

Determination of some sex hormones in health supplements by liquid chromatography with tandem mass spectrometry

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Abstract

The trend of using dietary supplements in daily life is increasingly popular. However, sex hormones are illegally mixed to improve product effectiveness such as promoting hair growth, developing chest muscles, preventing skin aging, promoting growth, etc. In this study, a liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was developed and applied to quantify sex hormones: progesterone (PG), testosterone (TS), and 17 β -estradiol (E2) in health supplements. A Symmetry C18 reversed-phase chromatography column (150 mm x 3.0 mm x 3.5 μ m) using a mobile phase system consisting of A: 10 mM HCOONH₄/0.1% HCOOH, B: ACN was used. Mass spectrometry conditions were set at positive ionization mode, precursor ion and two corresponding daughter ions were selected for each analyte. Quantification limits of PG and TS were 0.4 mg/kg and E2 was 4 mg/kg in liquid matrix, hard and soft capsules. The validated method was applied to detect sex hormones in 16 health supplement samples purchased at pharmacies in Hanoi, Vietnam and some online websites. Three samples were detected to be positive for sex hormones, including one sample containing a sex hormone (testosterone) banned from use in health supplements at a low content (about 0.5 mg/kg).

Keywords: Sex hormones, health supplement, LC-MS/MS.

1. INTRODUCTION

A health supplement is described as a dietary substance(s) for use by human beings to supplement the daily diet to maintain, enhance, and improve the functions of the body and reduce the risk of disease. However, many products have been illegally mixed with sex hormones to improve their effectiveness. It has been reported that early puberty and increased incidence of breast cancer and uterine cancer are closely related to hormone residues in food [1]. In Vietnam, the Ministry of Health has governed Circular

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10/2021/TTBYT regulating the list of substances banned from use in the production and trading of health supplements [2]. According to this circular, some hormones are banned from use, including sex hormones (testosterone). Meanwhile, 17β -estradiol is listed as a carcinogen according to the US National Toxicology Program [3]. Therefore, it is necessary to have a method to detect sex hormones in health supplements to ensure food safety and hygiene.

Hormones have been detected by some analytical methods such as enzyme-linked immunosorbent assay (ELISA) [4], micellar electrokinetic chromatography [5], high performance liquid chromatography (HPLC) [6-8], ultra-performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) [9], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [10-12], gas chromatography-tandem mass spectrometry (GC-MS/MS) [13-15]. Most of these studies involved biological samples (plasma, serum, urine), food samples of animal origin (pork, beef, milk), and environmental samples (wastewater). For health supplement samples, several studies have been performed using UPLC-MS/MS method [16] and high performance liquid chromatography-ion trap-time of flight tandem mass spectrometry (HPLC-IT-TOF-MS) method [17].

LC-MS/MS is an effective method, more commonly used to detect sex hormones in food because of its high sensitivity and diversity in sample processing. Nevertheless, in Vietnam, there have been no publications on the determination of sex hormones: progesterone (PG), testosterone (TS), and 17β -estradiol (E2) in health supplements by LC-MS/MS. This study aimed to propose a reliable, simple, fast, and inexpensive analytical method for the analysis of some sex hormones in health supplements by using LC-MS/MS method. Analytical conditions and sample processing conditions were investigated. The selected method was then validated and applied to real samples of different dosage forms.

2. MATERIALS AND METHODS

2.1. Chemicals and instrument

Hormone standards: testosterone (99.33%) and 17β -estradiol (95.88%) were purchased from Dr. Ehrenstorfer GmbH, while progesterone (99.85%) was from LGC. The stock solutions (2000 mg/L), and standard solutions of calibration curves were prepared with acetonitrile in volumetric flasks. Analytical grade chemicals used for LC-MS standard solvents and sample preparation including methanol, acetonitrile, formic acid, ammonium formate, acetic acid, ammonium acetate, and ethanol were from Merck KGaA.

The Sciex Triple Quad 6500 quadrupole liquid chromatography system (National Institute for Food Control, Vietnam) with a mass spectrometer detector was used. All compounds were separated using a Symmetry C18 column (150 mm x 3.0 mm x 3.5 μ m) and the corresponding pre-column VanGuard Cartridge (2.1 mm x 5 mm) were from Waters (USA).

