Research Article

Sadeem S. Alqahtani, Deema M. Bin Humaid, Sabreen H. Alshail, Dalal T. AlShammari, Hessa Al-Showiman, Nourah Z. Alzoman, Hadir M. Maher*

Development and validation of a high performance liquid chromatography/diode array detection method for estrogen determination: Application to residual analysis in meat products

<https://doi.org/10.1515/chem-2020-0118>
received May 18, 2020; accepted July 13, 2020

Abstract: In this work, an HPLC-DAD method was developed for the residual analysis of some estrogens such as estrone (E1), 17- β estradiol (E2), estriol (E3), natural estrogens, and 17- α ethinylestradiol (E4), an exoestrogen, in meat samples of different categories (chicken, $n = 155$, beef, $n = 124$, sheep, $n = 122$, and camels, $n = 40$), collected from the Saudi Market. Although banned, the use of E4 as a growth promoter in the black market is still encountered. Symmetry C18 column (3.5 μm , 4.6 mm $\times$ 150 mm) was used with a mobile phase consisting of 50% aqueous acetonitrile. Protein precipitation with acetonitrile was used for the sample preparation. The method was fully validated, as per the ICH guidelines, in the concentration ranges of 0.35–125 $\mu\text{g/g}$ (E1, E2), 0.188–125 $\mu\text{g/g}$ (E3), and 0.188–450 $\mu\text{g/g}$ (E4). The method allowed the trace analysis of estrogens with LOD values of 0.094 (E3, E4) and 0.126 $\mu\text{g/g}$ (E1, E2), and LOQ values of 0.188 (E3, E4) and 0.350 $\mu\text{g/g}$ (E1, E2). The analyzed samples contained different levels of estrogens. Within the same category, processed products contained the highest levels of E4, while the internal organs contained the least estrogen content. Finally, the estimated daily intake, $\mu\text{g/kg bw/day}$, of estrogens through the consumption of meat-based food products was calculated.

Key words: estrogens, food analysis, HPLC-DAD, meat products, Saudi market

1 Introduction

Estrogens are sex-related steroidal hormones which are known for their regulatory effect on the estrus cycle in mammals and more specifically on the menstrual cycle of humans [1]. It has been reported that estrogens are not only considered as female sex hormones, but they also play a significant role in controlling the male sexual functions [2]. Additionally, estrogens play an important role in regulating critical body functions, e.g., brain function, lipid, protein, and glucose homeostasis, blood coagulation, in addition to follicular growth and skeletal muscles' growth.

Estrogens are classified into two main groups, namely: natural estrogens, also known as endo-estrogens, and synthetic estrogens, known as exoestrogens. Estrone (E1), 17- β estradiol (E2), and estriol (E3) are the most common examples of natural estrogens. Synthetic estrogens include a large number of diverse compounds with estrogenic activities, e.g., pesticides, polychlorinated biphenyls, in addition to the well-known 17- α ethinylestradiol (E4), which is commonly used as a growth promoter [3].

The liability of finding estrogens in dietary meat samples is related to the presence of endogenous natural estrogens in different levels in meat-producing animals, as controlled by the estrus cycle, as well as the external use of estrogens, particularly E4, as a growth promoter [3]. Dietary intake of estrogen-containing food could result in the disturbance of different metabolic activities, including fat, sugar, and protein metabolism. Additionally, overexpression of estrogen receptors, as a result of an excessive estrogen intake, is related to the development of autoimmune diseases, as well as different types

* **Corresponding author: Hadir M. Maher**, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, 11495, P.O. Box 22452, Saudi Arabia; Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, El-Messalah, Alexandria, 21521, Egypt, e-mail: hadirrona@yahoo.com, tel: +20-3-4871317, fax: +20-3-4873273

Sadeem S. Alqahtani, Deema M. Bin Humaid, Sabreen H. Alshail, Dalal T. AlShammari, Hessa Al-Showiman, Nourah Z. Alzoman: Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, 11495, P.O. Box 22452, Saudi Arabia

of cancers, particularly breast, ovarian, and prostate cancers [4,5]. Recently, the role of estrogens in modulating metastatic cascades of cancer cells has been emphasized [6].

For the sake of human health, the use of hormones as growth promoters in animals is prohibited by European Union regulations (Directive 2008/97/EC, 2008) [7], with no maximum residue level because of the possibility of the presence of natural estrogens in meat samples. Despite such a legislation, the illegal use of estrogens as growth promoters in the black market is still encountered in the veterinary field.

According to the statistics of the “Organization for Economic Co-operation and Development, OECD” in 2018 [8], Saudi Arabia consumes a large amount of meat with poultry meat being the first source of meat, Saudi annual consumption of 40 kg/capita compared with world consumption of 14.2 kg/capita, followed by sheep meat, Saudi annual consumption of 5 kg/capita compared with world consumption of 1.7 kg/capita, followed by beef and veal, Saudi annual consumption of 3.5 kg/capita compared with world consumption of 6.5 kg/capita. Moreover, Saudi Arabia is considered the largest market of camel consumption all over the whole world. Camels are one of the most popular livestock animals in Saudi Arabia. The demand for camel meat seems to be increasing, particularly due to an increased awareness of the health benefits of camel meat related to less fat and less cholesterol content, relative to other types of red meat [9].

As a result of health hazards of residual estrogen, there is a demand for its determination in meat products, including chicken, beef, sheep, and camel, which are widely consumed by the Saudi population. Different analytical techniques have been applied for the determination of steroid hormones in biological samples/edible tissues. They include immunoassay [10], gas chromatography with mass spectrometric detector (GC-MS) [11,12], and liquid chromatography with mass spectrometric detector (LC-MS) [13–19]. Despite that immunoassay is widely used for measuring steroid hormones in biological matrices, an occasional lack of specificity is still a major drawback. Also, great sensitivity of GC-MS in estrogen analysis is still faced by researchers while performing the derivatization procedure that is required prior to the actual analysis. LC-MS/MS is characterized by its sensitivity and selectivity, and is thus widely used in biological analysis. HPLC with UV [20–24] or fluorescence [25,26] detection has also been used in food analysis and it is considered advantageous for its lower cost and ease of operation, relative to the more sophisticated LC-MS/MS instrument.

Residual determination of estrogens in different food stuffs has been performed in many countries, namely, China [14,15,17,19–23,26], Spain [15], Iran [25], and Egypt [24].

Since Saudi Arabia is considered among the highest consumers of meat all over the world, Saudis, along with residents and religious visitors, are susceptible to a high estrogen intake from ingested meat samples. Despite the importance of this issue to public health, the screening of estrogens in food samples from the Saudi market – to our knowledge – has not been performed until now. Therefore, this work aims at carrying out the residual analysis of estrogens in meat samples of different categories (chicken, beef, sheep, and camel) available in the Saudi market by the HPLC-DAD. The obtained results were statistically analyzed.

2 Experimental

2.1 Materials and reagents

Reference standards of estrone (E1), purity > 98%, 17- β estradiol (E2), purity > 98%, estriol (E3), purity > 98%, and 17- α ethinylestradiol (E4), purity > 98%, were purchased from MedChemExpress, China. Esomeprazole (ESM), purity > 98%, was supplied by Themis Laboratories Pvt. Ltd, Thane. The ESM was used as an internal standard (IS) all over the study.

