SIMULTANEOUS DETERMINATION OF SOME STEROID HORMONES IN MILK AND DAIRY PRODUCTS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

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Abstract

A sensitive method has been developed and validated for simultaneous determination of some steroid hormones (trenbolone, 17β-estradiol, progesterone, testosterone, estrone, melengestrol acetate and zeranol). Samples were hydrolyzed to release analytes, and analytes were then extracted from sample matrix using QuEChERS technique, detected and quantified by using reversed phase liquid chromatography coupled with tandem mass spectrometry. The tandem mass spectrometric detection was done by using ESI ion source working in positive mode and multiple reaction monitoring (MRM) quantitation. Validation results show that the method is sensitive and selective for analyzing seven steroid hormones on milk and dairy products. The quantitation limit of the method for these analytes ranged from 0.1 to 2.0 µg/kg. Method recovery was from 70.4 to 110.0 %. Relative coefficient of variation varied from 2.7 to 12%. The method was applied to determine steroid hormones in 40 milk and dairy product samples collected from different markets and commercial centers in Hanoi.

Keywords: LC-MS/MS, QuEChERS, steroid hormones, milk, dairy

1. INTRODUCTION

Milk and dairy products are a popular and important food group in human diet, providing nutritional ingredients to improve the physical development. For the majority of children, milk and dairy products are the main daily diets. However, to increase profits, hormones that stimulate milk production in livestock is increasingly abused, which result in unwanted residues of these hormones in bovine milk and tissues. Up to now, steroid hormones (estradiol, melengestrol acetate, progesterone, testosterone, trenbolone) have been detected in milk. Estradiol and progesterone are natural female hormones; testosterone is a natural male hormone; zeranol, trenbolone acetate, and melengestrol acetate are synthetic growth stimulants. There is clear evidence that certain hormones could cause endocrine disorders and other undesirable effects on humans [1].

Various analytical methods have been used to identify steroid hormones in milk and dairy products such as ELISA, GC-MS or LC-MS/MS. Among these, ELISA is a simple method and requires low investment in equipment. However, it could not simultaneously identify several steroid hormones and may raise false positives. GC-MS method has high sensitivity and accuracy but the sample

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preparation procedure is complicated and expensive due to the need of using different type of reagents to derivatize the analytes [2]. Compared to the above methods, the LC-MS/MS method has many outstanding advantages like simple sample preparation procedure, short analysis time, and, most importantly, the capacity of simultaneous analysis a large number of substances with high specificity, sensitivity and accuracy. Many publications have described the LC-MS/MS methods to simultaneously identify steroid hormones in milk and dairy product matrices [3-5]. Barreir et al. determined six natural sex hormones (pregnenolone, progesterone, dehydroepiandrosterone, estrone, testosterone, and androstenedione) in infant formula [3]. Up to now, there has been no official method for simultaneous determination of all seven sex hormones (estradiol, melengestrol acetate, progesterone, testosterone, trenbolone) in milk and dairy products in Vietnam.

This study aims at developing, validating and applying a method for simultaneous determination of seven common steroid hormones in milk and dairy products using LC-MS/MS, providing a technical solution for the control of these compounds in Vietnam.

2. MATERIALS AND METHODS

2.1. Standards and Materials

Standard substances of trenbolone, 17β-estradiol, progesterone, testosterone, estrone, melengestrol acetate, and zeranol were obtained from LGC (Germany).

Samples used for method development, validation and application were powdered milk, liquid milk, yogurt, whey, butter and cheese randomly purchased in various markets and commercial centers in Hanoi.

2.2. Reagents

Sodium chloride P.A. grade and acetic acid P.A. grade were purchased from Merck Chemical (Germany). Beta-glucuronidase enzyme 5000 U was purchased from Sigma Aldrich (Singapore) and the absorbents PSA, and C_{18} were provided by Agilent Technology (USA).

2.3. Instrumentation

LC-MS/MS 5000 QQQ system of SCIEX, USA was used in this study. Other equipment used includes shake incubator (Amerex, USA), pH meter (Metter Toledo, Switzerland), analytical balance (Metter Toledo, Switzerland), centrifuge (Hettich, Germany), vortex mixer (IKA, China), ultrasonic bath (Elma, Germany), etc.

