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Original Article

Determination of naturally occurring estrogenic hormones in cow's and river buffalo's meat by HPLC-FLD method

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ABSTRACT

This study was performed to measure and compare the levels of steroid hormones [estrone (E_1), 17β-estradiol (E_2), and estriol (E_3)] and their conjugated metabolites in cow's and river buffalo's meat in two distinct follicular and luteal phases. Moreover, the possible effect of a heating process on steroid hormone concentration was also investigated. The collected meat (*biceps femoris* muscle) samples were subjected to liquid extraction, enzymatical deconjugation, and C18 solid-phase extraction. Estrogens were analyzed using high performance liquid chromatography equipped with a fluorescence detector. In the follicular phase the levels of steroid hormones (E_1 and E_2) in either tested species were higher than the luteal phase. Moreover, in the present study, E_1 concentration (free and deconjugated value, 16.2 ± 1.1 ng/L) was found to be the highest phenolic estrogen in beef, while the dominant estrogen in muscle of river buffalo was E_2 (free and deconjucated value, 23.3 ± 1.3 ng/L). The study revealed that animal species influenced the concentration of hormones (E_1 and E_2) in the samples. The heating process did not significantly change ($p > 0.05$) the levels of estrogens. The further findings of the present study showed that E_3 (deconjugated form) was only detected in the buffalo's meat (15.8 ± 1.9 ng/L). These data suggest that although meat is one of the valuable nutrient sources for humans, there are, however, increasing concerns about the safety of meat due to the excessive presence of steroid hormones.

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1. Introduction

Among others, dietary composition is important factor, which might affect metabolism and the bioavailability of steroid hormones [1]. Human circulating estrogens are both produced by several organs such as the reproductive system, adrenal glands, and/or entered exogenously through diet and medication. Animal-derived foods contain either estrogens or their metabolites [2]. Since animal foods are rich of cholesterol, saturated fat, and proteins, and these are the precursors for the formation of steroid hormones [3], it is believed that the consumption of animal-derived foods directly and indirectly contribute to human circulating estrogen hormone concentrations. There are increasing experimental and epidemiological studies showing that changing a diet regimen from animal products to vegetables resulted in a significant reduction of estrogen concentration [4,5].

Although in the past decades the most serious meat safety concern focused on contaminated products, especially with bacterial pathogens, nevertheless there are parallel emerging evidence indicating other meat safety-threatening factors such as food additives, allergens, and antibacterial residues [6]. Another meat safety concern is the level of estrogens as there are increasing bodies of data suggesting a crucial role of estrogens in enhancing the cancer rate in meat consumers [3,7,8]. Recently, a 12-year follow-up study showed that greater red meat intake was strongly associated with an elevated risk of breast cancers in premenopausal women that were estrogen and progesterone receptor positive [9].

Hitherto, the levels of naturally occurring estrogens in animal edible tissues have been reported [10]. In this regard, due to the importance of quantification and more precise tracing of estrogens in human food several analytical methods also have been developed [11–13]. It is well established that for more intensive and large-scale analysis, the best way to trace elements and other compounds could be performed by screening assays including enzyme-linked immunosorbent assay technique. At the same time, it should be taken into account that due to very close structural similarity (only a group of hydroxyl) of steroid hormones using the enzyme-linked immunosorbent assay technique may result in a false-positive detection of the examined hormones; therefore, we decided to measure the level of hormones using a validated high performance liquid chromatography (HPLC) method. Since the level of estrogens could be verified upon nutritional, physiological, and other environmental factors, hence in this study the level of estrogens was measured in red meat from cattle in two distinct follicular and luteal phases. Moreover, as one of the main meat producing animals in Iran and some other countries of the south Asia is the river buffalo, therefore the level of naturally occurring estrogens was determined in this species as well.

