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Research Article

Determination of 8 steroid hormones in meat and meat products by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Abstract

Steroid hormones and endocrine compounds play an important role in regulating body physiology, however, they can pose potential risk to environmental and human health. In this study, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed to simultaneously analyze eight steroid hormones (i.e., boldenone, boldenone-17-alpha, progesterone, testosterone, melengestrol acetate, zeranol, trenbolone acetate, trenbolone-17-alpha) in meat and meat products. Samples were extracted using the QuEChERS method with acetonitrile (ACN) as solvent and and clean-up procedure using PSA and C18 sorbent combined with $MgSO_4$. The conditions for hormone separation on the LC-MS/MS system included a C18 reversed-phase column (2.1 × 150 mm, 3.5 µm) with a mobile phase of 0.1% formic acid and 10 mM ammonium formate in water and ACN using gradient elution and flow rate 0.5 mL/min. The analytical method had good specificity with a linear range ($R^2 > 0.99$) from 1-10 ng/mL, recovery of the substances reached 70.1-115%, relative standard deviation (RSD%) in the range of 1.23-12.1%. The limit of quantification of all eight substances was 1.0 µg/kg. The method was applied to determine 8 hormones in 20 meat and meat product samples in Hanoi. The analytical results showed that none of the 8 steroid hormone analytes were detected in all samples.

Keywords: *steroid hormone, LC-MS/MS, QuEChERS, meat and meat products.*

1. INTRODUCTION

Steroid hormones and natural or synthetic hormonal compounds play an important role in regulating physiological functions of the body, such as growth, reproduction and maintaining homeostasis. They are widely used in medicine, animal husbandry and sports [1]. In medicine, steroid hormones are used to treat endocrine disorders, cancer and osteoporosis [1, 2]. In addition, they are also commonly used in animal husbandry to stimulate growth and improve meat quality [1, 2]. Steroid hormones and hormonal compounds used in animal husbandry can be divided into three main groups: androgens and anabolic steroids (AAS), estrogens and estrogen-like compounds, and progestins [3]. However, if abused in animal husbandry, it will lead to hormone residues in meat and meat products, adversely affecting the health of consumers. They can cause endocrine disorders, affect the development of children, increase the risk of cancer, cardiovascular disease and infertility.

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Therefore, the Ministry of Health issued Circular No. 24/2013/TT-BYT dated August 14, 2013 on Regulations on Maximum Residue Limits (MRLs) of veterinary drugs in food. Some steroid hormones have MRLs ranging from 1-18 µg/kg depending on the substance and type of food [4]. In addition, the Ministry of Agriculture and Rural Development also issued Circular No. 10/2016/TT-BNNPTNT dated June 1, 2016 [5] on the list of veterinary drugs permitted for circulation and prohibited for use in Vietnam. Therefore, controlling hormone residues in food in general, meat and meat products in particular, is essential to protect public health.

Currently, a number of different methods have been used to analyze steroid hormones in food separately or simultaneously. Among them, chromatographic methods, including gas chromatography [6] and liquid chromatography [7-9], are commonly used. Because steroid hormones are thermally stable and have low volatility, derivatization is required before analysis on GC-MS/MS. The LC-MS/MS method has the advantages of not requiring derivatization, saving costs, high sensitivity and accuracy, and can simultaneously determine many steroid hormones. Therefore, in this study, the LC-MS/MS method was developed to simultaneously determine 8 hormones in meat and meat products.

2. MATERIALS AND METHODS

2.1 Research object/material

The substances selected for the study included eight steroid hormones (**Figure 1**), including: boldenone, boldenone-17-alpha, progesterone, testosterone, melengestrol acetate, zeranol, trenbolone acetate, trenbolone-17-alpha. These substances represent an important group of growth hormones. These hormones are not only banned or restricted in many countries, but also pose a risk of abuse in livestock farming to promote growth in livestock.

