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Analysis of anabolic steroid hormone residues in chicken meat from Iranian markets using LC-MS/MS

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ABSTRACT

This research aimed to investigate methyltestosterone and androstenedione hormones in chicken meat using the quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique and liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS). The optimization method was validated by assessing key parameters including recovery, limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation (RSD). Recovery values varied from 76.3% to 116.7%. The LOD and LOQ for both hormones were determined to be 0.16 and 0.50 ng/g, respectively. Analysis of 72 chicken meat samples revealed that the levels of both hormones were below the LOD. Therefore, the concentrations of these compounds were found to be below established regulatory limits, indicating no immediate health risk from these two hormones for consumers.

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1. Introduction

Hormones are biologically active chemical messengers that regulate a wide range of physiological processes by binding to specific receptors on target cells and modulating their cellular responses. They are essential for the regulation of physiological processes and play a pivotal role in growth, development, differentiation, and maturation in both humans and animals.

Anabolic androgenic steroids are the best-known sex hormones and therefore have major anabolic physiological functions such as increasing muscle mass and strength. For this reason, certain naturally occurring and synthetic steroid hormones, such as methyltestosterone and androstenedione, have been illicitly employed in livestock production as growth-promoting agents (1-3). This practice raises significant

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public health concerns due to potential hormone residues in edible tissues.

Growing concerns regarding the occurrence of steroid hormones in various animal-derived foods, including meat, milk, eggs, gizzards, and liver, have contributed to the implementation of restrictions and bans on hormonal use in livestock production since the 1960s. In 1980, the European Commission (EC) established stringent regulations prohibiting the administration of hormones to animals for fattening purposes, while allowing their use solely for therapeutic treatment. When used therapeutically, these hormonal agents should be administered under veterinary supervision, with animals and poultry being slaughtered only after completion of the prescribed withdrawal period. Beginning in the 1980s, the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the World Organization for Animal Health (formerly OIE) initiated evaluations of the safety of hormone use in meat-producing animals (4-6). Many studies have shown that prolonged or excessive exposure to steroids hormones may be adversely affect the liver, kidneys, and other organs of the body, and their residues may cause various diseases, including prostate, ovarian, and breast cancer (2, 5-7).

Over recent decades, considerable research has been conducted to investigate steroid hormones in various edible animal-derived tissues and products (including meat, edible giblet, milk, and eggs) (8-10). Several analytical techniques have been employed to detect and quantify hormones in food samples, including liquid chromatography–tandem mass spectrometry (LC-MS/MS), high-performance liquid chromatography (HPLC), and gas chromatography–mass spectrometry (GC-MS). Among these approaches, LC-MS/MS has been widely regarded as a highly suitable technique because of its high sensitivity and specificity, combined with relatively rapid analysis and efficient simultaneous determination of multiple compounds (11-13). Some researchers examined the applicability of LC-MS/MS for the sensitive determination of steroid hormone residues in food matrices. Owing to its high analytical precision and accuracy, this technique enables reliable detection and quantification of a broad range of steroid hormones present at trace levels in various food samples (13-15).

The study addresses a critical gap in food safety and public health, particularly in the context of the high per capita intake of chicken meat in Iran and globally. With the increasing demand for poultry products, ensuring the absence of harmful steroid hormones like methyltestosterone and androstenedione in meat is of paramount importance. This research not only highlights the potential risks associated with hormone residues in chicken meat but also emphasizes the necessity for stringent monitoring in markets where such products are sold. Moreover, the employing of LC-MS/MS represents a significant methodological advancement in the detection of these compounds. LC-MS/MS is renowned for its sensitivity, specificity, and ability to analyze complex matrices, making it an innovative approach for assessing food safety. By employing this state-of-the-art technique, the study contributes valuable data that can inform regulatory policies and enhance consumer protection against potential health risks associated with hormone contamination in poultry products. Considering that chicken is one of the major components of the Iranian

household food basket and its consumption is increasing day by day due to its cheapness compared to other animal proteins, and since no comprehensive study has been conducted on the status of chicken meat contamination with hormones, the present study was conducted for this purpose utilizing the QuEChERS (quick, easy, cheap, effective, robust and safe) method and LC-MS/MS.

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals and reagents used in the study were of analytical grade. Methyltestosterone (purity 97%) and androstenedione (purity 98%) standards were obtained from Sigma-Aldrich (St. Louis, Missouri, United States). Methanol (purity 99.9%), sodium acetate (CH_3COONa) (purity 99.0%), magnesium sulfate ($MgSO_4$) (purity 98.0%), acetonitrile (purity 99.9%), formic acid (purity ≥ 95%), as well as primary secondary amine (PSA) and C18 sorbents (>95% purity; HPLC reagent grade), were sourced from Merck (Darmstadt, Germany). Individual stock standard solutions of methyltestosterone and androstenedione were prepared at a concentration of 1 mg/mL in methanol. Mixed standard solutions were subsequently prepared at three concentration levels (Mix 1, 10 µg/mL; Mix 2, 1 µg/mL; and Mix 3, 0.1 µg/mL) using methanol as the solvent. All standard solutions were stored at −20 °C until analysis.

