Hindawi
Journal of Food Quality
Volume 2020, Article ID 5065386, 12 pages
https://doi.org/10.1155/2020/5065386

Review Article

Hormones and Hormonal Anabolics: Residues in Animal Source Food, Potential Public Health Impacts, and Methods of Analysis

Belachew B. Hirpessa,[1,2] **Beyza H. Ulusoy**,[1] **and Canan Hecer**[1]

[1]*Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Near East University, 99138 Nicosia/Lefkoşa, Cyprus*
[2]*Permanent Address: Veterinary Drug and Feed Authority, Quality Control and Residue Testing Center, P.O. Box 31303, Addis Ababa, Ethiopia*

Correspondence should be addressed to Belachew B. Hirpessa; bellnext@gmail.com

Received 3 February 2020; Revised 19 July 2020; Accepted 10 August 2020; Published 28 August 2020

Academic Editor: Susana Fiszman

The demand for nutritious food, especially food of animal origin, is globally increasing due to escalating population growth and a dietary shift to animal source food. In order to fulfill the requirements, producers are using veterinary drugs such as hormones and hormone-like anabolic agents. Hormones such as steroidal (estrogens, gestagens, and androgens), nonsteroidal, semisynthetic, and synthetic or designer drugs are all growth-promoting and body-partitioning agents. Hence, in food animal production practice, farm owners use these chemicals to improve body weight gain, increase feed conversion efficiency, and productivity. However, the use of these hormones and hormonal growth-promoting agents eventually ends up with the occurrence of residues in the animal-originated food. The incidence of hormone residues in such types of food and food products beyond the tolerance acts as a risk factor for the occurrence of potential public health problems. Currently, different international and national regulatory bodies have placed requirements and legislative frameworks, which enable them to implement residue monitoring test endeavors that safeguard the public and facilitate the trading activity. To make the tests on the animal-origin food matrix, there are different sample extraction techniques such as accelerated solvent extraction, supercritical fluid extraction, solid phase extraction, solid-phase microextraction, and hollow-fiber liquid-phase microextraction. After sample preparation steps, the analytes of interest can be assayed by screening and confirmatory methods of analysis. For screening, immunological tests such as ELISA and radioimmunoassay are used. Detection and determination of the specific residues will be done by chromatographic or instrumental analysis. Mainly, among high-performance liquid chromatography, liquid chromatography with mass spectrometry (LC-MS, LC-MS/MS), and gas chromatography with mass spectrometry (GC-MS and GC-MS/MS) methods, LC-MS/MS is being preferred because of easier sample preparation without a derivatization step and high detection and quantification capacity.

1. Introduction

The world has made significant progress in raising food consumption per person. In the last three and a half decades, it increased from an average of 2370 kcal/person/day to 2772 kcal/person/day (from 1970 to 2005/2007). It was 2860 kcal/person/day in 2015 and will be projected to 2960 and 3070 kcal/person/day by 2030 and 2050, respectively. Herewith, the most interesting point is all the food consumption growth have been accompanied by changes in composition and diets being shifted more towards primary livestock products. From food of animal origin, meat and milk together currently provide 22% of the total calories in the developing countries, up from 13% in the early 1970s [1–3].

Moreover, the world food economy is being increasingly driven by the demand shift of diets and food consumption patterns towards livestock products, which are associated with population growth, urbanization, and increasing incomes in the developing countries [3–5]. Because of this, a variety of animal species, including cattle, sheep, goats, birds, pigs, and fish, are kept for providing animal-origin protein-rich food and other nutritional requirements for the human

population. These foods are obtained from financial exploitations in which the animals' health must be guaranteed, thereby ensuring that food is harmless [6].

In order to maintain the well-being of animals and to improve the rate of weight gain and feed efficiency, as well as profit of the agribusiness, producers treat these animals with veterinary medicinal products (VMPs). These VMPs including antibiotics, hormones, and hormone-like anabolics are used for growth promotion, increasing weight-gain/meat yield and disease control in livestock production [7, 8], and such treatments can result in residues of the active ingredients, or their metabolites, entering the human food chain. Much higher residue levels may appear in the edible animal products when used unintentionally in overdoses or due to noncompliance with withdrawal periods [9, 10]. Nevertheless, in several countries, the safety of animal origin food has mainly been focused on avoiding the transmission of zoonotic diseases, less attention thus being paid to potentially present chemical residues, perhaps due to the course of the resulting disease [6].

However, concerns regarding the safety of livestock products and the prevalence of public health hazards have grown according to the increased use of hormones and hormonal anabolic substances [7]. These substances could be steroid hormones and nonsteroidal products and synthetic chemicals which mimic hormone functions and are known for their interference on the function of the endocrine system. In addition to endocrine disturbances, hormones such as estrogen are known for their carcinogenicity and genotoxic potential, and others such as diethylstilbestrol are reported to have mutagenic, carcinogenic, immunotoxic, and teratogenic properties [11, 12].

In recent years, an increase in education and consumer awareness has increased the demand for safe and healthy food and the need to know the foods they are eating are healthy and harmless. In addition, for this, objective information has to be presented. Hence, in order to assure the food is free from unwanted chemical residues such as hormones or to confirm that the residues are within the maximum limits of tolerance, standard analytical methods for detection and quantification are required [13]. Accordingly, continuous development of new and state-of-the-art multisample preparation techniques and residue detection and quantification methods become essentials [14].

Therefore, the main objectives of this article are to review about hormones and hormonal anabolic residues in in food of animal origin (milk and meat), to highlight on the potential public health impacts, and to summarize the latest and practically applicable methods for the detection and quantification of hormones and hormonal growth-promoter residues in animal-originated food matrixes.

2. Hormones and Hormonal Anabolic Products' Use and Residues

2.1. General Introduction. Hormones are endogenous biochemical messengers, which are produced in one kind of tissue to be released through the bloodstream and transported to their target organs to gradually stimulate, inhibit, or coordinate some physiological activities in a different tissue over a certain period of time [15, 16]. Hormones can be grouped as steroidal and nonsteroidal (protein hormones) and β-agonists (clenbuterol, cimaterol, ractopamine, salbutamol, and zilpaterol), and the steroidal hormones are further subgrouped as anabolic steroids and corticosteroids. Therefore, steroid hormones contain both the EGAs (estrogens, androgens, and gestagens), which are also known as the sex hormones or anabolic steroids, as well as the corticosteroids (Table 1) [27–29].

2.2. Hormonal Anabolics in Animal Production Practice Use and Residues. In general, hormone and hormonal anabolic substances are used in food animal production practice mainly because of their capacity to increase weight gain and to reduce the average feed intake in relation to the weight gain. The synergetic effects and ability to enhance nitrogen retention capacity and building up proteins are also reported in [30]. In addition, the synergistic effect of corticosteroids when combined with anabolic steroids or β-agonists has been described in [31].

Within the European Union, the use of hormones and other anabolic compounds for the purpose of fattening, production boosting, and growth promotion in farmed animals is completely banned, and residues are monitored [25]. However, it is allowed to use certain hormones for therapeutic and reproductive purposes under regulated conditions by authorized veterinarians. In such cases, the professionals are allowed on a transitional basis and under strict veterinary control to use 17-beta estradiol, testosterone, and progesterone and its derivatives for the treatment of gynecological disorders such as fetal maceration/mummification, pyometra in cattle, and estrus induction in cattle, horses, sheep, and goats (Table 2) [17, 25, 27].

