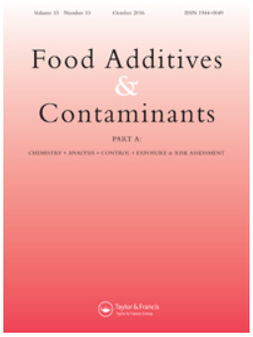

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Optimisation and validation of a new analytical method for the determination of four natural and synthetic hormones using LC-ESI-MS/MS

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ABSTRACT

A rapid liquid chromatographic-tandem mass spectrometric method was developed for the simultaneous determination of four natural and synthetic hormone residues (progesterone, testosterone, trenbolone acetate and zeranol) in animal tissue samples. Sample preparation was optimised to minimise time and solvent consumption. Meat samples were mechanically homogenised and digested in a procedure that gave similar recoveries to those enzymatically hydrolysed by *Helix pomatia*. Efficient extraction was achieved using acidified acetonitrile (1% acetic acid). Chromatographic conditions were optimised to minimise matrix effects. Analytes were separated using a C18 column with gradient elution using ammonium formate solution in methanol (MeOH)/water (1:9) and MeOH mobile phases. Finally, residues were qualitatively and quantitatively determined by electrospray ionisation tandem mass spectrometry in multiple reaction monitoring mode. Different parameters for LC-MS/MS (e.g., declustering potential and collision energy) were optimised using API 6500QT; all analytes were measured using positive-mode electrospray ionisation (ESI^+) except zeranol which was measured in negative mode (ESI^-). Due to LC-MS/MS signal enhancement/suppression, the determination of hormones was based on matrix-matched standard calculations. The method was validated for the four hormones on meat samples at different fortification levels and showed accepted performance criteria according to European Commission Decision 2002/657/EC. Decision limits and detection capabilities were estimated for all analytes.

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Liquid chromatography-mass spectrometry; homogenisation; mechanical digestion; method validation; hormone residues; meat

Introduction

Naturally occurring steroids such as progesterone and testosterone, as well as synthetic compounds such as zeranol that has high affinity for oestrogen receptors and trenbolone acetate that has affinity to androgen receptors (Jeong et al. 2010), are widely used as growth promoters in animal husbandry to increase bone density, muscular mass and improve the rate of protein deposition in livestock; others are used for clinical therapy and as contraceptives (Marchand et al. 2000; Shao et al. 2005; Yang et al. 2009).

An illegal or unsuitable use of these substances increases the risk of introducing harmful residues into the human food chain. Some hormones may have a carcinogenic effect like oestrogen, androgen and progesterone as it may lead to breast cancer, ovarian cancer and cell carcinoma in humans (Fu & Zhai 2010). Moreover, some synthetic growth promoters like zeranol and diethylstilbesterol have potential endocrine-disrupting properties (Schmidt et al. 2008).

For these reasons, European Commission Directive 96/22/EC prohibits in animal husbandry the administration of substances with a thyrostatic or a hormonal action (testosterone, progesterone, melengestrol acetate, zeranol, trenbolone acetate and 17β-estradiol). Moreover, Directive 96/23/EC divides all residues into groups A and B compounds, where group A comprises prohibited substances defined by Directive 96/22/EC and the banned compounds in Annex IV of the outdated Regulation (EEC) No. 2377/90 (which was replaced by Regulation 37/2010) for which no MRL could be established because of uncertainty about the risk, while group B contains substances with final and provisional MRLs.

Risk assessment of veterinary drugs involves assessing their toxicological and microbiological impact and identifying acceptable consumption levels of the compounds that should not be exceeded to limit the probability of human illness caused by the ingestion of livestock products containing residual veterinary drugs. From the point of view of risk management,

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MRLs are regarded as a monitoring tool for compliance, while the ADI is a decision point for human health impacts (Jeong et al. 2010). The European Union regulates the establishment procedure of these MRLs in foodstuffs of animal origin for both synthetic and natural hormones in Commission Regulation 37/2010. For reasons of ease, all pharmacologically active substances were listed alphabetically in one annex in two separate tables: one for allowed substances which were listed in annexes I–III of the outdated Regulation (EEC) No. 2377/90 and another for prohibited substances that were listed in annex IV to that regulation.

Commission Decision 2002/657/EC established minimum required performance limits (MRPLs) of analytical methods for substances which have no permitted limits. MRPL is the lowest content of an analyte in a sample that has to be detected and confirmed by an officially accredited laboratory (ISO 17025) using validated analytical methods. A LC or GC method coupled with mass spectrometric or infrared spectrometric detection is mandatory for confirmation of group A substances.

The analysis of these substances has been a challenging task because of their low levels and the complexity of biological matrices. Various techniques have been used throughout the previous years, such as TLC, which is simple and relatively inexpensive (Wortberg & Woller 1978) but is rarely used due to its low sensitivity and poor accuracy (Wang & Wang 2007). ELISA is suitable for the high-speed analysis for trace amounts of those compounds in a variety of samples (Hampl & Starka 1989), but it needs another confirmatory method and lacks the function of structural validation for the target analytes and cannot simultaneously determine multiresidues in complex matrices (Sokoll et al. 2004).

