.96



APPENDIX E Chromatographic condition

Chromatographic condition

Condition	
Analytical technique	LC-MS/MS
Ionization type	Positive Turbo spray
Detection method	Multiple Reaction Monitoring (MRM)
Parameter	
- Collision Gas (CAD)	6 psi
- Curtain Gas (CUR)	22 psi
- Ion source gas 1(GS1)	65 psi
- Ion source gas 2(GS2)	65 psi
- Ion spray voltage (IS)	5500.0 psi
- Temperature (TEM)	500 °C
- Scan dwell time	250 msec for all channels
Monitoring	
- Mestanolone	305.3/269.3
- Finasteride	373.5/355.4
Column type	C_8 100 x 4.6 mm, 5µm
Column oven	30°C
Autosampler temperature	20°C
Mobile phase	5 mM Ammonium formate : Acetonitrile
	0.0–2.0 min ACN = 10% 80% v/v
	2.0–6.0 min ACN = 80% v/v
	6.0–6.5 min ACN = 80% 100% v/v

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	6.5–8.0 min ACN = 100% v/v
	8.0–9.0 min ACN = 100% 10% v/v
	9.0–10.0 min ACN = 10% v/v
Flow rate	0.8 mL/min
Injection volume	10 µL
Retention time	
- Mestanolone	About 5.67 min
- Finasteride	About 5.04 min

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VITA

Miss Nion Vinarukwong was born on June 30th, 1982 in Bangkok, Thailand. She was an alumni of Triam Udom Suksa School (TU 61). In 2007, she accomplished the degree of Doctor of Veterinary Medicine (D.V.M.) with the Second Class Honors from the Faculty of Veterinary Science, Chulalongkorn University. After an achievement of D.V.M., she worked as a veterinarian in Vet 4 Small Animal Hospital in Bangkok, Thailand for 2 years. In 2009, she was accepted by the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University to pursue Doctor of Philosophy (Ph.D.) program in Veterinary Medicine and received a Ph.D. program scholarship of the Royal Golden Jubilee (RGJ) Ph.D. Program, the Thailand Research Fund and the CU. Graduate School Thesis Grant of Chulalongkorn University Fund.

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