2.2.1. Use in Fattening and Milk Production and Residues in the Products. There are exceptions and paradoxical practice worldwide regarding the use of hormones for fattening and milk production boosting purposes. For instance, in the US, estradiol is used in the form of silicone rubber implant which contains 25.7 or 43.9 milligrams (mg) of estradiol being coated with not less than 0.5 mg oxytetracycline powder. It is used to increase the rate of weight gain in suckling and pastured growing steers; for improved feed efficiency and increased rate of weight gain in confined steers and heifers at the dose of 25.7 mg implant for less than 200 days or 43.9 mg implant for every 400 days [18].

Besides, synthetic hormonal anabolic substances such as DES, hexestrol, and ethinylestradiol are still offered on the illegal market for body weight gaining purposes in animal fattening practice. Practically, these compounds are used as ear implants (released over a certain period), injectable (high concentrations in injection sites). Estradiol is used as a growth promoter in cattle and may produce two-fold to several ten-fold increases in the muscle tissue of treated animals [34].

From the xenobiotic hormonal anabolic products, with estrogenic effects, zeranol is used in beef cattle and sheep to

Table 1: Hormones and hormone-like compounds by their type, indications, and approval status.

Hormonal substances	Hormone types and active ingredients		Animal species/ prod.			Indications/ purposes				Reference and remark
	Natural	Synthetic/xenobiotic	Beef	Dairy	Sheep	WG	FE	ER	Milk	
Estrogens	17β-Estradiol*	Ethyl ester		*	*			*		[17]
	Estradiol	Estradiol benzoate	X			X	X	X		OTC in the US [18]
	Estrone	Stilbenes1								
	Resorcylic acid lactones	Zeranol	X		X	X	X			OTC in the US [19]
Gestagens	Progesterone	MLGA	X		X	X	X	X		OTC in the US [20]
		MPA								[21]
		19-Nortestosterone (nandrolone)								Illegal use in horse sports [22]
		17α-Methyltestosterone								
Androgens	Testosterone	DMT								
		TBA	X			X	X			OTC in the US [23]
		Testosterone propionate	X			X	X			OTC in the US [18]
G. hormones	BST	sBST or rBST		X					X	OTC in the US [24]
β-Agonists		Clenbuterol								[25]
		Ractopamine	X			X	X			[26]
		Zilpaterol	X			X	X			[26]

Note. X = approved for use by the FDA, * = approved in EU member countries for therapeutic use only, DES = diethylstilbestrol, Dienestrol, and hexestrol, MLGA = melengestrol acetate, MPA = medroxyprogesterone acetate, TBA = trenbolone acetate, DMT = desoxymethyltestosterone, sBST = synthetic bovine somatotropin, rBST = recombinant bovine somatotropin, WG = weight gain, FE = feed efficiency, ER = estrous regulation, Milk = increased milk production, OTC = over-the-counter drug, and EU = European Union.

increase the rate of weight gain, feed efficiency, and high-quality carcass (Table 1). In case of beef cattle production, it is used at the dose of 36 mg (one implant consisting of 3 pellets, each having 12 mg dose zeranol as an API). This implant dose is indicated to increase the rate of weight gain and improve feed conversion in weaned beef calves, growing beef cattle, feedlot steers, and feedlot heifers. In cattle, it will be discharged 65 days after implant with a residual effect of $\leq$2 ppb (μg/kg) in all organs and tissues (Table 2) [18, 19].

It can also be possible to mention the existence of possible practical use of other hormonal anabolics called "designer drugs." These are all kinds of new drugs but with structural analogue variations of the "old" forms. Some of the renowned examples of "designer drugs," which have similarities with steroid structures, are norbolethone, tetrahydrogestrinone (THG), and desoxymethyltestosterone (DMT) [35]. Vincent et al. [21] also reported the illegal use of medroxyprogesterone acetate (MPA) in swine production, which is also a "designer" drug.

However, gestagens are frequently employed as esters (melengestrol acetate (MLGA) or medroxyprogesterone acetate (MPA)), either alone or in combination with estrogens [16]. Melengestrol, as a synthetic progestogen, is administered orally as a feed additive to improve feed efficiency. The approved feeding doses are in a range of 0.25 ~ 0.50 mg/heifer per day during the fattening and finishing periods. Its activity is revealed via a high affinity for progesterone receptors as well as increases in prolactin secretion and the activation of estrogen receptors [36].

Well-known examples of androgens used in animal fattening are testosterone propionate in combination with estradiol benzoate or androgen-like xenobiotic such as 19-nortestosterone, 17β-methyl testosterone, boldenone, and trenbolone. In order to improve feed conversion efficiency and improve weight gain in heifers, a combination of testosterone propionate (200 mg) and estradiol benzoate (20 mg), as a single percutaneous ear implant [18]. In addition to the aforementioned anabolic agents, other analogues of androgens have been synthesized, e.g., stanozolol, 4-chlortestosterone, norethandrolone, and fluoxymesterone [37].

3. Potential Public Health Impacts of Hormones and Hormonal Anabolics

3.1. Public Health Impacts of Steroid Hormones in Animal Source Food

3.1.1. Estrogens. The concentration of naturally occurring estrogens in food varies from species to species along with its age, gender, and physiological status [38]. Milk is considered to be one of the potent sources of steroids including estrogens [39]. Results of large-scale epidemiological investigations evidenced that 17β-estradiol, as a mammary carcinogen, acts both as an initiator and promoter of breast carcinogenesis.

Estradiol has genotoxic potential by inducing micronuclei, aneuploidy, and cell transformation *in vitro* and oxidative damage to DNA and DNA single-strand breakage *in vivo* [40]. It is also concluded that 17β-estradiol is a Group I human carcinogen that has sufficient evidence for carcinogenicity to humans. The carcinogenicity of estradiol is found to be a result of its interaction with hormonal receptors because tumors largely occur in tissues possessing

Table 2: Maximum residue limit (MRL) and ADI of different sequentially selected hormones.

Pharmacologically active substance	Marker residue	Animal species	Target tissue	Maximum residue (MRL, μg/kg = PPb) EU*	Codex#	USA^	Remarks or notes
17β-Estradiol	Not applicable	All mammalian food-producing species	Muscle Liver Kidney Fat Milk (μg/L)	*No MRL required	#Unnecessary	0.12 0.24 0.36 0.48 —	#Residues resulting from use of the substance as a growth promoter with GAHP, unlikely to pose hazard to human health, ADI = unnecessary [32], 0–0.05 μg/kg BW [33]. ^Tolerance limit [18].
Clenbuterol hydrochloride	Clenbuterol	Bovine and equine	Muscle Liver Kidney Fat Milk (μg/L)	0.1 0.5 0.5 — 0.05	0.2 0.6 0.6 0.2 0.05	Prohibited use in food animals	*Agents acting on the nervous system. #Due to potential abuse MRLs recommended only associated with nationally approved therapeutic use^ in horses, in the case of COPD [24]
Progesterone	Not applicable	Bovine Ovine Caprinae Equidae (Female)	Muscle Liver Kidney Fat Milk	*No MRL required	#Unnecessary 21st	5^ 15^ 30^ 30^ -	*No entry (only for zoo technical use). [25]. #Residues resulting from use of the substance as a growth promoter with GAHP, unlikely to pose hazard to human health. ^Not in excess of natural concentration in the body of untreated animals
Testosterone	Testosterone	Bovine	Muscle Liver Kidney Fat	*No MRL	#Unnecessary	0.64 1.30 1.90 2.60	#Residues resulting from use of the substance as a growth promoter in accord. GAHP practice is unlikely to pose a hazard to human health ADI = 0–2 μg/kg BW [33]. ^Not for use in dairy or beef replacement heifers.
Trenbolone acetate	Trenbolone	Bovine	Muscle Liver	No MRL	2 10	^Not needed	#ADI: 0–0.02 μg/kg BW [33]. ^ADI: 0.4 μg/kg BW per day [23].
Zeranol	Zeranol	Bovine Ovine	Muscle Liver Muscle Liver Milk	*No MRL required (completely banned)	2 10 — — —	^Not needed 20 — —	*No entry [25]; #only for: bovine, ADI = 0–0.5 μg/kg body weight [32]. ^ADI = 0.00125 [19].