GC coupled to MS was used to determine steroid hormones, but unfortunately this technique generally requires pre-derivatisation processes for the analytes which would be tedious and time-consuming. Moreover, not all components can be easily derivatised (Barkatina et al. 2001; Dickson et al. 2003; Long et al. 2007; Trinh et al. 2011). However, further studies had shown the potential of microwave-assisted derivatisation (MAD) to enhance the process of derivatisation and reduce the time required (Bowden et al. 2009). HPLC combined with various ionisation techniques has been widely used for the determination of growth promoters, including reactive desorption electrospray ionisation (Huang et al. 2007), atmospheric pressure chemical ionisation and atmospheric pressure photoionisation (Leinonen et al. 2002), accurate mass time-of-flight (Nielen et al. 2007; Deng et al. 2011), and linear ion-trap mass spectrometry (Strahm et al. 2007). However, HPLC-electrospray ionisation tandem MS has been extensively utilised over the past decade due to its excellent sensitivity, high selectivity and specificity in many official methods (Blasco et al. 2007; Regal et al. 2009; Yang et al. 2009; Penning et al. 2010; Farke et al. 2011; Guedes-Alonso et al. 2013). Moreover, it gives a significant signal increase compared with atmospheric pressure chemical ionisation and atmospheric pressure photo-ionisation (Yunin et al. 2014).

The analytical process includes four main steps: sample collection, sample preparation, final analysis and evaluation of the results. However, the sample preparation step is a vital part of the analytical process and effective sample preparation is essential for achieving reliable results and maintaining instrument performance (Kinsella et al. 2009). Therefore, sample processing should ensure a fully representative test portion, i.e., it should consist of whole units of the commodity or portions removed from large units. Tissue preparation techniques can be categorised into mechanical, digestion or extraction instruments. Disruption of tissue may be achieved through enzymatic digestion with proteolytic enzymes such as subtilisin A (Daeseleire et al. 1991) or using a disruption apparatus including probe blenders, ultrasonic probes and stomachers. Extraction efficiency for them may be very good; however, the highest extraction for antibiotic residues from incurred tissue was achieved efficiently using probe blending (Kinsella et al. 2009).

Due to the extensive metabolism in animals after administration of drugs, the target residue for analysis is not always the parent drug but can be a metabolite. Free parent and metabolite residues can be extracted by organic solvents, H_2O or aqueous buffers. However, many residues may be present in conjugated forms (glucuronides or sulphates) and require liberation through enzymatic or chemical hydrolysis before extraction. Enzymatic hydrolysis guarantees milder conditions than acid or alkaline hydrolysis, and may be achieved using *Helix pomatia* juice (a mixture of β-glucuronidase and arylsulphatase) or *Escherichia coli* β-glucuronidase (Kinsella et al. 2009; Yang et al. 2009; Yunin et al. 2014). Despite the extensive use of *H. pomatia*, this can cause a conversion of steroids into other forms because it possesses oxidoreductase enzyme activity capable of converting the steroid 3-ol group to a 3-oxo group through oxidation (Vanluchene et al. 1982).

Several extraction methods have been introduced throughout the previous years, but lately the well-known QuEChERS methodology, which was developed

to determine different pesticide residues in food, was utilised in veterinary drug residues in several studies (Stubbings & Bigwood 2009; Aguilera-Luiz et al. 2012; Lopes et al. 2012).

The main aim of this investigation was to develop and establish a rapid analytical method based on the QuEChERS method to determine hormones in meat tissue. LC-MS/MS was used (in both positive- and negative-ionisation modes) to ensure high sensitivity, selectivity and specificity with low detection capability without a need either for a prior enzymatic digestion step or clean-up steps to prevent analyte loss and to ensure saving time. This fulfils the Egyptian Central Management for Veterinary Quarantine requirement for reporting result within 24 h from receiving a sample.

Material and methods

Materials and reagents

Organic solvents used such as acetonitrile (ACN) and methanol (MeOH) were all HPLC grade and purchased from Merck (Darmstadt, Germany). Acetic acid (glacial), 100%, was also purchased from Merck. Formic acid, 98–100%, and ammonia solution, 33%, were purchased from Honeywell Riedelx-de Haen (Seelze, Germany). Deionised water was generated by a Millipore water purification system (Millipore, Bedford, MA, USA). Sodium sulphate (anhydrous), fine powder, sulphatase and β-glucuronidase from *H. pomatia* were purchased from Sigma-Aldrich (Steinheim, Germany). Active ingredients reference standard of progesterone, testosterone, trenbolone acetate and zeranol were purchased from Dr. Ehrenstorfer (Augsburg, Germany); purities were > 95%.