2. Methods

2.1. Reagents and chemicals

Estrone (E_1), 17β-estradiol (E_2), and estriol (E_3), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Estrogens were dissolved in methanol to obtain 1.0 mg/mL, and stock solutions were stored at −20°C. Working solutions were prepared at appropriate concentrations and stored at 4°C. A standard solution containing 10 ng/mL of each individual estrogen was prepared from this working solution and was also stored at 4°C.

Methanol, acetonitrile, hexane, and dichloromethane (DCM) were of HPLC grade and obtained from Merck (Darmstadt, Germany). A β-glucuronidase/sulfatase-containing extract from *Helix pomatia* was obtained from Sigma-Aldrich. Analytical grade acetic acid was from Merck. Solid-phase extraction (SPE) cartridges C18 (500 mg; 3 mL) were purchased from J.T. Baker (Deventer, The Netherlands). Water was processed through a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Meat samples

Sixty adult cows ($n = 30$; age, 4–5 years; parity, ≥ 3) and river buffalos ($n = 30$; age, 4–5 years; parity, ≥ 3) were included in this study. The study was conducted during a 5-month period, from November to March in two consecutive years (2008 and 2009). The animals belong to nonpregnant Holstein × Friesian cows housed at the experimental farm facility of the Faculty of Agriculture (Urmia University, Urmia, West Azerbaijan, Iran) and nonpregnant river buffalos (*Bubalus bubalis*) kept at the national farm (Bakeri site, Urmia, West Azerbaijan, Iran). All cows and river buffalos were kept in similar diet conditions without any synthetic hormone administration. The basic diet of the cattle and river buffalo consisted of 35% of dry matter from grass hay and 65% of dry matter from a concentrate. The average composition of the concentrate mixture was corn seed (200 g/kg), soybean meal (200 g/kg), wheat bran (200 g/kg), barley (365 g/kg), salt (15 g/kg), and vitamin and mineral mixture (20 g/kg).

In the first step, an expert veterinarian in thriogenelogy, who was not aware of the study purpose, was asked to identify the reproductive phases in either species of animals using a transportable ultrasound device. In the ultrasonography, the periods of proestrus and estrus were diagnosed according to several parameters including increasing thickness especially in the uterine body, increasing uterine fluid accumulation and tonicity, minimum level of the uterine horns curl, the presence of preovulatory follicle, and estrus sign. Moreover, the diestrus cycle was characterized by a low level of uterine thickness and luminal fluid, and an elevated level of the uterine horns curl. Heterogeneous endometrial echotexture is reflective of uterine edema and excitement of the large corpus luteum associated with impending estrus and ovulation [14]. Immediately after slaughter, *biceps femoris* muscle samples were obtained from both proposed species as 15 samples from each phases (follicular or luteal): 15 follicular samples from cows and 15 follicular samples from buffalos and the same for luteal samples. Meat samples were frozen at −20°C until analysis.

2.3. Proximate composition analysis

Proximate composition (moisture, protein, and ash contents) of meat samples were analyzed using a standard method [15].

To determine the moisture content, the samples were dried in an air oven at 103°C to constant weight. Protein was determined based on the total N content using the Kjeldahl method. The ash content was assessed by incineration of the meat samples at 550°C for 5–6 hours in a muffle furnace. The total lipids from samples were assessed using Soxhlet apparatus. They were extracted with petroleum ether from the dried meat sample. The solvent was removed by evaporation and the residue of fat was weighed [16].

2.4. Preparing the raw and heated meat samples

Biceps femoris muscle meat (50 g) samples were thawed in ambient temperature and after removal of the outer surface 10 g of each sample were weighed accurately. The thawed samples were minced and chopped by means of a sterile scalpel blade at room temperature for 15 minutes. The homogenized meat samples were placed into screw-capped plastic tubes and cooked in a water bath until an internal temperature of 80°C was recorded for 1 hour [17]. The internal temperature of the meat samples was determined using a probe-type meat thermometer (model 450-ATT, Omega Engineering, Stamford, CT, USA). The cooked meat samples were then cooled to room temperature and used for further analysis. The raw meat samples from the corresponding animals and the same reproductive phase were also exposed to all procedures except the 1-hour 80°C heating step.