ADI = acceptable daily intake, BW = body weight, CAC = Codex Alimentarius Commission, CFR = Code Federal Regulations, COPD = chronic obstructive pulmonary diseases, GAHP = good animal husbandry practice, and MRL = maximum residue limit.

high levels of hormone receptors. Overall, estradiol is evaluated as a genotoxic carcinogen.

3.1.2. Zeranol. Research carried on human epithelial cell cultures through repeated zeranol treatments were shown to reduce cell doubling time, stimulate colony formation, and, most notably, induce expression of ER-β mRNA in the proliferation of human breast epithelial cell line (MCF-10A) and downregulate the tumor suppressor gene (P53) in tissues of rats and beef heifers [41]. Orally administered zeranol showed weak estrogenic effects in long-term toxicity studies using rats, dogs, and monkeys through changes in mammary glands and reproductive organs [42]. In several *in vitro* and *in vivo* genotoxic studies, zeranol and its metabolites, zearalenone and taleranol, were negative [34].

3.1.3. DES. Various reports suggested that DES has mutagenic, carcinogenic, and teratogenic properties, which have raised widespread public health concerns [43]. Its use in veterinary food products as growth stimulant for food-producing animals has been banned in several countries (since 1979 in the USA and 1981 in EU). Therefore, EU has proposed a minimum required performance limit (MRPL)

of 0.5–2.0 μg/kg to control its abuse in meat-producing animals [44].

3.1.4. Progesterone. In a study conducted on a postmenopausal woman, progesterone (200–300 mg/day/orally) and estradiol (1.5 or 3 mg/day/percutaneous) were administered for five years, and there was no evidence of endometrial hyperplasia or carcinoma after five years of estradiol and progesterone treatment [45]. However, some old reports showed that progesterone increased the incidences of ovarian, uterine, and mammary tumors in mice as well as mammary gland tumors in dogs, and these effects were regarded as hormone activity-related. These results from laboratory animals might be indications for considering progesterone as one of the potential causes of public health problems. In another study, progesterone has shown no evidence of genotoxicity [40, 46].

3.1.5. MLGA. It was found to be a low acute toxic chemical in rodents after oral administration. Melengestrol acetate was not a genotoxic chemical in a full range of *in vitro* and *in vivo* assays, including bacterial and mammalian cellular gene mutation assays. MLGA causes a progestational endpoint effect such as changed menstrual cycle on female cynomolgus monkeys [47].

3.1.6. β-Agonists. According to various research findings, when β-agonists, such as clenbuterol, accumulate beyond a certain concentration in the body, because of a higher affinity to receptors, they cause reactions triggering muscle tremors, tachycardia, and muscle pain.

Generally, hormones as a residue in food (mainly dairy and meat), after entering into the human body, may affect the endocrine system and could be regarded as endocrine-disturbing compounds (EDCs). This could be a potential risk factor for the increase of estrogen-dependent diseases such as breast cancer in women and may expose to other reproductive system problems [48, 49]. Different epidemiological studies have showed strong correlation between consumption of meat and dairy products and incidence rate of female breast, ovarian, and corpus uteri cancers. Specifically, 17β-estradiol (E2) is the most active estrogen and natural estrogen, which can be toxic and carcinogenic, even at low levels [49, 50].

4. Current Methods for Hormones and Hormonal Anabolic Residues' Analysis

4.1. Sample Preparation Methods (Extraction and Purification). Sample matrices, destined for the analysis of hormones and hormonal anabolic residues, should undergo a preparation process, which requires the extraction or purification and separation steps in order to detect and quantify the analytes of interest. Especially in the analysis of residues such as hormones from animal-originated food samples, the presence of matrix effect rendered the sample preparation steps to be laborious, time-taking, and reagent-consuming. Because of this, different methods of sample extraction and separation techniques have been developed [51–53]. In animal source food sample preparation, the actual sample components such as lipids, fats, and proteins will pose a matrix complexity on the analysis process of target of analytes. Additionally, the wide array of hormones and hormone-like compounds and the usual lowest levels (ng up to μg/kg) that should be detected and quantified make residue analysis for hormones a challenging task. To detect and determine the lowest residue levels, sample pretreatment activities, which allow preconcentration of the target analytes, are necessary, but they will also lead to the concentration of potential interfering matrix components [54].

Conventionally, animal-originated samples such as the muscle, fat, kidney, and liver will be grinded and/or freeze-dried and homogenized and then extracted with organic solvents (methanol and acetonitrile). After extraction with suitable organic solvents, a clean-up multistep process using liquid-liquid extraction (LLE) and/or liquid-solid extraction (LSE) is carried out. LSE is one of the most commonly used extraction techniques in the analysis of chemical residues such as veterinary drugs, steroid hormones, and pesticides, most of the time being performed in the form of solid-phase extraction (SPE) techniques [52, 54]. Alternatively, novel approaches for extraction using accelerated solvent extraction (ASE) technique or supercritical fluid extraction (SFE) methods are developed. In this review, we have tried to highlight more on those methods which are supposed to be novel, cost-effective, and time-saving, especially on methods related to animal-originated food matrix extraction, purification, and analysis [55].

In order to clean up the primary extract of the diluent and sample mixture, different sorbents can be used for solid-phase extraction and/or purification steps. For this reason, the inner wall surface of the SPE cartridges is made of sorbents such as C_8, ENVI-Chrom P, Si-NH_2, C_{18}, and Oasis HLB on which the interaction with the target molecules takes place and ends up with an output of clean aliquot. In a review done on chromatographic analysis of natural and synthetic estrogens in milk and dairy products, a number of extraction, purification techniques and determination methods had been summarized. In dairy product residue analysis, deproteinization is commonly performed as one of the first steps because, it provides matrix effects (e.g., ion suppression in MS detection), problem of contamination, blockage or irreversible damage on the HPLC pathway, and adsorption to the stationary phase or to the SPE columns (Table 3) [61]. Though purification is one of the critical steps to get the analyte of interest, based on the specificity of the method used, the aliquot might contain different purified components, each containing a restricted number of targets or the analyte of our interest and matrix compounds.

4.2. Innovative Matrix Extraction and Purification Techniques. Advanced novel extraction and purification methods of sample preparation are those methods, which are cost-effective (demand less organic reagents and labor), allow multiresidue analysis, and are relatively more specific.

Table 3: Determination of hormones and hormonal anabolics in food of animal origin using different techniques of sample preparation and analytical methods.