Preparation of hormone standards

Individual stock standard solutions (100 μg ml^{-1}) for all analysed hormones were prepared in MeOH and kept at –20 ± 2°C. Mixtures of working standard solutions (500 and 100 ng ml^{-1}) of the four hormones were prepared in ACN. Calibration mixtures solutions were prepared freshly in ACN at concentration levels of 1.0, 2.0, 4.0, 6.0 and 10.0 ng ml^{-1}.

LC mobile phase buffer

Ammonium formate 10 mM was prepared and adjusted to pH 4 by a pH meter (Thermo Electron Orion Star pH Bench Top) using formic acid and ammonia solution in MeOH–water (1:9).

Sample preparation

Homogenised sample (5.0 g) was weighed into a 50 ml polypropylene centrifuge tube, and 15 ml of acetonitrile (1% acetic acid) and 5.0 g of sodium sulphate were added. The sample was thoroughly homogenised with a rod homogeniser (Ultra-Turrax T25 Digital, Processing Equipment) for 1 min. The homogenate was then centrifuged at 5000 rpm for 10 min at 4°C, and the supernatant was filtered through acrodisc syringe filters (nylon membrane, diameter 25 mm, pore size 0.45 μm) and transferred to an injection vial. In the case of spiking sample, appropriate volumes were added and mixed with test portions at least 30 min before addition of the extracting solvent.

LC-MS/MS analysis

Separation was performed with an Agilent 1260 Series HPLC instrument coupled to an API 6500 Qtrap MS/MS from AB Sciex with electrospray ionisation (ESI) interface, using an XDB-C18 Eclipse-Plus column (4.6 × 150 mm, 5 μm particle sizes; Agilent, Richardson, TX, USA). The injection volume was 5 μl. The mobile phase A was 10 mM ammonium formate solution pH 4 in MeOH–water (1:9) and mobile phase B was MeOH. The 350 μl min^{-1} flow rate gradient mixing program (Start 100% A, 15–21 min 5% A and 35–38 min 100% A) was optimised to improve chromatographic separation and reduce the matrix effect as much as possible in 38 min run time. The ESI source was used in positive and negative modes; the N_2 nebuliser, curtain and other gas settings were optimised according to recommendations made by the manufacturer; the source temperature was 400°C and the ion spray potential was 5500 V in positive and –4500 V in negative modes. Declustering potential (DP) and collision energy (CE) were optimised via direct infusion using a Harvard Apparatus syringe pump by introducing individual hormones solutions into the MS instrument to allow optimisation of the MS/MS conditions, using concentrations that ranged between 0.01 and 1 μg ml^{-1}, starting at the lower concentration and passing through the higher ones depending on analyte sensitivity. Calibration curves were obtained by plotting the response of the analyte from externally standardised five calibration points prepared in ACN versus nominal concentrations added of the analyte.

Results and discussion

ESI-MS/MS performance

LC-MS/MS with ESI source and MRM mode provided a highly selective and sensitive method for the

determination of these hormones. According to the physicochemical characteristics of the target, three of the target analytes were ionised to $(M + H)^+$ forms in positive mode (progesterone, testosterone and trenbolone acetate) and one to the $(M - H)^-$ form in negative mode (zeranol). As shown in Table 1, each hormone has different DP and CE settings to get the best sensitivity; these parameters were collected to build up the acquisition method for the four hormones. MRMs shown in Table 1 are in accordance with those reported by Vanhaecke et al. (2011), Yang et al. (2009), Kaklamanos et al. (2009) and Shao et al. (2005).

LC-mobile phase performance

The buffer solution is necessary to improve ionisation and chromatographic separation of analytes related to their acidic dissociation constant. An ammonium formate buffer at 10 mM and pH 4 ensured a good sensitivity with enough separation for the four hormones which have similar polarities with octanol–water partition coefficients (log K_{ow}) of 3.3, 3.9, 3.77 and 3.6 for testosterone, progesterone, trenbolone acetate and zeranol respectively (Lai et al. 2000). Effective chromatographic separation with a long run time was required to separate matrix components in the hormone retention time window as a result of injecting the extracted sample without a clean-up step to prevent the loss of the target analytes during this step (Ferreira et al. 2012). The high sensitivity of the four hormones is illustrated in Figure 1, which shows (a) chromatograms for the most abundant transition of the four hormones for a meat samples spiked at 2.0 µg kg^{-1} concentration level and (b) a blank meat sample.