HPLC-grade acetonitrile was obtained from PanReac, E.U. Deionized water was obtained from the Millipore water purification system supplied with 0.2 μ m membrane filters such as Nihon and Millipore (Yonezawa, Japan).

2.2 Instrumentation and HPLC operating conditions

The instrument used was Waters HPLC system (USA), equipped with a 1,525 binary HPLC pump, 2,707 autosampler, 2,998 photodiode array detector, and Breeze™ 2 software for data manipulation.

Chromatographic analysis was performed using Symmetry C18, 3.5 μ m with dimensions of 4.6 mm $\times$ 150 mm (Waters, Ireland). The mobile phase consisted of acetonitrile/water mixture in the ratio of 50:50, v/v and adjusted at the flow rate of 1 mL/min. Before being introduced into the HPLC system, the mobile phase was

filtered through 0.45 μm membrane filters supported on a Millipore vacuum filtration system and then sonicated for degassing. The injection volume was 10 μL and the detection wavelength was adjusted at 220 nm.

2.3 Preparation of solutions and matrix-based calibration standards

Stock solutions (1,000 $\mu\text{g/mL}$) of each of E1, E2, E3, E4, and ESM (IS) were prepared in acetonitrile. Whenever needed, further dilutions were made in acetonitrile.

Matrix-based calibration standards were prepared by spiking estrogen-free meat samples (4.0 g) with standard solutions of the four estrogens, along with 50 μL ESM (IS), 1,000 $\mu\text{g/mL}$. Spiked samples with different concentrations of estrogens, 0.188–125 $\mu\text{g/g}$ (E3), 0.188–450 $\mu\text{g/g}$ (E4), and 0.350–125 $\mu\text{g/g}$ (E1, E2), were treated, as described later under “sample preparation”. Following the chromatographic analysis, the obtained peak area ratio of each analyte to the IS was related to the spiked concentrations to obtain the corresponding regression equation.

2.4 Application to estrogens' analysis in meat samples

2.4.1 Sample collection

The analyzed samples were categorized into four main categories: chicken category, beef category, sheep category, and finally camel category. Samples belonging to the four categories were purchased from the local Saudi market (Riyadh, Saudi Arabia), over a three-month period (January–March 2019). Chicken samples ($n = 155$) were classified into six groups, namely, breast muscles ($n = 24$), thigh muscles ($n = 44$), wings ($n = 20$), and internal organs ($n = 19$), in addition to processed samples including chicken burgers, sausages, nuggets, and mortadella ($n = 48$). Beef samples ($n = 124$) were classified into muscles ($n = 40$), internal organs ($n = 40$), and processed products ($n = 44$). By analogy, sheep samples ($n = 122$) were classified into muscles ($n = 41$), internal organs ($n = 40$), and processed products ($n = 41$). The last category (camel category, $n = 40$) comprised only two types of samples: muscles ($n = 20$) and internal organs ($n = 20$). The purchased samples were stored in a refrigerator at -4°C until the day of analysis, for not more than three days.

2.4.2 Sample preparation

Finely cut samples were blended using a Dison food chopper (Zhengzhou Dison Electric Co., Ltd., China) at the highest velocity for 2 min. Into a series of screw-capped test tubes, accurate weights of 4.0 g of blended meat samples were spiked with 50 μL ESM (IS), 1,000 $\mu\text{g/mL}$, and then mixed with 5.0 mL acetonitrile. The samples were sonicated for 15 min using a Branson 3510 ultrasonic cleaner (Branson Ultrasonics Corporation, CT, USA). The clear supernatants were separately transferred into clean vials and filtered using 0.45 μm membrane filters. Volumes of 10 μL of clean samples were introduced into the HPLC system for analysis.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Optimization of HPLC conditions

Although the LC-MS/MS is widely used nowadays to carry out the residual analysis of various drugs in different food matrices, the HPLC-UV/DAD is still of major interest. The simplicity of the use of HPLC-UV/DAD and its low price, compared with those of LC-MS/MS, are considered great advantages of the former technique, particularly in that the use of DAD detection impacts great specificity to the technique. The DAD offers the advantage of scanning the UV absorption spectrum at any point of the eluting compound and hence helps to assess the peak purity. This technique was previously used in the determination of estrogens in fishery samples [20,24], pork and chicken [21], dairy and meat samples [22], and milk samples [23].

3.1.1 Selection of optimum stationary phase and mobile phase

Different HPLC conditions were optimized for the purpose of obtaining a good separation between the analyzed estrogens, with a good response and within reasonable runtime. In this respect, both stationary and mobile phases were investigated.

Initially, the analysis was performed using a C 18 column, 10 μm (3.9×150 mm) with a mobile phase of acetonitrile/water mixtures. A complete overlap between

the E1 and E2 peaks was observed, with mobile phases containing 50–35% acetonitrile with a partial separation starting at 30–28% acetonitrile. Although a complete separation was achieved with 25% acetonitrile, an increased runtime was recorded, with E1 being eluted in almost 50 min as broad peaks. The same results were nearly obtained when replacing acetonitrile with methanol, a mobile phase of 45–43% aqueous methanol resulted in a partial overlap between the E1 and E2 peaks with a complete separation with 40% aqueous methanol with a runtime exceeding 55 min.

In an attempt to get an optimum separation within reasonable runtime, a C 18 column with a smaller particle size, 3.5 μm (4.6 mm $\times$ 150 mm), was tried. Fortunately, a mobile phase of 50% aqueous acetonitrile resulted in sharp, symmetric, well-resolved estrogens' peaks within reasonable runtime (<7 min). The analyzed estrogens were eluted in the following order: E3 (R_t 1.75 $\pm$ 0.05), E4 (R_t 4.30 $\pm$ 0.06), E2 (R_t 5.40 $\pm$ 0.09), and E1 (R_t 6.40 $\pm$ 0.08), being the last eluted compound. A lower acetonitrile content resulted in a decreased retention of the four estrogens with an increased runtime >45 min with 35% acetonitrile. A higher acetonitrile content (>50%) resulted in an accelerated elution of the estrogens, and E3 was eluted closer to the solvent front, which is generally not favored in the analysis of complex matrices (e.g., food analysis) with a partial overlap between the E2 and E4 peaks with 70% acetonitrile. Figure 1 shows the effect of acetonitrile% on the retention time of the studied estrogens using the two C 18 columns of different dimensions and particle sizes; 10 μm (3.9 $\times$ 150 mm) and 3.5 μm (4.6 $\times$ 150 mm).