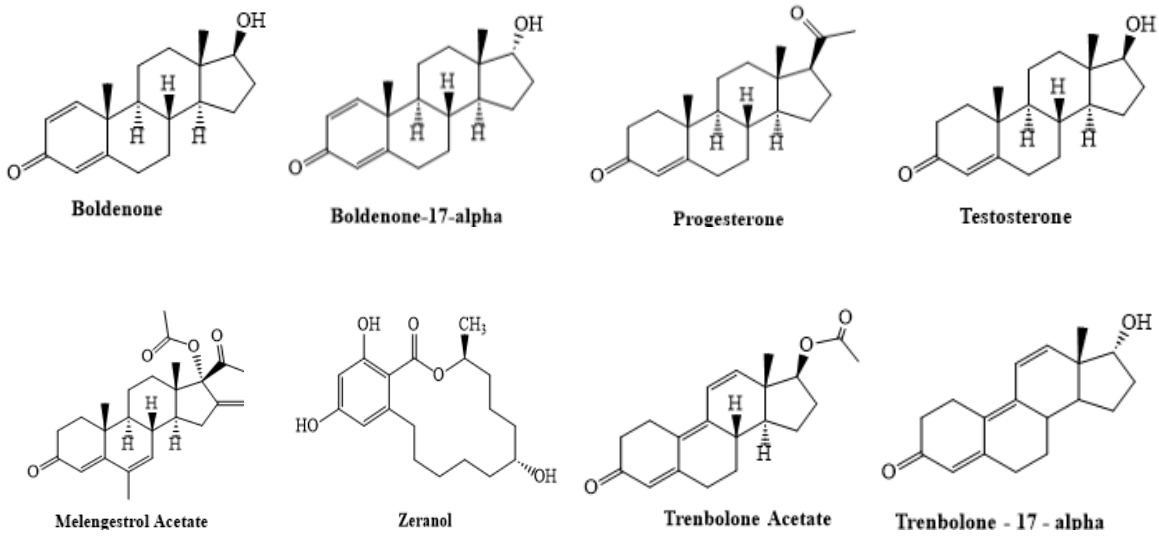

Figure 1. *Structural formulas of eight selected steroid hormones in the study*

The selected samples were pork, beef, chicken, ham, sausage, and Chinese sausage. These samples were randomly collected in Hanoi in March 2025, with a total of 20 samples including: 4 beef samples, 4 pork samples, 3 chicken samples, 3 Vietnamese pork paste samples, 2 sausage samples, 1 grilled pork paste sample, 1 lean pork paste sample.

2.2. Chemical reagents and standards

Chemicals reagents: methanol (CH_3OH) (Merck, Germany); acetonitrile (ACN, C_2H_3N) (Merck, Germany); acetone (C_3H_6O) (Merck, Germany); acetate buffer (pH 5.0); formic acid (HCOOH) (Merck, Germany); ammonium formate (NH_4HCO_2) (Merck, Germany); anhydrous magnesium sulfate ($MgSO_4$) (Xilong, China); anhydrous sodium chloride (NaCl) (Xilong, China); C18 adsorbent, particle size 40 µm (Agilent, USA); PSA adsorbent (Agilent, USA); sodium acetate (CH_3COONa) (Agilent, USA); sodium citrate

dihydrate ($C_6H_5Na_3O_7.2H_2O$) (Thermo scientific, USA); disodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7.1,5H_2O$) (Thermo scientific, USA); deionized water obtained from Milli-Q ultrapure water purifier (Integral, France).

Details of eight steroid hormone standards are presented in **Table 1**.

Table 1. *Information on the standards used in the study*

No.	Name of the standards	Purity	Lot.	Brand
1	Boldenone	97.24%	G1465886	LGC, UK
2	Boldenone-17-alpha (epiboldenone)	91.60%	G1429529	LGC, UK
3	Progesterone	99.85%	G1152711	LGC, UK
4	Testosterone	98.43 %	G1381985	LGC, UK
5	Melengestrol acetate	94.00 %	G1436669	LGC, UK
6	Zeranol (α-zearalanol)	99.78%	824746	HPC, Germany
7	Trenbolone acetate	95.04%	825343	HPC, Germany
8	Trenbolone-17- alpha	98.33%	826722	HPC, Germany

2.3. Instrument

The main equipment used in the study was a liquid chromatography system LC (SCIEX Exion LC 20AD) coupled with an AB SCIEX Triple Quad 6500+ mass spectrometry (MS/MS) detector (SCIEX), a reversed-phase chromatography column X Bridge C18 (2.1 x 150 mm, 3.5 µm, Waters) and a C18 VanGuard Pre-column (2.1 x 5 mm, 3.5 µm, Waters).

In addition, the study also used some equipment such as a centrifuge that can reach a speed of 6000 rpm with a 50.0 mL centrifuge tube (Mikro 200R, Hettich) and 13000 rpm with a 2 mL centrifuge tube (Extragene), a vortex shaker (IKA), an ultrasonic machine (Elma) and common laboratory equipment.

2.4. Research methods

2.4.1. Analytical method

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to simultaneously analyze eight steroid hormones in meat and meat products. This is a modern analytical method with high sensitivity, accuracy and selectivity, suitable for research purposes.