Matrix effect

The matrix effect was studied using ESI in both positive and negative modes to compensate for the deviation effect in the signal after matching the standard with the matrix. One significant drawback when ESI is used as an ionisation technique in MS is the matrix effect, which affects the ionisation efficiency of analytes, leading to suppression or enhancement of the signal depending on the analyte/matrix combination. Obviously, this affects quantification, unless matrix effects are minimised or compensated (Aguilera-Luiz et al. 2012). After calibrating the LC instrument using standards prepared in ACN, a matrix-matched standard was prepared by fortifying the final extract of a blank sample with a known concentration ($C_{mtrx-exp}$) and injecting it into the LC to be quantified in comparison with the solvent standard ($C_{mtrx-found}$). Equation (1) was then used to calculate the final result of the samples (C_s) according to the measured suppression or enhancement effect as follows:

$$Cs = Ci \times \frac{Vtot}{W} \times \frac{Cmtx - \exp}{Cmtx - found} \quad (1)$$

where C_i is the found concentration of sample in an injection (µg kg^{-1}); V_{tot} is the total volume of extraction (ml); W is the sample weight (g); $C_{mtrx-exp}$ is the expected concentration of matrix-matched standard; and $C_{mtrx-found}$ is the found concentration of matrix-matched standard.

Table 1. Molecular formula, precursor ion, product ions, declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP).

Compound	Molecular formula	Precursor ion (*m/z*)	Product ions (*m/z*)	DP (V)	CE (V)	EP (V)	CXP (V)
Progesterone	$C_{21}H_{30}O_2$	315	97	111	55	10	12
			109	116	31	10	12
Testosterone	$C_{19}H_{28}O_2$	289	97	111	53	10	12
			109	106	31	10	12
Trenbolone acetate	$C_{20}H_{24}O_3$	313	253	106	31	10	14
			91	96	69	10	10
Zeranol	$C_{18}H_{26}O_5$	321	277	−125	−30	−10	−15
			161	−120	−38	−10	−9

Optimisation of sample preparations

The whole laboratory sample (in most cases 1–2 kg) needs to be comminuted to ensure that the sample is homogeneous enough so that subsampling variability is acceptable. If this is not achievable, the use of larger test portions should be considered (SANCO/12571/2013). The following studies were performed to optimise the efficiency of the homogenisation process.

Degree of grinding significance (homogenisation)

Three different techniques were applied to investigate the effect of grinding degree on hormones recovery from incurred meat samples. Five meat samples incurred with progesterone were homogenised, and three replicates were analysed from each sample using the following techniques:

- Technique A: Meat samples were homogenised using a meat mincer with a mincer plate with hole size of 4.3 mm.
- Technique B: Grinding with the same mincer plate followed by freeze-drying where 25 g of each homogenised sample were weighted and lyophilised at −85°C (Top-press type vacuum