Sample (hormone)	Extraction and/or cleanup technique	Solvent for extraction	Assaying method and detectors used	LOD (μg/kg)	Reference
Beef meat (clenbuterol)	HF-SPME	Methanol (MeOH) and AA	HPLC-DAD	0.01–0.03 (μg/ml)	[56]
Pork (melted fat) (MPA)	SPE (C_{18} cartridge, 500 mg, 3 ml)	MeOH	HPLC-tandem MS	0.5	[57]
Meat (steroids and corticosteroids)	SFE and SPME SFE and SPE	CO_2, MeOH ACN, AA	HPLC-UV, (column 100 × 5 mm RP-C_{18})		[58, 59]
Kidney fat and meat (steroid hormones)	LSE and Si-NH_2 SPE	ACN hexane MeOH	GC-EI-IT-MS	0.5–5	[51]
Meat (steroid hormones)	LSEC18 SPE	ACN hexane MeOH: H_2O	GC-EI-IT-MS	0.1–0.4	[60]
Bovine milk (six types of estrogen)	Deproteinization Defatting (ASE) LLEC_{18}-SPE	Acetic acid MeOH:H_2OHexane ACN: H_2O	HPLC-ESI-MS/MS (positive mode) (ACN/water/AA)	0.005–0.01	[53, 61]
Milk (DES)	CNTs-HF-SPME	MeOH	HPLC-UV (C_{18})	5.1 μg/L	[43]
Yoghurt (17β-estradiol)	MIP	Acetic acid MeOH	HPLC-UV (C_{18})	0.03–013	[62]
Milk products Yoghurt Liquid ProbioticChees (estrogens)	HF-LPME	ACN plus acetic acid	HPLC-UV (C_{18} silica columns)	0.290.23–0.400.58	[55]

Note. AA = acetic acid, ACN = acetonitrile, EI = electron impact, ESI = electron spray ionization, GC = gas chromatography, H_2O = water, HF-LPME = hollow-fiber liquid-phase microextraction, HFPME = hollow-fiber solid-phase microextraction, HF-SPME = hollow-fiber solid-phase microextraction, HPLC = high-performance liquid chromatography, HPLC-DAD = HPLC with photodiode array detector, HPLC-UV = high-performance liquid chromatography-ultraviolet, IT = ion trap, LOD = limit of detection, LSE = liquid-solid extraction, MeOH = methanol, MIP = molecular imprinted polymer, MS = mass spectrometry, and SPE = solid-phase extraction.

There are various advanced sample extraction and purification techniques, with steps for the reduction or elimination of matrix interferences and for the enrichment of the selected analytes of interest to mention: Soxhlet extraction, accelerated solvent extraction, supercritical fluid extraction, solid-phase extraction (a routine sample preparation technique), solid-phase microextraction, hollow-fiber liquid-phase microextraction [55], microwave-assisted extraction, molecular imprinting polymer-solid phase extraction, and size-exclusion chromatography [62].

4.2.1. Accelerated Solvent Extraction (ASE). It is a technique, which enables to extract solid samples under high pressure and temperature; due to this, it is also called pressurized liquid extraction (PLE). One of the application areas of this extraction and purification technique is in food analysis, such as determination of hormone residue. Some of advantages of ASE are reduction of solvent use, fast extraction process (allows extraction of a large number of samples), and is a promising sample cleanup technique for steroid and nonsteroid hormones. So far, for screening tests of gestagens such as MLGA and MPA, ASE has been utilized for extraction of kidney fat samples. For instance, in the extraction of hormone residue from kidney fat, first the ASE vessels are filled containing the matrix with alumina and anhydrous sodium. Next, samples will be defatted with hexane before the gestagens were trapped on alumina. Finally, alumina was on-line extracted with acetonitrile followed by freezing of the extract to precipitate the remaining fat. For purification, the extract will be purified with C_{18} SPE. Addition of modifiers, to the extraction solvent, application of specific sorbents into the extraction cells, and the possibility to carry out repeated PLE with different extraction solvents are some of the complementary advantages PLE can offer. Automatic programmed rinsing steps between samples will alleviate concerns about cross-contamination [63].

4.2.2. Supercritical Fluid Extraction (SFE). SFE is a technique which utilizes supercritical fluid (substance above its critical temperature and pressure) instead of organic solvents as an extraction fluid. The main advantages are good solvating power, the high diffusivity, the low viscosity, the minimal surface tension, possibilities to manipulate pressure and temperature, and the use of modifiers, by doing so, changes the solvating power of the supercritical fluid. SFE technique had been used by different researchers, such as in the residue analysis of steroid hormones and corticosteroids [58], for the extraction of trenbolone from beef, and for the extraction of estrogenic and other anabolic agents from bovine tissue by using CO_2 as the supercritical fluid. A multianalyte, multimatrix method was also developed for the routine determination of steroids in animal tissues coupling SFE to SPE [59].

4.2.3. Solid-Phase Extraction (SPE). SPE is a routine sample preparation technique for extracting analytes from a

complex matrix. As reviewed by Barbara et al. [55], as one of the novel methods for sample extraction, SPE is easy to perform, has the ability to cope with large loads, gives high recoveries, consumes relatively small amounts of organic solvents, can be automated, has faster extraction than LLE, and has lower cost, and a wide range of stationary phases are available. Some of the stationary phases used for the preparation of the SPE cartridge include poly((-divinylbenzene-vinylpyrrolidone) resins (Oasis HLB), most successfully used in E2 extraction and C_{18}. For example, SPE packed with C_{18} (ODS, octadecyl silica) material as the stationary phase of 500 g with 3 ml capacity had been used for the determination of medroxyprogesterone acetate (MPA) in pork-origin product and serum (Table 3) [57]. Courant et al. [64] were able to extract and enrich the analytes of interest (estradiol or E2) using SPE from retail samples of milk and eggs; also, they were able to separate steroid hormones from milk, egg, and meat samples, respectively [65]. However, the nonselective sorbents (normally C_{18} silica) used in SPE often result in the coextraction of many matrix components. Repeated SPE is used in order to get better purification effects [62, 65].

4.2.4. Solid-Phase Microextraction (SPME). It is a method that allows analytes to be adsorbed onto the surface of a small fused-silica fiber coated with a suitable polymeric phase, placed in a syringe-like cartridge. Subsequently, analytes are desorbed into a suitable apparatus for separation and determination. This technique is based on the distribution of analytes of interest between an extraction phase (polymer) and the matrix [58]. Some of the critical advantages of SPME compared to solvent extraction are the reduction in the amounts of solvent used, the combination of sampling and extraction, and the ability to examine smaller sample sizes [66]. A recent research work done on the analysis of estrogenic compounds in dairy products by Barbara et al. [55] showed the use of the SPME-type LPME (liquid-phase microextraction technique) method that utilizes LLE technique which is simple, effective, and selective, specifically called hollow-fiber liquid-phase microextraction (HF-LPME). The HF-LPME method also found to consume low organic solvents, gives high percentage of the analytes of interest, and is low cost (Table 3) [67].

4.2.5. Molecularly Imprinted Polymer-Solid-Phase Extraction (MISPE). The combined use of MIPs with SPE technique has currently appeared as new selective sorbents for SPE of organic compounds in complex matrices. For example, herbicides and drugs can be selectively extracted from samples such as beef-liver extract, blood serum, and other biological samples. MIPs are synthetic cross-linked polymers that possess specific cavities designed for a target analyte (template). MIPs are introduced into the SPE procedure in order to improve the extraction efficiency and selectivity [68]. MIPs are a rapidly developing technique for the preparation of specific polymers, which can have specific recognition properties; by doing so, they allow specific analytes to be selectively extracted from complex matrices. Simply speaking, MIPs can recognise and bind the target analyte selectively as the antibody does. Furthermore, MIPs have advantages such as physical robustness, high strength, resistance to elevated temperatures and pressures, and inertness towards organic solvents, acids, or bases [69].

Researchers have also developed a multiresidue detection and quantification method of β-agonists in the urine matrix using MIP sample preparation. However, recently in food matrices, E2- (estradiol-) imprinted MIPs prepared by bulk polymerization have been used in offline MISPE with HPLC to determine E2 in spiked fishery samples [70]. MISPE techniques can be combined online and offline with LC-MS and HPLC, respectively. Offline MISPE combined with HPLC is easy and rapid to perform, but due to low packing quality of the molecularly imprinted solid phase extraction material, there will be channel formation and will lead to low number of theoretical plates of the material, which result in less separation of the analyte of interest. Furthermore, concentrations of E2 in dairy and meat samples are about ng/kg, much less than the detection limit (μg/L) of the reported offline MISPE with HPLC methods [70, 71].