2.5. Sample pretreatment

Following homogenization of the meat samples (10 g) with a Potter–Elvehjem apparatus (Krackeler Scientific Inc., Albany, NY, USA) with a Teflon pestle to determine the total amount of steroids, the samples were treated with β-glucuronidase/sulfatase overnight at 37°C with 25-mL acetate buffer (0.04 mol/L) at pH 5.3 [12]. Extra care was given to avoid any temperature changes which could result in degradation of the samples.

Quality control samples were prepared by spiking blank meat samples (prepared from clinically healthy and sexually immature calves from either species which were previously examined using a HPLC assay) and with a standard mixture of estrogens to final concentrations of 0.5 ng/g, 1 ng/g, and 5 ng/g for each estrogen, which proposed to assessment.

2.6. Sample preparation

To extract the estrogens, a 60-mL mixture of methanol and water (8:2, v/v) was added to all samples including the nonspiked, spiked, or β–glucuronidase/sulfatase-treated meat samples. Thereafter, the samples were vigorously mixed for 10 minutes at 500 rpm on a platform shaker and centrifuged at 2000*g* for 10 minutes at 4°C. To remove the fat part, the samples were extracted two times with 30-mL hexane. The remaining methanol/water part was extracted twice with 40-mL and 30-mL DCM, respectively. The DCM phases were combined, homogenized, and dried under a stream of N_2 gas.

2.7. SPE

The residue from chemical extraction was redissolved in 0.5 mL of methanol, and following vortex mixing, 4.5 mL of water was added. After homogenization, the solution was passed carefully through a C18 SPE column (500 mg; 3 mL), which was preactivated and conditioned with 5 mL of methanol and 5 mL of water, respectively.

The column was then washed with 5 mL of water and dried using N_2 gas. The estrogens were eluted with 4 mL of methanol, and the solvent of the collected fraction was evaporated under a stream of N_2 gas at room temperature. The resulting residue was dissolved in 200-μL mobile phase and a 20-μL liquate was analyzed with HPLC.

2.8. HPLC analyses

HPLC analysis of estrogens in meat samples was carried out according to the previously described method with minor modification [18]. The chromatographic system consisted of an Autosampler (Triathlon type 900, Germany) and dual pumps (Wellchrom HPLC pump, K-1001, KNAUER, Berlin, Germany). The HPLC column was a Nuleodur C18 (150 mm × 4.6 mm, 5 μm, gravity), which was eluted with mobile phase A consisting of a mixture of acetonitrile, water, and formic acid at a ratio of 40:60:0.4 (v/v/v) and with mobile phase B consisting of acetonitrile, water, and formic acid at a ratio of 90:10:0.4 (v/v/v). The flow rate was set at 200-μL/min using a linear gradient run as follows: 100% A for 1 minute, to 100% B in 9 minutes, and finally 100% B for 14 minutes. The HPLC column was equilibrated with 100% A for 10 minutes prior to the next injection. Estrogens were detected by means of a fluorescence detector (RF-10AXL; KNAUER) at an excitation and emission wavelength of 280 nm and 310 nm, respectively.

2.9. Data evaluation and statistical analyses

Quantification of concentrations was carried out using calibration curves, which was primarily obtained for each individual standard. Concentrations were corrected for the recovery of the corresponding analyte from the meat matrix. The limit of detection for each analyte was established by determining the signal-to-noise ratio at 3. For quality controls, evaluation of data was performed following the correction of peak areas for the "background" signals related to the naturally occurring estrogens. These "background" signals were obtained by the analysis of nonspiked ("blank") samples. Paired-samples *t* test at 95% confidence interval was used to determine differences between the level of estrogens in red meat from follicular and luteal phases in either species and also between two tested species of animals. The analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) software package.

