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Screening for estrogenic steroids in sheep and chicken by the application of enzyme-linked immunosorbent assay and a comparison with analysis by gas chromatography–mass spectrometry

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A total of 146 sheep urine and 87 chicken muscle samples collected from birds sold in the State of Kuwait local market (originating from Brazil, Denmark, France and Turkey) were tested for residues of two xenobiotic estrogenic compounds, namely diethylstilbestrol (DES); and ethinylestradiol. The samples were analyzed using enzyme-linked immunosorbent assay (ELISA) and gas chromatography–mass spectrometry (GC–MS). Data obtained showed that the levels of DES and ethinylestradiol in the urine ranged from not-detected (ND) to 1.2 and ND to 0.90 ppb, respectively. In the chicken muscle, the levels encountered were ND to 0.70 and ND to 0.30 ppb, respectively, indicating that some samples may show some activity in terms of a residue, as recommended by international organizations. In view of the results obtained by ELISA, the employment of a cut-off value of 0.30 ppb would make it reasonable to obtain low false-positive results, thus indicating that such a technique provides a fast and reliable method for the detection and screening of anabolic samples. All samples (both negative and positive) were subjected to GC–MS analysis for confirmatory purposes. The results obtained from the GC–MS analysis were found to be negative. These results show that the activity seen and reported above was due to the matrix of the samples, but not due to the active estrogenic compounds. Data on extraction recovery and coefficient of variation are also reported. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Steroid hormones have been used to improve the growth of cattle, sheep and other livestock. Many experimental studies have shown that estrogenic hormones, both natural and synthetic, increase body weight and protein deposition, and decrease fat deposition (Steen, 1985; Roche and Quirke, 1986; MacVinish and Galbraith, 1988; Cecave and Hancock, 1994; Mader, 1994; Turner *et al.*, 1995; Lone, 1997). The European Economic Community (EEC) has banned the use of anabolic hormones for growth promotion in food-producing animals and has effectively banned the import of meat from treated animals from other countries (Heitzman, 1993). The United States Food and Drug Administration (USFDA), on the other hand, allows the use of naturally occurring hormones (estradiol and testosterone) and synthetically derived hormones (zeranol and trenbolone) for animal production (Farber, 1991). Since the EEC has banned the use of any type of hormone in animal production, the black market is thriving and various mixtures and cocktails are available in the black market which contain all sorts of compounds, both hormonal and otherwise.

To detect the use of legal or illegal natural hormones or xenobiotic drugs, and to prevent the use of inappropriate therapeutic doses, veterinary and public health control laboratories are required to develop extensive programs for surveillance of the residue of these drugs in meat and meat-related products destined for human consumption. The method of choice should be accurate, sensitive, specific, and precise, so that both false-negative and false-positive results are eliminated. These methods are further divided into screening and

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confirmatory methods. In the beginning, radioimmunoassay (RIA) was the method of choice for screening; however, as analytical technology is continually changing and progressing, RIA has now been effectively and successfully replaced with enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA). For confirmatory purposes, methods based on high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) are usually used.

HPLC and GC–MS methods for the detection of estrogenic compounds, such as, stilbenes (Hoffmann and Blietz, 1983; Van Pateghem and Van Haver, 1986; Meyer, 1990), and estradiol-17β or ethinylestradiol (Arts *et al*., 1991; Daeseleire *et al*., 1991; Tsujioka and Ito, 1992; De Boever *et al*., 1993; Hendricks *et al*., 1993; Scippo *et al*., 1993, 1994; van Ginkel *et al*., 1993; Batjoens *et al*., 1994) have already been developed. Because Kuwait imports the majority of its meat and meat-related products from Asian, European and South American countries, and because of the availability of the cocktail and mixture of anabolic compounds in the rampant black markets of some of these countries, their use is quite widespread. In the present report, the results of a survey of the Kuwaiti market is presented regarding the presence of the residues of diethylstilbestrol (DES), and ethinylestradiol measured by a competitive ELISA.

MATERIALS AND METHODS

Urine was collected from Australian and Arabian breeds of sheep, kept at the animal keeping facility of the Kuwait Institute for Scientific Research, and from the central slaughterhouse in Kuwait City. Chicken muscle samples were collected from locally chilled and imported frozen carcasses. A total of 146 urine samples were collected from 95 Australian, and 51 Arabian sheep. Muscle samples were collected from 87 chickens which originated in France, Brazil, Denmark, and Turkey as well as two local companies.