In general, we can summarize that MIPs are a recently developing and practically promising technique, which subsequently requires investigation of some features, specifically for the potential application of MIPs in the cleanup of extracts of meat and milk matrices for the detection of hormones and hormone-like anabolic compounds. To mention, Shi et al. [62] had used MIP extraction technique in combination with high-performance liquid chromatography with ultraviolet detector (HPLC-UV) to determine 17β-estradiol (E2) in yogurt with a limit of detection (LOD) of 0.03–0.13 μg/kg (Table 3).

4.3. Methods of Hormone and Hormonal Residue Analysis in Animal Source Food. Residue detection and quantification of hormones and hormonal anabolic growth promoters are widely done by immunological and chromatographic methods of analysis. These methods may also be classified as screening and confirmatory methods of analysis. The chromatographic or instrumental means can carry out both screening and quantification assay. Because of this, they are becoming to replace screening methods of analysis of immunochemical-based methods such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Nopp et al. [14] have well summarized that the detection and quantification of steroid hormones in animal-originated food matrices have been dominated by chromatographic separation methods (GC or LC) coupled with different sensitive and specific detection systems such as mass spectrometer (MS). Generally, it is mentioned that GC coupled with single quad-MS or triple quad (GC-MS-MS) has been the most employed technique. Currently, liquid chromatographic (LC) system coupled with mass spectrometry as a detector (LC-MS/MS) is becoming as a more preferred technique [72, 73]. In this review, both means of analysis can be summarized, attention given to the chromatographic methods.

4.3.1. Immunological Methods of Analysis. Immunological methods are highly sensitive and easy to perform, but the instability of natural antibodies limits their applications to some extent. Sample pretreatment, such as LLE or SPE, is necessary to ensure the accuracy and repeatability of immunological methods [74]. Immunoassays are often used for inspection of hormonal residues in animal source food (meat and milk) as a screening test. These types of screening methods are primarily designed to avoid false-negative results and minimize the number of samples that need to be confirmed. One of the flawed characters of immunoassay tests such as ELISA is the lack of specificity or the possibility of cross-reaction [75, 76].

The immunological methods are based on the interaction of the antigen and antibody, which is very specific for a particular residue. The most usual technique consists of the ELISA, and the detection system is usually based on enzyme-labeled reagents. There are different formats for the specific agent (antigen) quantification, such as the double-antibody or sandwich-ELISA tests and direct competitive ELISA tests [77]. Radioimmunoassay (RIA) is based on the measurement of the radioactivity of the immunological complex. Other assays have enhanced detectability by using luminescence detectors if using chemiluminescent compounds or a fluorimeter in the case of fluorescent compounds [78].

Today, there are many different types of ELISA kits commercially available for a large number of hormones and hormonal anabolic substances summarized in-group in Tables 1 and 2 such as the steroids (EGAs), β-agonists, corticoids, stilbenes, and resorcylic acid lactones. These kits allow the analysis of a large number of samples per kit, do not require sophisticated instrumentation, the results are available in a few hours, and are quite specific and sensitive [75]. For instance, in case of banned hormone analysis such as DES, radioimmunoassay (RIA), fluorescence immunoassay (FIA), and enzyme-linked immunosorbent assay (ELISA) were developed. Compared to RIA and FIA, ELISA has the advantages of safety, speediness, reliability, sensitivity, and low cost [74]. Yang et al. [43] also developed a competitive indirect chemiluminescence enzyme immunoassay (CLEIA) based on a polyclonal antibody and horseradish peroxidase-labeled secondary antibody chemiluminescence system to detect DES residues in seafood. This assay allows the rapid screening of DES residues. The CLEIA is a combination of sensitive chemiluminescence detection and specific immunosorbent assay. In CLEIA, enzyme labels are detected by chemiluminescent (CL) substrates, such as the luminol/peroxide/enhancer system for horseradish peroxidase (HRP) substrates for alkaline phosphates [79]. The CLEIA is a rapid assay that has a large linear dynamic range, high sensitivity and specificity, involves small sample volumes, and does not create any radioactive pollution [80].

4.3.2. Chromatographic Methods of Analysis. Chromatographic methods, including LC (HPLC), LC-MS, LC-MS/MS, GC-MS, and GC-MS/MS, are able to provide screening and quantification of the target analyte. However, extensive sample preparation is necessary in order to allay matrix effect and increase accuracy of the analysis. LC or HPLC with UV, DAD, and FL detector assay is simple, rapid, and widely available in most laboratories, but with relatively limited sensitivity [71]. For instance, in order to reduce the detection limits of HPLC, effective enrichment of trace E2 from a large amount of samples is very important. With this regard, as it is summarized in Table 3, Shi et al. [62] have utilized an advanced extraction method (MIP), and Yang et al. [81] also used carbon nanotube-hollow-fiber solid-phase microextraction (CNT-HF-SPME) so as to improve the detection limit of HPLC-UV.

Regarding β-agonist residue analysis in food of animal origin, a very recent research work done so far and summarized in this particular review showed that these chemicals can be specifically and selectively analyzed by automated online SPE coupled to LC-tandem mass spectrometry [82]. Methods such as HPLC coupled with DAD have also been reported in the determination of ractopamine and clenbuterol residues in beef after using graphene oxide hollow-fiber solid-phase microextraction (HF-SPME) as a sample extraction technique [56].

Concerning GC-MS methods, they are sensitive, accurate, and more accessible, but derivatization is needed [72]. Fuh et al. [60] have determined residual anabolic steroids in meat by gas chromatography-ion trap-mass spectrometry (Table 3). In this method, they have used the technique called gas chromatography-electron impact-ion trap-mass spectrometry (GC-EI-IT-MS) for the determination of residual anabolic steroid in beef, pork, and chicken meat and visceral organs such as the kidney and liver. Extraction of the analytes of interest was done by ACN, then isolated and preconcentrated by SPE, and finally, the isolates were derivatized with N-methyl-N-trimethylsilytrifluoroacetamide prior to GC-EI-IT-MS measurement. Positive mode was used for detection [83, 84].

In the analysis of hormones in animal-originated food samples, LC-mass spectrometry (LC-MS), particularly LC-MS/MS methods, is recently used and is known to provide the lowest LOD with high selectivity, sensitivity, and accuracy without the need for derivatization [72]. However, it is not accessible in all laboratories for routine analysis due to the high cost of equipment (initial capital) and the requirement of a skillful operator. This method is able to conduct screening and quantification assay of the compounds by producing precursors and product ions and finally quantifying and detecting each compound by using charge-to-mass ratio of each molecule, which is specific as a fingerprint for any target molecules captured on the mass detector [85, 86].

5. Conclusion and Recommendations

The alarmingly escalating demand for animal source food due to population growth, as well as the ever-increasing intensive production system, is forcing producers to use legal and/or illegal VMPs. Moreover, dietary shift for animal source food and some preferences such as the desire for cholesterol-free lean meat are pushing the livestock farming

industry to use hormones and other growth promoters. Because of the above reasons, there is unprecedented use of hormones and other hormonal anabolic compounds. In different countries, the compounds are legally used for the treatments of clinical cases, reproduction, and productivity improvement in the meat and dairy industry by setting limits. However, others such as the European Union countries strongly prohibit the use of hormones and hormonal growth promoters and anabolic drugs in farmed animals destined for human food. Albeit of these facts, due to the potential health impacts to the public in general, the use of these products is strictly controlled and legally regulated by the national regulatory authorities and international legislative frameworks. Hence, sensitive, selective, and accurate method of analysis that will be preceded by effective and time-saving sample preparation techniques should be developed, summarized, and used.

Based on the aforementioned conclusion, the following recommendations are forwarded:

(1) Public awareness programs and community mobilization campaigns are needed to alert the farming community, food professionals, and industry owners about the negative consequences and the potential public health impacts related to the use and misuse of hormones and hormonal anabolics in animals raised for human food.

