



A Fast Quantitative Multi-analyte Method for Growth Promoters in Bovine Meat Using Bead-Disruption, 96-well SPE Clean-up and Narrow-Bore UHPLC-MS/MS Analysis

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Abstract

A new method for detecting low levels of growth promoters in bovine meat was developed with the following goal: easy, fast and sensitive analysis of a wide range of compounds, with reduced consumption of chemicals and disposables. Several classes of growth promoters were included, i.e. resorcylic acid lactones (RALs) and steroids, the latter including corticosteroids and gestagens. For sample treatment, 0.5 g of homogenised bovine meat was simultaneously disrupted and extracted in a bead-ruptor machine. The organic extraction solvent was further processed by solid-phase extraction (SPE) clean-up using 96-Well Oasis® HLB Plates. Six SPE washing steps were applied to remove matrix compounds after which the growth promoters were eluted and analysed using UHPLC-MS/MS. To achieve lower detection levels and to reduce LC-solvent consumption, a narrow-bore column with an internal diameter of 1 mm was used, instead of the conventional 2.1 mm. During analysis, the mass spectrometer was operated in negative and positive ionisation mode (ion switching). The newly developed method was validated according to the Commission Decision 2002/657. The results demonstrate that the method meets the criteria as established in this Commission Decision. The precision of the method for exogenous steroids varies between 85 and 115%, the CC α for the compounds ranges from 0.1–0.9 $\mu\text{g kg}^{-1}$ and the expanded measurement uncertainty was lower than 36%. Compared to our current in-house methods with analysis times of 2 days for a maximum of 24 samples, the new method offers improved sample throughput (96 samples in less than 24 h) and lower detection limits.

Keywords Bovine meat · Growth promoters · Bead-disruption · UHPLC-MS/MS · Multi-analyte analysis · 96-wells SPE

Introduction

In Europe, the use of growth promoters in animal production is strictly forbidden (Council Directive 96, 1996; Directive C, 1996). A zero-tolerance policy is applied for these substances, which means in practice that the analysis has to be performed at the lowest concentration possible (Committee ES, 2012). There are several classes of growth promoters including stilbenes, steroids and beta-agonists. There is ongoing debate on

the risks for humans when steroids are used as growth promoters (Committee ES, 2012). Endocrine disruption is regarded as one of the main health issues related to the use of hormonal growth promoters. Therefore, it is expected that the EU ban on using growth promoters for fattening will not be lifted in the near future which makes it necessary to improve current control methods so that a larger number of compounds can be detected at low concentrations in food (Koster et al., 2011). Routine analysis of meat samples is mainly carried out using methods based on LC-MS/MS or GC-MS/MS. However, in order to comply with the latest views on acceptable concentrations described in the EFSA opinion (Panel, 2013), a decrease of the limits of detection of current analytical methods is needed.

Current routine methods of analysis of growth promoters in meat typically consist of processing 2–5-g portions of meat by either a digestion step with the enzyme subtilisin (Blasco et al., 2007; Daeseleire et al., 1991), an ultrasonic disruptor (Kinsella et al., 2009) and lyophilisation/freeze drying,

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followed by supercritical fluid extraction (Marchand et al., 2000; Stolker et al., 1998), defatting (Barkatina et al., 2001; Stolker & Brinkman, 2005), several LLE (liquid-liquid extraction) steps or a few SPE (solid-phase extraction) steps (Bovee et al., 2009; Deceuninck et al., 2013), and sometimes even HPLC fractionation (van Ginkel et al., 1992), followed by mass spectrometric detection. Some methods include a deconjugation step (Malone et al., 2009), although there is a negligible amount of phase II conjugates present in meat (Maume et al., 2001). Often, significant background signals due to interfering compounds such as proteins, salts and fatty acids (Antignac et al., 2005) are observed. Typically, processing 24 samples takes approximately two working days with our current methods, using large amounts of chemicals and organic solvents. Recent literature demonstrates that sample clean-up can in principle be more efficient. For example, smaller aliquot of samples of meat can be processed to reduce matrix effects, so less matrix compounds will be introduced during the final analysis (Stahnke et al., 2012). Also, the use of 96-wells format increase the efficiency of sample clean-up (O'Brien et al., 2009; Pinel et al., 2008). In a recent paper of Luo et al. (Luo et al., 2016), the use of hollow-fibre liquid-phase microextraction (HF-LPME) was combined in a 96-wells format to extract 16 steroids from urine, milk and plasma in only a few hours. Another fast method is based on a modified Quick Easy Cheap Effective Rugged Safe (QuEChERS) approach (Attalah et al., 2016) to extract four steroids from meat. This is a fast method and applicable in many laboratories, although sensitivity and number of compounds need to be improved to widen the applicability of the method. Besides optimising sample clean-up procedures, efficiency can also be increased by the application of full-scan accurate mass measurement (HRAM) instead of triple-quad measurements as described by Lynn Vanhaecke et al. (Vanhaecke et al., 2013). In this study, it is demonstrated that the performance of HRAM rivals the performance of triple-quad measurements in combination with a traditional clean-up. The use of HRAM opens the door to the detection of an unlimited number of compounds and it can pursue the perspective of untargeted strategies and retrospective analyses.

In spite of the advances made during the past years, the current described methods for the analysis of steroids in samples of muscle tissue lack the sensitivity which is necessary to comply with the latest EFSA opinion (Panel, 2013). Also, they are relatively time consuming, detect single classes of compounds and are environmental unfriendly. The objective of this study was to redesign our current methods in such a way that the sensitivity is increased, the workload is reduced and the use of chemicals is decreased by miniaturisation. Although not the explicit goal of this study, the principles as described by Gałuszka et al. (Gałuszka et al., 2013) to make analytical methods 'greener' were adopted by implementing miniaturisation, resulting in reduction of the amount of chemicals. The

developed method was validated by measuring the precision, linearity, decision limit ($CC\alpha$), detection capability ($CC\beta$) and measurement uncertainty according to the guidelines established by the European Union (Commission E, 2002). Method optimisation and validation are both described.

Experimental

Solvents and Reagents

Methanol (MeOH), water, acetonitrile (ACN) and formic acid (FA) (all ULC/MS grade) were obtained from Actua-All Chemicals (Oss, The Netherlands); ammonia (25%), acetic acid (100%) and ethanol from Merck Millipore (Darmstadt, Germany); and ammonium formate from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least $18.2 \text{ M}\Omega \text{ cm}^{-1}$ (Merck Millipore).