3. Results

Results of proximate analysis of cattle and buffalo meat are shown in Table 1. No significant differences ($p > 0.05$) were

Table 1 – Proximate composition of cattle and buffalo's meat.

	Moisture	Protein	Total lipids	Ash
Buffalo meat	75.1 ± 0.73	20.2 ± 0.68	0.8 ± 0.33[a]	1.01 ± 0.04
Cattle meat	74.8 ± 0.25	20.0 ± 0.29	2.8 ± 0.25[b]	1.00 ± 0.08

[a,b] Mean ± standard error of the mean in each column with different letters are significantly different ($p < 0.05$).

found for moisture, protein, and ash between samples from the two species. Based on our results, the fat content of cattle meat was significantly ($p < 0.05$) higher than those found in the meat of the river buffalo.

In the present study, two kinetic elements of recovery percentage and limit of detection level for analyzed estrogens in red meat were estimated as important perquisites. Our findings showed the highest and lowest recovery percentages for E_1 and E_3 in meat samples from river buffalo, respectively. No significant differences ($p > 0.05$) were found between samples from the two examined species (Table 2). We found that the lowest concentration of E_1 and E_2, which could be detected by the used method, was lower than that of E_3 (Table 2). The HPLC chromatogram for three analyzed estrogens both in standard solution and in the meat samples is depicted in Figure 1.

The concentration of free estrogens in red meat samples were measured and the results showed that the level of free E_1 in either examined species in the follicular phase was significantly ($p < 0.05$) higher than that in the luteal phase. Our findings indicated that E_2 levels only in the samples from river buffalo in the follicular phase was significantly ($p < 0.05$) higher than that in luteal phase and there was no significant differences between E_2 concentrations from two phases of cow samples. We failed to show any detectable E_3 in the meat samples from both tested species and in either reproductive phase (Table 3).

The level of all three estrogens after deconjugation processes was measured and the obtained results indicated that E_1 concentration in both species was remarkably lower in the luteal phase samples than that in follicular samples, while E_2 levels in the samples from river buffalo was found to be significantly ($p < 0.05$) lower in the luteal phase than in the follicular phase. Unlike the samples from the cows, in which the E_3 level was lower than the detection level, we found a considerable amount of E_3 in the river buffalo samples and in either reproductive phase. Overall, all three measured steroid hormone levels in river buffalo samples were higher than those in cow samples (Table 4).

To evaluate the heating effect on the steroid hormones level in meat samples, we established an applicable procedures providing 80°C for 1 hour. Comparing the concentrations of free and total (free + conjugated) estrogens in the heated and nonheated samples revealed that there were no significant ($p > 0.05$) differences between them in the cow's and in the river buffalo's meat samples (Tables 5 and 6).

Table 2 – High performance liquid chromatography analyses of estrogens in red meat.

	Recovery (%)		LOD (ng/kg)
	Cow	River buffalo	
E_1	70.86 ± 9.47	75.53 ± 13.17	5
E_2	68.53 ± 7.51	72.21 ± 16.1	5
E_3	59.11 ± 4.73	55.39 ± 8.91	10

Recoveries calculated from three experiments and are presented as averaged values ± standard deviations.
E_1 = estrone; E_2 = 17β-estradiol; E_3 = estriol; LOD = limit of detection.

4. Discussion

Concentrations of phenolic estrogen hormones in cow's and river buffalo's muscles were determined in two follicular and luteal cyclic phases. It has been uncovered that in the follicular phase the levels of steroid hormones (E_1 and E_2) in either tested species were higher than that in the luteal phase. Moreover, we report here that the E_1 concentration was found to be the highest phenolic estrogen in cow's muscle, while in the muscles of the river buffalo the E_2 concentration was the dominant estrogen. The heating process did not significantly change ($p < 0.05$) the level of estrogens.