3.1.2 Selection of the IS

The IS method is the method of choice, compared with the external standard method, particularly in the analysis of complex matrices (e.g., food samples). The IS should yield a comparable response to the analytes, with a closer retention time. Another important point is that it should not be an integral part, or even likely be present as an impurity/additive, in the analyzed matrix. In this respect, different compounds were tried. Some of them were eluted too early, before the elution of the least retained estrogen E3, e.g., caffeine, paracetamol, emtricitabine, salicylic acid, ascorbic acid, methyltrihydroxybenzoate, gallic acid, isoconazole, and valsartan. Some compounds showed an overlap with or incomplete separation from E3, e.g., lovastatin, hydrochlorothiazide, ornidazole, and benzoic acid. Other compounds were

Figure 1: The effect of acetonitrile % on the retention time of the studied estrogens using C 18 column, 10 μm (3.9 mm $\times$ 150 mm) (a) and C18 column, 3.5 μm (4.6 mm $\times$ 150 mm) (b).

eluted too late, after the elution of the most retained estrogen E1, namely glibenclamide, gliclazide, and ibuprofen. In spite of the fact that parabens, methyl, ethyl, and propyl parabens, were eluted within the runtime as sharp, well-defined peaks, they could not be used as IS due to the likeliness of their occurrence as preservatives in the analyzed samples. Finally, ESM was eluted at 2.20 min as a sharp, symmetric, well-resolved peak with reasonable response, compared with the analytes. Thus, it was selected as the IS in the proposed method for the determination of estrogen residues in the analyzed meat samples. Figure 2 shows a typical HPLC chromatogram of a standard mixture of the studied estrogens with their corresponding absorption spectra.

3.2 Sample preparation

In this method, protein precipitation (PPT) was used as a sample preparation technique. Acetonitrile was used for the purpose of cleaning-up as well as for extraction of

estrogens from meat samples. Although other works used more advanced sample preparation techniques, e.g., solid phase extraction (SPE) [17,20,22,24,25] or liquid-liquid extraction (LLE) [13], PPT is still considered advantageous in many ways [23,26]. Compared with SPE and LLE, PPT is simpler, less time-consuming, and requires less cost since it does not require any special apparatus or particular gases, machinery (supplies, pumps, syringes,..., etc.). Moreover, LLE requires the use of toxic organic solvents and the procedure is tedious and time-consuming. The use of acetonitrile for

PPT [23,26] and extraction [20,21,23,24,26] of estrogens from edible samples was previously applied.

3.3 Method validation

With reference to the international conference on harmonization (ICH) guidelines [27], different validation parameters were evaluated, namely, linearity, limits of detection (LOD) and of quantitation (LOQ), extraction recovery, precision, and accuracy.

Figure 2: A typical HPLC chromatogram of a standard mixture of 10 µg/mL of each of the studied estrogens (a), estriol (E3), 17 α -ethinylestradiol (E4), 17 β estradiol (E2), and estrone (E1), with the internal standard (IS) ESM, 10 µg/mL. The absorption spectra of the studied estrogens and the IS are also shown (b). The mobile phase consisted of acetonitrile/water mixture in the ratio of 50:50, v/v and adjusted at the flow rate of 1 mL/min.

3.3.1 Linearity

The method's linearity was evaluated by analyzing estrogen-free samples of the four analyzed meat categories: chicken, beef, sheep, and camel, spiked with different concentrations of estrogens, along with the IS. The obtained peak area ratio of each estrogen to the IS was related to the spiked concentrations to derive the matrix-based calibration graphs and the corresponding regression equations. Linearity was assessed in the concentration ranges of 0.188–125 µg/g (E3), 0.188–450 µg/g (E4), and 0.350–125 µg/g (E1, E2) with good correlation coefficients (r values ≥ 0.9981), as shown in Table 1.

3.3.2 LOD and LOQ

Values of LOD and LOQ were selected, based on the concentrations providing a response of three times or ten times signal-to-noise ratio (S/N), respectively. As seen in Table 1, LOD values of 0.094 (E3, E4) and 0.126 µg/g (E1, E2), and LOQ values of 0.188 (E3, E4) and 0.350 µg/g (E1, E2) were obtained. The LOD and LOQ values were sufficiently low to allow the residual analysis of estrogens in different meat samples.

3.3.3 Extraction recovery studies

Extraction recovery of the sample preparation technique was assessed by analyzing estrogen-free meat samples which had been spiked with the analyzed estrogens at three different concentration levels, 25, 12.5, and 6.25 µg/g. Extraction recovery values were calculated by relating the responses obtained from spiked samples to those obtained from standard solutions with the same nominal concentrations. High values of extraction recovery % ranging from 74.89 to 91.38 of the four estrogens indicated the efficiency of the sample preparation technique in the determination of estrogens in meat samples.

3.3.4 Precision and accuracy

Precision and accuracy were evaluated by analyzing spiked samples, at the same concentration levels as those used in "recovery studies," three times on the same day or on three successive days for intra-day and inter-day levels, respectively. The obtained responses were compared with the matrix-based calibration standards to derive the %

recovery and errors for assessing the accuracy and % RSD for assessing the precision. Table 2 reveals that high values of recovery% (85.28–114.58) and RSD% (0.11–13.93) were obtained for all the analyzed estrogens, indicating a high degree of accuracy and precision of the proposed method, respectively.

3.3.5 Stability of solutions

Stock and standard solutions of the analyzed estrogens were found to be stable, when stored refrigerated (-4°C) for one month.

3.4 Method's applicability to the residual analysis of estrogens in meat samples

The proposed method was applied to the determination of estrogen residues (E1, E2, E3, and E4) in meat samples of the four different categories, chicken, beef, sheep, and camel, collected from the Saudi market.

3.4.1 Occurrence of estrogens in meat samples

3.4.1.1 Chicken samples

The developed HPLC-DAD method was applied for the determination of estrogen residues in chicken samples ($n = 155$), grouped as breast muscles ($n = 24$), thigh muscles ($n = 44$), and wings ($n = 20$), internal organs including liver, spleen, kidney, and heart ($n = 19$), in addition to processed products including chicken nuggets, chicken burger, mortadella, and sausages ($n = 48$). HPLC chromatograms of selected samples are shown in Figure 3a.

Occurrence of estrogen residues in different groups of chicken samples is summarized in Table 3. Figure 4a also illustrates the occurrence of different estrogens (E1, E2, E3, and E4) among different chicken groups. The results revealed that the processed products showed a much higher estrogen content, compared with other groups, followed by wings, breast and thigh muscles, and that the least estrogen content was noticed with the internal organs, notably E4.

Processed products showed positive results for the analyzed samples, the highest existence was for E4 (52.08%, mean 22.36 µg/g) followed by E2 (35.41%, mean 2.92 µg/g), then E1 (16.67%, mean 0.33 µg/g), and the least occurrence was for E3 (8.33%, mean 3.46 µg/g).

Table 1: Matrix-based calibration parameters for the determination of estrogens in different meat categories using the proposed HPLC-DAD method