Standards

ADD (androsta-1,4-diene-3,17-dione), betamethasone, 17 β -boldenone, clobetasol, isoflupredone, medroxyprogesterone-acetate, methylboldenone, 17 α -methyltestosterone, 17 α -nortestosterone, 17 β -nortestosterone, 17 α -testosterone, 17 β -testosterone and 17 β -trenbolone were obtained from Steraloids (Newport, USA); chlormadinone-acetate, dexamethasone, flumethasone, megestrolacetate, melengestrolacetate, prednisolone, methylprednisolone, triamcinolone-acetonide, α -zearalenol, β -zearalenol, zearalenone, α -zeranone and β -zeranone from Sigma-Aldrich (St. Louis, USA); α -trenbolone and 16 β -OH-stanozolol from NMI (Australia, Sydney); 17 α -boldenone from the EURL (Wageningen, the Netherlands); stanozolol from Sequoia Research Products (Pangbourne, UK) and zearalanone from TRC (Toronto, Canada).

The deuterium-labelled standards 17 α -boldenone-d3, 17 β -boldenone-d3, megestrolacetate-d3, methylboldenone-d3, medroxyprogesterone-d3, 17 α -methyltestosterone-d3, 17 β -nortestosterone-d3, 16 α -OH-stanozolol-d3, 17 α -trenbolone-d3, 17 β -trenbolone-d3, triamcinolone-acetonide-d6, α -zearalanone-d4/ β -zearalanone-d4, α -zearalenone-d4 and β -zearalenone-d4 were obtained from the EURL (Wageningen, the Netherlands); melengestrolacetate-d3, prednisolone-d8 (d7 major), zearalanone-d6 and zearalenone-d6 from TRC (Toronto, Canada); dexamethasone-d4 from Cachesyn (Mississauga, Canada); stanozolol-d3 from Cerilliant (Round Rock, USA) and 17 β -testosterone-d3 from NMI (Australia, Sydney).

Materials

The extraction plate manifold, Oasis HLB 96-well plate (60-mg sorbent per well, 60- μm particle size), 96-well collection plates (2 ml), 96-well PTFE/silicone seal with pre-slit, as well as the Acquity UPLC BEH C_{18} column (1.7 μm , 1.0 \times 100 mm) were purchased from Waters (Milford, MA, USA). The bead-ruptor machine and polypropylene reaction tubes were purchased from Omni International (NW Kennesaw, USA), and beads from BioSpec Products (Bartlesville, USA), the evaporator for 96-well plates from Biotage (Uppsala, Sweden) and the centrifuge from Heraeus (Hanau, Germany). The UHPLC-MS/MS system consisted of a Waters ACQUITY UPLC[®] I-Class System connected to a Waters triple quadrupole mass spectrometer Xevo TQS with an ESI interface (Milford, MA, USA). For this study, only bovine meat was used.

Sample Extraction and Clean-up

Comparisons of Digestions and Primary Extractions from Bovine Meat

To investigate whether the bead-ruptor extracts the components efficiently from the meat, several common extraction procedures (Blasco et al., 2007; Daeseleire et al., 1991; Kinsella et al., 2009; Barkatina et al., 2001) were tested and the outcomes were compared. With 1 g of meat, the following extraction procedures were tested: 2 ml TRIS buffer, pH 9.5, with 10 mg subtilisin for 16 h; 2 ml acetate buffer, pH 5.2, with 10 μl Helix SMP for 16 h; 2 ml phosphate buffer, pH 7.8, with 10 μl beta-glucuronidase for 16 h; 2 ml TRIS buffer, pH 9.5; 2 ml acetate buffer, pH 5.2, for 16 h; 2 ml phosphate buffer, pH 7.8, for 16 h; ultrasonic disruptor in 2 ml water for 30 s; bead-disruption in 2 ml 60/40 acetonitrile/ H_2O for 2 min. The eight different digested meat samples were then subjected to visual inspection by microscopic examination ($\times 200$), examining the amount of intact muscle tissue, the nuclei and the general status of the meat. Three types of sections were made using 100 μl of the final digest: without addition of modifiers (native) and with addition of haematoxylin or Newmans containing TBS, as colouring methods. As visual inspection of the treated meat samples might be difficult and subjective, the extraction efficiency also was determined by measuring the concentration of endogenous $17\alpha/\beta$ -testosterone for each tested primary extraction technique. After extraction, all extracts were processed with the method as described in the next section, using a high throughput 96-wells purification procedure.

Primary Extraction from Meat by Bead-Disruption

To 500 mg of homogenised bovine meat sample or standard, 2 $\mu\text{g kg}^{-1}$ of (isotope-labelled) internal standard mixture (see

Table 1 for compounds included in the internal standard mixture) was added. A calibration curve was prepared by spiking blank meat samples with standards (see Table 1 for compounds included in the standard mixture) at concentrations of 0, 0.2, 0.5, 1.0, 2.5, and 5.0 $\mu\text{g kg}^{-1}$ and 1 ml of extraction solvent, i.e. 60% acetonitrile, were added. Bead-disruption was carried out on the bead-ruptor machine using the following program: speed, 5.65 m s^{-1} ; cycle time, 0.75 min; number of cycles, 2 and pause dwell, 0.50 min. Subsequently, the tubes were centrifuged for 10 min at 3452 g and the supernatant was transferred into a clean glass tube, while avoiding transferring the fat layer as much as possible. To dilute the transferred supernatant to a final content of 20% acetonitrile (to prevent the loss of analytes during application onto the Oasis HLB sorbent), 2 ml of water was added.

SPE 96-wells Purification

An Oasis HLB 96-well plate was first conditioned with 1 ml methanol, followed by 1 ml water. Samples were transferred in two portions to the SPE 96-well plate and each well was subsequently washed with 1 ml water. Next, six consecutive washing steps of 1 ml each were applied using different solvents, i.e. 60% methanol in water/2% formic acid, 20% acetonitrile in water/2% formic acid, 20% acetonitrile in water, 10% acetonitrile in water/2% ammonia, 50% methanol in water/2% ammonia, and 60% methanol in water. Elution of analytes was performed with 1 ml acetonitrile, and the eluate was collected in a 96-well collection plate already containing 20 μl DMSO per well as a keeper. The plate was placed in a 96-well plate evaporator and dried until only the DMSO was left. Then, 30 μl of 10% methanol was added and the plate was gently shaken. For analysis, 10 μl of the final residue was injected into the UHPLC-MS/MS system.