2.2. Samples collection

In total, chicken meat (72 samples) was gathered from two different brands (36 samples per brand) in Tehran from July to December 2023. Approximately 100–150 g of lean chicken samples were collected, quickly transported to the laboratory in polyethene bags, and kept at -20 °C.

2.3. Sample preparation

Sample preparation was performed using the QuEChERS method with some modifications (16-19). Briefly, approximately 0.5 g of sample was added to the internal standard mixture. Subsequently, 3 mL of deionized water was added, and the sample was vortex-mixed for 30 s. Then, 5 mL of acetonitrile was added, and the mixture was vortexed for 1 min. Then, 0.75 g of CH_3COONa and 3 g of $MgSO_4$ were added, and the solution was vortexed again for 1 min and centrifuged at 8500 rpm for 10 min. After centrifugation, 1 mL of the resulting supernatant was added to $MgSO_4$ (0.45 g), PSA sorbent (0.075 g), and C_{18} sorbent (0.075 g). The mixture was vortexed for 1 min and subsequently centrifuged at 5000 rpm for 15 min. A 4-mL aliquot of the clarified supernatant was then transported to a vial and evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 500 µL of methanol and 500 µL of deionized water and vortex-mixed thoroughly. Finally, 20 µL of the prepared extract was injected into the LC-MS/MS . system for analysis

LC–MS/MS analyses .2.4

The LC-MS/MS analysis was performed using a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC System UHPLC+Focused (San Jose, CA, USA) coupled to a Thermo Scientific TSQ Quantum Access MAX triple-quadrupole mass spectrometer (QQQ; San Jose, CA, USA). The instrumental parameters and operating conditions were established in accordance with previously published methods (16-21)

The separation was achieved using a reversed-phase C_{18} column (100 mm × 2 mm; BlueOrchid 175-108, KNAUER). The mobile phase consisted of solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in acetonitrile). The gradient elution program was as follows: 40% B and 60% A at 0 min, maintained for 5 min; 100% B at 5 min, maintained for 5 min; 40% B and 60% A at 11 min, maintained for 1 min; and 60% B and 40% A at 22 min. The flow rate was set at 0.8 mL/min, and the injection volume was 20 µL.

For the simultaneous determination of the target compounds, electrospray ionization (ESI) operated in positive-ion mode was employed in combination with multiple reaction monitoring (MRM) (22). Table 1 shows the additional information of the LC-MS/MS ,+[instrument (including parent ion [M+H daughter ions (m/z), CE(eV), DP(eV) and EP(eV)).

3. Results

3.1. Method optimization

To optimize the mass spectrometric conditions and establish the precursor ions, product ions, and collision energies for the MRM method, individual standards were prepared at a concentration of 5 µg/mL and directly injected into the mass spectrometer. The optimized mass spectrometric parameters are summarized in Table 1. Representative chromatograms showing the identification of methyltestosterone and androstenedione based on their precursor and daughter ions are presented in Fig 1 and 2, respectively. The selection of the HPLC mobile phase considerably influences separation efficiency, MS/MS response, analyte ionization, and method sensitivity. In the present study, gradient elution using a water–acetonitrile mixture resulted in higher peak intensities and shorter retention times, whereas the water–methanol mixture provided improved peak separation but produced broader peaks and longer retention times (Fig 3 and 4) (23, 24). The addition of 1% formic acid was used to optimize the mobile phase and enhance the ionization of the target compounds (24).

To account for matrix effects, matrix-matched calibration curves were prepared using five concentration levels (0.5, 1, 2, 5, and 7 ng/g) of the mixed standard spiked into blank samples. The resulting calibration curves showed excellent linearity, with all coefficients of determination (R^2) exceeding 0.999.

Method validation was performed using blank chicken meat samples with the target steroid hormones at five concentration levels (0.75, 1.25, 3, 4.5, and 6 ng/g), followed by analysis under the optimized LC–MS/MS conditions. Recovery performance was examined for methyltestosterone and androstenedione across the evaluated concentration range, including both lower and higher levels. The precision of the analytical procedure was determined based on inter-day measurements conducted over five consecutive days at a spiking concentration of 0.1 µg/mL. The resulting recovery RSD values were all below 9.51%. In positive electrospray ionization mode, the method provided mean recovery values ranging from 76.3% to 116.7% for the investigated hormones (Table 2).