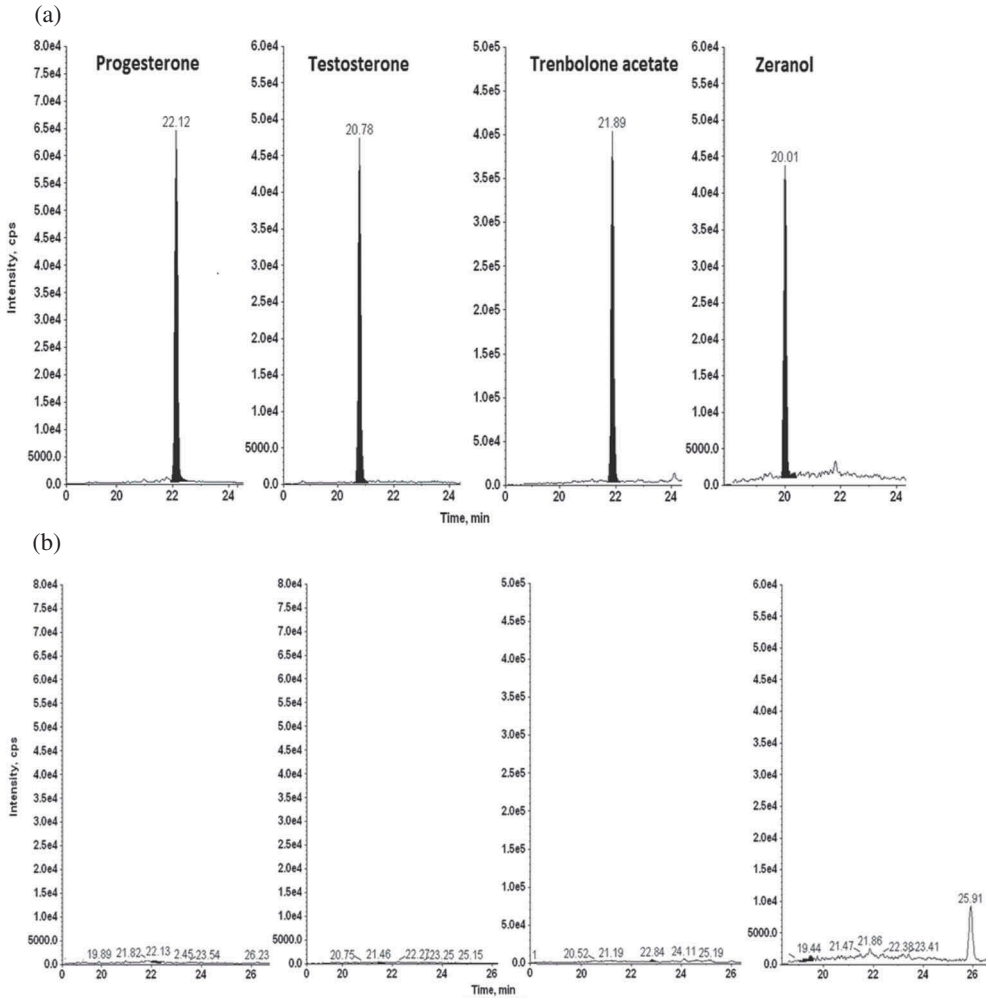

Figure 1. (a) Chromatograms for the most abundant transition of the four hormones for meat samples spiked at a 2.0 μg kg^{-1} concentration level and (b) a blank meat sample.

freeze-dryer, Ilshin, model FD 8512). Each whole lyophilised sample was weighed, and an equivalent weight of the fresh sample was taken and continued through the same steps.

- Technique C: Two consecutive grinding processes using a mincer plate with a hole size of 3 mm. These samples were then extracted with acidified ACN, analysed and quantified.

The effect of different homogenisation techniques on the extraction of hormones showed an overall decrease in the RSD for technique C over techniques A and B and this trend was more prominent in higher concentrations. For example, RSD was 78% for the highly concentrated sample, which is then decreased in technique C to 5% for the same sample, as shown in Table 2.

The perforations of grinding plates vary from 1 to 13 mm. Meat is compressed by a rotating feeding auger, pushed through the cutting system and extruded through holes in the grinding plates after being cut by revolving star knives. Simple equipment has only one star knife and a grinder plate, but normally a series of plates and rotary knives are used. The degree of mincing is determined by the size of the holes in the last grinding plate (Heinz & Hautzinger 2007). Therefore, homogenisation was not sufficient in technique A giving a high RSD, where a mincer plate with holes sized 4.3 mm was used. In technique B, adding the extra step of freeze-drying after grinding with a mincer plate with holes sized 4.3 mm improves homogeneity. As described in some research papers, freeze drying was preferred because lyophilisation permits grinding of the sample to a fine powder, which results in increased interaction between sample and solvent, better extraction yield and more efficient deconjugation (Marchand et al. 2000). However, mincing twice through a plate with smaller holes (3 mm) in technique C results in a superfine

Table 2. Mean results (μg kg^{-1}), standard deviation (SD) and relative standard deviation (RSD) for different techniques of homogenisation.

Grinding technique	Replicates	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
A: Using a mincer plate with a hole size of 4.3 mm	Mean	2.54	5.33	0.90	26.02	11.62
	SD	0.42	2.89	0.10	20.39	3.68
	RSD	16%	54%	11%	78%	32%
B: Using a mincer plate with a hole size of 4.3 mm followed by a freeze-dryer	Mean	2.83	6.88	1.20	12.41	8.28
	SD	0.37	1.23	0.13	1.62	1.31
	RSD	13%	18%	11%	13%	16%
C: Using a mincer plate with a hole size of 3 mm, twice in succession	Mean	1.73	4.33	0.86	37.82	5.38
	SD	0.19	0.13	0.03	1.74	0.34
	RSD	11%	3%	3%	5%	6%

grinding of the samples ensuring a sufficient homogeneity of the whole sample without the need to add an extra step like freeze-drying, thus saving time.

Significance of enzymatic hydrolysis

After achieving sufficient sample homogenisation, the effect of enzyme addition was studied; two of the previously incurred samples were chosen for this comparison enzyme test.

In this comparison, the effect of the enzymatic hydrolysis on the conjugated portion of the hormone (progesterone) in meat was studied by weighing three replicates from each of the two homogenised incurred samples, then fortifying the other three hormones (testosterone, trenbolone acetate and zeranol) externally at 6.0 μg kg^{-1} at least 30 min prior to addition of the enzymes. Since the four hormones have the same conjugation behaviour, variation in the recovery of progesterone between different techniques will reflect the behaviour of the remaining hormones that had been added externally. Furthermore, each replicate underwent a different test: the first was performed without enzyme addition; the second was carried out by adding 100 μl of β-glucuronidase enzyme from *H. pomatia* after adjusting the pH to 5.0 with phosphate buffer then vortexing for 1 min and incubating overnight at 45°C. While in the third, 100 μl of sulphatase enzyme were added together with 100 μl of the β-glucuronidase enzyme to enhance its activity, after adjusting the pH to 5.5 with phosphate buffer and incubating overnight at 45°C. These replicates were then extracted with acidified ACN, analysed and quantified.

Results in Table 3 show that the difference between the three procedures was minimal for the four hormones. For example, the difference in concentrations for sample A between the two procedures of adding glucuronidase and sulphatase and that without the enzyme step was low and can be neglected.