Diethylstilbestrol

Extraction of urine

The urine (0.5 ml) was diluted with 3 ml of sodium acetate buffer (50 mM; pH 4.8), and 8 μl of β-glucuronidase/arylsulfatase (Boehringer) (5.5:2.6 U/ml at 38°C) was added. The contents were incubated for 3 h at 37°C. The hydrolyzed products were purified by C_{18}-Bakerbond separation columns. For this, the columns were rinsed with 2 ml of 100% methanol and then equilibrated with 2 ml of 20 mM TRIS buffer (pH 8.5) containing 20% methanol. The sample was then added to the column, and the column was rinsed with 2 ml of 20 mM TRIS buffer (pH 8.5) containing 20% methanol. Afterwards, a second rinsing was performed with 2 ml of 40% methanol/water. Lastly, the sample was eluted with 1 ml of 80% methanol/water. The eluate-containing sample was diluted with 3 ml of 80% methanol and 4 ml of distilled water. Two 20 μl samples were employed for the estimation of DES.

Extraction of muscle

Fat and connective tissue were removed from the muscle and 5 g of the minced muscle was homogenized with 10 ml of 67 mM phosphate buffer (pH 7.2). Homogenate equivalent to 3 g of the muscle was extracted with 8 ml of tertiary butylmethylether (TBME), after thoroughly shaking. The contents were centrifuged at 3000 rpm for 10 min. The residue was extracted again with TBME. The supernatants were mixed and dried, and the residue was dissolved in 1 ml of 70% methanol. To this, 3 ml of petroleum ether was added and the contents were thoroughly mixed. The mixture was centrifuged and the hydrocarbon layer was discarded. The methanolic solution was dried, dissolved in 1 ml of dichloromethane and extracted once with 3 ml of 1 M NaOH. The extract was neutralized with 300 μl of 6 M phosphoric acid, and the mixture was loaded on a C_{18} column for elution. The method of preparation, the rinsing of the column and the elution of DES were the same as described for urine.

Estimation of DES (Meyer, 1990)

A Ridascreen ELISA kit was obtained from R-Biopharm GmbH, Germany. The standards used contained 0, 13, 25, 50, 100, and 200 pg DES ml^{-1} of buffer, whereas the antibody used had cross-reactivities with other related compounds, as indicated by the manufacturer's literature and shown in Table 1.

The standard and sample microwells were analyzed in duplicate. To the marked microwells, 50 μl of the diluted enzyme conjugate (peroxidase–DES conjugate) solution was added. To the microwells, marked standards, 20 μl each of the standard solution, were then added. To the sample microwells, 20 μl of the extract was added in duplicate. Then, 50 μl of the antibody was added, and after mixing, the contents were incubated at room temperature for 2 h. The contents of the wells were then discarded, and 200 μl of distilled water added. After rinsing, the water was also discarded. Then, 50 μl of the substrate (urea peroxide) and 50 μl of chromagen (tetramethylbenzidine) were added, and after incubating for 30 min at room temperature, 100 μl of the stop solution (1 M sulphuric acid) was added. After mixing, the absorbance was read at 450 nm. Color was stable for 60 min.

Table 1. Cross-reactivities of diethylstilbestrol (DES) antibody with various compounds

Compound	Cross-reactivity(%)
DES	100.0
Hexestrol	22.0
Dienestrol	8.0
DES-glucuronide	68.0
17β-estradiol	< 0.01
All other related steroids	< 0.01

Table 2. Cross-reactivities of ethinylestradiol antibody with various compounds

Compound	Cross-reactivity(%)
Ethinylestradiol	100.0
Estradiol-17β	0.7
Trenbolone	0.03
Nortestosterone-17β	0.005
Estrone	0.002
Estradiol-17α	< 0.001
All other similar steroids	< 0.001

Ethinylestradiol

The extraction and estimation procedures for both the urine and the muscle tissue were the same as those used for DES. The cross-reactivity of the ethinylestradiol antibody with related compounds, as indicated by the manufacturer's literature (R-Biopharm, GmbH, Germany), is shown in Table 2.