2.2. Sample preparation

Samples of health supplements were randomly purchased from pharmacies in Hanoi (Vietnam) and online websites. The samples are expected to be processed following the synthesis procedure from reference [17] and the routine procedure of the National Institute of Food Control (Vietnam). Each sample was prepared by mixing 10 g of sample. The expected procedure is described as follows: 1.0 g of sample was weighed accurately into a 50 mL tube, and 20 mL of acetonitrile was added. The sample tube was vortexed for 1 min, ultra-sonicated at 27°C for 15 min, and centrifuged at 6000 rpm for 5 min. After centrifugation, the supernatant was collected, made up to 20 mL, and passed through a 0.22 µm syringe filter before analysis by LC-MS/MS.

2.3. Method development

Standard solutions of PG (200 ng/mL), TS (200 ng/mL), and E2 (500 ng/mL) were injected directly into LC-MS/MS system for investigation of method conditions. Referring to the documents [1, 9-11], the ionization mode, parent ion, and daughter ions of the three analytes along with optimal equipment parameters were investigated and selected. Based on references [9, 11, 12, 17], the chromatography column expected to be used is C18 column. The mobile phase is expected to follow the gradient: mobile phase A (HCOOH 0.1%/H₂O) and B (ACN). Effects of mobile phase composition, ionization mode and optimal equipment parameters were subsequently investigated.

2.4. Method validation

The analytical method was validated according to the instructions of AOAC 2016 [18], ICH 2005 [19], and the monograph guidance document [20] on blank with the following criteria: selectivity, linearity, detection and quantification limits, repeatability, and recovery.

3. RESULTS AND DISCUSSION

3.1. Investigation of LC-MS/MS conditions

The ionization mode, precursor ion, daughter ions, and optimal equipment parameters were investigated and selected [21]. The results of parent ion, daughter ion, and optimal parameters for each fragment are tabulated in Table 1.

Table 1. Optimum conditions for analyte fragments

<i>Analyte</i>	<i>Precursor ion (m/z)</i>	<i>Daughter ion (m/z)</i>	<i>CE (eV)</i>	<i>Type</i>
Progesterone	315	97	26	Quantitation
		109	30	Qualification
Testosterone	289	97	26	Quantitation
		109	26	Qualification
17β-Estradiol	255	159	20	Quantitation
		173	8	Qualification

The tandem mass spectrometer main working parameters were optimized as follows: positive ionization mode, ion spray voltage: 5500 V, source temperature: 400°C, curtain gas: 35 psi, collision gas: 7 psi, ion source gas 1 and 2 were both adjusted to 40 psi.

In this study, we investigated seven mobile phase systems (A, B) including (1) 0.1% HCOOH, ACN; (2) 0.1% CH₃COOH, ACN; (3) 10 mM HCOONH₄, ACN; (4) 10 mM CH₃COONH₄, ACN; (5) 0.1% CH₃COOH, MeOH; (6) 10 mM HCOONH₄/0.1% HCOOH, MeOH; (7) 10 mM HCOONH₄/0.1% HCOOH, ACN. As shown in Figure 1, the mobile phase system (1) gives bad, unbalanced peaks of PG and E2, and high background noise. The peak of PG when using (2) and (3) is unstable, and the signal is low. (4) gives good shapes of PG and TS, however, the E2 peak is not symmetrical and appears background noise at the base of the peak. PG peak is not good in (5). Hormone peaks obtained by (6) and (7) were comparable, but the signal of (6) was relatively smaller. Therefore, (7) was selected as the mobile phase because it provides good and suitable signals for three hormones.