2.4.2. Sample preparation method

In this study, the QuEChERS method was used to process samples with the advantages of being fast, easy to perform, low cost, effective, stable and safe. Through reference to the document [10], the expected sample processing procedure is carried out as follows: The meat sample is removed from the skin, bones and fat, then chopped and homogenized using a sample homogenizer, the sample is stored at -18°C until analysis. Weigh accurately about 5.0 g of the homogenized sample on an analytical balance (with an accuracy of 0.1 mg) into a 50 mL centrifuge tube. Add 5.0 mL of water, vortex for 1 minute. Add 10.0 mL of extraction solvent (solvents tested: ACN, 0.1% HCOOH/ACN and 1% HCOOH/ACN), vortex for 3 minutes. Add the extraction salt mixture (testing the salts: 1g NaCl combined with 4g anhydrous $MgSO_4$; 6 g $MgSO_4$ with 1.5 g CH_3COONa; 1 g NaCl, 4 g $MgSO_4$, 1 g $C_6H_5Na_3O_7.2H_2O$, 0.5 g $C_6H_6Na_2O_7.1.5H_2O$), vortex for 1 minute and centrifuge at 6000 rpm for 5 minutes. Transfer 1.0 mL of the extract into the d-SPE tube containing the cleaning salt mixture (testing the adsorbent in the d-SPE composition: 0.15 g $MgSO_4$ combined with 0.05 g C18; 0.05 g PSA and 0.025 g C18, 0.025 g PSA), vortex for 1 minute, then centrifuge at 13000 rpm for 5 minutes. Transfer 600 µL of upper extract layer into a sample vial and analyze on the LC-MS/MS system.

2.4.3. Method validation

The validation of the analytical method was performed according to the guidelines of the Association for Official Analytical Cooperation (AOAC) [11]. The validation parameters included: specificity, detection limit (MDL), quantification limit (MQL) of the method, construction of a standard curve and evaluation of linearity, accuracy (assessed through recovery), precision (assessed through repeatability with relative standard deviation (RSD%) values).

2.4.4. Data processing method

The data and results were processed using Microsoft Excel 2019 software. The content of analytes (8 steroid hormones) in the sample was calculated according to the formula:

$$X = \frac{C \times V \times k}{m}$$

Where:

X is the analyte content in the sample (µg/kg);

V is the volume of the extract (mL);

C is the analyte concentration calculated from the standard curve (µg/L);

k is the dilution factor;

m is the sample mass (g).

3. RESULTS AND DISCUSSION

3.1. Investigate on selecting conditions for analyzing 8 steroid hormones by LC-MS/MS

Based on the reference [12] and the structure of the analytes, MS/MS conditions were investigated with ESI electrospray ionization technique by injecting non-column standard solutions of each analyte into the mass spectrometer system in turn with a concentration of 50.0 ng/mL. The selected parent ions were further fragmented into daughter ions in Selected Reaction Monitoring (SRM) mode. Since some substances in the steroid hormone group have many isomers and homologues, some substances may have the same quantitative ion and qualitative ion, for example boldenone with boldenone-17-alpha (**Figure 2**), therefore, in addition to the characteristic spectral fragments, retention time is also used to accurately identify isomers or substances with similar molecular weight or similar fragmentation patterns. The analytical conditions for 8 steroid hormones on the LC-MS/MS instrument are presented in **Table 2**.

Table 2. *Retention time and analytical conditions of 8 steroid hormones by LC-MS/MS*

Analyte	t_R	ESI	Molecule weight (g/mol)	Parent s ion (m/z)	Daughter ion (m/z)	CE (V)	CXP (V)	Note
Boldenone	5.02	(+)	286.4	287.2	121.1 135.1	31 19	4 4	Qualitative Quantitative
Boldenone-17-alpha	4.86	(+)	286.4	287.0	135.0 121.0	23 35	4 4	Qualitative Quantitative
Progesterone	5.63	(+)	314.5	315.0	97.0 109.0	55 31	4 4	Qualitative Quantitative
Testosterone	5.02	(+)	288.4	289.0	97.0 109.0	53 31	4 4	Qualitative Quantitative
Melengestrol acetate	5.59	(+)	396.5	397.0	337.0 279.0	20 20	18 21	Qualitative Quantitative
Zeranol (alpha zeranol)	5.42	(-)	322.4	321.0	277.3 303.3	-32 -32	-19 -19	Qualitative Quantitative
Trenbolone acetate	5.51	(+)	312.4	313.0	253.0 91.0	31 69	4 4	Qualitative Quantitative
Trenbolone-17-alpha	4.85	(+)	270.4	271.0	253.0 199.0	28 29	4 4	Qualitative Quantitative