UHPLC-MS/MS Analysis

Chromatographic separation was performed on an UPLC BEH C_{18} , 100 mm \times 1.0 mm, 1.7- μm analytical column. Mobile phase A consisted of water/acetonitrile/ammonium formate 1 M/formic acid 900/100/2/0.02 (v/v/v/v), and mobile phase B of water/acetonitrile/ammonium formate 1 M/formic acid 100/900/2/0.02 (v/v/v/v). Column temperature was kept at 60 $^{\circ}\text{C}$, vial tray temperature was 20 $^{\circ}\text{C}$, flow rate was 0.15 ml/min and injection volume was 10 μl . All analytes were eluted using the following linear gradient: 0–0.2 min 80% A, 0.2–3.2 min 70% A, 3.2–7.5 min 50% A, 7.5–8.6 min 0% A and 8.6–10.0 min 80% A.

The MS system switched between positive and negative ion ionisation during analysis, and the capillary voltage was set on 3.0 kV, with a cone gas flow of 150 l per hour. The source temperature was 150 $^{\circ}\text{C}$, and the desolvation temperature was 400 $^{\circ}\text{C}$. The flow of the desolvation gas was 800 l per

Table 1 Monitored MRMs of the UHPLC-MS/MS analysis

Component	I.S. ID	Pre-cursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)	ESI +/-	I.S. (component ID)	Rt (min)
17 α / β -Trenbolone		271.20	199.10 253.20	30 30	25 20	+ +	1	3.56/3.10
17 α / β -Trenbolone-d3	1	274.20	256.20	30	20	+		3.51/3.07
17 α / β -Nortestosterone		275.20	109.10 145.10	30 30	25 20	+ +	2	4.71/3.64
17 β -Nortestosterone-d3	2	278.20	109.10	30	25	+		3.67
17 α / β -Boldenone-d3	3	290.20	121.10	25	25	+		4.63/3.52
17 α / β -Boldenone		287.20	121.10 135.10	25 30	25 15	+ +	3	4.67/3.56
Methylboldenone-d3	21	304.20	121.10	20	25	+		4.12
Methylboldenone		301.20	121.10 149.10	20 20	25 15	+ +	21	4.17
17 β -Testosterone-d3	4	292.20	97.10	30	25	+		4.41
17 α / β -Testosterone		289.20	97.10 109.10	30 30	25 25	+ +	4	5.50/4.45
17 α -Methyltestosterone-d3	5	306.10	97.10	35	25	+		5.10
17 β -Methyltestosterone		303.20	97.10 109.10	30 30	25 30	+ +	5	5.12
16 β -OH-Stanozolol-d3	6	348.25	81.10	50	45	+		3.02
16 β -OH-Stanozolol		345.20	81.10 95.10	30 30	45 40	+ +	6	3.04
Androstadienedione (ADD)		285.20	121.10	25	25	+	2	4.00
Clobetasol		411.20	391.20 355.10	20 20	5 13	+ +	10	5.12
Megestrolacetate-d3 (MGA-d3)	7	388.25	328.20	35	5	+		7.52
Megestrolacetate (MGA)		385.24	325.20 267.20	35 35	13 18	+ +	7	7.54
Melengestrolacetate-d3	8	400.20	337.20	35	5	+		7.86
Melengestrolacetate		397.24	337.20 279.10	35 35	13 20	+ +	8	7.88
Medroxyprogesterone-acetate-d3 (MPA-d3)	9	390.25	330.20	35	5	+		7.89
Medroxyprogesterone-acetate (MPA)		387.25	327.20 123.10	35 35	15 25	+ +	9	7.91
Chlormadinone-acetate (CMA)		405.18	345.20 309.10	35 35	13 15	+ +	7	7.84
Triamcinolone-acetonide-d6	10	441.30	421.30	40	8	+		3.25
Triamcinolone-acetonide		435.30	415.30 339.20	40 40	8 13	+ +	10	3.28
Zearalenone-d6	11	323.20	175.10	50	23	-		5.43
Zearalenone		317.20 317.20	175.08 131.15	44 44	24 30	- -	11	5.47
α / β -Zearalenol-d4	12/13	323.20	160.10	45	32	-		4.32/3.29
α / β -Zearalenol		319.20	275.20 160.15	30 30	21 30	- -	12/13	4.33/3.34
α / β -Zearalanol-d4	14/15	325.20	281.10	50	20	-		4.06/3.16
α / β -Zearalanol		321.20	277.20 259.20	56 56	20 26	- -	14/15	4.09/3.18
Zearalanone-d6	16	325.20	281.10	50	20	-		5.34
Zearalanone		319.20	275.20 205.20	30 54	21 22	- -	16	5.39
Dexamethasone-d4 ^a	17	441.20	363.10	20	15	-		2.78

Table 1 (continued)

Component	I.S. ID	Pre-cursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)	ESI +/-	I.S. (component ID)	Rt (min)
Prednisolone ^a		405.20	329.10	20	15	–	18	1.87
			280.10	20	35	–		
Methylprednisolone ^a		419.20	309.00	20	30	–	18	2.17
			343.10	20	18	–		
Dexamethasone/betamethasone ^b		437.20	307.00	20	30	–	17	2.80/2.75
			361.10	20	15	–		
Prednisolone-d7 ^a	18	412.20	336.13	20	15	–		1.80
Flumethasone ^a		455.20	379.10	20	20	–	17	2.78
			305.00	20	30	–		
Isoflupredone ^a		423.20	347.10	20	15	–	17	1.81
			293.00	20	30	–		
Stanozolol-d3	19	332.20	81.10	20	40	+		5.53
Stanozolol		329.20	81.10	20	40	+	19	5.55
			121.10	20	40	+		

I.S. internal standard, *Rt* retention time in minutes

^a Formic acid adduct

^b Due to incomplete separation of dexamethasone and betamethasone, these compounds are quantified cumulative

hour. The LM 1 resolution was 3.0 in ESI-positive and 2.8 in ESI-negative mode. The HM 1 and HM 2 resolution was 15. The ion energy 1 was set to 1.0 in ESI-positive and 1.9 in ESI-negative mode. The ion energy 2 was set to 1.0 in ESI-positive and 1.3 in ESI-negative mode. Table 1 gives an overview of the measured transitions, and the associated cone voltages for each compound are included.