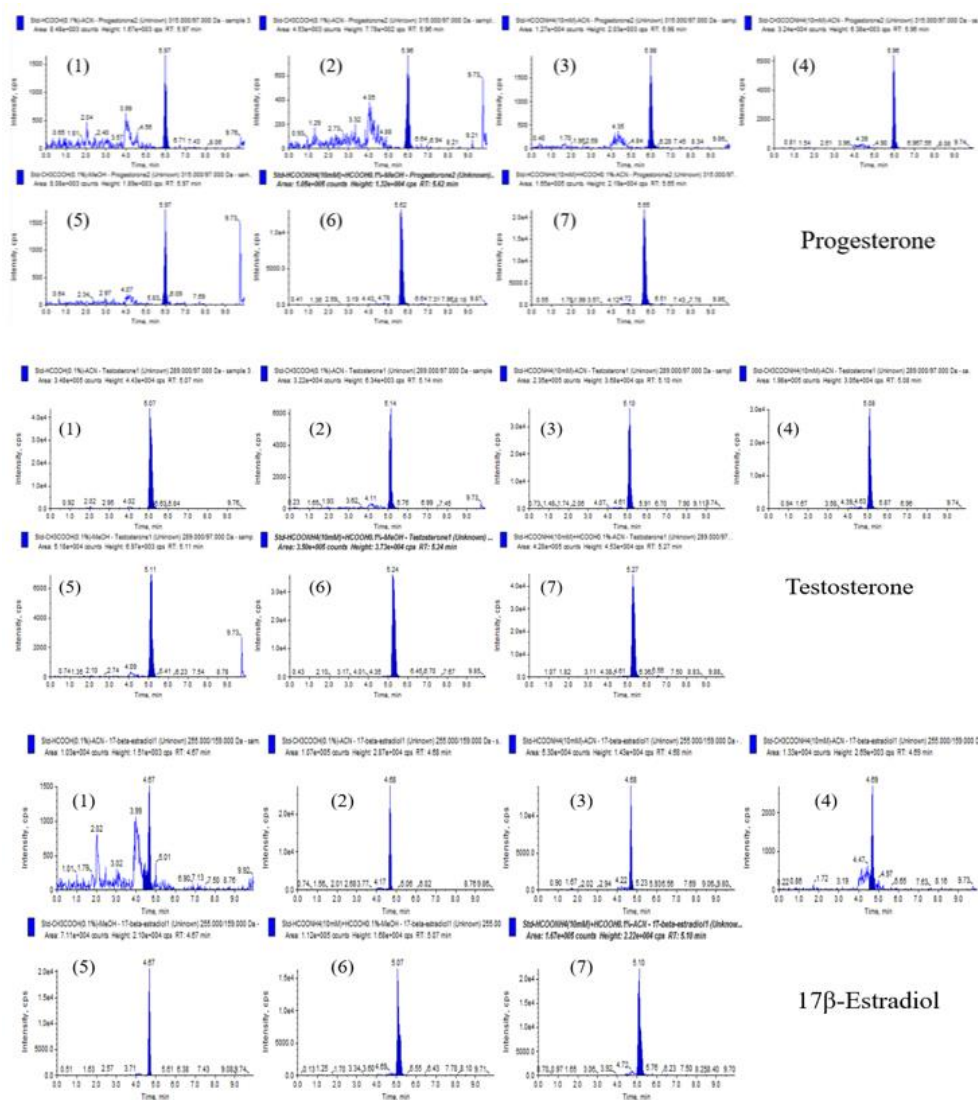


Figure 1. Effects of mobile phase on the determination of PG, TS, and E2

The mobile phase system (A: 10 mM HCOONH₄/0.1% HCOOH, B: ACN) was set at a flow rate of 0.5 mL/min, the injection volume of 10 μ L and was adjusted as following (0 - 1.5 min: 90:10 A/B, 1.5 - 3 min: 90 \rightarrow 0:10 \rightarrow 100 A/B, 3 - 7 min: 100% B, 7 - 8 min: 0 \rightarrow 90:100 \rightarrow 10 A/B, 8 - 10 min: 90:10 A/B).

3.2. Investigation of sample preparation procedure

The expected procedure was performed with 10 samples, looking for a positive sample. The investigation was tested on samples that were confirmed positive for PG. Sample processing in the next steps will be changed according to previously investigated conditions. We performed the investigation under different conditions, each condition performed sample processing and analysis repeated three times.

3.2.1. Effects of extraction solvent

In this study, we investigated three solvents including acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH). Extraction solvent investigation chart are illustrated in Figure 2.