(2) The use of hormones and hormonal anabolic growth promoters should always be under the concerned senior vet professionals, and strict regulatory monitoring programs have to be in place.

(3) Analytical methods that are sensitive, selective, accurate, and cost-effective in terms of time and reagent consumption should be selected, developed, validated, and used.

(4) It is advised first to use immunological methods of analysis as a screening tool to limit the number of samples that need confirmatory tests. Then, the assaying process should be augmented with excellent sample preparation methods of latest technology to avoid the sample matrix effect and increase the concentration of analyte of interest.

(5) Research works done so far on the use of hormones and hormonal anabolics should be further summarized and synthesized, and the potential public health impacts of these compounds on consumers have to be deeply studied.

Conflicts of Interest

All the authors declare that there are no conflicts of interest.

References

[1] M. J. Boland, A. N. Rae, J. M. Vereijken et al., "The future supply of animal-derived protein for human consumption," *Trends in Food Science & Technology*, vol. 29, no. 1, pp. 62–73, 2013.

[2] FAO, *World Agriculture Towards 2030/2050: The 2012 Revision*, pp. 23–26, FAO, Rome, Italy, 2012.

[3] FAO, "Food system, urbanization and dietary changes," in *The State of Food and Agriculture Leveraging Food Systems for Inclusive Rural Transformation*FAO, Rome, Italy, 2017.

[4] C. Delgado, M. Rosegrant, and S. Meijer, "Livestock to 2020: the revolution continues," in *Proceedings of the Paper Presented at the Annual Meetings of the International Agricultural Trade Research Consortium (IATRC)*, pp. 18-19, Auckland, New Zealand, January 2001.

[5] C. Delgado, "Rising demand for meat and milk in developing countries: implications for grass lands-based livestock production,""Rising demand for meat and milk in developing countries: implications for grass lands-based livestock production," in *Grassland: a Global Resource*, D. A. McGilloway, Ed., pp. 29–39, Wageningen Academic Publishers, Wageningen, Netherlands, 2005.

[6] C. L. Maria and T. Mary, "Chemical residues in animal food products: an issue of public health," in *Public Health-Methodology, Environmental and Systems Issues*IntechOpen, London, UK, 2012.

[7] S.-H. Jeong, D.-J. Kang, M.-W. Lim, C.-S. Kang, and H.-J. Sung, "Risk assessment of growth hormones and antimicrobial residues in meat," *Toxicological Research*, vol. 26, no. 4, pp. 301–313, 2010.

[8] X.-T. Tan, Z.-M. Li, L.-G. Deng, S.-C. Zhao, and M.-L. Wang, "Analysis of 13 kinds of steroid hormones in raw milk using modified QuEChERS method combined with UPLC-QTOF-MS," *Journal of Integrative Agriculture*, vol. 15, no. 9, pp. 2163–2174, 2016.

[9] K. N. Woodward, "The toxicity of particular veterinary drug residues,,""The toxicity of particular veterinary drug residues,," in *Pesticide, Veterinary and other Residues in Food*, H. David and Watson, Eds., pp. 175–223, Woodhead Publishing Ltd., New York, NY, USA, 2004.

[10] N. Bülent, Ç. Hilal, A. Ali, and H. Hamparsun, "The presence of some anabolic residues in meat and meat products sold in Istanbul," *Turkish Journal of Veterinary and Animal Sciences*, vol. 29, no. 3, pp. 691–699, 2005.

[11] E. Diamanti-Kandarakis, J.-P. Bourguignon, L. C. Giudice et al., "Endocrine-disrupting chemicals: an endocrine society scientific statement," *Endocrine Reviews*, vol. 30, no. 4, pp. 293–342, 2009.

[12] C. Donovan, "If FDA does not regulate food, who will? a study of hormones and antibiotics in meat production," *American Journal of Law and Medicine*, vol. 41, no. 2-3, pp. 459–482, 2015.

[13] N. VanHoof, D. Courtheyn, D. J. P. Antignac et al., "Multiresidue liquid chromatography/tandem mass spectrometric analysis of beta agonists in urine using molecular imprinted polymers," *Rapid Communication in Mass Spectrometer*, vol. 19, no. 19, pp. 2801–2808, 2005.

[14] H. Noppe, B. Le Bizec, K. Verheyden, and H. F. De Brabander, "Novel analytical methods for the determination of steroid hormones in edible matrices," *Analytica Chimica Acta*, vol. 611, no. 1, pp. 1–16, 2008.

[15] H. Galbraith, "Hormones in international meat production: biological, sociological and consumer issues," *Nutrition Research Reviews*, vol. 15, no. 2, pp. 243–314, 2002.

[16] P. Annamaria, "Steroid hormones in food producing animals," in *A Bird's-Eye View of Veterinary Medicine*IntechOpen, London, UK, 2012.

[17] EU/EC, "Amending council directive 96/22/EC concerning the prohibition on the use in stockfarming of certain

substances having hormonal or thyreostatic action and of beta agonists," *European Community Council (ECC) Directive 2003/74/EC of 22 September 2003, Official Journal of European Commissions*, vol. 262, pp. 17–21, 2003.

[18] U S–FDA, "Tolerances for residue of new animal drugs in food," in *Animal Drugs, Feeds, and Related Products, Implantation or Injectable Dosage form New Animal Drugs, Code of Federal Regulation (CITE: 21CFR 522)*Vol. 6, FDA, Silver Spring, MD, USA, 2017.

[19] U S–FDA, "Implantation or injectable dosage form new animal drugs, zeranol," in *Animal Drugs, Feeds, and Related Products, Code of Federal Regulation (CITE 21CFR 556.760)* Vol. 6, FDA, Silver Spring, MD, USA, 2017.

[20] U S–FDA, "Tolerances for residue of new animal drugs in food," in *Animal Drugs, Feeds, and Related Products, Code of federal regulation (CITE: 21CFR 556)*Vol. 6, FDA, Silver Spring, MD, USA, 2017.

[21] V. Porphyre, M. Rakotoharinome, T. Randriamparany, D. Pognon, S. Prévost, and B. Le Bizec, "Residues of medroxyprogesterone acetate detected in sows at a slaughterhouse, Madagascar," *Food Additives and Contaminants: Part A*, vol. 30, no. 12, pp. 2108–2113, 2013.

[22] M. S. Bahrke and C. E. Yesalis, "Abuse of anabolic androgenic steroids and related substances in sport and exercise," *Current Opinion in Pharmacology*, vol. 4, no. 6, pp. 614–620, 2004.

[23] U S–FDA, "Tolerances for residue of new animal drugs in food, trenbolone," in *Animal Drugs, Feeds, and Related Products, Code of Federal Regulation (CITE: 21CFR 556.739)* Vol. 6, FDA, Silver Spring, MD, USA, 2017.

[24] U S–FDA, "Oral dosage form new animal drugs, clenbuterol syrup," in *Animal Drugs, Feeds, and Related Products, Code of Federal Regulation (CITE 21CFR 520.452)*Vol. 6, FDA, Silver Spring, MD, USA, 2017.

[25] EU/EC, "Pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin," *Official Journal of European Commissions*, vol. 37, pp. 1–72, 2010.

[26] FSIS, *United States National Residue Program for Meat, Poultry, and Egg Products 2015 Residue Sampling Plans*, United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS), Office of Public Health Science, Washington, DC, USA, 2015.

[27] D. Courtheyn, B. Le Bizec, G. Brambilla et al., "Recent developments in the use and abuse of growth promoters," *Analytica Chimica Acta*, vol. 473, no. 1-2, pp. 71–82, 2002.