Data Processing

UHPLC-MS/MS data was automatically processed using MassLynx V4.1 software from Waters. All peak integrations were manually checked and baseline corrected if necessary. Recovery losses during sample clean-up were corrected by the use of suitable internal standard compounds as denoted in Table 1.

Method Validation

The described method was validated as a quantitative screening method according to the Commission Decision 2002/657/EC (European Commission 2002) (Commission E, 2002) and was performed for all compounds. For all compounds, the $CC\alpha$, $CC\beta$, precision, repeatability and measurement uncertainty (MU) were determined. The decision limit ($CC\alpha$) and detection capability ($CC\beta$) were determined by spiking at three different concentration levels (1, 2 and 3 $\mu\text{g kg}^{-1}$) in different blank meat samples. Eight different bovine meat samples were obtained from routine control programs in the Netherlands. One of these meat samples was used for the matrix-matched standard calibration curve, the other meat

samples for the validation. Each individual concentration was fully processed in seven-fold as if it was an unknown sample and this set of samples was analysed on 1 day. From the obtained calibration curve, the y-intercept and slope were calculated ($y = ax + b$) and the $CC\alpha$, $CC\beta$ and MU were calculated according to ISO11843 (Van Loco & Beernaert, 2003). For maximum residue level (MRL) compounds, the $CC\alpha$ was calculated as MRL plus 1.64 times the standard deviation of the within-laboratory reproducibility. $CC\beta$ MRL was calculated as the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility. The expanded MU was calculated using a coverage factor of $k = 2$ and a confidence level of 95%. The repeatability was determined from the obtained dataset using the approach as described in ISO 5725 (Guide I. 5725–1: accuracy (trueness and precision) of measurement methods and results. Part 1: general principles and definitions, 1994). The overall precision was calculated by taking the square root of the sum of the squares of the precision on each spiking level divided by 3. The stability of the extracts was assessed by reinjecting samples after 1-week storage at $-20\text{ }^{\circ}\text{C}$.

Results and Discussion

Optimisation of the Digestion and Primary Extraction from Meat

The primary digestion can be performed in three different ways: chemical, enzymatic, or mechanical. These three digestive procedures were tested, and the remaining muscle fibres

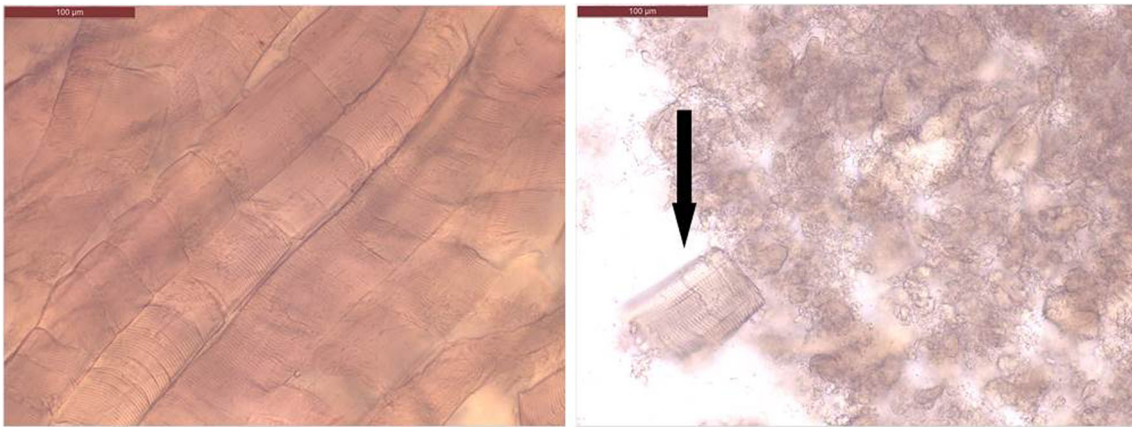


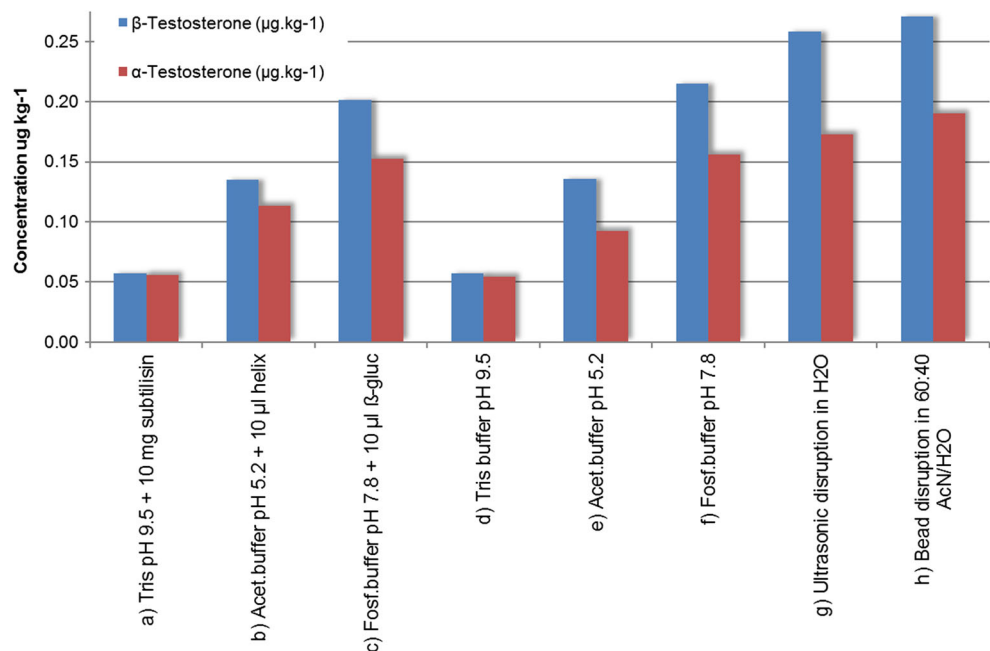
Fig. 1 Microscopic examination ($\times 200$, native) of intact meat (left) and bead-disrupted meat (right). The transversal striping of the muscle fibres as seen in the left picture is still partly visible (arrow) between the debris of the meat extracted by bead-disruption