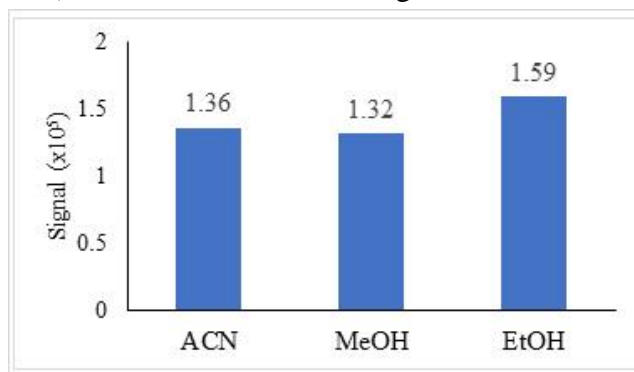


Figure 2. Extraction solvent investigation chart

As shown in Figure 2, the response of the analyte when extracted with EtOH is the highest while MeOH gave the lowest analytical signal. Analytes are more soluble in EtOH than MeOH and ACN. The difference between this study with previously published methods (reference [6, 12]) is the use of extraction solvent (EtOH) for the analysis process.

3.2.2. Effects of ultrasonication time

The effects of ultrasonication time on sample extraction efficiency are illustrated in Figure 3. The values were investigated including 5, 15, 30, 60, and 120 minutes, respectively.

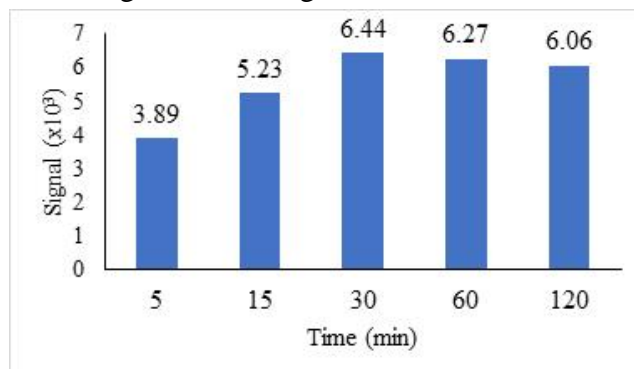


Figure 3. Ultrasonic time investigation chart

After examination, the signal of the analyte increased to its highest at 30 minutes the response was higher. Therefore, 30 min was selected as the optimal ultrasonication time. Longer ultrasonication time can dissolve the maximum amount of analyte into the solvent. However, too long ultrasonication can disperse many impurities into the extract, affecting the analytical signal.

3.2.3. Effects of ultrasonic temperature

In this experiment, we applied different temperatures of room temperature (27°C), 40°C, 60°C, and 80°C in the ultrasonic process. The effects of ultrasonication investigation on sample extraction efficiency are illustrated in Figure 4.

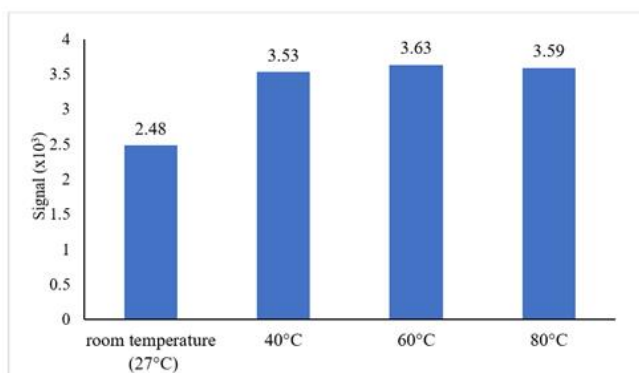


Figure 4. Ultrasonic temperature investigation chart

The effects of ultrasonication temperature are presented in Figure 4. The response of the analyte at 60°C and 80°C is not significantly different. Increasing the temperature will increase the diffusion of the analyte into the solvent, so more analytes can be dissolved. However, more temperature increases, more solvent evaporates, and many impurities can be generated, reducing the analytical signal. These results indicate that an ultrasonication temperature of 60°C is suitable for a good compromise.