2.4. Method development and validation

Firstly, the mass spectral conditions were optimized to identify the precursor and product ions. Two transitions were selected for each compound. Then, liquid chromatographic conditions (chromatographic column, mobile phase composition and gradient program) were selected. Finally, sample preparation steps (pH condition, enzyme content, hydrolysis temperature, hydrolysis time, extracting salt, dispersion sorbent) were investigated and optimized.

Referred to previous publications [3-5], the hydrolyzed process combined with QuEChERS methodology was selected. The proposed procedure was as follows: about 10 g of the homogenized sample was accurately weighed into a 50 mL centrifuge tube. Ten mL of acetate buffer (pH = 5.2) was added into the tube, and mixed by vortex within one minute, followed by adding 150 μL β-glucuronidase 5000 U/5mL, and the tube was mixed again within one minute. After the addition of 10 mL of acetonitrile and the salt mixture (4 g $MgSO_4$ anhydrous and 1 g NaCl), the tube was shaken vigorously within one minute and centrifuged within five minutes at 6000 rpm. One mL of the supernatant was pipetted into a 2 mL centrifuge tube containing adsorption mixture (150 mg $MgSO_4$ and 50 mg C18). The tube was then covered, shaken and centrifuged within three minutes at 13000 rpm. The extraction solution was filtered through a 0.2 μm membrane prior to being analyzed by LC-MS/MS.

The method was validated in terms of selectivity, the limit of detection, the limit of

quantification, linearity, precision (repeatability), trueness (recovery). Validation data were assessed and compared to European regulations (EC/657/2002) and AOAC [6, 7].

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS condition optimization

3.1.1. Selection of mass spectral conditions

Precursor and product ions were determined by using direct injection into the tandem mass spectrometry system, using a sample needle of 1 mL for directly injecting 1 µg/mL of hormone standard solutions. The product ions which appear with the highest intensity and stability were used for the quantitation and the other ones which have lower intensity were used to confirm the purpose. The optimized MS/MS conditions were shown in Table 1.

Table 1. *Analysis conditions of seven steroid hormonesby LC-MS/MS*

Analyte	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Collision cell exit potential (volts)	Note
Trenbolone	271	253	27	16	Quantified
		199	35	24	Identified
17β-estradiol	255	133	19	16	Quantified
		159	20	18	Identified
Progesterone	315	109	30	18	Quantified
		97	26	22	Identified
Testosterone	289	97	26	18	Quantified
		109	26	18	Identified
Estrone	271	159	20	20	Quantified
		157	20	18	Identified
Melengestrol Acetate	397	337	20	18	Quantified
		279	20	20	Identified
Zeranol	321	277	-30	-16	Quantified
		303	-30	-18	Identified
Progesterone – d9	324	113	30	16	Internal standard
	324	100	22	20	

3.1.2. Investigation into liquid chromatography conditions

The analyzed steroid hormones are compounds with low polarity; hence, the C18 reverse phase chromatographic column is selected due to its suitability. Reverse phase chromatographic column Symmetry C18 (150mm x 3.0 mm id; 3.5µm) and corresponding pre-columns from Waters were used to separate the hormones in the study.

The investigation of the mobile phase system with two components: (A) water with the added substrate and (B) acetonitrile or methanol was used for research. Prepare a mixture of seven hormones of 10 µg/L concentration to investigate the composition and the mobile phase gradient. The results of the survey of mobile phase were presented in Table 2.

Table 2. Investigation of mobile phase conditions

Mobile phase	Signal intensity			
	A: Water (acetic acid 0.1%)&B: Acetonitrile	A: Water (acetic acid 0.1%)& B: Methanol	A: Water (formic acid 0.1%)&B: Acetonitrile	A: Water (formic acid 0.1%)&B: Methanol
Trenbolone	29,700	9,800	50,367	3,600
17β-estradiol	19,800	3,700	23,200	No peak
Progesterone	28,400	8,500	51,400	2,100
Testosterone	81,300	7,600	27,200	1,800
Estrone	11,500	5,500	26,300	1,500
Melengestrol acetate	15,900	6,300	25,800	1,700
Zeranol	14,500	4,300	43,000	No peak

The optimized mobile phase gradient condition was specified as followed: initial 30% B (five minutes); increase to 90% from five to nine minutes, maintain within 1 minute; decrease to 30%. Total analyzed time was 15 minutes.