were microscopically examined. The chemical extraction with 2 ml phosphate buffer, pH 7.8, did almost not affect the meat, i.e. the extracts still contained intact muscle fibres. Chemical extraction at mild acid (2 ml acetate buffer, pH 5.2) or alkali conditions (2 ml TRIS buffer, pH 9.5) showed more destruction of the muscle tissue, but the residue still contained visible cell nuclei. Enzymatic digestion with 2 ml phosphate buffer, pH 7.8, with 10 μ l beta-glucuronidase was not very effective either; the residue contained intact muscle fibres. Enzymatic digestion with 2 ml TRIS buffer, pH 9.5, with 10 mg subtilisin and 2 ml acetate buffer pH 5.2 with 10 μ l Helix SMP was effective, and most of the meat was digested. Mechanical digestion was carried out by means of a bead-ruptor machine. This bead-disruptor is specifically designed for grinding, lysing and homogenising biological samples prior to extraction. In ‘bead beating’, a large number of minute glass, ceramic or

steel beads are vigorously agitated by shaking. Disruption occurs by the crushing action of the beads as they collide with the tissue. Another mechanical digestion technique is by use of an ultrasonic disruptor whereby a high-frequency energy disrupts the cells. Microscopic examination showed that the bead-disruption in 2 ml 60/40 acetonitrile/H₂O and by use of an ultrasonic disruptor in 2 ml water both contained some visible cell nuclei and remnants of muscle fibres the rest of tissue was digested. Figure 1 shows microscopic photographs of intact meat and meat treated by bead-disruption in acetonitrile/water.

As an alternative approach for determination of the extraction efficiency, the levels of endogenous concentrations of 17 α - and 17 β -testosterone were determined. Figure 2 shows the concentrations for each extraction technique tested (a–h). From Fig. 2, it can be concluded that the highest

Fig. 2 Determination of the concentration of endogenous 17 α - and 17 β -testosterone after different digestion and primary extraction techniques: a–h as described in the text above and the ‘Experimental’ part



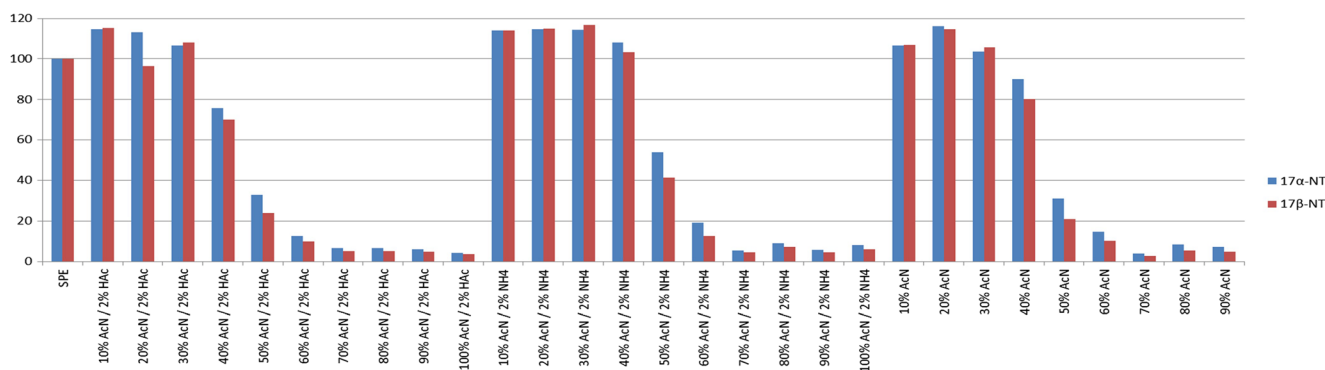


Fig. 3 Example of an elution profile of 17- α / β -nortestosterone in acetonitrile/water, acid and alkaline compositions

concentrations were obtained by use of the bead-ruptor (h) for both testosterone isomers compared to the other digestion and extraction techniques (a–f). Based on the microscopic results, it was expected that the enzymatic digestion with 2 ml TRIS buffer, pH 9.5, with 10 mg subtilisin and 2 ml acetate buffer pH 5.2 with 10 μ l Helix SMP would have given the highest yield since the meat was completely digested. The lower yield of the two enzymatic digestions compared to that of the mechanical digestion could be explained by the fact that enzymatic digestion takes 16 h at elevated temperatures resulting in losses of the compounds. The most efficient digestion, timewise, was using the bead-ruptor since 24 samples can be simultaneously processed in 2 min resulting in minimal losses during digestion as depicted in Fig. 2.

Optimization of a 96-well SPE Extraction Method

For a more efficient workflow and to increase the sample throughput, solid-phase extraction using single SPE columns was converted to a 96-well plate format. To obtain the highest possible recoveries, the influence of the 96-well clean-up materials on the analyte recoveries was investigated. It turned out

that large losses of compounds occurred at the solvent-evaporation step in the 96-well collection plates. Most probably, compounds were adsorbed to the plastic material of the 96-well plate, hindering reconstitution. These recovery losses were plate and brand independent. To prevent these losses, a small volume of dimethylsulfoxide (DMSO) was added before the evaporation step, i.e. DMSO serving as a ‘keeper’ solvent. After addition of DMSO as the keeper, losses were minimal (see supplementary information).

Due to the efficient workflow in the 96-well format, it is easy to test numerous and different washing steps. The following washing solvents were tested whereby the organic solvent percentage was increased from 10 to 90% in steps of 10%: methanol/water/formic acid (2%); methanol/water/ammonia (2%); methanol/water; acetonitrile/water/formic acid (2%); acetonitrile/water/ammonia (2%) and acetonitrile/water. For each compound, the corresponding elution profile was constructed. Figure 3 shows an example of such an elution profile.