3.2.4. Effects of sorbents

Two cleaning agents were investigated in this study including activated carbon and C18 (Figure 5). The purpose of using cleaning agents is to remove remaining impurities and adsorb substances that affect the analyte. Noppe et al. used Oasis HLB solid phase extraction column [12], while some others used SPE C18 solid phase extraction column [10, 11].

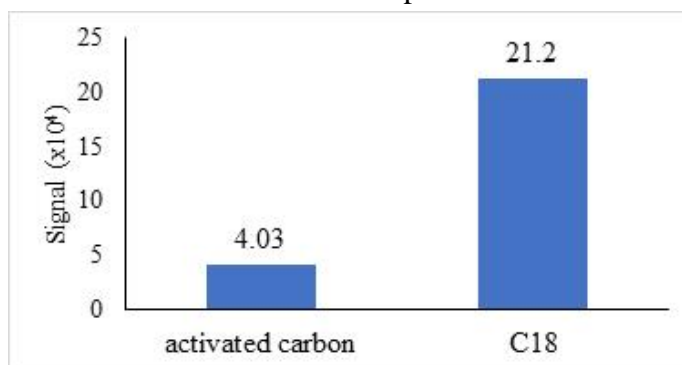


Figure 5. Cleaning agent investigation chart

It has been recognized that the signal of using activated carbon is significantly lower than C18. Activated carbon adsorbed the analyte. As a result, it reduced the amount of analyte remaining in the extract after cleaning. C18 was chosen to obtain the best cleaning efficiency.

Considering these investigations, a sample preparation procedure was provided. 1.0 g of homogenized sample was weighed accurately into a 50 mL tube, and 20 mL of absolute ethanol was added. The sample tube was vortexed for 1 min, ultrasonicated at 60°C for 30 min, and centrifuged at 6000 rpm for 5 min. After centrifugation, the aliquot was collected and titrated to 20 mL. Then 1.0 mL of this was drawn into a tube containing 100 mg C18, vortexed for 1 min, and centrifuged at 13000 rpm for 5 min. The aliquot was collected and passed through a 0.22 µm syringe filter before analysis by LC-MS/MS.

3.3. Matrix effect

The matrix effect is the influence of one or more components in the sample matrix on the determination of the concentration of the analyte. The matrix effect can be detected through an increase or decrease in the signal of the analyte in the sample matrix compared to the analyte with the same concentration in the solvent. Calibration curves on solvent and three blank matrices (hard capsule, soft capsule, liquid) for the analyte were established. The matrix effect on the signal of the analyte is determined by comparing the slopes of the two standard curves according to the following formula, where a_b is the slope of the calibration curve prepared by fortifying blank matrix extracts after sample preparation at five concentration levels (concentration of PG and TS from 20 ng/mL to 200 ng/mL, E2 from 0.2 µg/mL to 2 µg/mL) and a_s is the slope of the calibration curve constructed in pure solvent at the same concentrations.

$$ME = \frac{a_b - a_s}{a_s} \times 100\%$$

The matrix effect (Table 2) exceeds $\pm 20\%$ of the AOAC requirement, so the sample matrix influences the determination of analyte content. As a result, using a standard curve based on blank to calculate analytical results is necessary.

Table 2. Evaluation of the matrix effect of the analytical method

<i>Analytes</i>	<i>Parameters</i>	<i>Pure solvent</i>	<i>Hard capsule</i>	<i>Soft capsule</i>	<i>Liquid matrix</i>
Progesterone	a	5550	6730	6760	7070
	ME (%)		21.26	21.80	27.39
Testosterone	a	62700	54400	74100	76500
	ME (%)		-13.24	18.18	22.01
17β-Estradiol	a	2230000	1700000	1580000	1680000
	ME (%)		-23.77	-29.15	-24.66

3.4. Method validation

3.4.1. Selectivity

The method selectivity was evaluated by using chromatograms of PG, TS, and E2 in blank, standard, sample, and standard addition sample (Figure 6). All of the procedural blanks had undetectable signals of the three analytes., indicating that our procedure is free of contamination. The retention time of the analyte in the spiked sample corresponds to the one in the standard sample (< 0.1 min). All analytes have one parent ion and two daughter ions, and the "identification points" (IP) score is 5 (IP score is calculated according to Commission Implementing Regulation (EU) 2021/808 [22]). The ion ratio of the two daughter ions obtained in the spiked sample is comparable to the one in the standard sample, the difference is within the allowable limit, satisfying the European Council requirement.