GC–MS

GC–MS of the estrogenic steroids was performed according to van Ginkel *et al.* (1989). GC–MS analysis was carried out with a Shimadzu QP-5000 gas chromatograph/mass spectometer equipped with a SE-52 fused silica capillary column (25 m × 0.25 mm i.d.). The analysis conditions were as follows: initial temperature 100°C, final temperature 280°C, at 20°C min^{-1}. For extract purification, immunoaffinity chromatography (IAC) offered a very specific and selective clean-up of the extract. The prepared immunoaffinity gel was obtained commercially from the Laboratory of Hormonology (Marloie, Belgium). GC–MS, selected ion mode (ion 474 and 412), were used for the detection of ethinylestradiol and DES, respectively. Multiple ion mode was used for confirmation, ions 459, 446, 353 for ethinylestradiol and ions 397, 383 for DES. For quality control, a deuterated internal standard (d_6-diethylstilbestrol) was incorporated and its absolute recovery monitored.

RESULTS AND DISCUSSION

DES

DES, a synthetic compound with potent anabolic properties, has been shown to be carcinogenic and its use was banned in the late 1970s in livestock production. The standard curve for the DES assay is given in Fig. 1. Sheep urine was spiked from 0.5 to 5 ppb. The results, however, are presented up to the 2.5 ppb level because, beyond that limit, the values obtained were out of the range of the standard curve in Fig. 2. For later experiments, the spiking was at a level below 3 ppb. The results are presented in Table 3. The mean recoveries

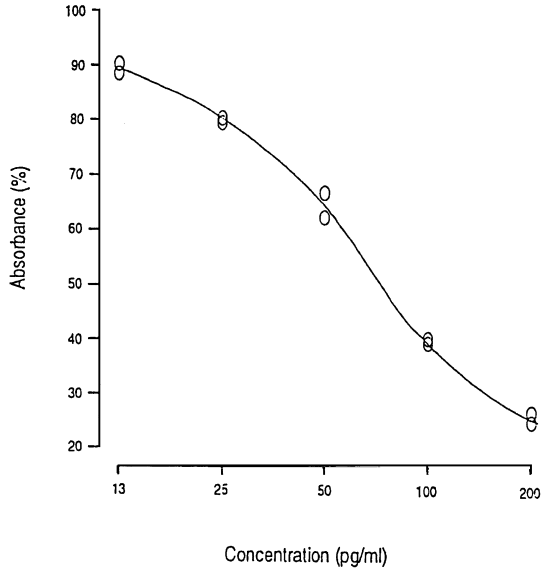

Fig. 1. Diethylstilbestrol calibration curve.

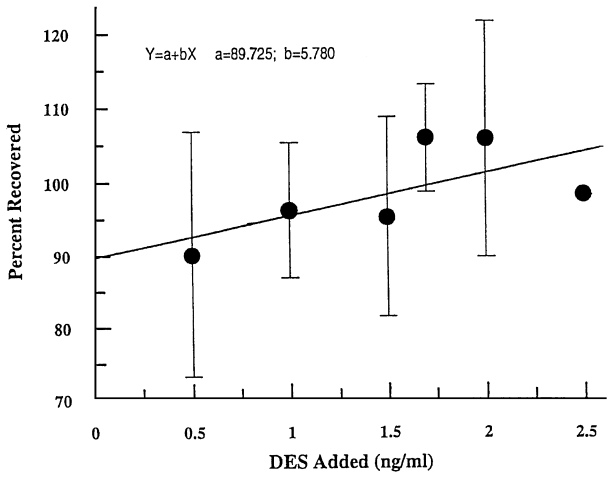

Fig. 2. Percentage of recovery of diethylstilbestrol (DES) from spiked sheep urine.

obtained for this xenobiotic compound were from 90 to 106%. The CVs for these recoveries ranged from 6.3 to 18.6. This shows that inter-assay variation existed, possibly due to the changes in the reagent batches and/or a slight shift in the standard curve.

In addition to the recovery and screening of the sheep urine, recovery from the spiked samples of chicken muscle was also studied. For this, chicken muscle samples were spiked with DES concentrations ranging from 0.20 to 1.5 ppb. These spiked muscle samples were then subjected to the method described. At each concentration, a minimum of four to eight extractions were made to ascertain the recovery from the spiked samples. The results of these recovery experiments are presented in Table 4. The recoveries seemed to decrease as the concentrations increased. For example, for samples spiked with 0.20 ppb DES, the mean recovery was 77% with a CV of 17.8, whereas for samples spiked with 1.5 ppb

Table 3. Effect of spiking sheep urine on the accuracy of diethylstilbestrol (DES) determination using enzyme-linked immunosorbent assay (ELISA)