Next, for each solvent mixture, the critical breakthrough washing condition was selected. That is, the composition with the lowest percentage of organic modifier determines the

Table 2 Overview of experimental design to optimise the SPE washing procedure. The numbers in the table represent the tested sequence in the washing procedure. The amount of numbers per combination represent the amount of washing steps. All washing solvents were applied in portions of 1 ml. Exception in this table is combination A, where no wash steps were performed

Combination	60% MeOH/HAc	50% MeOH/NH ₃	60% MeOH	20% ACN/HAc	20% ACN/NH ₃	20% ACN
A	–	–	–	–	–	–
B	–	–	1	–	–	2
C	1	–	–	2	–	–
D	–	1	–	–	2	–
E	1	3	2	–	–	–
F	–	–	–	1	3	2
G	1	5	6	2	4	3
H	1	2	–	–	–	–
I	–	–	–	1	2	–
J	1	–	–	–	2	–

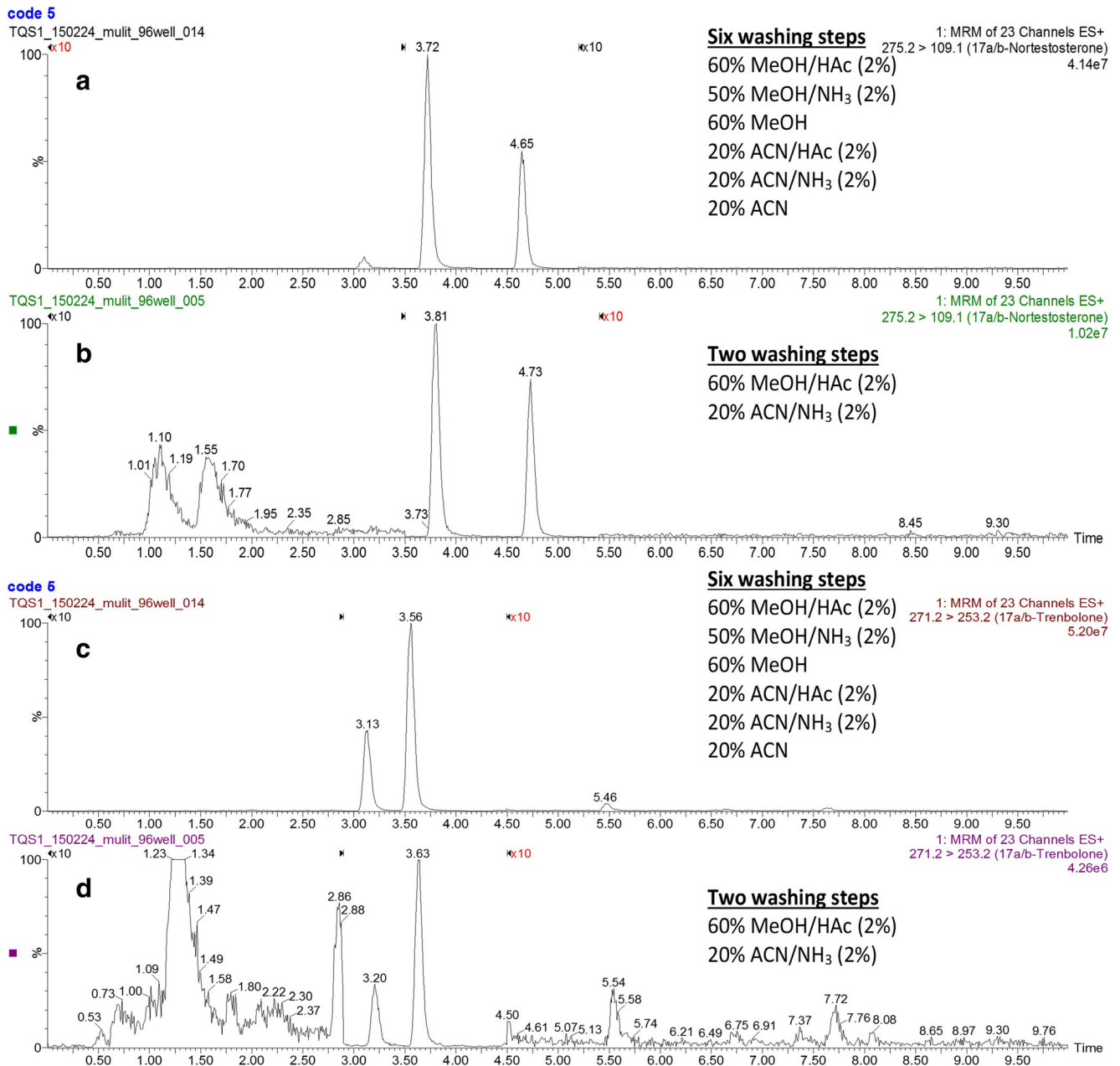


Fig. 4 Example of two combinations of washing steps in meat matrix. Extracted ion chromatograms of α/β -nortestosterone (**a, b**) and α/β -trenbolone (**c, d**) after application of two different washing procedures during the SPE 96-well plate clean-up: (1) combination G, use of six

consecutive washing steps and (2) combination J, use of two washing steps. Retention time windows before the first peak and after the last peak were amplified by factor 10 to demonstrate the difference in background noise in the samples

critical breakthrough condition for that washing solvent (washing step) in the whole method. The following critical washing conditions were determined: 60% in methanol/water/formic acid; 50% in methanol/water/ammonia; 60% in methanol/water; 20% in acetonitrile/water/formic acid; 20% in acetonitrile/water/ammonia and 20% in acetonitrile/water. After determination of these critical compositions, an experimental design was constructed to test the optimal number and order of the selected washing solvents (Table 2).

The chromatograms for all combinations were visually evaluated, for some of the combinations interfering peaks were visible from the meat extracts, making integration more difficult which will eventually result in unreliable quantification. It was noted that the cleanest chromatograms were obtained using a combination of a protic and an aprotic organic solvent, e.g. methanol and acetonitrile. So, combinations E and H (only protic) and F and I (only aprotic) were less effective in removing interfering peaks. Washing combinations

with only neutral (combination B), only acid (combination C) or only alkaline (combination D) washing solvents were also unsuccessful in removing all interfering peaks. The optimum number and order of solvents were found to be a combination of acid and alkaline protic and aprotic solvents. At the end, the optimal washing condition consisted of six different solvents in the following order: 60% methanol/formic acid, 50% methanol/ammonia, 60% methanol, 20% acetonitrile/formic acid, 20% acetonitrile/ammonia and 20% acetonitrile. Figure 4 shows that this comprehensive washing procedure (Table 2 combination G) produced lower background signals compared to the two washing steps method with two different solvents (Table 2 combination J) and also resulted in minimal losses during sample clean-up for most of the compounds.