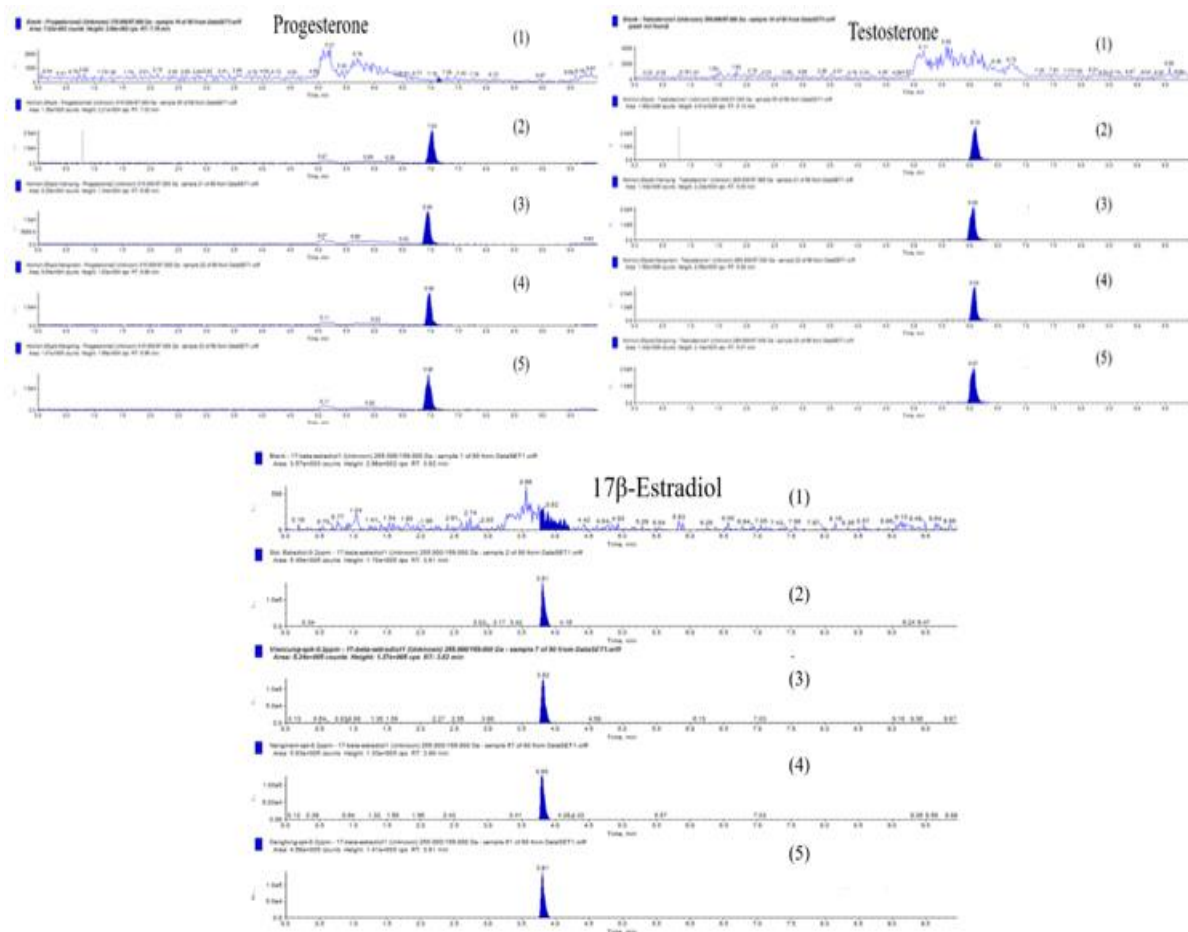


Figure 6. Chromatograms of PG, TS and E2 in (1) blank; (2) standard; spiked sample on (3) hard capsule; (4) soft capsule; (5) liquid matrix