3.2. Measurement of hormones in chicken samples

In the present study, the levels of the investigated hormones in all samples were below the LOD (< 0.16 ng/g). This LOD is above the standard limit set by EC. The LC–MS/MS procedure employed in this study provided adequate analytical sensitivity and reliable

confirmatory capability through multiple reaction monitoring (MRM), while the RSD values obtained for the recovery experiments remained below 9.51%.

Table 1. Precursor and product ions of hormones

Compound	Parent ion [M+H]+	daughter ions (m/z)	CE(eV)	DP(eV)	EP(eV)
Methyltestosterone	303.4	97.2 109.2	29	36	9
Androstenedione	287.3	97.1 109.1	29	36	9

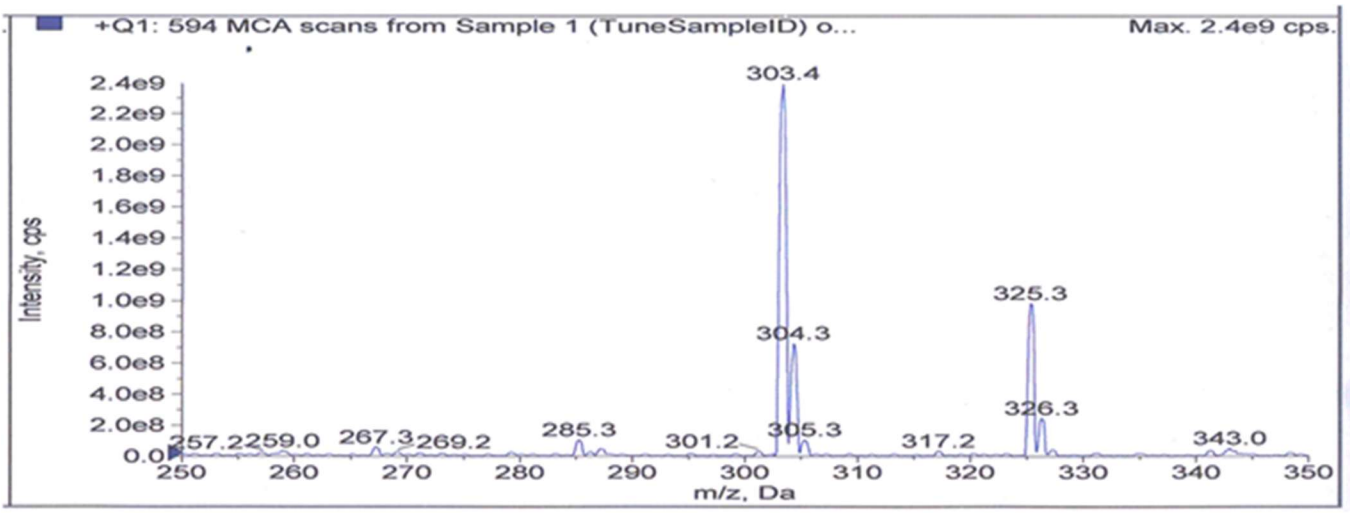
CE: Collision Energy, DP: Declustering Potential and EP: Entrance Potential

Table 2. Results obtained from the determination of hormone in chicken meat samples