[28] A. A. M. Stolker and U. A. T. Brinkman, "Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals-a review," *Journal of Chromatography A*, vol. 1067, no. 1-2, pp. 15–53, 2005.

[29] E. S. E. Hafez, M. R. Jainudeen, and Y. Rosnina, "Hormones and growth factors in reproduction,""Hormones and growth factors in reproduction," in *Reproduction in Farm Animals*, B. Hafez and E. Hafez, Eds., pp. 108–120, Wiley-Blackwell, Lindon, UT, USA, 7th edition, 2013.

[30] M. E. Dikeman, "Effects of metabolic modifiers on carcass traits and meat quality," *Meat Science*, vol. 77, no. 1, pp. 121–135, 2007.

[31] H. F. De Brabander, B. Le Bizec, G. Pinel et al., "Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals," *Journal of Mass Spectrometry*, vol. 42, no. 8, pp. 983–998, 2007.

[32] CAC, "Maximum residue limits (MRLs) and risk management recommendations for residues of veterinary drugs in foods," in *Codex Alimentarius Commission (CAC)/MRL 2-2017*, pp. 1–84, CAC, Japan, 2017.

[33] JECFA, "Evaluation of certain veterinary drug residues in food," in *Joint FAO/WHO Expert Committee on Food Additives (JECFA)*vol. 52, WHO Technical Report Series893 pages, WHO Technical Report Series, Geneva, Switzerland, 2000.

[34] P. E. Strydom, L. Frylinck, J. L. Montgomery, and M. F. Smith, "The comparison of three β-agonists for growth performance, carcass characteristics and meat quality of feedlot cattle," *Meat Science*, vol. 81, no. 3, pp. 557–564, 2009.

[35] W. Karpiesiuk, A. F. Lehner, C. G. Hughes, and T. Tobin, "Preparation and chromatographic characterization of tetrahydrogestrinone, a new "Designer" anabolic steroid," *Chromatographia*, vol. 60, no. 5-6, pp. 359–370, 2004.

[36] G. A. Perry, W. V. Welshons, R. C. Bott, and M. F. Smith, "Basis of melengestrol acetate action as a progestin," *Domestic Animal Endocrinology*, vol. 28, no. 2, pp. 147–161, 2005.

[37] S. Poelmans, *Application of GC–and LC–MS in the analysis and metabolization studies of steroids in livestock and aquatic invertebrates*, Ph.D. Thesis, p. 196, Ghent University, Ghent, Belgium, 2006.

[38] A. Daxenberger, D. Ibarreta, and H. H. Meyer, "Possible health impact of animal oestrogens in food," *Human Reproduction Update*, vol. 7, no. 3, pp. 340–355, 2001.

[39] S. Hartmann, M. Lacorn, and H. Steinhart, "Natural occurrence of steroid hormones in food," *Food Chemistry*, vol. 62, no. 1, pp. 7–20, 1998.

[40] IARC, *Monographs on the Evaluation of Carcinogenic Risks to Humans: Hormonal Contraception and Postmenopausal Hormone Therapy*, Vol. 72, IARC Press, Lyon, Francs, 1999.

[41] S. Liu and Y. C. Lin, "Transformation of MCF-10A human breast epithelial cells by zeranol and estradiol-17 beta," *The Breast Journal*, vol. 10, no. 6, pp. 514–521, 2004.

[42] JECFA, "Toxicological evaluation of certain veterinary drug residues in food: estradiol-17β, progesterone, and testosterone," in *Joint FAO/WHO Expert Committee on Food Additives (JECFA)*Vol. 43, WHO Food Additives Series, Geneva, Switzerland, 2000.

[43] J. Y. Yang, Y. Zhang, H. T. Lei et al., "Development of an ultrasensitive chemiluminescence enzyme immunoassay for the determination of diethylstilbestrol in seafood," *Analytical Letters*, vol. 46, no. 14, pp. 2189–2202, 2013.

[44] F. Taşçi, "Determination of diethylstilbestrol residue in raw meat sold in Burdur, Turkey," *Journal of Applied Biological Sciences*, vol. 8, no. 3, pp. 32–34, 2014.

[45] D. L. Moyer, B. d. Lignieres, P. Driguez, and J. P. Pez, "Prevention of endometrial hyperplasia by progesterone during long-term estradiol replacement: influence of bleeding pattern and secretory changes ** supported in part by Besins-Iscovesco laboratories, Paris, France," *Fertility and Sterility*, vol. 59, no. 5, pp. 992–997, 1993.

[46] M. J. Seraj, A. Umemoto, M. Tanaka, A. Kajikawa, and K. Monden, "DNA adduct formation by hormonal steroids in vitro," *Mutation Research/Genetic Toxicology*, vol. 370, no. 1, pp. 49–59, 1996.

[47] JECFA, "Toxicological evaluation of certain veterinary drug residues in food," in *Melengestrol Acetate*Vol. 45, WHO Food Additives Series, Geneva, Switzerland, 2000.

[48] J. D. Yager and N. E. Davidson, "Estrogen carcinogenesis in breast cancer," *New England Journal of Medicine*, vol. 354, no. 3, pp. 270–282, 2006.

[49] D. Ganmaa and A. Sato, "The possible role of female sex hormones in milk from pregnant cows in the development of breast, ovarian and corpus uteri cancers," *Medical Hypotheses*, vol. 65, no. 6, pp. 1028–1037, 2005.

[50] F. Massart, J. C. Harrell, G. Federico, and G. Saggese, "Human breast milk and xenoestrogen exposure: a possible impact on human health," *Journal of Perinatology*, vol. 25, no. 4, pp. 282–288, 2005.

[51] S. Impens, K. De Wasch, M. Cornelis, and H. F. De Brabander, "Analysis on residues of estrogens, gestagens and androgens in kidney fat and meat with gas chromatography-tandem mass spectrometry," *Journal of Chromatography A*, vol. 970, no. 1-2, pp. 235–247, 2002.

[52] B. Shao, R. Zhao, J. Meng et al., "Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography-tandem mass spectrometry," *Analytica Chimica Acta*, vol. 548, no. 1-2, pp. 41–50, 2005.

[53] H. Malekinejad, P. Scherpenisse, and A. A. Bergwerff, "Naturally occurring estrogens in processed milk and in raw milk (from gestated cows)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 26, pp. 9785–9791, 2006.

[54] C. Blasco, C. Van Poucke, and C. Van Peteghem, "Analysis of meat samples for anabolic steroids residues by liquid chromatography/tandem mass spectrometry," *Journal of Chromatography A*, vol. 1154, no. 1-2, pp. 230–239, 2007.

[55] S. R. Barbara, A. R. María, H. B. Javier, and R. D. Miguel, "Analysis of estrogenic compounds in dairy products by hollow-fiber liquid-phase micro extraction coupled to liquid chromatography," *Journal of Food Chemistry*, vol. 149, pp. 319–325, 2014.

[56] T. Gao, N. Ye, and L. Jian, "Determination of ractopamine and clenbuterol in beef by graphene oxide hollow fiber solid-phase microextraction and high-performance liquid chromatography," *Analytical Letters*, vol. 49, no. 8, pp. 1163–1175, 2016.

[57] L. Giannetti, D. Barchi, F. Fiorucci et al., "High-performance liquid chromatography-tandem mass specrometry validation of medroxyprogesterone acetate in products of pork origin and serum," *Journal of Chromatographic Science*, vol. 43, no. 7, pp. 333–336, 2005.

[58] K. Kureckova, B. Maralikova, and K. Ventura, "Supercritical fluid extraction of steroids from biological samples and first experience with solid-phase micro extraction–liquid chromatography," *Journal of Chromatography B*, vol. 770, no. 1-2, pp. 83–89, 2002.