The necessity of enzymatic hydrolysis of hormone glucuronides or sulphates in tissues is a controversial issue (Noppe et al. 2008). In some references this procedure was required, while others assumed that the conjugated proportion could be omitted (Hartmann et al. 1997; Yang et al. 2009), since the percentage of cleavable conjugated testosterone in muscle tissue was lower than 20% and that of conjugated 17β-estradiol was lower than 5% (Hoffman & Rattenberger 1977; Marchand et al. 2000), of conjugated estrogens it ranged from 3% to 5% (Dunn et al. 1977) and of conjugated progesterone it was about 5% (Estergreen et al. 1977).

Therefore, we can conclude from the results of both homogenisation and enzymatic hydrolysis studies that superfine grinding with the mincer plate with a hole size of 3 mm two times increases the recovery so it is very near those with enzyme addition, taking in consideration that all analytical steps were kept the same. This strongly suggests that the mechanical digestion grinding technique assists in cell lysis and thus deconjugation of hormones can be achieved, which indicates that the developed extraction conditions can recover hormones quantitatively. It is clear that the use of glucuronidase and sulphatase enzymes has no significant effect. Moreover, it takes more time and consumes more reagents.

Sample extraction

The use of an appropriate organic solvent is required to enrich the target compounds, making analytes more suitable for separation and detection. The presence of analytes in tissues and their interactions are important considerations when deciding how best to develop a method for analysis with highly efficient extraction. During metabolism, steroids generally become more hydrophilic either by reduction, further hydroxylation or esterification with glucuronic or sulphuric acid (Makin et al. 2010), so a solvent with suitable polarity must totally disrupt the binding of the steroid to protein and must extract the steroid of interest quantitatively and leave behind non-specific interfering substances. We studied the efficiency of extraction

Table 3. Enzyme test results (μg kg^{-1}).

	Without enzyme		With glucuronidase enzyme		With glucuronidase plus sulphatase enzymes	
	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
Progesterone	6.45	5.35	6.70	4.74	6.75	5.04
Testosterone	5.14	5.24	5.18	5.54	4.96	5.09
Trenbolone acetate	5.67	5.59	5.71	6.00	5.40	5.26
Zeranol	5.51	5.48	5.21	5.42	5.39	5.52

through a comparison between four polar solvents: ACN, ACN with 1% acetic acid, MeOH and MeOH with 1% acetic acid. Twelve replicates of a sample with incurred progesterone were weighed out and fortified by a spiking standard solution containing the other three targeted compounds before addition of the extracting solvent. Each group of three replicates was extracted with a different solvent from the four studied solvents. The fortification level was 6.0 μg kg^{-1}. The 12 samples were then taken through our standard procedure.

Results shown in Table 4 clearly demonstrate that ACN, and ACN with 1% acetic acid, gave very similar recoveries (extraction efficiency) for the four compounds, and higher than MeOH and MeOH containing 1% acetic acid. Extraction efficiency for the three fortified hormones reflect the percentage of the actual concentration of a hormone recovered during the analytical procedure. In the case of progesterone which is incurred in the sample, extraction efficiency was calculated by assuming the highest mean concentration as 100% efficiency for ease of comparison. The matrix effect was calculated for all analytes by calculating the percentage of the found concentration for a matrix-matched standard point against its expected value.

Since recoveries between ACN and ACN (1% acetic acid) were very close, it is preferable to choose the acidified ACN as adding acetic acid to ACN in the extraction and the extra advantage of increased polarity will give future flexibility to include more polar hormones in the method without adding extra steps.

The same point of view is supported by other studies which reported that using acidified ACN with 1% acetic acid was the best extracting solvent (Stubbings & Bigwood 2009) which can improve the extraction of certain compounds (Anastassiades et al. 2003). Other method development activities indicated that acetonitrile was a more selective extraction solvent (Blasco et al. 2007).

On the other hand, some methods used MeOH as their extracting solvent (Yang et al. 2009). However, it may decrease the extraction of some hormones and gives a dirty extract yield because MeOH can extract many matrix compounds (Lopes et al. 2012)

Method validation

The developed analytical method was validated according to the performance criteria specified in Decision 2002/657/EC for quantitative confirmation. Linearity was evaluated using calibration points at five concentration levels ranging from 1.0 to 10.0 ng ml^{-1}. According to the criteria of European Union regulations in Decision 2002/657/EC, fortification with the analytes should be done at concentrations levels equivalent to 1, 1.5 and 2 times the MRPL, which was set as 1.0 μg kg^{-1}. Therefore, evaluation of intraday precision (repeatability) and interday precision (reproducibility) was studied by spiking blank meat samples at three concentration levels (1.0, 1.5, 2.0 μg kg^{-1}) during 3 consecutive days. Recovery was performed at the same levels. Finally, estimation of the uncertainty (U) was carried out using data derived from the validation of the method. The validation protocol involves performance criteria of the analytical methods as follows.