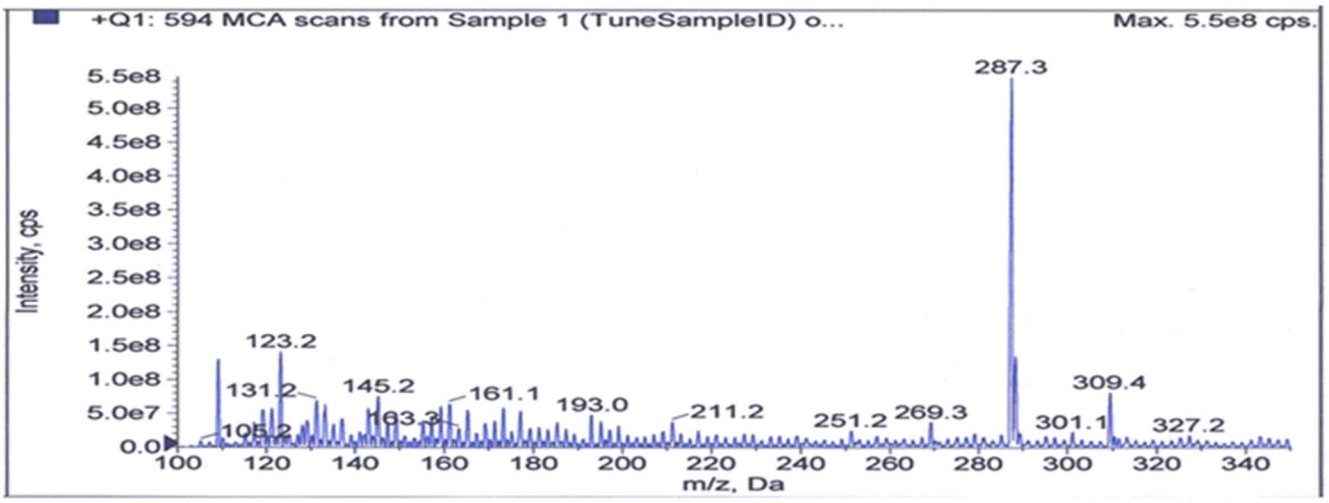
Hormone	Amount in all samples	LOD (ng/g)	LOQ (ng/g)	Spiking Level (ng/g)	Recovery (mean) %	RSD %
Methyl testosterone	***ND or <LOD**	0.16	0.5	0.75	76.3692	5.21
				1.25	102.2715	7.23
				3	111.1720	9.51
				4.5	93.2249	6.54
				6	105.1773	6.33
Androstenedione	***ND or <LOD**	0.16	0.5	0.75	116.7001	6.21
				1.25	80.8810	8.76
				3	102.4505	8.32
				4.5	92.5432	7.56
				6	107.4718	8.31

*** non detected**

N=72

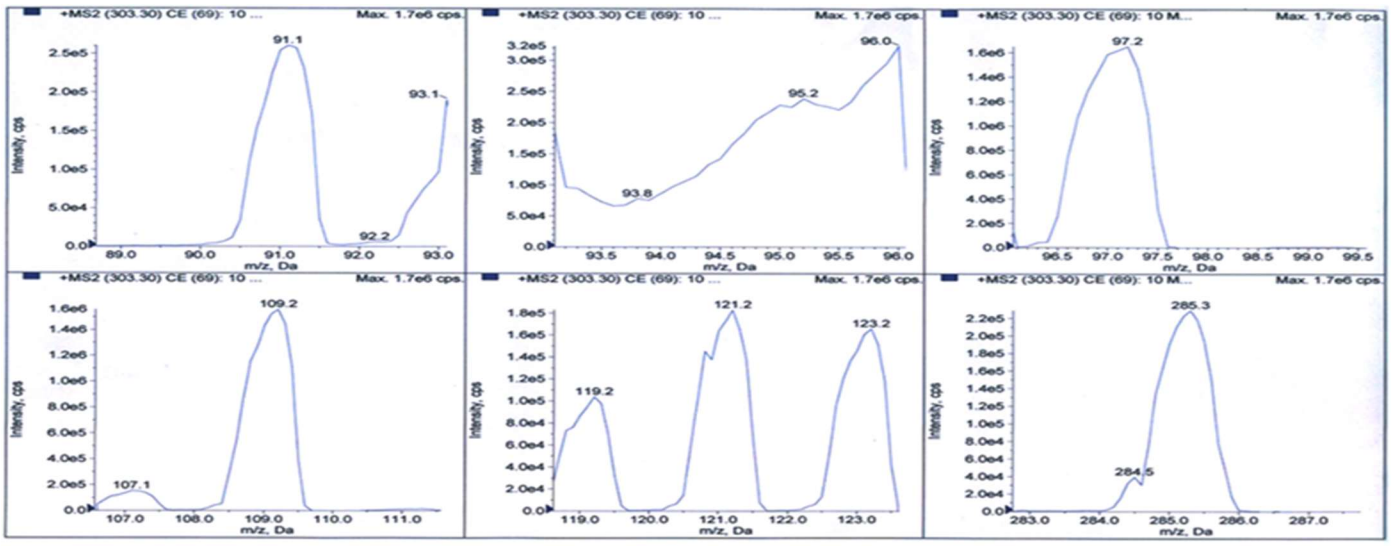


(A)