[59] A. A. M. Stolker, P. W. Zoontjes, and L. A. Van Ginkel, "The use of supercritical fluid extraction for the determination of steroids in animal tissues," *The Analyst*, vol. 123, no. 12, pp. 2671–2676, 1998.

[60] M.-R. Fuh, S.-Y. Huang, and T.-Y. Lin, "Determination of residual anabolic steroid in meat by gas chromatography-ion trap-mass spectrometer," *Talanta*, vol. 64, no. 2, pp. 408–414, 2004.

[61] B. Socas-Rodríguez, M. Asensio-Ramos, J. Hernández-Borges, A. V. Herrera-Herrera, and M. Á. Rodríguez-Delgado, "Chromatographic analysis of natural and synthetic estrogens in milk and dairy products," *TrAC Trends in Analytical Chemistry*, vol. 44, pp. 58–77, 2013.

[62] Y. Shi, D.-D. Peng, C.-H. Shi, X. Zhang, Y.-T. Xie, and B. Lu, "Selective determination of trace 17β-estradiol in dairy and meat samples by molecularly imprinted solid-phase extraction and HPLC," *Food Chemistry*, vol. 126, no. 4, pp. 1916–1925, 2011.

[63] H. Hooijerink, E. O. Van Bennekom, and M. W. F. Nielen, "Screening for gestagens in kidney fat using accelerated solvent extraction and liquid chromatography electrospray tandem mass spectrometry," *Analytica Chimica Acta*, vol. 483, no. 1-2, pp. 51–59, 2003.

[64] F. Courant, J.-P. Antignac, D. Maume, F. Monteau, F. Andre, and B. Le Bizec, "Determination of naturally occurring oestrogens and androgens in retail samples of milk and eggs," *Food Additives and Contaminants*, vol. 24, no. 12, pp. 1358–1366, 2007.

[65] F. Courant, J.-P. Antignac, J. Laille, F. Monteau, F. Andre, and B. Le Bizec, "Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. determination of steroid hormones in milk, egg, and meat samples," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 9, pp. 3176–3184, 2008.

[66] K. Ridgway, S. P. D. Lalljie, and R. M. Smith, "Sample preparation techniques for the determination of trace residues and contaminants in foods," *Journal of Chromatography A*, vol. 1153, no. 1-2, pp. 36–53, 2007.

[67] B. Socas-Rodríguez, M. Asensio-Ramos, J. Hernández-Borges, and M. Á. Rodríguez-Delgado, "Hollow-fiber liquid-phase microextraction for the determination of natural and synthetic estrogens in milk samples," *Journal of Chromatography A*, vol. 1313, pp. 175–184, 2013.

[68] V. Pichon, "Selective sample treatment using molecularly imprinted polymers," *Journal of Chromatography A*, vol. 1152, no. 1-2, pp. 41–53, 2007.

[69] F. G. Tamayo, E. Turiel, and A. Martín-Esteban, "Molecularly imprinted polymers for solid-phase extraction and solid-phase microextraction: recent developments and future trends," *Journal of Chromatography A*, vol. 1152, no. 1-2, pp. 32–40, 2007.

[70] M. Jiang, Y. Shi, R.-l. Zhang et al., "Selective molecularly imprinted stationary phases for Bisphenol A analysis prepared by modified precipitation polymerization," *Journal of Separation Science*, vol. 32, no. 19, pp. 3265–3273A, 2009.

[71] T. Jiang, L. Zhao, B. Chu, Q. Feng, W. Yan, and J.-M. Lin, "Molecularly imprinted solid-phase extraction for the selective determination of 17β-estradiol in fishery samples with high performance liquid chromatography," *Talanta*, vol. 78, no. 2, pp. 442–447, 2009.

[72] Z. Zeng, R. Liu, J. Zhang, J. Yu, L. He, and X. Shen, "Determination of seven free anabolic steroid residues in eggs by high-performance liquid chromatography-tandem mass spectrometry," *Journal of Chromatographic Science*, vol. 51, no. 3, pp. 229–236, 2013.

[73] I. M. Zuchowska, B. Wozniak, and A. Posyniak, "Determination of hormone residues in milk by gas chromatography-mass spectrometry," *Food Analytical Methods*, vol. 10, no. 3, pp. 727–739, 2017.

[74] D. A. Pape-Zambito, A. L. Magliaro, and R. S. Kensinger, "17β-Estradiol and estrone concentrations in plasma and milk during bovine pregnancy," *Journal of Dairy Science*, vol. 91, no. 1, pp. 127–135, 2008.

[75] W.-J. Wang, Y. Ling, T. Xu, H.-B. Gao, W. Sheng, and J. Li, "Development of an indirect competitive ELISA based on polyclonal antibody for the detection of diethylstilbestrol in water samples," *Chinese Journal of Chemistry*, vol. 25, no. 8, pp. 1145–1150, 2007.

[76] B. P. Shankar, P. B. H. Manjunatha, S. Chandan, D. Ranjith, and V. Shivakumar, "Rapid methods for detection of veterinary drug residues in meat," *Veterinary World*, vol. 3, no. 5, pp. 241–246, 2010.

[77] U. Yücel, N. Atasoy, Ö. İşleyici, and İ. Türe, "Determination of some anabolic hormone residues in cattle meat consumption in Van, Turkey," *International Journal of Advanced Research*, vol. 6, no. 8, pp. 129–139, 2018.

[78] A. Roda, A. C. Manetta, O. Portanti et al., "A rapid and sensitive 384-well microtitre format chemiluminescent enzyme immunoassay for 19-nortestosterone," *Luminescence*, vol. 18, no. 2, pp. 72–78, 2013.

[79] D. Knopp, "Immunoassay development for environmental analysis," *Analytical and Bioanalytical Chemistry*, vol. 385, no. 3, pp. 425–427, 2006.

[80] G. J. Zheng, L. Q. Feng, H. Chen et al., "Magnetic microparticle chemiluminescence immunoassay method for determination of estriol in human urine," *Chinese Journal of Analytical Chemistry*, vol. 39, no. 1, pp. 62–65, 2011.

[81] Y. Yang, J. Chen, and Y.-P. Shi, "Recent developments in modifying polypropylene hollow fibers for sample preparation," *TrAC Trends in Analytical Chemistry*, vol. 64, pp. 109–117, 2015.

[82] J. Mi, S. Li, H. Xu, W. Liang, and T. Sun, "Rapid analysis of three β-agonist residues in food of animal origin by automated on-line solid-phase extraction coupled to liquid chromatography and tandem mass spectrometry," *Journal of Separation Science*, vol. 37, no. 17, pp. 2431–2438, 2014.

[83] S. Croubels, E. Daeseleire, S. De Saeger, P. Van Eenoo, and L. Vanhaecke, "Hormone and veterinary drug residue analysis in food, feed, biological and environmental matrices," *Analytical and Bioanalytical Chemistry*, vol. 407, no. 15, pp. 4339–4342, 2015.

[84] R. T. Cristina, F. Hanganu, R. Lazăr et al., "Prevalence of steroid hormone residues by GC-MS/MS screening in animal matrices in Romania," *Romanian Biotechnological Letters*, vol. 22, no. 1, pp. 12155–12162, 2017.

[85] B. Wozniak, I. Matraszek–Zuchowska, A. Klopot, and A. Posyniak, "Fast analysis of 19 anabolic steroids in bovine tissues by high performance liquid chromatography with tandem mass spectrometry," *Journal of Separation Science*, vol. 42, no. 21, pp. 3319–3329, 2019.

[86] R. Gadzała-Kopciuch, J. Ricanyova, and B. Buszewski, "Isolation and detection of steroids from human urine by molecularly imprinted solid-phase extraction and liquid chromatography," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, no. 11-12, pp. 1177–1184, 2009.