		Linearity range (µg/g)	Regression equation	<i>r</i>	LOD (µg/g)	LOQ (µg/g)
Chicken category						
E1	Breast muscles	0.350–125	$y = 0.0002 + 0.0048x$	0.9998	0.126	0.350
	Thigh muscles	0.350–125	$y = -0.0052 + 0.0027x$	0.9992	0.126	0.350
	Wings	0.350–125	$y = 0.0038 + 0.0088x$	0.9990	0.126	0.350
	Internal organs	0.350–125	$y = -0.0012 + 0.0057x$	0.9974	0.126	0.350
	Processed products	0.350–125	$y = -0.0071 + 0.0089x$	0.9987	0.126	0.350
E2	Breast muscles	0.350–125	$y = 0.0066 + 0.0074x$	0.9971	0.126	0.350
	Thigh muscles	0.350–125	$y = 0.0012 + 0.0051x$	0.9981	0.126	0.350
	Wings	0.350–125	$y = -0.0003 + 0.0055x$	0.9990	0.126	0.350
	Internal organs	0.350–125	$y = -0.0006 + 0.0074x$	0.9978	0.126	0.350
	Processed products	0.350–125	$y = -0.0078 + 0.0091x$	0.9983	0.126	0.350
E3	Breast muscles	0.188–125	$y = -0.0038 + 0.0082x$	0.9955	0.094	0.188
	Thigh muscles	0.188–125	$y = -0.0028 + 0.0022x$	0.9921	0.094	0.188
	Wings	0.188–125	$y = 0.0092 + 0.0066x$	0.9992	0.094	0.188
	Internal organs	0.188–125	$y = 0.0052 + 0.0094x$	0.9991	0.094	0.188
	Processed products	0.188–125	$y = -0.0038 + 0.0081x$	0.9985	0.094	0.188
E4	Breast muscles	0.188–450	$y = 0.0028 + 0.0060x$	0.9981	0.094	0.188
	Thigh muscles	0.188–450	$y = 0.0088 + 0.0099x$	0.9975	0.094	0.188
	Wings	0.188–450	$y = -0.0026 + 0.0086x$	0.9986	0.094	0.188
	Internal organs	0.188–450	$y = -0.0068 + 0.0052x$	0.9993	0.094	0.188
	Processed products	0.188–450	$y = -0.0050 + 0.0088x$	0.9958	0.094	0.188
Beef category						
E1	Muscles	0.350–125	$y = -0.0092 + 0.01258x$	0.9985	0.126	0.350
	Internal organs	0.350–125	$y = -0.0088 + 0.00918x$	0.9992	0.126	0.350
	Processed	0.350–125	$y = -0.0056 + 0.0089x$	0.9994	0.126	0.350
E2	Muscles	0.350–125	$y = 0.0009 + 0.0084x$	0.9968	0.126	0.350
	Internal organs	0.350–125	$y = 0.0019 + 0.0075x$	0.9958	0.126	0.350
	Processed	0.350–125	$y = 0.0022 + 0.0158x$	0.9939	0.126	0.350
E3	Muscles	0.188–125	$y = -0.0063 + 0.0066x$	0.9991	0.094	0.188
	Internal organs	0.188–125	$y = -0.0074 + 0.0238x$	0.9990	0.094	0.188
	Processed	0.188–125	$y = 0.0151 + 0.0165x$	0.9985	0.094	0.188
E4	Muscles	0.188–450	$y = 0.0012 + 0.0287x$	0.9975	0.094	0.188
	Internal organs	0.188–450	$y = -0.0025 + 0.0127x$	0.9993	0.094	0.188
	Processed	0.188–450	$y = 0.0037 + 0.0098x$	0.9970	0.094	0.188
Sheep category						
E1	Muscles	0.350–125	$y = 0.0007 + 0.0015x$	0.9990	0.126	0.350
	Internal organs	0.350–125	$y = 0.0008 + 0.0019x$	0.9995	0.126	0.350
	Processed	0.350–125	$y = 0.0016 + 0.0036x$	0.9985	0.126	0.350
E2	Muscles	0.350–125	$y = 0.0038 + 0.0022x$	0.9979	0.126	0.350
	Internal organs	0.350–125	$y = 0.0005 + 0.0019x$	0.9987	0.126	0.350
	Processed	0.350–125	$y = 0.0202 + 0.0048x$	0.9955	0.126	0.350
E3	Muscles	0.188–125	$y = 0.0332 + 0.0115x$	0.9981	0.094	0.188
	Internal organs	0.188–125	$y = 0.0092 + 0.0157x$	0.9990	0.094	0.188
	Processed	0.188–125	$y = 0.0089 + 0.0208x$	0.9958	0.094	0.188
E4	Muscles	0.188–450	$y = 0.0128 + 0.0099x$	0.9967	0.094	0.188
	Internal organs	0.188–450	$y = 0.0299 + 0.0198x$	0.9991	0.094	0.188
	Processed	0.188–450	$y = 0.0408 + 0.0880x$	0.9908	0.094	0.188
Camel category						
E1	Muscles	0.350–125	$y = 0.0032 + 0.0099x$	0.9977	0.126	0.350
	Internal organs	0.350–125	$y = 0.0077 + 0.0367x$	0.9986	0.126	0.350
E2	Muscles	0.350–125	$y = 0.0084 + 0.0258x$	0.9991	0.126	0.350
	Internal organs	0.350–125	$y = 0.0081 + 0.0090x$	0.9995	0.126	0.350
E3	Muscles	0.188–125	$y = 0.0112 + 0.0968x$	0.9955	0.094	0.188
	Internal organs	0.188–125	$y = 0.0257 + 0.0999x$	0.9966	0.094	0.188
E4	Muscles	0.188–450	$y = 0.0012 + 0.0112x$	0.9978	0.094	0.188
	Internal organs	0.188–450	$y = 0.0091 + 0.0086x$	0.9952	0.094	0.188

r: correlation coefficient, LOD: limit of detection, LOQ: limit of quantitation.

Table 2: Average results^a for evaluating the precision and accuracy for the determination of estrogens in different meat categories using the proposed HPLC-DAD method