Optimization of the UPLC Conditions

To increase the sensitivity and reduce the organic solvent usage, a column with an internal diameter of 1.0 mm was used instead of the commonly used 2.1 mm. From a theoretical point of view, the sensitivity should increase quadratic with linear reduction of the column diameter when the same mass amount of sample is injected. In this case, switching from a 2.1-mm column to a column with an ID of 1.0 mm would theoretically result in a factor of 4 increase in sensitivity. It should be noted that this factor of 4 can only be reached if all LC connections are made properly and the dead volume is reduced to a minimum. Figure 5 gives an example of α/β -nortestosterone whereby the same amount was injected on a 2.1- and 1.0-mm column. Comparing the signal to noise ratio, but also the peak height, demonstrates that there is indeed an

increase in sensitivity, in general, the sensitivity of this column was approximately a factor of 4 better. In addition, the amount of organic solvents used was reduced, as the flow was decreased from 0.6 ml/min on a 2.1-mm column to 0.15 ml/min on a 1-mm column, this is a 75% reduction of organic solvent.

Validation

The method was validated as a screening method according to the guidelines set in the Commission Decision 2002/657 (Commission E, 2002). The validation results for precision, CC α , CC β , linearity, recovery and measurement uncertainty are presented in Table 3. The method was validated for all compounds as if they were forbidden substances. However, this is not the case for dexamethasone, betamethasone, prednisolone and methylprednisolone which are permitted for therapeutic purposes. There are MRLs established for these compounds. The MRL for dexamethasone and betamethasone is 0.75 $\mu\text{g kg}^{-1}$; for prednisolone, 4 $\mu\text{g kg}^{-1}$ and for methylprednisolone, 10 $\mu\text{g kg}^{-1}$. The CC α and CC β values were calculated at the MRL as well (see Table 3). As meat contains certain endogenous hormones or unintended contaminants, it is impossible to determine the performance characteristics for these compounds. During validation, 17 α -testosterone ($n = 7$, average concentration 0.23 $\mu\text{g kg}^{-1}$) and 17 β -testosterone ($n = 7$, average concentration 1.45 $\mu\text{g kg}^{-1}$) were detected in the blank samples. From literature, it is also known that prednisolone (Arioli et al., 2010; De Clercq et al., 2015), α/β -zearalenol and zearalenone originating from *Fusarium* can be found in blank samples (Kennedy et al., 1998; Seeling et al., 2005; Price & Fenwick, 1985) as well as 17 α/β -nortestosterone (de

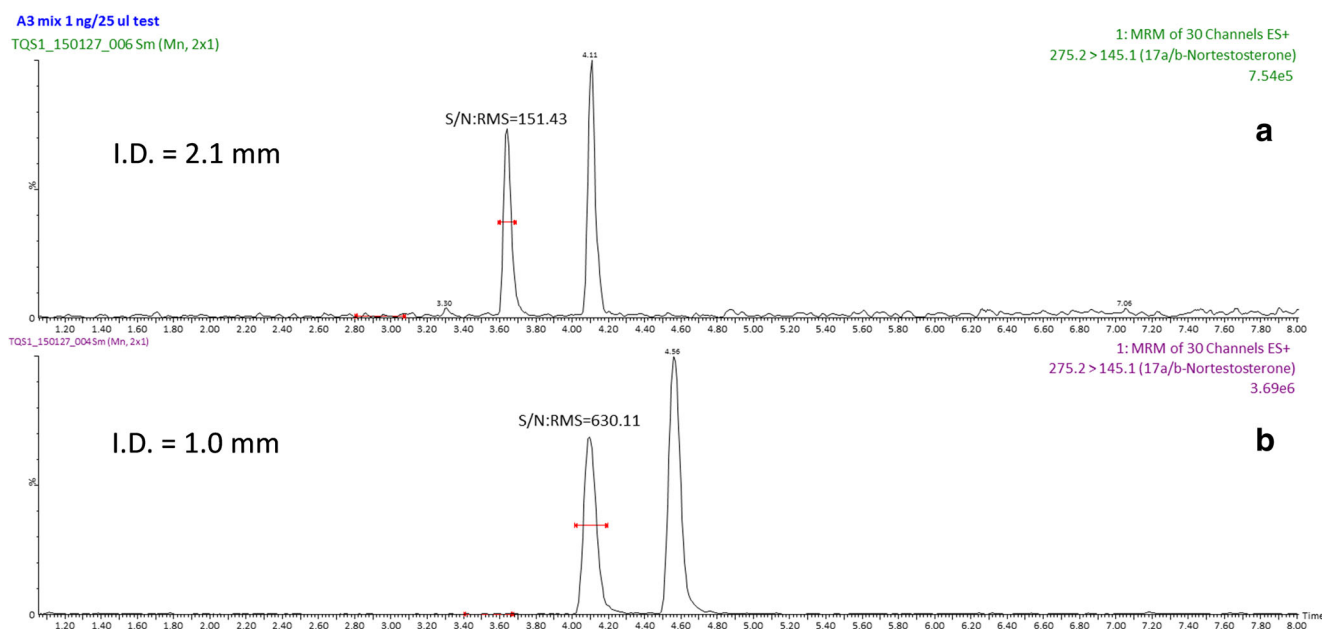


Fig. 5 Extracted ion chromatograms of 17 α/β -nortestosterone analysed with Acquity BEH C₁₈, 1.7 μm , 2.1 \times 100 mm (a) and Acquity BEH C₁₈, 1.7 μm , 1.0 \times 100 mm (b)

Brabander et al., 1994). In the blank samples used in this validation study, these endogenous compounds were not detected, except for 17 α / β -testosterone. This results in higher measurement uncertainties, in particular for 17 α / β -testosterone. Therefore, it cannot be neglected that the validation results could be influenced by background concentrations of these compounds. 17 α / β -Testosterone, prednisolone, methylprednisolone and α / β -zearalenol have higher CC α values than 1 $\mu\text{g kg}^{-1}$ (Table 3). All other growth promoters have a CC α in the range of 0.1–0.9 $\mu\text{g kg}^{-1}$ (Table 3). The measurement uncertainty for these growth promoters is lower than 36%. The precision for these exogenous compounds is within the range of 85–115%, which is considered as acceptable for the intended use of this method and in compliance with the requirements as

described in the EU Commission Decision 2002/657/EC. All matrix-matched calibration curves resulted in a $R^2 > 0.99$ in the range from 0.2 to 5 $\mu\text{g kg}^{-1}$ except for β -zearalenol, α -zearalenol, prednisolone and methylprednisolone. The overall sensitivity of these compounds is lower compared to that of the other compounds resulting in a lower correlation coefficient. The recovery for most compounds is between 30 and 55%, which is acceptable at residue levels. The recoveries for the resorcylic acid lactones, prednisolone and isoflupredone are low, 8–20%. The poor recovery of these compounds is due to the extensive washing procedure. The specificity was tested by the analysis of eight different blank meat samples: apart from the naturally occurring compounds, no chromatographic peaks were present at the expected retention time of the individual