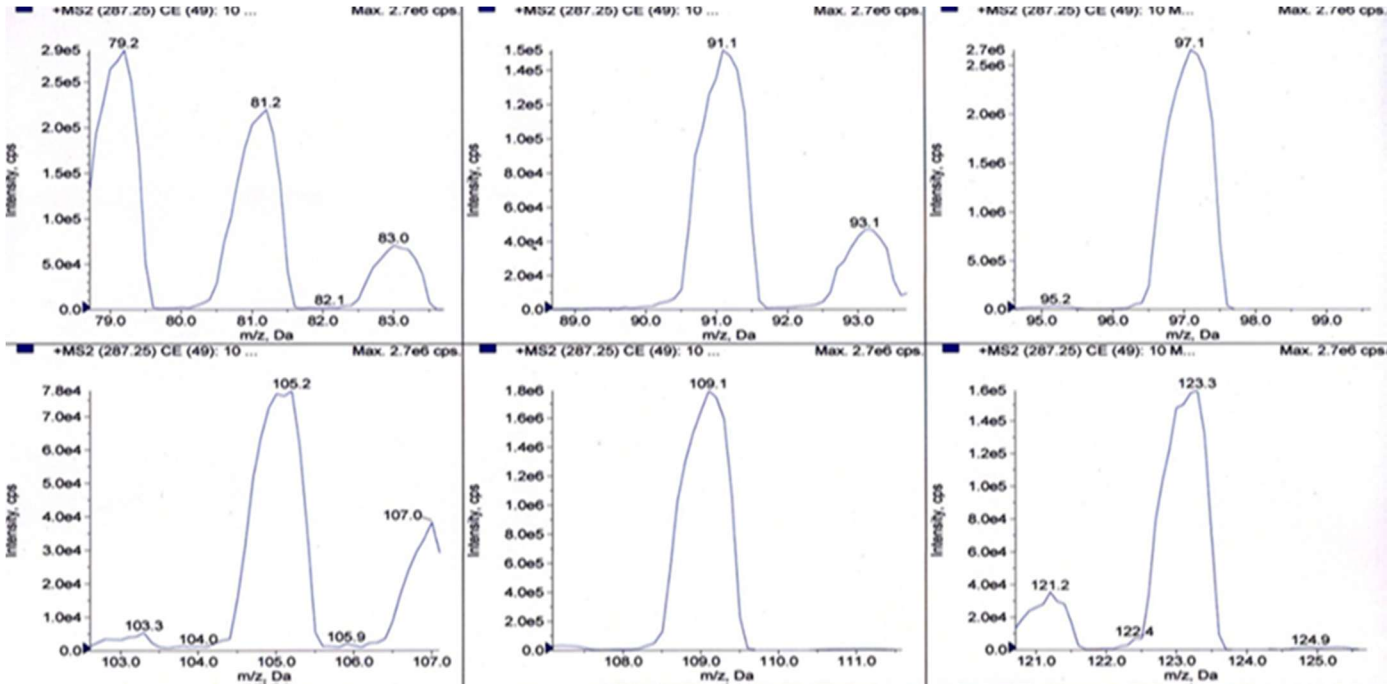


(B)

Figure 1. A) Chromatogram of identification methylestosterone parent ion. B) Chromatogram of identification androstenedione Parent ion



(A)



(B)

Figure 2. A) Chromatogram of identification methylestosterone daughter ions. B) Chromatogram of identification androstenedione daughter ions.

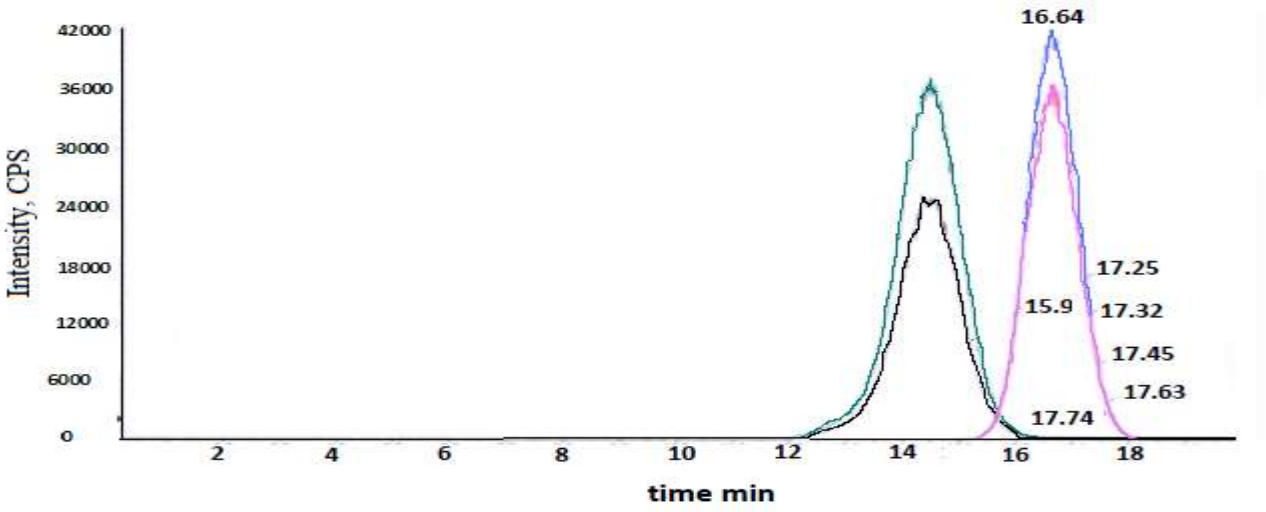


Figure 3. Chromatogram of separation of two hormones with mobile phase, water and methanol