The first necessary statement that must be addressed is the source of tissues which release estrogens during the two tested cyclic phases. Undoubtedly, the main source of estrogenic hormones in the entire body including the muscles of the female animals is the reproduction system and more precisely the ovaries. During the estrous cycle in cows the follicular phase is a short period (24 hours), during which the level of estrogens is enhanced and followed by the luteal phase that is a rather long period and characterized by a dramatic increase of progesterone. It seems the first finding of the current study confirms the previous data indicating high estrogen levels during the follicular phase in comparison with the luteal phase. The produced estrogens by follicular cells in the follicular phase are transported in the blood stream to all parts of the cow's/buffalo's bodies. Therefore it seems normal to have high steroid hormone concentrations in the follicular phases. At the same time the reason for the presence of steroidal hormones in muscle tissue during the luteal phase (the main hormone is progesterone) could be explained by the fact that due to the short period of the follicular period (maximum 36 hours) in either tested animals, a few days is required to distribute the synthesized steroidal hormones in the entire body. However, other sources of steroidal hormones in the body could not be excluded.

We found in this study there are some differences in the levels of E_1 and E_2 between the two studied species; however, the basic pattern of changes in the hormone profiles of river buffaloes during the estrous cycle closely resembles that of cattle [19]. In this regard, it has to be taken into account that the reproductive system is not the only source of steroid hormones in the entire body and in addition to the adrenal glands, the capability of skeletal muscles in locally synthesizing sex steroid hormones from circulating dehydroepiandrosterone or testosterone has been reported [20]. Therefore, it would be acceptable to have the various levels of measured