Table 3 Overview of the validation results (MU is measurement uncertainty); displayed value represents the average of the precision at three measured levels (1.0, 2.0 and 3.0 $\mu\text{g kg}^{-1}$), R^2 = correlation coefficient

Identification	Precision (%)	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	CC α_{MRL} ($\mu\text{g kg}^{-1}$)	CC β_{MRL} ($\mu\text{g kg}^{-1}$)	R^2	Recovery (%)	MU (%)
17 β -Testosterone ^a	199.3	7	14	–	–	0.9995	51	542
17 β -Nortestosterone ^a	101.4	0.23	0.47	–	–	0.9985	50	12.8
17 β -Boldenone	103.9	0.15	0.31	–	–	0.9998	42	10.9
17 α -Trenbolone	99.1	0.16	0.32	–	–	0.9994	41	9.0
17 α -Testosterone ^a	121.1	0.73	1.46	–	–	0.9991	52	51.3
17 α -Nortestosterone ^a	101.8	0.24	0.48	–	–	0.9997	48	13.5
17 α -Boldenone	99.4	0.23	0.45	–	–	0.9991	37	13.7
17 β -Trenbolone	96.7	0.26	0.52	–	–	0.9995	49	12.7
16 β -OH-Stanozolol	102.3	0.10	0.20	–	–	0.9996	29	7.3
α -Zearalenol	103.6	0.53	1.07	–	–	0.9990	8	29.7
Androstadienedione	100.2	0.16	0.31	–	–	0.9993	48	11.3
β -Zearalenol ^a	117.7	1.33	2.67	–	–	0.9962	6	89.5
β -Zearalenol	90.2	0.71	1.41	–	–	0.9814	9	30.2
α -Zearalenol ^a	61.2	1.96	3.93	–	–	0.9453	19	76.6
Clobetasol	87.4	0.54	1.08	–	–	0.9956	54	36.4
Chlormadinone-acetate	101.2	0.31	0.63	–	–	0.9990	46	18.4
Dexamethasone-FA adduct + betamethasone-FA adduct	90.5	0.93	1.86	1.34	1.94	0.9922	30	28.8
Flumethasone-FA adduct	102.7	0.12	0.23	–	–	0.9995	41	8.0
Isoflupredone-FA adduct	105.3	0.33	0.65	–	–	0.9979	13	23.7
Megestrolacetate (MGA)	109.3	0.20	0.40	–	–	0.9993	45	13.8
Medroxyprogesterone-acetate (MPA)	113.4	0.29	0.58	–	–	0.9988	47	16.5
Triamcinolonacetone	101.5	0.29	0.58	–	–	0.9993	41	18
Prednisolone-FA adduct ^a	109.8	1.58	3.15	4.48	6.96	0.9884	8	92
Stanozolol	97.1	0.10	0.21	–	–	0.9987	40	6.3
Melengestrolacetate (MLA)	103.5	0.16	0.32	–	–	0.9995	46	11
Methylprednisolone-FA adduct	95.7	1.97	3.49	10.79	11.57	0.9722	39	74.6
Methylboldenone	103.2	0.54	1.09	–	–	0.9978	46	40.0
Methyltestosterone	100.4	0.14	0.28	–	–	0.9993	52	9.7
Zearalanone	90.6	0.54	1.09	–	–	0.9976	26	36.1
Zearalenone ^a	96.7	0.30	0.60	–	–	0.9987	18	15.5

^a Compounds can occur naturally in samples of meat

checked growth promoters. This indicates the absence of interfering compounds that might result in false positive outcomes. The selectivity of the MRM transitions was assessed according to the description of Berendsen et al. (Berendsen et al., 2013). All measured transitions were found to be selective. Sample extracts were found to be stable for at least 1 week.

The results of the validation study confirm that the newly developed 96-well high throughput clean-up procedure in combination with the optimised UHPLC-MS/MS analysis results in a greener method for the fast and broad detection of growth promoters in meat, showing acceptable performance characteristics that fulfil the performance criteria as established in CD 2002/657/EC.

Conclusions

An easy, fast and sensitive method for broad analysis of different growth promoters, with reduced consumption of chemicals and disposables, was developed. The compounds analysed with this method are different classes of steroids, corticosteroids, gestagens and resorcylic acid lactones. So far, no other published method is able to analyse these different classes of compounds in meat in a single method at levels below $1.0 \mu\text{g kg}^{-1}$ as recommended by EFSA (Panel, 2013). Overall, this new method offers an increased sample throughput: sample pre-treatment, analysis of 96 samples and analysis of calibration and control samples in less than 24 h. As a result of the reduction in analysis time and consumables needed for sample clean-up, the costs for analysis are substantially reduced. The precision of the method for exogenous steroids varies between 85 and 115%, the $CC\alpha$ for the compounds ranges from 0.1–0.9 $\mu\text{g kg}^{-1}$ and the expanded measurement uncertainty was lower than 36%. The validation proved that the method is reliable and sensitive. The method can be used as a routine screening method to detect abuse of growth promoters in cattle fattening.

Furthermore, the method can be extended to include other species and matrices. An interesting future application could be the use of this clean-up method in combination with full-scan accurate mass spectrometric measurement to obtain a true generic method which is capable of analysing an unlimited number of growth promoters in meat.

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Compliance with Ethical Standards

Conflict of Interest Frederike van Tricht declares that she has no conflict of interest. Martien Essers declares that he has no conflict of interest. Maria Groot declares that she has no conflict of interest. Saskia Sterk declares that she has no conflict of interest. Marco Blokland declares that

he has no conflict of interest. Leen van Ginkel declares that he has no conflict of interest.

Ethical Approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Not applicable

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