3.4.2. Limits of detection and quantification

Limits of quantifications (LOQs) of PG, TS, and E2 were determined using signal-to-noise ratios equal to 10. Spike samples at 5 ng/mL, 20 ng/mL, 50 ng/mL levels (with PG and TS), and 0.2 µg/mL, 0.4 µg/mL, 0.6 µg/mL levels (with E2) were determined to obtain

LOQs. LOQs of PG, TS and E2 were 0.4, 0.4 and 4.0 mg/kg, respectively. Limits of detections (LODs) were estimated to be 0.12 mg/kg for PG, TS and 1.21 mg/kg for E2.

3.4.3. Linearity

Calibration curves were used to determine linearity: blank extracts of various matrices were fortified with increased concentrations ranging from 20 to 200 ng/mL for PG, TS and from 0.2 to 2 µg/mL for E2. As a result, the calibration curve equations have correlation coefficients of $0.995 \leq R \leq 1$, and the deviation of standard points is not more than 15%. We can assume that the analytes have a linear dependence between their peak area and concentration.

3.4.4. Precision and trueness

The precision of measured values refers to how close the agreement is between repeated measurements. Trueness refers to the closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value. In this study, we evaluate precision by repeatability and trueness by recovery. We used liquid matrix, hard and soft capsules, which were spiked with known amounts of analytes at different levels to estimate method repeatability and recovery. Three spikes of PS and TS (20, 40, and 100 ng/mL) and E2 (0.2, 0.4, and 1 µg/mL) were performed six times per matrix. The recovery of all analytes (Table 3) meets the requirement of AOAC (80%-110%) [18]. Relative standard deviations (RSD) of PG and TS are both smaller than 15% and E2 is smaller than 11% (Table 3). These RSDs satisfy the requirement of AOAC [18].

Table 3. Method precision and recovery of PG, TS and E2 in different matrices

Parameter	Progesterone			Testosterone			17β-estradiol					
	Conc. (ng/mL)	Sample matrix			Conc. (ng/mL)	Sample matrix			Conc. (µg/mL)	Sample matrix		
		1	2	3		1	2	3		1	2	3
RSD (%)	20	2.03	0.85	1.70	20	3.12	0.91	1.16	0.2	1.54	1.39	2.16
	40	1.63	0.60	1.84	40	2.66	0.75	1.99	0.4	1.51	2.42	2.95
	100	2.71	1.02	1.09	100	2.79	1.17	1.41	1	5.17	4.30	2.32
Recovery (%)	20	104.3	105.5	101.8	20	103	98.5	104.8	0.2	103.4	103.1	101.5
	40	103.6	104.2	99.3	40	103.3	97.6	99.9	0.4	104.1	101.3	101.3
	100	102.1	100.5	95.8	100	105.1	103.7	104.2	1	91.1	99.7	101.5

1: Hard capsule, 2: Soft capsule, 3: Liquid matrix

3.5. Method application for determination of sex hormones in health supplements

Results of 16 health supplements randomly collected at pharmacies in Hanoi (Vietnam) and some online websites are summarized in Table 4.

Table 4. Analytical results of three hormones in real samples

<i>Sample ID</i>	<i>Form</i>	<i>Volume of extract solvent (mL)</i>	<i>Weighing mass (g)</i>	<i>Content (mg/kg)</i>
M1	Tablet	20	1.0036	ND
M2	Hard capsule	20	1.0088	PG = 0.75
M3	Tablet	20	1.0064	ND
M4	Hard capsule	20	1.0028	ND
M5	Tablet	20	1.0044	TS (about 0.5)
M6	Tablet	20	1.0054	ND
M7	Tablet	20	1.0082	ND
M8	Tablet	20	1.0086	ND
M9	Tablet	20	1.0082	ND
M10	Tablet	20	1.0056	ND
M11	Soft capsule	20	1.0058	ND
M12	Hard capsule	20	1.0076	ND
M13	Hard capsule	20	1.0054	PG = 0.60
M14	Hard capsule	20	1.0050	ND
M15	Hard capsule	20	1.0024	ND
M16	Liquid	20	1.0064	ND

ND: Not detected

The results are evaluated by comparing retention time, parent fragment, daughter fragment, qualitative and quantitative fragment ratio to determine the presence of the analyte, the hormone content in real samples is then calculated based on the calibration curve. Out of 16 analyzed samples, we detected 2 samples positive for progesterone with detectable content of 0.75 mg/kg and 0.60 mg/kg, respectively; 1 sample was positive for testosterone with a detectable content of about 0.5 mg/kg.