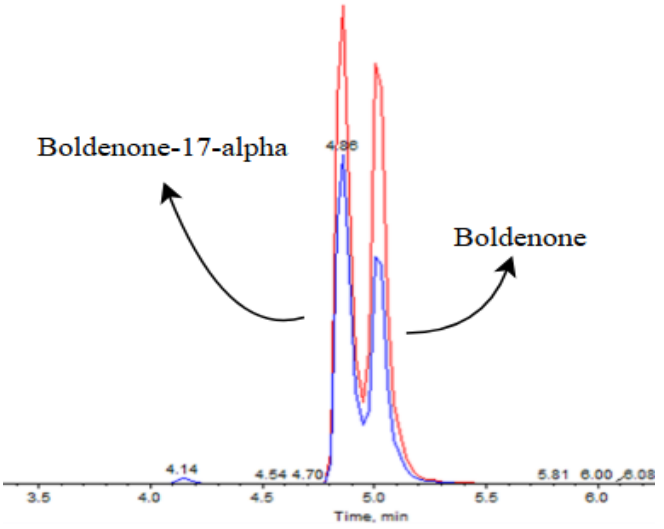

Figure 2. *Chromatogram of separation of two isomers boldenone and boldenone-17-alpha*

Through reference [12] and investigate on the LC-MS/MS equipment system, the mobile phase system selected for use includes 2 channels: channel A: 0.1% HCOOH and 10 mM NH_4HCO_2 in water and channel B: ACN, flow rate is 0.5 mL/min, sample injection volume is 10 µL. Total analysis time is 10 minutes. The mobile phase gradient program is shown in **Table 3**.

Table 3. *Mobile phase gradient program for analysis of 8 steroid hormones*

Time (min)	Channel A (%)	Channel B (%)
0 - 2.01	100.0	0
2.01 - 8.00	0	100.0
8.00 - 10.0	100.0	0

3.2. Optimizing sample processing conditions

3.2.1. Investigation of extraction solvent

Based on the sample treatment procedure described in section 2.4.2, the extraction solvent investigate was conducted with a standard-spiked blank sample at a concentration of 20 µg/kg. The selected extraction solvents for the survey included: ACN, 0.1% HCOOH/ACN and 1% HCOOH/ACN. The results of the survey on the recovery of analytes when using the extraction solvents are presented in **Figure 3**.

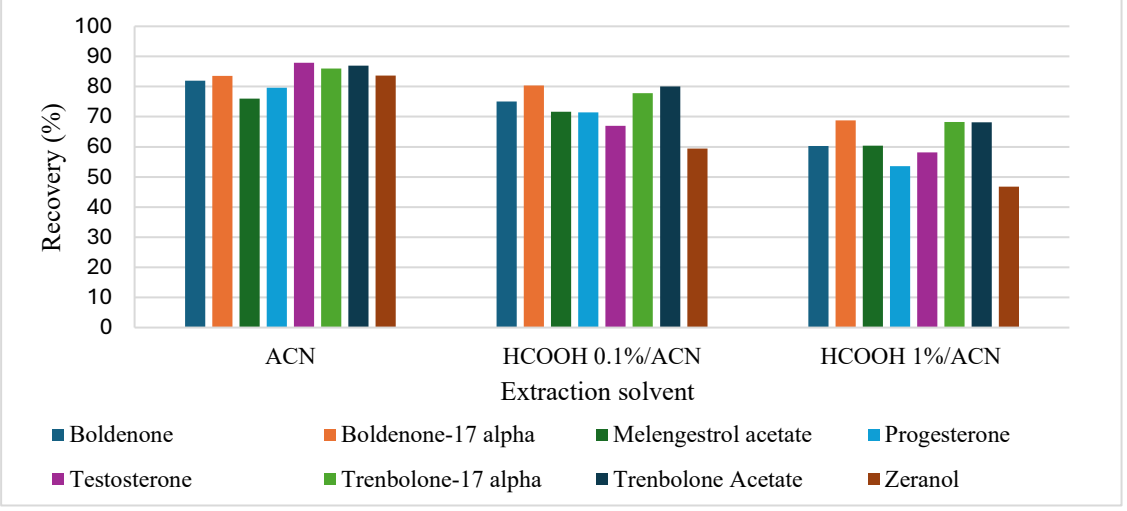

Figure 3. *Results of extraction solvent investigation*

The results in **Figure 3** show that ACN gives a high stable yield (76-88%) for 8 steroid hormones. When HCOOH is added to ACN, the recovery yield of many substances decreases significantly, especially at a concentration of 1% HCOOH/ACN, zeranol decreases from 83.7% to 46.75%. This may be because when adding HCOOH, the pH decreases, reducing the solubility in ACN, making it difficult for the analyte to completely transfer to the organic phase. Therefore, ACN was chosen as the extraction solvent for the following experiments.