3.2. Investigation into the sample preparation

The selected sample was a children's supplement drink which was detected with four substances (trenbolone, progesterone, testosterone, estrone). The sample was spiked with the standard solution of three undetectable substances in the sample (17β-estradiol, melengestrol acetate, zeranol). Then, the whole sample was shaken and stored for 24 hours at the temperature of 2 ÷ 8°C before processing.

3.2.1. Selection of pH of buffer solution

Carry out the tests with different pH values (4.6, 5.0 and 5.2) of the buffer solution. The result was given in Figure 1.
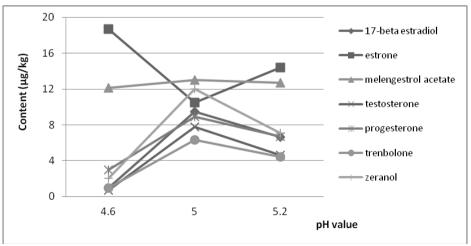

Figure 1. Comparison the pH of buffer solution

The results in Figure 1 showed that the obtained results at three pH values were different. The contents of six hormones (except estrone) at pH = 4.6 were lower than those of other pH values.

The obtained content of substances at pH = 5.2 was lower than that at pH = 5.0 (with exception of melengestrol acetate, whose content was a bit higher at pH = 5.2). Moreover, better stability of analysis results were observed at pH = 5.0. Therefore, pH = 5.0 is the optimal value of the acetate buffer in this study.

3.2.2. Evaluation of enzyme amount

Preliminary trials were done with 4 different amounts of enzyme: no. 25, 50, 100, 150 µL β-glucuronidase 5,000 U solution/5 mL water. Results of these expriments were presented in Figure 2.
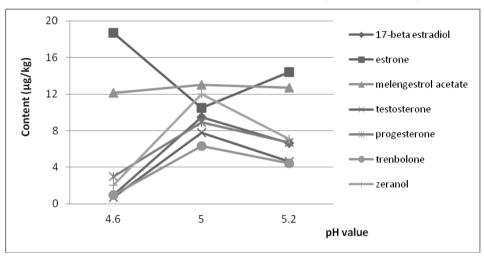

Figure 2. *Evaluation of enzyme amount*

The results in Figure 2 pointed out that the recovery of the substances changed while changing the added amount of enzyme. The lowest efficiency was observed at 25 µL of enzyme solution then it increased rapidly when using 50, 100 µL of enzyme solution and changed not significantly when increased to 150 µL β-glucuronidase solution 5,000 U/5 mL of water. These results demonstrated that the most suitable amount of enzyme was 100 µL. This amount was sufficient for complete ydrolysis of the sample. In order to both ensure the efficiency of the method and to save costs, 100 µL β-glucuronidase solution of 5,000 U/5 mL was selected as the optimal amount of enzyme.

3.2.3. Selection of components of the salt mixture in the extraction

Preliminary trials were done on three types of salt mixture: four g $MgSO_4$ and one g NaCl (mixture 1); four g $MgSO_4$ and one g CH_3COONa (mixture 2); four g $MgSO_4$, one g NaCl, one g sodium citrate and 0.5 g sodium citrate tribasic dehydrate (mixture 3).
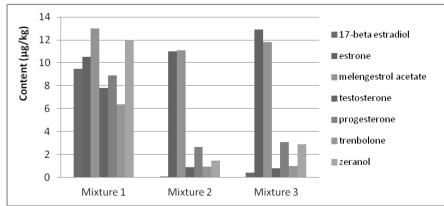

Figure 3. *Diagram for investigation of salt mixture in the extraction*

The results in Figure 3 pointed out that there were no major difference in estrone and melengestrol contents when changing the salt mixtures. Perhaps these two hormones were not

significantly affected by the choice of salt mixtures. Meanwhile, the content of other five hormones are greatly affected by the presence of acetate and citrate salts. When acetate and citrate salts were present in the salt mixture, the concentration of these five hormones decreased drastically. It was probable that these two salts had changed the pH of the sample solution, rendered it not suitable for the extraction of these steroid hormones. So the salt mixture 1 (4 g $MgSO_4$ anhydrous, 1 g NaCl) is the most suitable one for the extraction process.