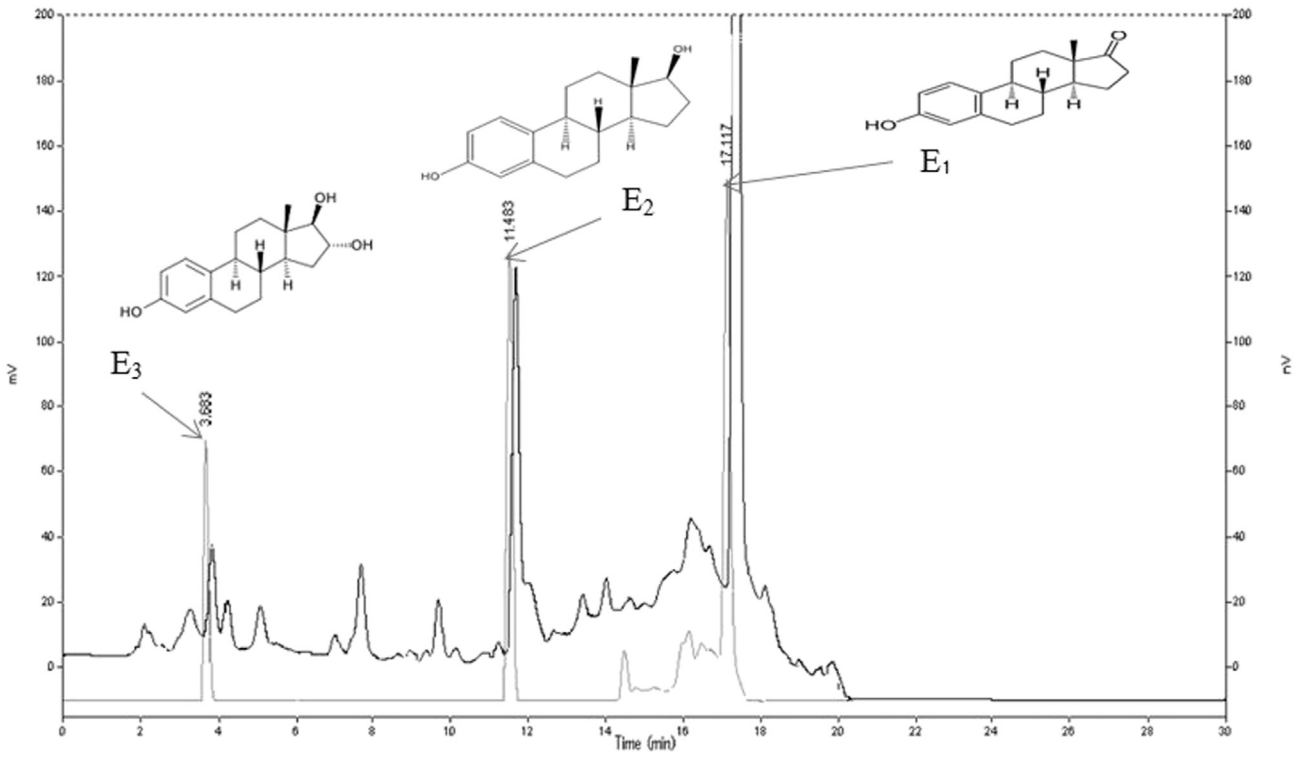

Figure 1 – High performance liquid chromatography chromatograms of E_1 (estrone), E_2 (17β-estradiol), and E_3 (estriol). The solid chromatograms represent the meat sample estrogens and the light chromatograms show the estrogens in standard solution.

estrogens in the muscles of the two different species of animals. There are increasing data indicating notable differences between cattle and buffalo in terms of the genetic variations, nutrition requirements, reproductive performance, milk, and the composition of milk [21,22]. Thus, differences in sex estrogen levels in edible tissues such as muscle, as reported in this study, could also be expected between cow and buffalo.

Another reason for having differences in E_1 and E_2 levels between the examined animals might be related to their muscle fat contents and equally to the solubility of tested hormones in lipophilic or hydrophilic media. According to the results obtained in the current study, the mean values for the fat content of cattle and buffalo meat was 2.8% and 0.8%, respectively. There are numerous reports indicating that buffalo's muscle fat contents are approximately 3.5 fold lower than that in cow's muscles [23], supporting our findings in this study. Thus it might be reasonable to say that the higher fat content in cow's meat the higher lipophilic estrogens such as E_1, and the lower fat content in buffalo's meat the higher hydrophilic estrogens such as E_2. Our previous report showed that there is a strong positive correlation between the lipophilicty of estrogens and the fat content of milks [18]. Another finding of the present study, which showed that E_3 was only detectable in buffalo's meat, may confirm this fact

Table 3 – Free estrogens (ng/kg) in raw red meat from cows and river buffaloes in follicular and luteal reproductive phases.

Species	Cow		River buffalo	
	FP	LP	FP	LP
E_1	13.07 ± 0.44	5.45 ± 0.3*	9.19 ± 0.32[a]	5.5 ± 0.2*
E_2	6.4 ± 1.4	5.9 ± 0.7	11.8 ± 1.3[a]	8.4 ± 1.7[b]
E_3	nd	nd	nd	nd

* Significant differences ($p < 0.05$) between the level of estrogens in red meat from follicular and luteal phases in either species.
E_1 = estrone; E_2 = 17β-estradiol; E_3 = estriol; FP = follicular phase; LP = luteal phase; nd = not detected.
[a,b] Mean ± standard error of the mean in each column with different letters are significantly different ($p < 0.05$).

Table 4 – Sum of free and deconjugated estrogens (ng/kg) in raw meat from adult cows and buffalos.

Species	Cow		River Buffalo	
	FP	LP	FP	LP
E1	16.2 ± 1.1	9.2 ± 0.3[a]	17.6 ± 2.2	10.3 ± 0.9[a]
E2	8.7 ± 0.7	8.1 ± 1.71	23.3 ± 1.3[b]	13.9 ± 1.7[a,b]
E3	nd	nd	15.8 ± 1.9[b]	12.8 ± 1.9[b]

[a] Mean with different letters in the same row are significantly different between follicular and luteal phases in each specie ($p < 0.05$).
[b] Mean with different letters in the same row were found significantly different between the levels of estrogens from follicular and luteal phases in two different species of animals ($p < 0.05$).