3.2.2. Investigation of extracted salt composition

The extraction salt mixture plays an important role in enhancing the polarity of the aqueous phase, while preventing the degradation of hormones by maintaining a stable pH throughout the extraction process using the QuEChERS method. Specifically, the salt mixture includes $MgSO_4$ which acts as a dehydrating agent and NaCl which helps increase the electrolytic capacity of the extract, supporting the substances to be extracted into the solvent better and separated from the aqueous phase. In addition, this mixture also has a combination of sodium citrate dibasic sesquihydrate buffer salts ($C_6H_6Na_2O_7.1.5H_2O$) and sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7.2H_2O$), which are added to stabilize the pH and optimize the extraction efficiency. In this study, the salt mixture components investigated include: (1) 1.0 g NaCl combined with 4.0 g anhydrous $MgSO_4$ and (2) 1.5 g CH_3COONa with 6.0 g anhydrous $MgSO_4$. At the same time, a buffer salt mixture consisting of (3) 1.0 g NaCl with 4.0 g $MgSO_4$, 1g $C_6H_5Na_3O_7.2H_2O$ and 0.5 g $C_6H_6Na_2O_7.1.5H_2O$ was also investigated. The results of the recovery of 8 steroid hormones on a blank sample using different extraction salt mixtures are shown in **Figure 4**.

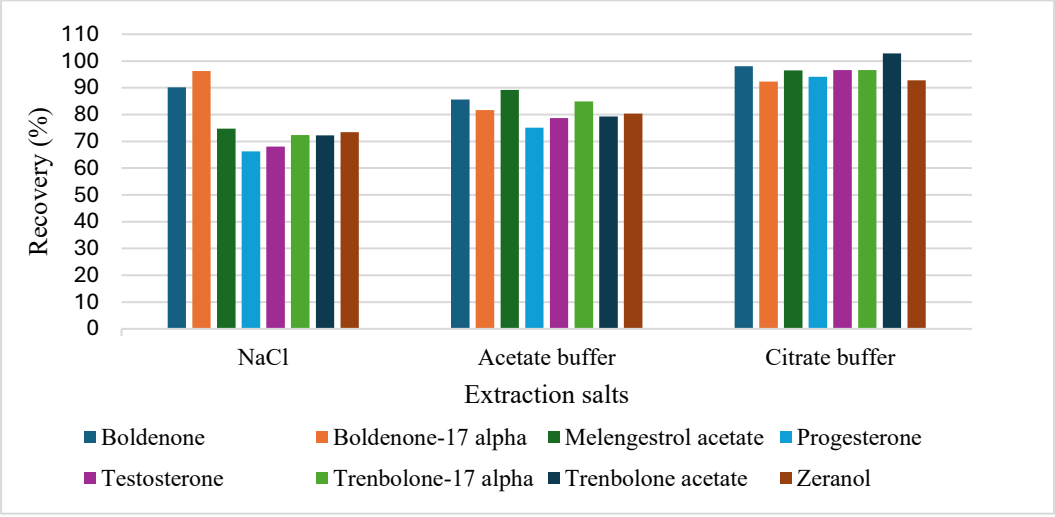

Figure 4. *Results of extracted salt composition investigation*

The results from **Figure 4** show that when using NaCl (1), the recovery of most compounds reached ~80%, however, melengestrol acetate and testosterone had lower recovery efficiencies than 70%. At the same time, the recovery between analytes when using NaCl (1) extraction salt varied quite significantly (68.1-96.2%). Meanwhile, citrate buffer (3) gave higher and more stable recovery efficiencies, with most compounds reaching over 92.25%, indicating good extraction ability. Although acetate buffer (2) showed stability between the recovery efficiencies of the analytes, it was lower than that of citrate buffer (3). Overall, the citrate buffer mixture provided an optimal pH environment, which improved the recovery efficiencies of 8 steroid hormone compounds compared to other extraction salts, so it will be selected for use in subsequent surveys.

3.2.3. Investigation of adsorbents in d-SPE composition

The d-SPE component in the sample treatment process plays a role in removing substances that are co-dissolved with the analyte, increasing the purity of the sample matrix, and reducing the influence of co-dissolved substances on the detection of the analyte on the device. The d-SPE mixture includes $MgSO_4$ to help remove water mixed in the organic solvent layer, C18 powder is a reverse-phase, hydrophobic adsorbent, helping to remove non-polar substances such as lipids, while PSA acts as a primary and secondary amino acid adsorbent used to remove polar substances such as fatty acids and organic acids. Therefore, it is necessary to