Estrogen	Type of matrix	Intra-day level (<i>n</i> = 3)			Inter-day level (<i>n</i> = 9)		
		Mean% recovery ± SD	RSD%	<i>E_r</i> %	Mean% recovery ± SD	RSD%	<i>E_r</i> %
Chicken category							
E1	Breast muscles	106.19 ± 8.60	8.10	6.19	109.25 ± 5.76	5.27	9.25
	Thigh muscles	100.16 ± 0.56	0.56	0.16	104.64 ± 1.93	1.84	4.64
	Wings	107.27 ± 0.33	0.31	7.27	108.43 ± 1.67	1.54	8.43
	Internal organs	101.33 ± 1.15	1.14	1.33	108.10 ± 7.63	7.06	8.10
	Processed products	90.47 ± 8.38	9.26	9.33	90.30 ± 0.62	0.70	9.70
E2	Breast muscles	105.52 ± 6.62	6.28	5.52	109.05 ± 4.81	4.41	9.05
	Thigh muscles	94.29 ± 2.83	3.00	−5.71	95.39 ± 8.11	8.50	−4.61
	Wings	107.79 ± 0.52	0.48	7.79	109.22 ± 0.48	0.44	9.22
	Internal organs	90.39 ± 0.10	0.11	−9.61	89.35 ± 2.25	2.52	−10.65
	Processed products	109.80 ± 5.57	5.07	9.80	110.47 ± 8.47	7.66	10.47
E3	Breast muscles	105.82 ± 1.17	1.01	5.82	106.46 ± 2.10	1.98	6.46
	Thigh muscles	96.57 ± 0.10	0.11	−3.43	96.38 ± 0.32	0.33	−3.62
	Wings	88.27 ± 4.88	5.53	−11.73	85.43 ± 8.67	10.15	−14.57
	Internal organs	87.92 ± 7.89	8.97	−12.08	85.59 ± 9.05	10.57	−14.57
	Processed products	109.55 ± 4.25	3.88	9.55	111.19 ± 7.54	6.78	11.19
E4	Breast muscles	99.58 ± 10.89	8.10	6.19	90.16 ± 12.56	13.93	−9.84
	Thigh muscles	112.27 ± 4.99	0.56	0.16	114.27 ± 0.83	0.73	14.27
	Wings	101.55 ± 3.08	0.31	7.27	95.43 ± 2.67	2.80	−4.57
	Internal organs	109.19 ± 3.60	10.94	9.19	103.19 ± 6.66	6.45	3.19
	Processed products	111.16 ± 7.56	6.80	11.16	91.16 ± 8.05	8.83	−8.84
Beef category							
E1	Muscles	105.33 ± 1.15	1.09	5.33	107.55 ± 3.35	3.11	7.55
	Internal organs	112.47 ± 8.38	7.45	12.47	98.30 ± 10.12	10.30	−1.70
	Processed	89.52 ± 10.55	11.79	5.52	88.58 ± 7.77	8.77	−11.42
E2	Muscles	112.88 ± 4.33	1.14	1.33	108.98 ± 4.52	4.15	8.98
	Internal organs	88.47 ± 2.88	9.26	−10.48	86.30 ± 3.58	4.15	−13.7
	Processed	99.00 ± 3.58	3.62	−1.00	90.89 ± 10.25	11.28	−9.11
E3	Muscles	87.55 ± 5.55	6.34	−12.54	86.88 ± 6.55	7.54	−13.12
	Internal organs	87.00 ± 4.25	4.89	−13.00	95.30 ± 4.33	4.54	−4.70
	Processed	103.39 ± 1.25	1.21	3.39	101.35 ± 7.25	7.15	1.35
E4	Muscles	105.22 ± 8.22	7.81	5.22	107.47 ± 6.25	5.82	7.47
	Internal organs	110.89 ± 6.61	5.96	10.89	112.33 ± 3.88	3.45	12.33
	Processed	92.57 ± 3.35	3.62	−7.43	90.05 ± 4.25	4.72	−9.95
Sheep category							
E1	Muscles	107.93 ± 7.12	6.60	7.93	109.55 ± 8.25	0.91	9.55
	Internal organs	104.55 ± 3.25	3.11	4.55	88.25 ± 5.22	5.92	−11.75
	Processed	109.22 ± 1.89	1.73	9.22	111.52 ± 7.77	6.97	11.52
E2	Muscles	101.08 ± 5.08	5.03	1.08	105.10 ± 6.88	6.55	5.10
	Internal organs	89.58 ± 2.33	2.60	−10.42	88.21 ± 1.58	1.79	−11.79
	Processed	90.33 ± 4.87	5.39	−9.67	107.89 ± 5.11	4.74	7.89
E3	Muscles	107.02 ± 8.02	7.49	7.02	112.10 ± 2.38	2.12	12.10
	Internal organs	88.44 ± 1.11	1.26	−11.56	85.28 ± 1.58	1.85	−14.12
	Processed	106.21 ± 3.28	3.09	6.21	103.75 ± 3.88	3.74	3.75
E4	Muscles	102.58 ± 7.77	7.75	2.58	112.18 ± 5.55	4.95	12.18
	Internal organs	93.59 ± 9.97	10.65	−6.41	95.66 ± 5.23	5.47	−4.34
	Processed	112.01 ± 8.22	7.34	12.01	104.05 ± 8.58	8.25	4.05
Camel category							
E1	Muscles	90.25 ± 6.62	7.34	−9.75	92.55 ± 9.88	10.68	−7.45
	Internal organs	92.58 ± 7.77	8.39	−7.42	88.58 ± 5.22	5.89	−11.42
E2	Muscles	111.20 ± 5.21	4.69	11.22	96.25 ± 6.98	7.25	−3.75
	Internal organs	107.25 ± 2.55	2.38	7.25	114.58 ± 1.25	1.09	14.58
E3	Muscles	107.58 ± 8.25	7.67	7.58	102.88 ± 3.58	3.48	2.88

Table 2: Continued

Estrogen	Type of matrix	Intra-day level ($n = 3$)			Inter-day level ($n = 9$)		
		Mean% recovery $\pm$ SD	RSD%	$E_r\%$	Mean% recovery $\pm$ SD	RSD%	$E_r\%$
E4	Internal organs	108.78 $\pm$ 4.58	4.21	8.78	89.52 $\pm$ 1.58	1.76	-10.48
	Muscles	107.66 $\pm$ 1.85	1.72	7.66	87.20 $\pm$ 1.48	1.70	-12.80
	Internal organs	105.44 $\pm$ 7.55	7.16	5.44	88.36 $\pm$ 4.44	5.02	-11.64

^a Results obtained as an average of three concentration levels for each analyte: 1.25, 12.5, and 100 $\mu\text{g/g}$. RSD%: relative standard deviation percentage. $E_r\%$: Percentage relative error.

It is noteworthy to mention that all analyzed groups showed negative results ($<\text{LOD}$) for E2 and E3, except for the processed products. The results also revealed that about 40% of wing samples contained E1 (mean 0.71 $\mu\text{g/g}$) and that about 25% of the samples contained E4 (mean 0.29 $\mu\text{g/g}$). Regarding breast and thigh muscles, although almost 30% (breast) and 34% (thigh) of the analyzed samples showed positive response for E1, the average content was not more than 1.37 $\mu\text{g/g}$ for breast muscles and 0.71 $\mu\text{g/g}$ for thigh muscles. Similarly, the content of E4 did not exceed 1.02 (breast) and 0.72 $\mu\text{g/g}$ (thigh), accounting for nearly 21% and 34% of the analyzed breast and thigh samples, respectively.

It is clear from Table 3 and Figure 4a that despite thigh muscles and the internal organs having 0.70 and 0.77 $\mu\text{g/g}$ as the mean E1 content, the E4 mean content in the internal organs was much less, 0.028 $\mu\text{g/g}$, compared with 0.72 $\mu\text{g/g}$ in the internal organs and thigh muscles, respectively.

3.4.1.2 Beef samples

Determination of estrogen residues in beef samples ($n = 124$) was carried out using the proposed HPLC-DAD method. Samples were categorized into three main groups: muscles ($n = 40$), internal organs ($n = 40$), and processed products ($n = 44$). HPLC chromatograms of some beef samples are presented in Figure 3b. Results of the analysis of beef samples are summarized in Table 4. As in chicken meat, the highest E4 estrogen content was found in the processed products, as compared with muscles and internal organs as shown in Figure 4b (54.55%, mean 34 $\mu\text{g/g}$). E2 occurred in nearly 11.36% of the processed products (mean 0.48 $\mu\text{g/g}$), and E1 being of the least frequency and content, 2.27% (mean 0.03 $\mu\text{g/g}$). The absence of E3 in all the analyzed samples was also noticed. Muscles and internal organs had only E2 and E3, being the least in the internal organs.

3.4.1.3 Sheep samples

Representative chromatograms of the analyzed sheep samples are shown in Figure 3c. As per chicken and beef, the highest E4 content was found in nearly 61% of processed products (mean 61 $\mu\text{g/g}$), compared with only 0.16 and 0.25 $\mu\text{g/g}$ of muscles and internal organs. E1 was only found in the processed products (4.88%, mean 0.17 $\mu\text{g/g}$). E2 was found in the three groups in nearly 4.88% of the analyzed muscles (mean 0.06 $\mu\text{g/g}$) and processed products (mean 0.09 $\mu\text{g/g}$), while at a higher frequency in the internal organs (15%, mean 0.15 $\mu\text{g/g}$). It was also noticed that none of the analyzed processed products contained E3, which was found in 0.37 $\mu\text{g/g}$ in only one sample of the 41 analyzed muscles. On the other hand, 22.5% of the analyzed internal organs contained E3 in an average content of 0.49 $\mu\text{g/g}$. Results of the analysis are summarized in Table 5 (Figure 4c).