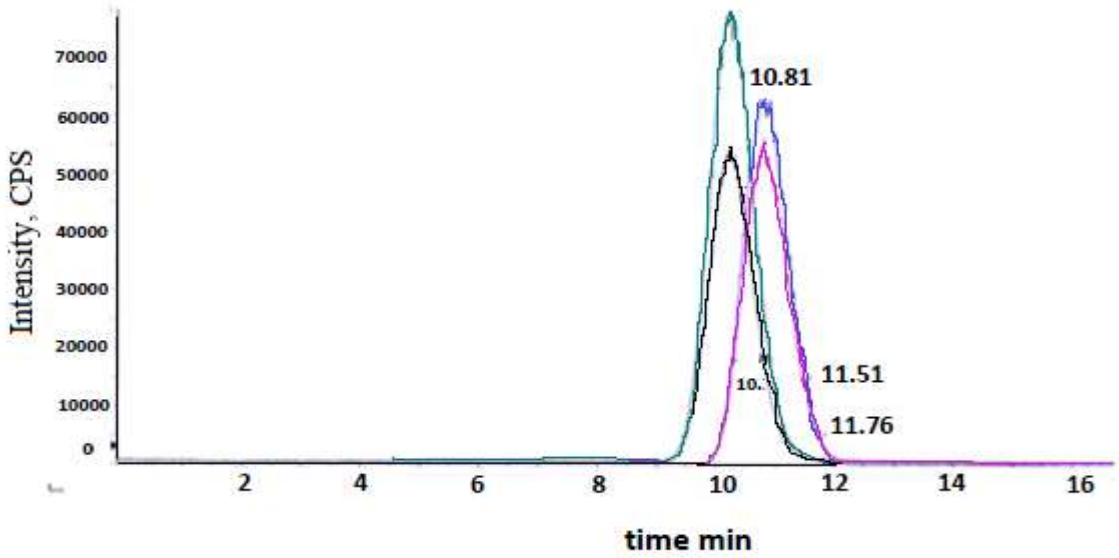


Figure 4. Chromatogram of separation of two hormones with mobile phase, water and acetonitrile

Acetonitrile was selected as the extraction solvent to facilitate the recovery of the target compounds and the removal of lipids from the chicken meat matrix. For subsequent sample clean-up and purification, C18 and PSA sorbents were employed, and the resulting mean recovery was 96.5%.

4. Discussion

4.1. Method optimization

Bussche et al. (25) investigated growth-promoting substances in meat using QqToF-MS and reported that the RSDs obtained for repeatability and reproducibility were below 20%, and the recovery rate was 85.2 - 109.9%, which was somewhat similar to our results. Another study (26) demonstrated that ESI offered greater analytical sensitivity than APCI and was associated with lower mass errors for the steroid hormones examined. In conventional analytical procedures, the extraction and purification of solid food matrices are generally achieved using organic solvents, commonly through liquid–liquid extraction and liquid–solid extraction. Also, they stated recovery and LOD were 70- 103% and 1- 2 ng/g, respectively (which were slightly higher than our findings). Fuh et al. (2004) (LOD= 0.1 to 0.4 ng/g and recovery= 79.3 to 104.3%, which were slightly lower than our findings) (27) and Blasco et al. (2007) (LOD= 0.5 ng/g, which was slightly lower than our findings) (23) utilized acetonitrile for extraction of hormones and Shao et al. (2005) (LOD= 0.001 to 0.12 ng/g and recovery= 64% to 104%, which were slightly lower than our findings) (28), Impens et al. (LOD=2-50 ng/g, which was higher than our findings) (29), Marchand et al. (the results of the method validation are not mentioned) (30) and Seo et al. (LOD= 0.1–0.4 ng/g and recovery= 68–106%, which were slightly lower than our findings) (31) employed methanol as the extraction solvent for the analysis.

In another study, an acetonitrile-based QuEChERS procedure was used to extract target compounds from chicken, beef, and pork samples. The subsequent clean-up involved the use of aluminium oxide and Florisil. The authors evaluated the performance of the QuEChERS procedure against a "dilute and shoot" approach for the analysis of meat matrices and obtained more favorable analytical results with QuEChERS (26).