investigate and optimize the amount of C18 adsorbent, PSA and the amount of $MgSO_4$ in the d-SPE composition to obtain the best extraction efficiency. First of all, the conditions selected for the study include: 0.05 g C18; 0.05 g PSA and 0.025 g C18 0.025 g PSA when combined with 0.15 g $MgSO_4$ in the d-SPE mixture. The results of the survey of the recovery efficiency of the analytes when using different amounts of sorbents are shown in **Figure 5**. The results showed that the sample treatment process using the mixture of PSA and C18 adsorbents in d-SPE provided the highest and most stable recovery efficiency, ranging from 85.7% to 95.4% for all analytes. When compared with the use of PSA or C18 alone, the recovery efficiency decreased and was almost the same, both < 85%. Meanwhile, the combination of both adsorbents enhanced the ability to effectively remove both polar and non-polar impurities, clean the sample matrix, cause less interference and increase the accuracy in analysis. Therefore, the mixture of PSA and C18 was chosen in this study.

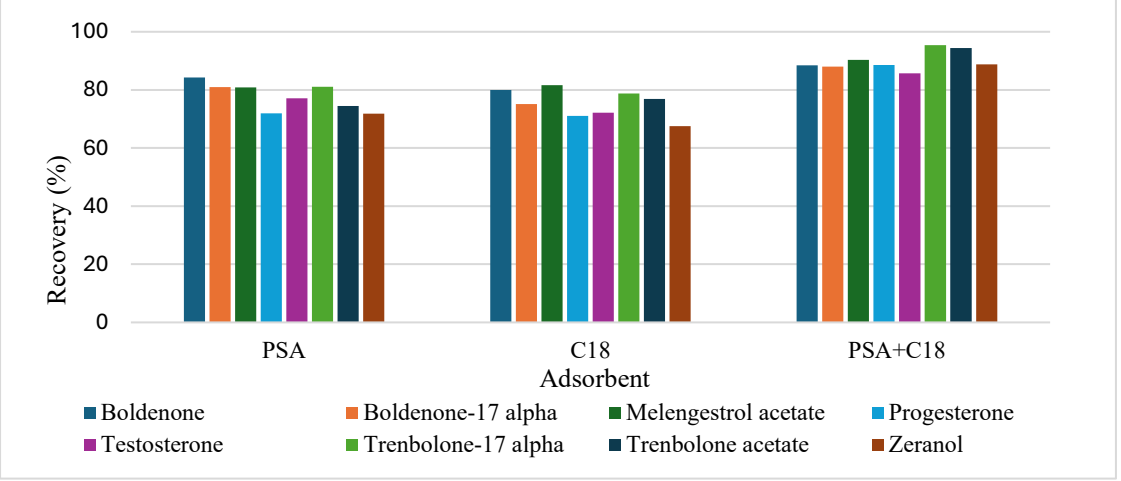

Figure 5. *Results of adsorbents in the d-SPE composition investigation*

Based on the investigation results obtained, the sample processing procedure for simultaneous analysis of eight steroid hormones is shown in **Figure 6**.