3.4.1.4 Camel samples

Camel samples were divided into only two groups, muscles and internal organs, since no processed products were found in the commercial market. Results of the analysis are shown in Table 6 (Figure 4d). It was clear that none of the analyzed estrogens were detected in the internal organs. No residual E2 was found in the analyzed muscle samples and E4 was found in 5% of the samples, mean 0.17 $\mu\text{g/g}$. Also, E1 and E3 were only found in one of the analyzed muscles ($n = 20$) in the levels of 0.12 $\mu\text{g/g}$ (E1) and 0.03 $\mu\text{g/g}$ (E3).

A general outlook at Figure 4 revealed that samples of the four categories, chicken, beef, sheep, and camel, contained different levels of estrogens. Among the same category, the processed products contained the highest levels of E4, indicating that this growth promoter is still illegally used, with accumulation in the lipids constituting the major part of processed samples. Moreover, samples of the sheep category showed maximum abundance (61%) of

Figure 3: Typical HPLC chromatograms of selected samples of each meat category showing the estrogen found in each sample. (a) Chicken category: Burger chicken (5.5 µg/g E4, 0.50 µg/g E2) and Mortdella chicken (225 µg/g E4, 0.40 µg/g E2). (b) Beef category: Burger (250 µg/g E4) and Mortdella (70 µg/g E4). (c) Sheep category: Muscles (E2) and Burger (3.39 µg/g E2). (d) Camel category: Muscles (2.40 µg/g E1) and Muscles (3.36 µg/g E4).

Table 3: Occurrence of estrogen residues ($\mu\text{g/g}$) in chicken samples

		Breast muscles ($n = 24$)	Thigh muscles ($n = 44$)	Wings ($n = 20$)	Internal organs ($n = 19$)	Processed products ($n = 48$)
E1	Number of positive samples (% of total)	7 (29.17)	15 (34.09)	8 (40)	9 (47.37)	8 (16.67)
	Mean ($\mu\text{g/g}$)	1.37	0.70	0.71	0.77	0.33
	Mean of positive samples ($\mu\text{g/g}$)	4.70	2.04	1.75	1.63	1.97
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	1.15–22.21	0.87–4.01	0.81–3.06	0.73–4.03	0.54–4.55
E2	Number of positive samples (% of total)	—	—	—	—	17 (35.41)
	Mean ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	2.92
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	8.25
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	0.39–40.38
E3	Number of positive samples (% of total)	—	—	—	—	4 (8.33)
	Mean ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	3.46
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	41.50
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	10.67–74.25
E4	Number of positive samples (% of total)	5 (20.83)	15 (34.09)	5 (25)	2 (10.53)	25 (52.08)
	Mean ($\mu\text{g/g}$)	1.02	0.72	0.29	0.028	22.36
	Mean of positive samples ($\mu\text{g/g}$)	4.89	2.10	1.16	0.57	42.93
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD	<LOD	0.59
	Range of positive samples ($\mu\text{g/g}$)	3.05–7.65	0.32–7.41	0.28–1.74	0.51–0.64	0.53–349.62

Figure 4: Occurrence of estrogens (µg/g) in different categories of meat samples: (a) chicken, (b) beef, (c) sheep, and (d) camel. The same scale of the y-axis was used for comparison.

E4 with the highest levels (mean 61 µg/g). Also within the same category, the internal organs contained the least estrogen content. This matches what was previously reported on the distribution of estrogen receptors (ER), being the highest in the mammary glands (ER α) or the ovary (ER β) and the lowest in the internal organs; liver, spleen, heart, lung, and kidney.

3.4.2 Calculation of the estimated daily intake (EDI) of estrogens from meat consumption

In order to provide a clear picture of estrogen exposure as a result of meat consumption among the Saudi

population, the corresponding EDI was calculated. Based on the dietary pattern, estimated daily meat consumption, and the average body weight [28,29] in different age groups, the EDI was calculated using the average content of each type of estrogen (E1, E2, E3, and E4) found in each meat category, as summarized in Tables 3–6. As can be seen from Table 7, estrogen exposure as a result of meat consumption decreases in relation to the progression of age with the greatest exposure in the age group of 10–15 years. This could be attributed to the largest fast-food consumption among this young age group since the high content of estrogens, particularly E4, in processed products results

Table 4: Occurrence of estrogen residues ($\mu\text{g/g}$) in beef samples

		Muscles ($n = 40$)	Internal organs ($n = 40$)	Processed products ($n = 44$)
E1	Number of positive samples (% of total)	—	—	1 (2.27)
	Mean ($\mu\text{g/g}$)	<LOD	<LOD	0.0263
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	1.1589
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	1.1589
E2	Number of positive samples (% of total)	11 (27.5)	1 (2.5)	5 (11.36)
	Mean ($\mu\text{g/g}$)	3.7622	0.0097	0.4793
	Mean of positive samples ($\mu\text{g/g}$)	13.6806	0.3864	4.2174
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	6.6150–15.5793	0.3864	0.6101–7.2065
E3	Number of positive samples (% of total)	—	—	—
	Mean ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
E4	Number of positive samples (% of total)	6 (15)	1 (2.5)	24 (54.55)
	Mean ($\mu\text{g/g}$)	0.6226	0.0315	33.8985
	Mean of positive samples ($\mu\text{g/g}$)	4.1506	1.2602	62.1472
	Median ($\mu\text{g/g}$)	<LOD	<LOD	1.0890
	Range of positive samples ($\mu\text{g/g}$)	1.5514–10.6992	1.2602	0.7338–360.2211

in a high total estrogen intake. It is well known that hormonal changes occurring during the age of adolescence are responsible for the resulting changes in the body shape and psychological disturbances. Improper appearance, as a result of hormonal imbalance, can badly affect the adolescents' psychology and may

proceed to depression and even suicide in some cases [30]. In this respect, a particular concern should be paid to male adolescents where increased incidence of breast enlargement, called "Gynecomastia," is partly related to the intake of exogenous estrogens in the age of puberty. Being a significant cause of psychological distress, it is

Table 5: Occurrence of estrogen residues ($\mu\text{g/g}$) in sheep samples

		Muscles ($n = 41$)	Internal organs ($n = 40$)	Processed products ($n = 41$)
E1	Number of positive samples (% of total)	—	—	2 (4.88)
	Mean ($\mu\text{g/g}$)	<LOD	<LOD	0.17
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	3.42
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD	1.27–5.59
E2	Number of positive samples (% of total)	2 (4.88)	6 (15)	2 (4.88)
	Mean ($\mu\text{g/g}$)	0.06	0.15	0.09
	Mean of positive samples ($\mu\text{g/g}$)	1.16	0.97	1.94
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	0.54–1.78	0.39–2.32	0.50–3.39
E3	Number of positive samples (% of total)	1 (2.44)	9 (22.5)	—
	Mean ($\mu\text{g/g}$)	0.009	0.49	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	0.37	2.17	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	0.37	0.21–8.68	<LOD
E4	Number of positive samples (% of total)	9 (21.95)	5 (12.5)	25 (60.98)
	Mean ($\mu\text{g/g}$)	0.16	0.25	60.98
	Mean of positive samples ($\mu\text{g/g}$)	0.71	2.02	206.20
	Median ($\mu\text{g/g}$)	<LOD	<LOD	0.91
	Range of positive samples ($\mu\text{g/g}$)	0.40–1.61	1.22–2.47	0.34–419.65