Guedes-Alonso et al. (32) reported LOD values ranging from 0.14 to 49.0 ng/g, which were slightly lower than those obtained in the present study. They also assessed methanol and acetonitrile as extraction solvents for steroid hormones in fish tissue. The use of acetonitrile resulted in recoveries below 15%, while a 50:50 (v/v) acetonitrile–water mixture produced substantial signal suppression exceeding 30%. In contrast, recoveries above 50% were achieved with both pure methanol and a 50:50 (v/v) methanol–water mixture. However, the latter solvent system was associated with approximately 36% signal suppression. Based on these extraction efficiency and matrix-effect findings, pure methanol was ultimately selected as the organic solvent for microwave-assisted extraction. To minimize interference from phospholipids and proteins, Phenomenex Phree cartridges were subsequently employed for sample clean-up. Using this procedure, the recovery obtained from muscle tissue extracts was approximately 56%.

The validation parameters obtained in this study (recoveries: 76.3–116.7%, RSD <9.51%, LOD=0.16 ng/g) are comparable to, and in some aspects superior to,

those reported in prior literature. This demonstrates the robustness and high sensitivity of the optimized QuEChERS-LC-MS/MS method, positioning it as a reliable method for the surveillance of hormone residues in poultry. This consistency underscores the reliability and validity of our method for detecting steroid hormones in chicken meat samples. Additionally, we have focused on specific aspects and parameters that enhance the accuracy and sensitivity of our results, such as optimizing extraction and analytical conditions. Furthermore, we have examined the limitations of existing techniques and discussed the utilization of LC-MS/MS techniques in assessing food safety. This approach could serve as a new standard for monitoring the quality of meat products in both Iranian markets and other countries.

4.2. Measurement of hormones in chicken samples

In our study, no contamination was found (not detected) at the allowable level of EC in all samples collected from grocery stores and main food supply locations, which may be due to reasons such as the high cost of providing hormones by chicken farms, strict standards in Iran, and continuous monitoring by related organizations. Based on the results of the present research, it can be inferred that the amounts of anabolic hormones in meat chicken samples from Iran are currently below the limits of detection, suggesting effective monitoring practices in meat production. However, further comprehensive studies are needed to evaluate all stages of meat production in Iran.

A review of previous studies:

Shahbazi et al. measured steroid hormone concentrations [estriol (E3), 17β-estradiol (E2), and estrone (E1)] and their conjugated metabolites in cow meat and river buffalo samples, and reported that the E1 level (free and deconjugated value, 16.2±1.1 ng/L) was found to be the highest phenolic estrogen in beef, while the dominant estrogen hormone in river buffalo muscle was E2 (free and deconjugated value, 23.3 ± 1.3 ng/L). Also, they stated that E3 (deconjugated form) was only identified in the meat of buffalo (15.8 ± 1.9 ng/L) (33). Courant et al. measured hormones (steroids) in meat, egg, and milk samples, and reported that targeted androgens and estrogens existed at analogous concentrations (0.01–0.1 ng/g) in the evaluated samples of milk and muscle. The same compounds existed at about 10-fold higher levels (i.e., in the 0.1–1 ng/g range) in the samples of kidney and eggs (34). Hartmann et al. measured anabolic and catabolic steroid hormones (androsterone, dehydroepiandrosterone, epitestosterone, dihydrotestosterone, androstenedione, testosterone, pregnenolone, progesterone and hydroxyprogesterone) in meat and reported these were 0.06, 0.13, 0.07, <0.06, 0.19, <0.02, 1.51. 0.10 and <0.10 ng/g, respectively (35).

Lu et al. (2014) measured 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) in the litter of poultry and reported that the E2 level was as high as 719 ng/g in the top layer of the litter pile and tended to be lower in deeper layers; they also reported that E2 levels were significantly reduced over time (36). López-García et al. measured steroid hormones (testosterone, prednisolone, cortisone, hydrocortisone, acetate, prednisone, testosterone propionate, progesterone, cortisone-21-acetate, 17α-methyltestosterone, melengesterol hydrocortisone-21-acetate, medroxyprogesterone and 6α-methylprednisolone)