Spiking concentration (ppb)	*n*	Mean recovery (%)	Standard deviation	Coefficient of variation
0.5	4	90	16.8	18.6
1.0	4	96	9.2	9.6
1.5	4	95	13.6	14.3
2.0	4	106	7.2	6.3
2.5	1	98	–	–

Table 4. Effect of spiking chicken muscle on the accuracy of diethylstilbestrol (DES) determination using enzyme-linked immunosorbent assay (ELISA)

Spiking concentration (ppb)	*n*	Mean recovery	Standard deviation	Coefficient of variation
0.2	4	68	12.1	17.8
0.3	8	59	9.4	16.0
0.5	5	64	11.3	17.7
0.7	7	68	8.9	13.1
1.0	7	57	10.2	17.9
1.5	5	49	7.6	15.4

DES, the recovery was 49% and the CV was 15.4. A similar situation was reported by Van Pateghem and Van Haver (1986). These authors spiked meat samples with DES (0.1–1.0 ng g^{-1}) and studied the recovery by first chromatographically cleaning the meat extracts and then applying RIA for estimation. The amounts recovered from spikings of 0.1, 0.5 and 1.0 ng g^{-1} were 115, 64 and 67% of the spiked level, respectively.

The recovery of DES from animal tissues has been described in the literature, and the values obtained in the current study compare favorably with the published values. Reuvers *et al.* (1991) described a chromatographic method for the determination of DES in meat (muscle) samples. In this method, using bovine muscle, the authors reported an average recovery of 63–68% with mean values of 66% and a CV of 21.4. These values and the recovery values obtained by the present study are comparable. However, the average CV achieved in the present study, i.e. 16.3, is lower than that obtained by Reuvers *et al.* (1991). The detection limit in the study of Reuvers *et al.* (1991) was 0.10–0.20 ng g^{-1}. However, they were of the opinion that, with that detection limit, other compounds could interfere with the analysis and false-positive results could be obtained. Heitzman (1992), while describing the reference methods for the anabolic steroids used in veterinary production, indicated average recovery figures of 67% with CV values of 12.2, 18.0 and 14.5 for DES, dienestrol and hexestrol, respectively. The recovery levels obtained in the current study are comparable and, in some cases, better than those reported in the literature. However, the method is still being improved for the optimization of recovery and the minimization of intra- and inter-assay variation.

After the recovery experiments were completed, screening of the sheep urine samples from various sources for DES was undertaken. During these studies, a total of 146 urine samples (95 from Australian sheep and 51 from Arabian sheep) were analyzed. Of the 95 Australian samples, 45 samples were below the detection limit and 50 samples showed positive results. The concentration of these samples ranged from 0.20 to 0.90 ppb. With the Arabian sheep, 33 samples produced positive results. The concentrations for these samples were 0.20–1.2 ppb. At an average recovery rate of 98% for urine, the mean lower limit of detection for the method employed in the present studies was 0.2 ppb for the standard hormone. In order to decide on the preferred cut-off limits and for application in the future, it was decided to take into consideration positive samples over 0.5 ppb and in the range 0.5–0.7 ppb. The total number of samples analyzed was 146. The number of ELISA positive > 0.5 ppb was 12 (8.3%) and > 0.7 ppb was 4 (2.7%). Therefore, by employing a cut-off limit of 0.7 ppb, a very low percentage of false-positive samples, with probability to avoid false-negative results would ensue. The EC guidelines for the maximum permissive dose of DES in biological samples is 2 ppb (Meyer, personal communication). Thus, the levels obtained in the present study, both from the Australian and Arabian sheep urine, were well below the limits set for this compound. Therefore, the samples were considered negative. However, in order to validate the limit set, we also analyzed all the 146 samples of urine by GC–MS. The GC–MS could not detect any activity in any of the urine samples, confirming the rejection limit determined with the ELISA method.

In addition to the urine from the Australian and Arabian sheep, muscle samples from chicken were collected from the local co-operative markets and analyzed for residues of DES. A total of 87 muscle samples were analyzed. Seventy-one of 87 muscle samples analyzed showed activity over the detection limit of the method (0.1 ppb). Values of the positive samples obtained ranged from 0.1 to 0.7. A cut-off limit of 0.5 ppb will reduce the false-positive samples to 4 (4.6%) which is considered as reasonable. For confirmation, like urine, all ELISA positive samples were analyzed by GC–MS and none of the samples were found to be positive.