Table 6: Occurrence of estrogen residues ($\mu\text{g/g}$) in camel samples

		Muscles ($n = 20$)	Internal organs ($n = 20$)
E1	Number of positive samples (% of total)	1 (5%)	—
	Mean ($\mu\text{g/g}$)	0.1198	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	2.3962	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	2.3962	<LOD
E2	Number of positive samples (% of total)	—	—
	Mean ($\mu\text{g/g}$)	<LOD	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	<LOD	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	<LOD	<LOD
E3	Number of positive samples (% of total)	1 (5%)	—
	Mean ($\mu\text{g/g}$)	0.0259	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	0.5174	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	0.5174	<LOD
E4	Number of positive samples (% of total)	1 (5%)	—
	Mean ($\mu\text{g/g}$)	0.1680	<LOD
	Mean of positive samples ($\mu\text{g/g}$)	3.3600	<LOD
	Median ($\mu\text{g/g}$)	<LOD	<LOD
	Range of positive samples ($\mu\text{g/g}$)	3.3600	<LOD

recommended to limit the EDI of estrogens as low as possible [31]. Moreover, increased hormonal levels in puberty result in the prevalence of an ovarian cyst with a peak frequency in the age of 15 years [32].

3.4.3 Comparison of meat-related estrogen exposure among the Saudi population with worldwide estimations

In a previous Korean study concerned with the determination of estrogens in muscle tissues of beef cattle, the concentrations of the estrogens E1, E2, and E3 were below $0.1 \mu\text{g/kg}$ [12]. Also, E1, E2, and E3 determination in buffalo's and cow's meat in Iran showed that E1 was the highest in beef muscles (up to 16.2 ng/L), that E2 was dominant in buffalo muscles (up to 23.3 ng/L), and that E3 was only found in buffalo muscles (15.8 ng/L) [25]. Analysis of estrogens has also been conducted on fish samples, with variations in the obtained results ranging from the total absence of E1 and E3 [14] and of E1, E2, and E3 [26], to $0.775\text{--}11.884 \mu\text{g/kg}$ for E2 [24]. Determination of estrogens in milk and dairy products has gained much attention. In a study conducted on colostrum powder, E1 was found in the fat fraction, $7.59 \mu\text{g/L}$, while in defatted milk and colostrum powder, E1 ($5.51\text{--}15.0 \mu\text{g/kg}$) and E2 ($2.28\text{--}3.3 \mu\text{g/kg}$) were detected with the total absence of E3 in different types of milk and colostrum powders [13]. In

China, the absence of E1, E2, and E3 in all the analyzed milk samples was reported [15,18], while the estrogen concentration range of $0.05\text{--}3.2 \mu\text{g/kg}$ was found in milk samples that were analyzed in a different study [17]. For dairy products, estrone, 17β -estradiol, estriol, and 17α -ethynylestradiol were absent in all the analyzed samples [16]. In addition, determination of estrogens in eggs revealed the total absence of the analytes, E1 and E2, in the analyzed samples [19]. The current study revealed that the concentrations of estrogens in the analyzed meat samples were maximum in the processed products of chicken, beef, and sheep (up to $5.59 \mu\text{g/g}$ E1, $40.38 \mu\text{g/g}$ E2, $74.4 \mu\text{g/g}$ E3, and even up to $419.56 \mu\text{g/g}$ E4). It is obvious that these levels are considered to be much higher than those found in previous estimations in other countries. Thus, for the sake of health benefits, particular attention should be paid to monitor the estrogenic content in meat products available in the Saudi market.

3.5 Comparison of the performance of the proposed method with previous literature

The analytical performance of the proposed method was compared with those of HPLC-UV methods, previously

Table 7: EDI, $\mu\text{g/kg bw/day}$, of estrogens through the consumption of meat-based food products by the Saudi adult based on different age groups (years)

Compound	Meat category ^a	10–15	16–24	25–39	>40
E1	Chicken	0.36	1.3	1.13	0.911
	Red meat	0	0	0	0
	Processed	0.15	0.04	0.03	0.009
E2	Chicken	0	0	0	0
	Red meat	0.08	1.16	1.08	1.06
	Processed	1.39	0.33	0.25	0.08
E3	Chicken	0	0	0	0
	Red meat	0.0002	0.003	0.002	0.002
	Processed	0.5	0.125	0.09	0.03
E4	Chicken	0.12	0.44	0.37	0.3
	Red meat	0.017	0.23	0.22	0.21
	Processed	14.35	3.402	2.56	0.855
Σ Estrogens		17.01	7.1	5.8	3.5

^a Red meat (beef, sheep), chicken meat (breast, thigh, and wings), and processed meat (chicken, beef, and sheep).

applied in the determination of estrogen residues in edible tissues [20–24]. Regarding the four studied estrogens, E1, E2, E3, and E4, the proposed method enabled their simultaneous determination in meat samples, compared with only one of the cited estrogens in previous studies, E2 [24,25]. Despite that ref. [16] described the determination of the four estrogens in fishery samples, only spiked samples were analyzed. Regarding the sample preparation technique, this study employed PPT as a simple, efficient, and less-costly preparation technique where acetonitrile was used as both clean-up and extraction solvents. On the contrary, other reports used more complicated and tedious sample preparation techniques: molecularly imprinted solid phase micro-extraction [16,25] or PPT followed by stir bar sorptive extraction procedure [24] that needs many optimization procedures and is thus more complicated. Another important point is that only 4 g of the analyzed sample was sufficient to determine the estrogens' residues, compared with 500 g in some of the previously reported HPLC-UV methods [25]. Finally, the high-throughput analysis was assessed by the short runtime (10 min), compared with nearly 22 min [21,22] or even up to 40 min [20].

4 Conclusion

An HPLC-DAD method for the determination of four estrogen residues in meat products has been developed

and validated. PPT was used as a simple sample preparation technique. Moreover, the use of DAD impacts the advantage of specificity compared with the universal UV detector and is also less expensive and easily applicable, compared with the mass spectrometric detectors.

Four estrogenic compounds (E1, E2, E3, and E4) were determined in meat samples of different categories (chicken, $n = 155$, beef, $n = 124$, sheep, $n = 122$, and camels, $n = 40$) available in the Saudi market.

The content of estrogens in the analyzed meat samples was maximum in the processed products of chicken, beef, and sheep (up to $5.59 \mu\text{g/g}$ E1, $40.38 \mu\text{g/g}$ E2, $74.4 \mu\text{g/g}$ E3, and even up to $419.56 \mu\text{g/g}$ E4). These high levels have pointed out that, for the sake of health benefits, particular attention should be paid to monitor the estrogenic content in meat products available in the Saudi market.

Acknowledgments: The authors would like to extend their appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the research group project no. RGPVPP-331.

Conflict of interest: Authors declare no conflicts of interest.