Table 5 – Free estrogens in raw and heated red meat from cow and river buffalo (ng/kg).

Species	Cow		River buffalo	
	Raw meat	Heated meat	Raw meat	Heated meat
E_1	8.4 ± 1.1	7.1 ± 0.9	9.8 ± 1.7	7.7 ± 1.2
E_2	6.9 ± 0.8	6.6 ± 0.4	9.5 ± 1.6	8.6 ± 1.5
E_3	nd	nd	nd	nd

No significant ($p > 0.05$) differences were found between raw and heated meat samples.
E_1 = estrone; E_2 = 17β-estradiol; E_3 = estriol; nd = not detected.

that the low lipophilicity of E_3 and the low fat content of buffalo's meat resulted in having a detectable amount of E_3 in buffalo's but not cow's meat.

An interesting finding of the current study is that the rate of glucuronidation for either tested estrogens (E_1 and E_2) in both follicular and luteal phases was found to be significantly ($p < 0.05$) higher in buffalo's meat than that in cow's meat. It is well known that glucuronidation requires uridine diphosphoglucuronic acid and a glucuronyl transferase. Glucuronic acid is attached to a hydroxyl group on the steroid molecules [24]. Formation of the glucuronide conjugates results in a more hydrophilic compound. Therefore, it could be concluded that either the glucuronyl transferase activity in buffalo's meat should be more than cow's meat or due to the higher hydrophilicity of buffalo's meat the glucuronide steroids are easily detectable.

Besides remarkable differences of cattle and buffalo meat fat and glucuronyl transferase activity, the concentration of steroid hormones in different animals can be varied depending upon several factors including physiological condition, age, castration, gestation, diseases, as well as nutritional, seasonal, and geographical conditions [11]. Hence, further research should be focused on the determination of animal hormone differences based on mentioned parameters.

Ultimately in this study we showed that heating processes was not able to significantly alter the level of phenolic estrogens in meat. Previous reports also showed that estrogenic compounds are heat resistance [25].

Undoubtedly meat is one of the valuable nutrient sources for humans; there are, however, increasing concerns about the safety of meat due to the excessive presence of steroid hormones.

Table 6 – Sum of free and deconjugated estrogens (ng/kg) in heated meat from adult cows and buffalos.

Species	Cow		River Buffalo	
	FP	LP	FP	LP
E1	15.3 ± 0.1	8.4 ± 0.5[a]	16.4 ± 3.1	9.7 ± 0.9[a]
E2	8.2 ± 0.5	7.4 ± 0.6	22.9 ± 0.6[b]	12.4 ± 2.1[a,b]
E3	nd	nd	15.1 ± 0.8[b]	11.9 ± 0.4[b]

[a] Mean with different letters in the same row are significantly different between follicular and luteal phases in each specie ($p < 0.05$).
[b] Mean with different letters in the same row were found significantly different between the levels of estrogens from follicular and luteal phases in two different species of animals ($p < 0.05$).

This report and previous reports [12,13] indicate that meat from various origins and with different fat content might be one of the risk factors in human foods. In this regard, there are reports with increasing warning indicating that long-term exposure to the estrogens may increase the risk of certain cancers such as breast, prostate, and corpus uteri cancers [26–28].

As one of the known risk factors for estrogen-receptor dependent cancers could be frequent exposure to estrogens, hence regular monitoring of these compounds is recommended. The results of the present study provides important information about the presence of steroid hormones in cow's meat and for the first time demonstrates that these compounds exist in buffalo's meat in higher concentrations. Moreover, the differences in the concentrations of estrogens in meat between the two examined species in addition to genetic, nutritional, and physiological differences might be attributed to the fat content of the meat samples. In addition, our data showed that heating processes cannot significantly reduce the levels of estrogens.

Conflicts of interest

The authors have nothing to disclose.

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