Ethinylestradiol

The standard curve for ethinylestradiol is presented in Fig. 3. Sheep urine samples were spiked with different standard concentrations of ethinylestradiol, i.e. from 0.5 to 3.0 ppb. The recovery data for this steroid are presented in Table 5. The recoveries obtained ranged from 76 to 108%. The average recovery ($n = 21$) for all the concentrations used was 85 ± 11.8, and the CV was 13.6. The recovery decreased with increases in the spik-

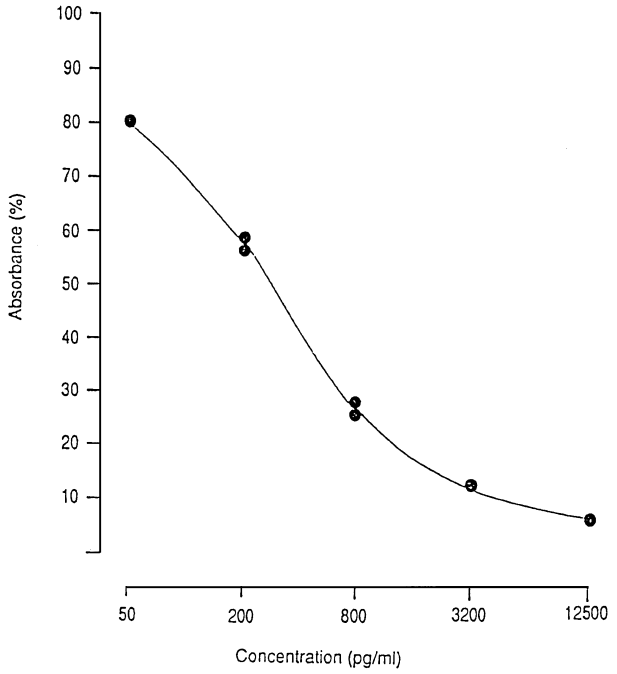

Fig. 3. Ethinylestradiol calibration curve.

ing concentration of the compound. However, the recovery seemed to plateau after reaching a level of 1.5 ng ml^{-1}, because further increases in the spiking concentration did not seem to decrease the recovery (ANOVA; $p > 0.05$).

Apart from the studies on urine, chicken muscle was also used in the recovery experiments for ethinylestradiol. Chicken leg muscle was spiked with six different concentrations ranging from 0.20 to 1.5 ppb. The mean recovery for samples analyzed for these concentrations was from 51 to 85% with an overall mean value of 72% ($n = 31$). The CV for these values ranged from 4.98 to 12.7 with an overall mean of 9.6 (Table 6). It appears that somewhere between the spiking levels of 0.5 and 1.0 ppb, a plateau of maximum recovery was reached. For these spiking concentrations, the mean recovery was 83.6 ± 6.3 with a CV of 7.6 ($n = 17$). These values are quite acceptable, keeping in mind that the muscle extract was not cleaned prior to EIA by HPLC or TLC.

Ethinylestradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol) is a synthetic derivative of the natural estrogenic hormone, estradiol-17β(1,3,5(10)-estratriene-3,17β-diol). Therefore, several recently developed methods are available for the measurement of the estradiol in the blood, urine and tissues of livestock (Tsujioka and Ito, 1992; Hendricks *et al.*, 1993; Scippo *et al.*, 1993, 1994; van Ginkel *et al.*, 1993; De Boever *et al.*, 1993; Batjoens *et al.*, 1994). These methods can be adapted for the determination of ethinylestradiol. Using specific HPLC–RIA techniques, Arts *et al.* (1991), established reference values for the concentrations of estradiol-17β, testosterone, and progesterone and their metabolites in treated and untreated calves. Using spiked urine samples, these authors showed that the recovery of estradiol-17β was around 104 ± 10.5% (CV = 10.13) for a dose range of 0.03–0.10 μg litre^{-1} (ppb). This value is slightly better than the overall mean values obtained in the present study for the dose range of 0.5–3.0 ppb. However, for the comparable dose range (i.e. a spiked level of up to 0.1 ppb), our recovery values of 100 ± 11.5% (CV = 11.5) were closer to the values obtained by Arts *et al.* (1991). Similarly, Daeseleire *et al.* (1991) described a GC–MS method for the detection of the illegal use of ethinylestradiol in cattle urine and described the recovery of radioactive ethinylestradiol in various steps of the method. The recovery of the steroid from the C_{18} column was 99% ($n = 5$), 73 ± 3.3% ($n = 5$) for the combination of the C_{18} and the NH_2 columns, and 95% ($n = 1$) for the HPLC column prior to the GC–MS analysis. All of these values compare fairly well with our system, keeping in mind that no HPLC steps were employed to clean the sample extracts in our study.