and their metabolite (17α-hydroxyprogesterone) in 3 samples of meat (beef, pork and chicken) and reported two hormones, progesterone and hydrocortisone, were determined in a beef and pork sample at 1.7 and 2.8 ng/g, respectively (26). Donna et al. measured ketosteroid hormones (testosterone, progesterone, androsterone, pregnenolone, cortisone, hydrocortisone, trenbolone acetate and melengestrol acetate) in meat samples from four countries (Italy, Poland, Netherland and France) and reported that these hormones were ranged Nd-15.97 ng/g in Italy, Nd-6.76 ng/g in Poland, Nd-24.81 ng/g in Netherland and Nd-8.18 ng/g in France (37). Van Cruchten et al. measured 19-nor-4-androstenedione and nandrolone in pig muscle and reported these were not detected in all samples (38). Wang et al. analyzed steroid hormones (corticosterone, estrone, estriol, medroxyprogesterone, progesterone, 17-αestradiol, 17-α-hydroxyprogesterone, testosterone and 19-nortestosterone) in seven frozen tissues from fish samples belonging to pisces (mackerel, cod, whitebait, hairtail, and yellow croaker), cephalopoda (squid) and crustacea (shrimp), and reported the testosterone was found in yellow croaker (0.45 ± 0.03 ng/g). Also, 19-nortestosterone and 17-αEstradiol were found with the contents of 0.30 ± 0.010 and 39 ± 0.03 ng/g in the shrimp. 17-α-hydroxyprogesterone, 17-α-estradiol, and Corticosterone were found in the hairtail at levels of 0.26±0.01, 0.44±0.02, and 0.57±0.03 ng/g, respectively (39). Ahmadkhaniha et al. (40) measured endogenous androgenic steroids (testosterone) in beef using three methods and reported that this hormone ranged from 0.12 to 1.43 ng/g (40). Seo et al. (31) measured synthetic and anabolic steroid hormones in meat and reported that no samples were detected to contain abnormal levels of synthetic or natural hormones. Goyon et al. (41) assessed steroid hormones (estrogens [17² - estradiol, estrone, estriol and 17±-estradiol,], androgens [testosterone, 4-androstenediol, 5-androstenediol and 4-androstenedione], progestogens [17±-hydroxyprogesterone and progesterone] and glucocorticoids [cortisol and cortisone]) in bovine milk and reporting the average concentration of the hormones analyzed (4-androstenedione = 0.684 ng/g, estrone = 0.159 ng/g, 17±-estradiol = 0.031 ng/g, 17² - estradiol = 0.006 ng/g, 17-hydroxyprogesterone = 0.214 ng/g, progesterone = 15.486 ng/g, cortisol = 0.235 ng/g and cortisone = 0.112 ng/g) was comparable to data available in the literature. Also, in all samples, androstenediols, testosterone, and estriol were not detected at their respective LOQ (41).

Steroid hormones detected in chickens may originate from several sources, including their therapeutic administration by veterinarians, non-prescription use by poultry producers, and endogenous production by the animals themselves through the gonads and adrenal glands (42). While our study found that the levels of these hormones in chicken meat from Iran were below the limits of detection, it is important to recognize that high intake of such hormones may pose various complications to human health. Short-term effects may include gynecomastia, hair loss in men, facial hair growth in women, menstrual irregularities, and voice deepening in women. Long-term exposure can result in more serious health issues, including liver tumors, abnormal levels of cholesterol, heart disorders, testicular shrinkage in boys, and changes in behavior and mood (43-45). Given the high proportion of chicken

in the dietary habits of the Iranian population, routine assessment of steroid hormone concentrations in food remains crucial. Although our findings indicate low hormone levels, the potential side effects associated with higher levels warrant ongoing monitoring (46). Therefore, utilizing precise and accurate measuring devices such as LC-MS/MS is essential for ensuring food safety and protecting public health.

5. Conclusion

Given the significant role of chicken in the global diet, monitoring hormone residues is a crucial food safety objective. This study successfully used the sensitive QuEChERS-LC-MS/MS method, which demonstrated its effectiveness in detecting residues of methyltestosterone and androstenedione in poultry meat with high sensitivity and selectivity. Remarkably, our findings revealed no detectable levels of these hormones in any of the samples tested. These results not only align with the standards set by the European Commission (EC) but also suggest that the contamination levels of chicken meat marketed in Iran are comparable to those found in other countries. As a result, we conclude that the absence of these two hormones in chicken meat indicates that its consumption poses no significant risk (regarding these two hormones) to human health. The significance of this study lies in its contribution to food safety and public health knowledge, particularly in regions where chicken is a dietary staple. Future research should focus on expanding the analysis to other types of poultry and meat products, as well as investigating the long-term health impacts of hormone exposure from various food sources. Additionally, continuous monitoring and regulatory assessments are recommended to ensure ongoing compliance with safety standards.

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Author's contributions

Nabi Shariatifar Design of study, Supervision, Conceptualization, Writing- Reviewing and Editing, Methodology, Validation. **Mojtaba Moazzen**: Writing-Original draft, Design of study, Methodology. **Serveh Anboyeh**: Data curation, Visualization, Investigation, Software, Validation, Methodology, writing- Original draft, Design of study.

Declaration of competing interest

The authors declare that have no competing interests.

Data availability

Data is provided within the manuscript or supplementary information files

Ethics and consent to participate

Not applicable. This study used slaughtered chicken samples, which did not include a code of ethics or informed consent to participate.

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