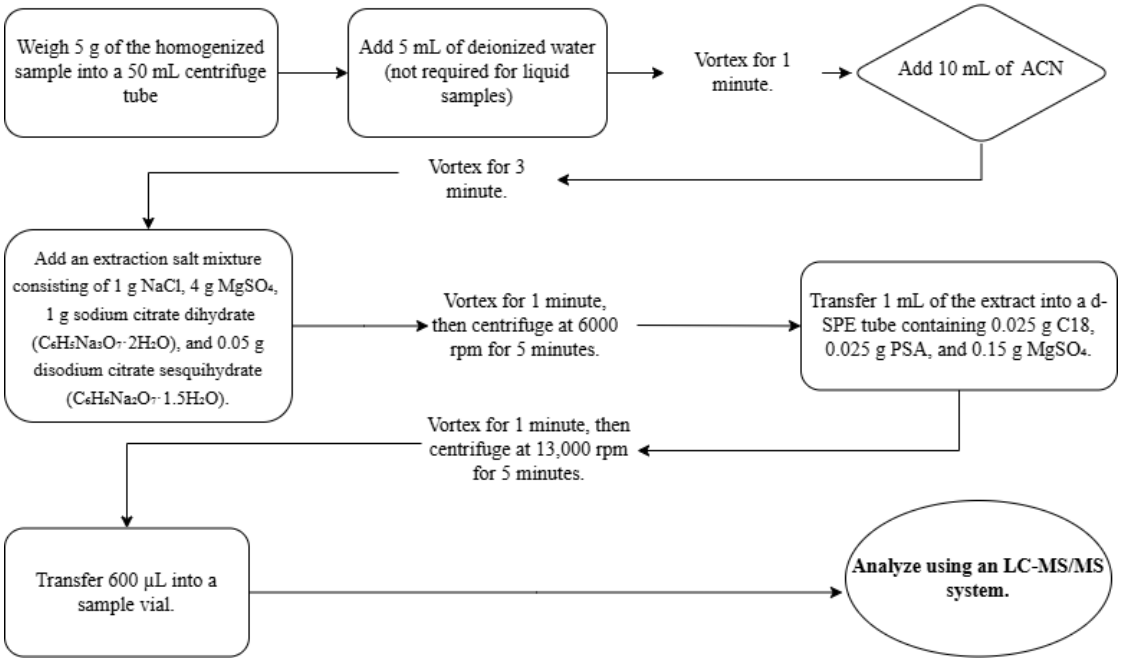

Figure 6. *Sample preparation procedure for simultaneous analysis of 8 steroid hormones in meat and meat products using LC-MS/MS method*

3.3. Method validation

3.3.1. Selectivity

In the chromatographic method using mass spectrometry (MS) detector, the IP score (identification point) is an important value to evaluate the specificity. According to the IP score calculation, 1 parent ion gets 1 point and each daughter ion gets 1.5 IP points [13]. According to the results in **Table 2**, each analyte has 1 parent ion and 2 daughter ions, so 4 IP points are obtained, so the method has specificity that meets the IP score requirement of the LC-MS/MS method (IP ≥ 4). In addition, the specificity of the method is also determined by analyzing the blank sample, standard sample and spiked sample with at least 6 repetitions [13]. From the obtained results, it can be seen that the blank sample does not show any analyte signals, the retention times of 8 analytes in the standard sample and spiked sample are not statistically different (<1%). Therefore, the method has good specificity, meeting the analytical requirements.

3.3.2. Limit of detection and limit of quantification

The detection limit (MDL) and quantification limit (MQL) of the method were determined based on the signal/noise ratio (S/N) [13]. The MDL was determined by analyzing blank samples spiked with decreasing concentrations of the mixture of 8 analytes until a signal/noise ratio (S/N) of 3 was obtained [13]. The MQL was determined by multiplying the S/N ratio by 10 times. The MDL and MQL values of the 8 analytes were 0.3 and 1.0 µg/kg, respectively. According to Circular 24/2013/TT-BYT [4], the maximum allowable limit of zeranol, trebolone acetate is 2 µg/kg, melengestrol acetate is 1 µg/kg in meat, it can be seen that this method has achieved sensitivity to detect and quantify analytes to meet food safety control requirements.

3.3.3. Calibration curve and linearity

Based on the selected analytical conditions, the standard curves of 8 steroid hormones were constructed with a linear dependence between the peak area and the concentration of the analytes in the range of 1.0 to 10.0 µg/kg. To eliminate the influence of the sample matrix, the standard curves of the analytes were constructed on a blank sample. The results of the standard curve equations of the 8 substances together with the correlation coefficients are shown in **Table 4**.

Table 4. *Standard curve equation and correlation coefficient on meat sample matrix*

Analyte	Calibration curve equation	Correlation coefficient (R^2)
Boldenone	y = 63323x + 33302	0.9988
Boldenone-17-alpha	y = 51561x + 2547.3	0.9992
Progesterone	y = 37404x + 7444	1.000
Testosterone	y = 39789x + 7264.4	0.9991
Melengestrol acetate	y = 235472x + 211661	0.9996
Zeranol (alpha zeranol)	y = 14381x + 15673	0.9997
Trenbolone acetate	y = 160528x + 12339	0.9921
Trebolone-17-alpha	y = 18187x + 15371	0.9938

The results in **Table 4** show that the standard curve equations of the 8 substances all have correlation coefficients $R^2 > 0.99$. Therefore, in the investigated concentration range, there is a linear dependence between the peak area and the corresponding concentration. The deviation at all concentration points does not exceed ± 15%, meeting the requirements of AOAC [11].

3.3.4. Precision and accuracy of the method

The accuracy (assessed by recovery (H%)) and precision (assessed by repeatability with relative standard deviation (RSD%)) were based on the analysis of spiked blank samples at 3 concentration levels of 1.00; 3.00 and 10.0 µg/kg. Each concentration level was analyzed 6 times following the sample treatment procedure in **Figure 6**. The precision and accuracy results are summarized in **Table 5.**

Table 5. *Precision and accuracy assessment results*

Analyte	RSD%	H (%)
Boldenone	2.32-3.82	80-114
Boldenone-17-alpha	2.86-6.8	83.6-106
Progesterone	2.90-4.18	80.7-114
Testosterone	1.79-12.07	83-115
Melengestrol acetate	3.09-9.75	80-115
Zeranol (alpha zeranol)	4.55-10.23	88.4-114
Trenbolone acetate	2.17-2.79	80.7-115
Trebolone-17-alpha	1.23-8.74	70.1-113

The results obtained in **Table 5** show that the recovery of the substances is in the range of 70.1 - 115% and the RSD is in the range of 1.23 - 12.1%. According to the regulations of AOAC [11], the analytical method has met the requirements of accuracy and precision, and can be applied to analyze the residues of 8 steroid hormones in real samples.