Specificity

Specificity was studied by observing any interference (signals, peaks or ion traces) in the region of interest where the target analyte is expected to elute by analysing some representatives blank samples ($n \geq 20$). The absence of a signal, above a signal-to-noise ratio of 3, at the retention times of the target compounds showed that the method is free of interference at the compound-specific retention time window and no interference between individual compounds as shown in Figure 1(b). In some cases, minor matrix components were observed, but these were chromatographically resolved from the compounds of interest and did not hinder quantification or identification. Therefore, the newly developed method was found to be specific.

Selectivity

Tandem mass spectrometry itself as a detection technique offers a high degree of selectivity. A system of identification points (IPs) was used to interpret the data, according to the criteria of Directive 96/23/EC.

Table 4. Extraction solvent test.

	Progesterone				Testosterone				Trenbolone acetate				Zeranol			
Solvent	ACN	Acidified ACN	MeOH	Acidified MeOH	ACN	Acidified ACN	MeOH	Acidified MeOH	ACN	Acidified ACN	MeOH	Acidified MeOH	ACN	Acidified ACN	MeOH	Acidified MeOH
Matrix effect (%)	53.0	49.5	49.5	48.5	95.0	90.0	102.0	99.0	84.0	80.0	54.5	40.7	84.5	85.0	104.5	110.0
Mean concentration (μg kg^{-1}) (n = 3)	12.28	12.34	7.78	6.07	5.98	6.22	4.44	4.13	6.16	5.98	3.40	3.48	5.39	5.57	3.85	3.58
Extraction efficiency (%)	99.5	100	63.0	49.2	99.8	103.7	74.0	68.9	102.8	99.6	56.7	58.1	89.9	92.7	64.1	59.7

ACN: acetonitrile; MeOH: methanol.

A minimum of four IPs is required: precursor (1 IP) and product ions (1.5 IP/ion) of each analyte, as presented in Table 1. As a result, the minimum required amount of IPs, set at four, was achieved for every compound and identification of the four hormones extracted from meat tissue was unambiguous.

Calibration curves

The linearity of the chromatographic response was evaluated with five calibration points prepared in a solvent, i.e., 1.0, 2.0, 4.0, 6.0 and 10.0 ng ml^{-1}, which covers the range for the recommended concentration of compounds. Good linearity was obtained with correlation coefficients (R^2) ≥ 0.999.

Recovery

According to the criteria of Decision 2002/657/EC, if no certified reference material is available, recoveries have to be determined by spiking 18 identical blank samples with target compounds at levels of 1.0, 1.5 and 2.0 μg kg^{-1} divided over six sets (n = 6 each level) on 1 day. Recoveries for the fortified samples are reported in Table 5. Overall recoveries for the four hormones at a level of 1.0 ng ml^{-1} were between 71% and 85%, at a level of 1.5 ng ml^{-1} were between 80% and 101%, and at a level of 2.0 ng ml^{-1} were between 72% and 93%. All calculated mean recoveries fulfilled the criteria put forward in Decision 2002/657/EC, stating that an analyte mass fraction below 1.0 ng ml^{-1} should comply with a mean recovery range of 50–120%, while a mass fraction between 1.0 and 10.0 ng ml^{-1} requires a mean recovery range of 70–110%.

Precision

Repeatability and within-laboratory reproducibility were determined by fortifying a set of six blank samples with the target compounds at each of the three levels (1.0, 1.5 and 2.0 μg kg^{-1}) and repeated on two other occasions at the same conditions to calculate the overall repeatability (RSD_r) for all hormones. All measurements were found to be below 15% for the three fortified levels and repeated on two other occasions with different operators. The within-laboratory reproducibility (RSD_R) was found to be below 18%, as reported in Table 5.

Decision limits (CCα)

According to Decision 2002/657/EC, a CCα was used instead of the LOD. For prohibited substances, an α-

value equal to 1% is required. In the case of substances for which no permitted limit has been decided, CCα can be established by analysing at least 20 blank materials per matrix to calculate the signal-to-noise ratio during the time window in which the analyte is expected. The minimum concentration (at which the analyte can be reliably detected) was established by comparing measured signals from extracted spiked samples of known low concentrations of analyte with those of blank samples. A signal-to-noise ratio of 3 is considered acceptable for estimating the CCα (or decision limit). Based on these criteria, the decision limit was 0.3 μg kg^{-1} for progesterone and trenbolone acetate, 0.9 μg kg^{-1} for testosterone, and 1.0 μg kg^{-1} for zeranol.

Detection capability (CCβ)

According to Decision 2002/657/EC, CCβ can be established by analysing at least 20 blank samples fortified with the analytes at the decision limit. The decision limit plus 1.64 times the standard deviation (SD) of the measured content equals the detection capability. Based on this equation (CCβ = CCα + 1.64 SD), the decision capabilities were 0.37, 0.34, 1.02 and 1.18 μg kg^{-1} for progesterone and trenbolone acetate, testosterone and zeranol respectively.