3.3. Method validation

3.3.1. Selectivity

The blank, standard and spiked samples with concentration of each hormone at 10 µg/kg in milk and butter matrices were analyzed using the optimized method. An example of chromatograms of testosterone are presented in Figure 4.
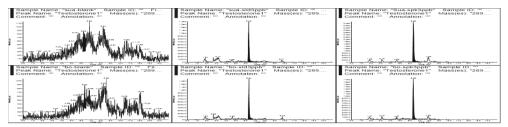

Figure 4. Chromatograms of blank, standard and spiked sample (testosterone)

The given chromatograms in Figure 4 revealed that there was no signal within the retention time window of the analyte. Retention time of compound in standard and spiked sample have the deviation that did not exceed 5%, which conformed to Europe regulation (EC/657/2002).

Number of identification points (IPs) and ion ratio of seven hormones were gathered in Table 3.

Table 3. Number of IPs and ion ratioof seven steroid hormones

Analyte	Precusor ion	Produc Ions	IPs	Standard sample ion ratio	Spiked sample ion ratio	% deviation
Trenbolone	271	253/199	4	34.7	31.5	± 20%
17β-estradiol	255	133/159	4	63.8	54.1	± 25%
Progesterone	315	109/97	4	40.9	43.1	± 20%
Testosterone	289	97/109	4	29.5	33.8	± 20%
Estrone	271	159/157	4	43.6	47.0	± 20%
Melengestrol acetate	397	337/279	4	58.4	51.6	± 25%
Zeranol	321	277/303	4	31.8	28.5	± 20%

The results in Table 3 confirmed that number of IP points and the ion ratio of seven hormones all met the Europe regulations for analysis using tandem mass spectrometry (EC/657/2002).

3.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

The spiked samples at the concentration of 0.1 µg/kg for trenbolone, progesterone; at 1 µg/kg for 17β-estradiol, testosterone, estrone and melengestrol acetate), and at 2 µg/kg for zeranol were analyzed and the ratio of S/N for each analyte was calculated. The results of LOD and LOQ were summarized in Table 4.

Table 4. *LOD and LOQ value of seven steroid hormones*

Hormone (µg/kg)	Trenbolone	17β-estradiol	Progesterone	Testosterone	Estrone	Melengestrol acetate	Zeranol
LOD	0.1	1.0	0.1	1.0	1.0	1.0	2.0
LOQ	0.3	3.0	0.3	3.0	3.0	3.0	6.0

In comparison to previous studies, the obtained LOD and LOQ value of this study was significantly better. LODs varied from 0.3 to 6.0 µg/kg, guaranteed the method sensitivity for the intended application.

3.3.3. Linearity

Linearity of steroid hormones was determined by analyzing different standard solutions with the concentration from 0,3 to 30 mg/L (trenbolone, progesterone), from 3 to 300 mg/L (17β-estradiol, testosterone, estrone, melengestrol acetate; from 6,0 to 300 mg/L (zeranol) in blank matrices. All the calibration curves have the coefficients of determination (R^2) higher than 0.995 which meet the criteria.

3.3.4. Repeatability and recovery

The repeatability and recovery of the method are validated by analyzing the spiked samples at three different concentrations with six replicates at each concentration. Results were shown in Table 5.