3.4. Real sample analysis

The method was applied to analyze the content of 8 steroid hormones in 20 meat and meat product samples (including: beef, pork, chicken, Vietnamese pork paste, sausage, grilled pork past, lean pork paste). The results showed that 8 analytes were not detected in all samples (with MDL of 0.3 µg/kg). In the previous study with a larger number of samples than 200, only a few samples detected Progesterone and testosterone at levels higher than MQL in the range of 1.5-35 µg/kg [12]. To demonstrate the applicability of the method to real samples, representative samples were spiked at concentrations of 1 and 50 µg/kg and analyzed according to the procedure selected in **Figure 6**. The chromatograms illustrating the analysis of some real samples and spiked samples are shown in **Figure 7**. The results showed that the recovery efficiency of all substances in the spiked samples was in the range of 81.0 - 107%, meeting the requirements of AOAC [11]. This result demonstrated the applicability of the method to real sample matrices.

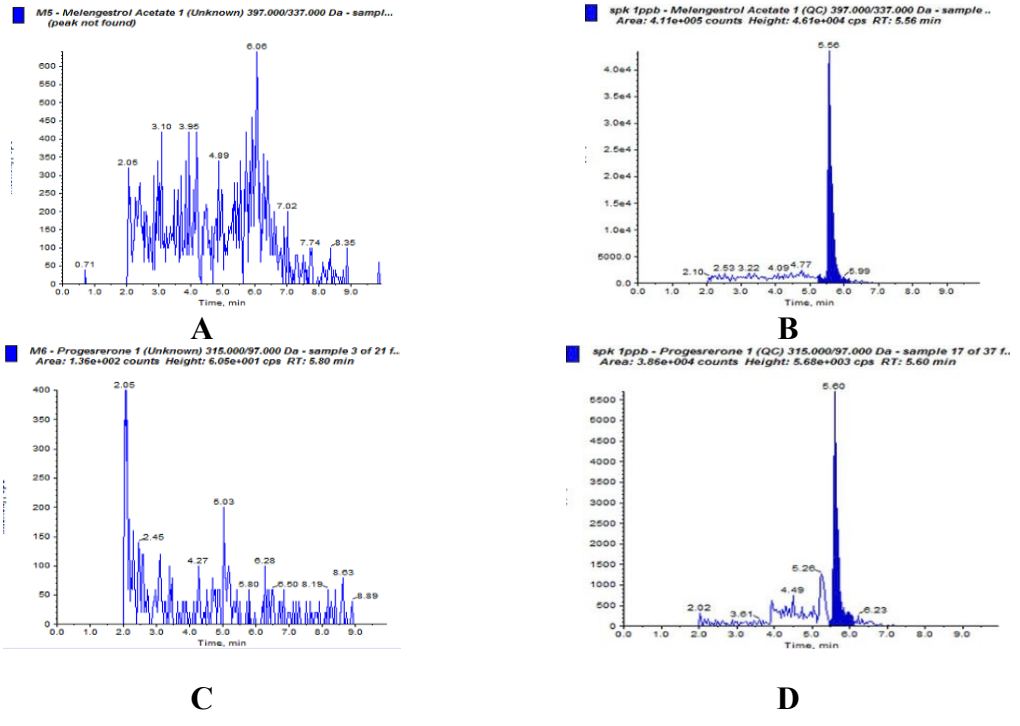

Figure 7. *Chromatograms of actual and spiked samples. A: Vietnamese pork paste, B: Vietnamese pork paste sample spiked (melengestrol acetate), C: Sausage sample, D: Sausage sample spiked (progesterone)*

4. CONCLUSIONS

The study has successfully developed a process to simultaneously determine 8 steroid hormones in meat samples and meat products, with high efficiency and accuracy. The method has been applied to analyze the content of 8 steroid hormones in 20 meat samples and meat products (beef, pork, chicken, Vietnamese pork paste, sausage, grilled pork past, lean pork paste) randomly collected from markets in Hanoi. Initial results showed that no residual steroid hormones were detected in any sample with an MDL of 0.3 µg/kg. The method will continue to be applied to test these substances in different sample subjects, as well as expand the research to other steroid hormones in particular and endocrine compounds in general.

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