References

- [1] Findlay JK, Liew SH, Simpson ER, Korach KS. Estrogen signaling in the regulation of female reproductive functions. In: Habenicht UF, Aitken R, editors. *Handbook of Experimental Pharmacology*. Vol. 198. Berlin, Heidelberg: Springer. Fertility Control. 2010. p. 29–35.
- [2] Schulster M, Bernie AM, Ramasamy R. The role of estradiol in male reproductive function. *Asian J Androl*. 2016;18:1–6.
- [3] Leung KC, Johannsson G, Leong GM, Ho KK. Estrogen regulation of growth hormone action. *Endocr Rev*. 2004;25:693–721.
- [4] Adeel M, Song X, Wang Y, Francis D, Yang Y. Environmental impact of estrogens on human, animal and plant life: a critical review. *Env Int*. 2017;99:107–19.
- [5] Patel S, Homaei A, Rajud AB, Meher BR. Estrogen: the necessary evil for human health, and ways to tame it. *Biomed Pharmacother*. 2018;102:403–11.
- [6] Nair S, Sachdeva, G. Estrogen matters in metastasis. *Steroids*. 2018;138:108–16.
- [7] Directive 2008/97/EC of the European Parliament and of the Council of 19 November 2008 amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists. *OJ*. 2008;L318:9–11.

- [8] OECD Agriculture Statistics: OECD-FAO Agricultural Outlook (Edition 2018), <https://data.oecd.org/agroutput/meat-consumption.htm> (accessed Feb 2019).
- [9] Albalawi S. A Comparative study on the chemical composition and cholesterol content of fresh camel, beef and goat meat. *SJST*. 2015;15:73–80.
- [10] Xin TB, Liang SX, Wang X, Li HF, Lin JM. Determination of estradiol in human serum using magnetic particles based chemiluminescence immunoassay. *Anal Chim Acta*. 2008;267:277–84.
- [11] Hartmann S, Steinhart H. Simultaneous determination of anabolic and catabolic steroid hormones in meat by gas chromatography-mass spectrometry. *J Chromatogr B Biomed Sci Appl*. 1997;704:105–17.
- [12] Seo J, Kim HY, Chung BC, Hong J. Simultaneous determination of anabolic steroids and synthetic hormones in meat by freezing-lipid filtration, solid-phase extraction and gas chromatography-mass spectrometry. *J Chromatogr A*. 2005;1067(1–2):303–9.
- [13] Farke C, Rattenberger E, Roiger SU, Meyer HHD. Bovine colostrum: determination of naturally occurring steroid hormones by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J Agr Food Chem*. 2011;59:1423–7.
- [14] Wang H, Zhou X, Zhang Y, Chen H, Li G, Xu Y, et al. Dynamic microwave-assisted extraction coupled with salting-out liquid–liquid extraction for determination of steroid hormones in fish tissues. *J Agric Food Chem*. 2012;60:10343–51.
- [15] Qu X, Su C, Zheng N, Li S, Meng L, Wang J. A survey of naturally-occurring steroid hormones in raw milk and the associated health risks in Tangshan City, Hebei Province, China. *Int J Env Res Public Health*. 2018;15(1):38.
- [16] Socas-Rodríguez B, Herrera-Herrera AV, Hernández-Borges J, Rodríguez-Delgado MA. Multiresidue determination of estrogens in different dairy products by ultra-high-performance liquid chromatography triple quadrupole mass spectrometry. *J Chromatogr A*. 2017;1496:58–67.
- [17] Liu H, Lin T, Cheng X, Li N, Wang L, Li Q. Simultaneous determination of anabolic steroids and β -agonists in milk by QuEChERS and ultra high performance liquid chromatography tandem mass spectrometry. *J Chromatogr B*. 2017;1043:176–86.
- [18] Yan W, Li Y, Zhao LX, Lin JM. Determination of estrogens and bisphenol A in bovine milk by automated on-line C-30 solid-phase extraction coupled with high-performance liquid chromatography-mass spectrometry. *J Chromatogr A*. 2009;1216:7539–45.
- [19] Wang QL, Zhang AZ, Pan X, Chen LR. Simultaneous determination of sex hormones in egg products by ZnCl₂ depositing lipid, solid-phase extraction and ultra performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Anal Chim Acta*. 2010;678:108–16.
- [20] Hu YL, Wang YY, Chen XG, Hu YF, Li GK. A novel molecularly imprinted solid-phase microextraction fiber coupled with high performance liquid chromatography for analysis of trace estrogens in fishery samples. *Talanta*. 2010;80:2099–105.
- [21] Hu C, He M, Chen B, Hu B. Determination of estrogens in pork and chicken samples by stir bar sorptive extraction combined with high-performance liquid chromatography–ultraviolet detection. *J Agric Food Chem*. 2012;60:10494–500.
- [22] Shi Y, Peng DD, Shi CH, Zhang X, Xie YT, Lu B. Selective determination of trace 17 β -estradiol in dairy and meat samples by molecularly imprinted solid-phase extraction and HPLC. *Food Chem*. 2011;126:1916–25.
- [23] Yan Q, Yang L, Li S. Solid-phase extraction combined with high performance liquid chromatography-diode array detector for rapid determination of estrogens in milk. *Trop J Pharm Res*. 2015;14:2077–82.
- [24] Afifi R, Elnwishy N, Hannora A, Hedström M, Mattiasson B, Omran H, et al. SPE and HPLC monitoring of 17- β -estradiol in Egyptian aquatic ecosystems. *J Liq Chromatogr Rel Technol*. 2016;39:428–34.
- [25] Shahbazi Y, Malekinejad H, Tajik H. Determination of naturally occurring estrogenic hormones in cow's and river buffalo's meat by HPLC-FLD method. *J Food Drug Anal*. 2016;24:457–63.
- [26] Li G, Dong L, Wang A, Wang W, Hu N, You J. Simultaneous determination of biogenic amines and estrogens in foodstuff by an improved HPLC method combining with fluorescence labeling. *LWT Food Sci Technol*. 2014;55:355–61.
- [27] International Conference on Harmonization. Validation of Analytical Procedures: Text and Methodology. Geneva, Switzerland; 2005. <http://www.ich.org/LOB/media/MEDIA417.pdf>.
- [28] Moradi-Lakeh M, El Bcheraoui C, Afshin A, Daoud F, AlMazroa MA, Al Saeedi M, et al. Diet in Saudi Arabia: findings from a nationally representative survey. *Public Health Nutr*. 2016;20:1075–81.
- [29] Azzeh FS, Bukhari HM, Header EA, Ghabashi MA, Al-Mashi SS, Noorwalia NM. Trends in overweight or obesity and other anthropometric indices in adults aged 18–60 years in western Saudi Arabia. *Ann Saudi Med*. 2017;37:106–13.
- [30] Manceaux P, Jacques D, Zdanowicz N. Hormonal and developmental influences on adolescent suicide: a systematic review. *Psychiatr Danub*. 2015;27:S300–304.
- [31] Lemaine V, Cayci C, Simmons PS, Petty P. Gynecomastia in adolescent males. *Semin Plast Surg*. 2013;27:56–61.
- [32] Emeksiz HC, Derinöz O, Akkoyun EB, Pınarlı FG, Bideci A. Age-specific frequencies and characteristics of ovarian cysts in children and adolescents. *J Clin Res Ped End*. 2017;9:58–62.