Heitzman (1992) reported data on the recovery of 2 μg kg^{-1} ethinylestradiol added to the kidney tissue and analyzed by GC–MS after preliminary extraction. The mean recovery level for five determinations was reported to be 1.26 μg kg^{-1} ethinylestradiol. This value was equivalent to a recovery percentage of 63 with a CV of 15.5%, which is lower than the recoveries obtained in the present study.

Once reasonable recoveries were obtained, studies on the screening of the sheep urine and muscle were started. A total of 146 urine samples (95 Australian and 51

Table 5. Effect of spiking sheep urine on the accuracy of ethinylestradiol determination using enzyme-linked immunosorbent assay (ELISA)

Spiking concentration (ppb)	*n*	Mean recovery (%)	Standard deviation	Coefficient of variation
0.5	3	108	37.2	34.4
1.0	3	92	29.7	32.3
1.5	3	79	11.6	14.7
1.7	3	77	5.9	7.7
2.0	3	76	6.5	8.6
2.5	3	87	17.4	20.1
3.0	3	79	16.5	21.0

Table 6. Effect of spiking chicken muscle on the accuracy of ethinylestradiol determination using enzyme-linked immunosorbent assay (ELISA)

Spiking concentration (ppb)	*n*	Mean recovery (%)	Standard deviation	Coefficient of variation
0.2	4	61	7.8	12.7
0.3	6	51	5.5	10.7
0.5	8	84	4.2	5.0
0.7	5	85	8.8	10.4
1.0	4	81	6.7	8.3
1.5	4	72	7.5	10.5

Arabian) were analyzed for ethinylestradiol. Of the 95 Australian samples analyzed, 14 samples showed activity over the detection limit of the method of 0.1 ppb and ranged from 0.15 to 0.93, while nothing could be detected in the rest. In the Arabian sample group, 13 samples out of 58 showed activity over the detection limit, while the rest (i.e. 45 samples) did not show any activity. The maximum permissible dose of ethinylestradiol in the biological samples according to EEC guidelines should be 'zero-tolerance', yet in practice this is translated into an action value of 2 ppb in urine and 0.5 ppb in muscle for screening and confirmation. In order to confirm the ELISA positives, all the urine samples, both from the Australian and the Arabian sheep were also analyzed by GC–MS and the data obtained were found to be negative. It was preferred to decide on the cut-off limit for the ELISA method, in order to cut down the false-negative results. Therefore, by adopting a cut-off limit of $0.50\,ng\,ml^{-1}$, the number of ELISA positives were reduced from 18.5% at $0.1\,ng\,ml^{-1}$ to 1.4%.

In addition to the urine samples taken from the Australian and the Arabian sheep, 87 muscle samples from chickens originating from France, Brazil, Denmark, Turkey and two local companies were also analyzed for residues of ethinylestradiol. Only 11 samples showed activity over the detection limit (0.1 ppb) of the method, with levels ranging from 0.10 to 0.3 ppb. As is quite clear from these concentrations, they are considered low compared with EC guidelines for the maximum allowed limits of 0.5 ppb (Meyer, personal communication). The same samples, however, were subjected to GC–MS analysis and were also found to be negative. Therefore, to reduce the number of false-positives obtained in the ELISA method, a cut-off limit of $0.30\,ng\,ml^{-1}$ would be desirable to implement. In such a case, the ELISA positives were reduced from 11 (12.6%) to 1 (1.2%) and clearly demonstrated that employing a cut-off value of 0.3 ppb would be reasonable to obtain low false-positive results.

The results of the present study clearly demonstrate that the competitive ELISA method used is useful for the screening of xenobiotic estrogenic compounds in particular, and that this methodology can be further extended and adapted for other anabolics, both natural and xenobiotic anabolic compounds, used in the livestock industry. However, any suspect sample(s) must be analyzed by the GC–MS method, as has been the case in the present study, to confirm the results and exclude the possibility of false-positive samples.

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