3.6. Research limitations and orientations

Testosterone, 17 β -estradiol, and progesterone have very low oral bioavailability, so the oral pharmaceutical form is usually in ester form (Testosterone Undecanoate, Methyl testosterone, Estradiol valerate, Estradiol acetate,...) or prepared as microparticles (micronized Estradiol, micronized Progesterone,...). Recently, some health supplements contain animal ingredients, they have been processed and extracted, the remaining Testosterone content is very low, which can affect the results. As a result, we need to research this issue more and compare total extraction and free-form extraction. The objective of this study is the analysis of these free hormones on health supplement matrices. This is an initial study, subsequent study will approach the process of analyzing sex hormones in different forms and in different matrices such as blood, and urine.

5. CONCLUSION

The present study has successfully developed an LC-MS/MS method for the determination of sex hormones in health supplements with limits of quantification (LOQs) of progesterone, testosterone (0.4 mg/kg), and 17 β -Estradiol (4 mg/kg) on different matrices (liquid, hard and soft capsule). The validated method was applied to analyze health supplement samples of different preparations. Analytical results showed that 3/16 samples were positive for sex hormones, the contents of those hormones were then quantified. This is an initial study, subsequent studies will continue to investigate the sample preparation and cleaning process to minimize the influence of the sample matrix on the analytical signal and develop analytical methods to detect other sex hormones in health supplements such as (estrone, estriol, and methyltestosterone).

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Xác định một số hormon sinh dục trong thực phẩm bảo vệ sức khỏe bằng phương pháp sắc ký lỏng khối phổ hai lần

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Tóm tắt

Xu hướng sử dụng thực phẩm bảo vệ sức khỏe (TPBVSK) ngày càng trở nên phổ biến hơn. Tuy nhiên, các hormon sinh dục được trộn trái phép nhằm nâng cao hiệu quả sản phẩm như thúc đẩy mọc tóc, phát triển cơ ngực, ngăn ngừa lão hóa da, thúc đẩy tăng trưởng,... Trong nghiên cứu này, phương pháp sắc ký lỏng khối phổ 2 lần LC-MS/MS được phát triển và ứng dụng để định lượng các hormon sinh dục: progesteron (PG), testosterone (TS) và 17 β -estradiol (E2) trong TPBVSK. Các khảo sát bao gồm điều kiện xử lý mẫu và điều kiện phân tích. Nghiên cứu sử dụng cột sắc ký pha đảo Symmetry C18 (150 mm x 3,0 mm x 3,5 μ m). Điều kiện khối phổ chế độ ion hóa dương, chọn được ion mẹ và hai mảnh ion con tương ứng cho chất phân tích. Phương pháp có thể định lượng PG, TS ở hàm lượng 0,4 mg/kg và E2 ở hàm lượng 4 mg/kg trên nền viên cứng, nang mềm và dạng lỏng. Phương pháp sau khi thẩm định đã được áp dụng để phát hiện các hormon sinh dục trong 16 mẫu TPBVSK được mua tại các nhà thuốc tại Hà Nội (Việt Nam) và một số trang mạng online. Kết quả phát hiện 3 mẫu dương tính với hormon sinh dục, trong đó có một mẫu chứa hormon sinh dục bị cấm sử dụng trong TPBSVK (testosterone) theo thông tư 10/2021/TT-BYT với nồng độ rất thấp (khoảng 0,5 mg/kg).

Từ khóa: Hormon sinh dục, thực phẩm bảo vệ sức khỏe, LC-MS/MS.