Stability of the analytes in solution

Fresh stock solutions of analytes were prepared and diluted to a concentration of 10 ng ml^{-1}. Appropriate volumes were then dispensed into vials, labelled and stored at four different storing conditions (–20, 4, 20°C in the dark and 20°C in the light) according to the scheme provided in Decision 2002/657/EC. Stability of the compounds was followed up by injecting the aliquots for 15 weeks. The aliquots were identified and quantified for this period, and the concentration of the analytes was calculated for every injection time in each aliquot by using freshly prepared solution at the time of analysis as 100%:

$$\text{Analyte Remaining } (\%) = \text{Ci} \times 100/C_{\text{fresh}}$$

where C_i is the concentration at time point; and C_{fresh} is the concentration of the fresh solution.

Results showed the high stability of compounds throughout the first 5 weeks at the four stored temperature conditions. However, at week 6, degradation was observed for trenbolone acetate stored at RT (20°C) in the light, with 84% of the analyte remaining. On week 15 degradation starts for trenbolone acetate stored under the other conditions with 79.5%, 79% and 71% remaining for the –20, 4 and 20°C (dark) storage conditions respectively. The other three compounds (progesterone, testosterone and zeranol) were still stable after 15 weeks.

Stability of the analytes in the matrix

Stability of the analytes was tested according to Decision 2002/657/EC criteria. A sample with incurred progesterone was analysed, and the concentration of progesterone was determined while the sample is still fresh. The tissue was stored at –20°C. Further aliquots of the incurred sample were taken and analysed in three replicates after 1, 2, 4 and 20 weeks. The results were close to each other, indicating a high stability of the analyte in the sample stored at –20°C.

Measurement uncertainty

Uncertainty was calculated for the worst case between the four compounds according to the EURACHEM guidelines (EURACHEM/CITAC Guide 2012). The most important variables contributing to the uncertainty of measurement were the reproducibility and bias of the analytical procedure. The possible uncertainties from the preparation of the stock solutions (weighing, volumetric flask, chemical purity of the analyte standards and solvents) and micropipette use were not significant. The calculated expanded uncertainty was ±38.8%.

Table 5. Mean recoveries, repeatability and within-laboratory reproducibility for the determination of the four hormones fortified at 1.0, 1.5 and 2.0 μg kg^{-1}.

Compound	MRPL (μg kg^{-1})	Repeatability, CV (%)[a]	Within-laboratory reproducibility, CV (%)[b]	Recovery (%)[c]
Progesterone	1.0	10.9	18.1	85.0
	1.5	11.9	7.11	98.2
	2.0	12.9	10.9	92.7
Testosterone	1.0	12.2	17.8	73.2
	1.5	11.1	8.1	100.5
	2.0	4.5	14.2	85.2
Trenbolone acetate	1.0	10.0	7.4	76.0
	1.5	12.4	11.7	84.5
	2.0	14.8	8.5	72.0
Zeranol	1.0	12.1	10.9	71.0
	1.5	11.2	6.4	79.7
	2.0	9.1	11.8	84.8

Notes: [a]RSD_r for 18 replicates for 3 days at each level.
[b]RSD_R for 18 replicates by three different operators at each level.
[c]Six replicates at each level.

Analysis of real samples

The developed method was applied to the analysis of more than 200 real samples collected from different markets in Egypt. To ensure the reliability of the results when the proposed method is applied, an internal quality control was used. Among the analysed samples progesterone and testosterone were detected at different concentrations levels, some below the LOQ. Progesterone and testosterone were found to be above the LOQ in several samples, ranging from 2.0 to 35 and from 1.2 to 5.0 μg kg^{-1} respectively. The results were not high enough to conclude that their levels were compliant or non-compliant because food-producing animals naturally secrete these hormones and there are no MRLs for progesterone and testosterone in animal-derived food. Besides, the concentration of naturally occurring steroids in food products of animal origin depends on the type of animal product, the species and its gender, the feed, castration, gestation, disease, age, medication and physiological condition. However, when finding a high concentration, we report it to the appropriate authority for them to take suitable action.

Conclusions

A specific, sensitive and reliable LC-MS/MS method was developed that simultaneously identifies and quantifies four hormones (trenbolone acetate and zeranol are synthetic, progesterone and testosterone are natural) in meat tissue. These hormones only were included because they are specified by national regulations for the imported meat. The proposed homogenisation technique for the animal tissue samples is novel. The method is based on a modified QuEChERS method approach, and it can be considered as rapid since it has no enzymatic hydrolysis or clean-up steps, achieving the requirements of the Egyptian Central Management for Veterinary Quarantine for reporting results within 24 h of receiving a sample. Consumption of solvents is low due to the simple analytical procedure. The method was fully validated according to the criteria laid down by Decision 2002/657/EC. Data obtained showed satisfactory precision and trueness. All calculated CCα and CCβ values ranged from 0.3 to 1.0 μg kg^{-1}, which meets the low levels recommended by the European Commission. The inclusion of new hormones is under investigation. Furthermore, the method has been successfully applied in routine analysis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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