Table 5. *Results of repeatabilityand recovery*

No.	Hormone	R (%)	RSD (%)
1	Trenbolone	83.7 ÷ 106.0	5.4 ÷ 11.0
2	17β-estradiol	77.3 ÷ 105.0	5.7 ÷ 8.4
3	Progesterone	76.0 ÷ 93.3	4.0 ÷ 9.2
4	Testosterone	83.3 ÷ 102.0	5.8 ÷ 7.0
5	Estrone	76.3 ÷ 110.0	4.6 ÷ 7.0
6	Melengestrol acetate	97.3 ÷ 110.0	2.7 ÷ 5.0
7	Zeranol	70.4 ÷ 101.0	3.5 ÷ 12.0

The obtained results showed that the method have the recovery in the range of 70.4 ÷ 110%, and the relative deviation in the range of 2,7 ÷ 12,0% which conformed with the AOAC International's regulation.

3.4. Application for analyzing steroid hormone in milk and dairy products

The method was applied for the determination of seven steroid hormones in 40 milk and dairy products collected from different markets in Hanoi. The results are listed in Table 6.

Table 6. *Testing of hormone in milk and dairy product samples*

Analyte	Milk Powdered		Liquid Milk		Other Dairy Products	
	Number of detected sample	Content (µg/kg)	Number of detected sample	Content (µg/kg)	Number of detected sample	Content (µg/kg)
Trenbolone	0/10	-*	1/10	< 0.3	8/20	0.44 ÷ 34
17β-estradiol	0/10	-	0/10	-	0/20	-

Progesterone	8/10	3.5 ÷ 12	8/10	1.9 ÷ 13	11/20	0.63 ÷ 34
Testosterone	0/10	-	1/10	< 3.0	0/20	-
Estrone	5/10	11 ÷ 22	8/10	3.5 ÷ 26	2/20	3.1 ÷ 3.6
Melengestrol acetate	0/10	-	0/10	-	0/20	-

*Not detected

Analysis on 40 samples detected the presence of estrone in five powdered milk samples, eight liquid milk samples and two dairy products; that of progesterone in eight powdered milk samples, eight liquid milk samples and eleven dairy products. Especially, trenbolone was detected in one liquid milk sample and eight dairy products; testosterone was detected in one liquid milk sample which requires a more stringent control on steroid hormones content in milk and dairy products.

4. CONCLUSION

Method for simultaneous determination of seven steroid hormones (estrone, 17β- estradiol, progesterone, testosterone, trenbolone, melengestrol acetate, zeranol) was developed and validated, completely satisfied the regulation of AOAC International and Europe. The method could be applied to determine steroid horme in milk powderd, liquid milk or dairy products.

Acknowlegdement

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Tóm tắt

XÁC ĐỊNH ĐỒNG THỜI MỘT SỐ HORMON STEROID TRONG SỮA VÀ CÁC SẢN PHẨM TỪ SỮA BẰNG KỸ THUẬT LC-MS/MS

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Phương pháp xác định đồng thời một số hormon steroid (gồm trenbolone, 17β-estradiol, progesterone, testosterone, estrone, melengestrol acetate và zeranol) đã được xây dựng dựa trên quy trình xử lý mẫu thủy phân kết hợp với chiết QuEChERS và phân tích bằng kỹ thuật LC-MS/MS. Hệ thống sắc ký lỏng với cột C18 pha đảo, detector khối phổ ba tứ cực nguồn ESI (+) và chế độ theo dõi ion MRM đã được sử dụng trong nghiên cứu. Kết quả thẩm định cho thấy phương pháp có độ nhạy và chọn lọc cao đối với 7 hormon trên nền mẫu sữa và các sản phẩm sữa. Phương pháp cho phép xác định các hormon ở mức hàm lượng thấp với giới hạn định lượng trong khoảng 0,1 ÷2,0 µg/kg. Quy trình có độ thu hồi trong khoảng 70,4 ÷ 110,0%, độ lệch chuẩn tương đối trong khoảng 2,7 ÷ 12,0%. Phương pháp đã được ứng dụng để xác định hàm lượng 7 hormon steroid trong 40 mẫu sữa, sản phẩm sữa được thu thập trên thị trường Hà Nội.

Từ khóa: LC-MS/MS, QuEChERS, hormon steroid, sữa