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Surveillance study of a number of synthetic and natural growth promoters in bovine muscle samples using liquid chromatography tandem mass spectrometry

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4 **Surveillance study of a number of synthetic and natural growth**
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6 **promoters in bovine muscle samples using liquid chromatography**
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8 **tandem mass spectrometry**
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14 Edward Malone^{a,b}, Chris Elliott^b, Glenn Kennedy^c David Savage^a and Liam Regan^a
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24
25

26 **Abstract**
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28

29 A simple, new method permitting the simultaneous determination and confirmation of
30 trace residues of twenty four different growth promoters and metabolites using liquid
31 chromatography mass spectrometry has been developed and validated. The
32 compounds were extracted from bovine tissue using acetonitrile; sodium sulphate was
33 also added at this stage to aid with purification. The resulting mixture was then
34 evaporated to approximately 1 mL and subsequently centrifuged at high speed and an
35 aliquot injected onto the LC-MS/MS system. The calculated CC α values ranged
36 between 0.11 and 0.46 $\mu\text{g kg}^{-1}$, calculated CC β were in the range 0.19 to 0.79 $\mu\text{g kg}^{-1}$.
37 Accuracy, measurement of uncertainty, repeatability and linearity were also
38 determined for each analyte. The analytical method was applied to a number of bovine
39 tissue samples imported in to Ireland from third countries. Levels of progesterone were
40 found in a number of samples at concentrations ranging between 0.28 $\mu\text{g kg}^{-1}$ and
41 30.30 $\mu\text{g kg}^{-1}$. Levels of alpha and beta testosterone were also found in a number of
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3 samples at concentrations ranging between 0.22 to 8.63 $\mu\text{g kg}^{-1}$.and 0.16 to 2.08 $\mu\text{g kg}^{-1}$
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respectively.

Keywords: Bovine tissue; Mass Spectrometry; Steroids; Imported, Survey

Introduction

In recent years there has been an increase in global concern relating to the adverse effects that may result from exposure to chemicals that could interfere with the endocrine system. A number of classes of compound have been highlighted in this regard, they include dioxins, phyto-oestrogens, Bisphenol A, hormones, etc. of these hormones continue to be in general the most biologically active. Hormonally active growth promoters have been shown to be very effective for promotion of muscle growth in farm animals; particularly bovine ruminants, veal calves and pigs (Lu et al. 1976, WHO 1982, Meissonnier et al. 1983, Berende et al. 1983). Within the EU, treatment of animals intended for human consumption with substances exhibiting thyreostatic, androgenic, oestrogenic or gestagenic effects is a regulatory offence (Council Directive EC 96/22 1996). The use of these substances is controlled through national residue control plans in each EU member state (Council Directive EC 96/23 1996). The EU inspection programs focus on matrices that are more suitable for the detection of prohibited substances, especially if the animals are still on farm, these matrices urine, faeces or hair. With regard to slaughtered animals, the more favoured matrices are bile, retina, fat, blood, liver and kidney. Rarely is muscle tissue sampled for the detection of steroidal type growth promoters. Muscle is usually only tested in the case of import controls or in monitoring programs of meat sampled in butcher shops or supermarkets. As a result, data on steroid concentrations in muscle meat

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3 samples is rare. Data on levels of natural hormones in meat is even rarer because of the
4
5 absence of “legal natural levels” for these hormones in compliance testing. A restricted
6
7 controlled use of some specified anabolic steroids is permitted in the USA, Canada,
8
9 Australia, New Zealand and in some countries in South America, Asia and Africa. The
10
11 EU imports large amounts of beef each year from these third countries, 524,000 tonnes
12
13 in 2005 alone (Eurostat 2005). However when beef is imported in to the EU, it should
14
15 meet the same standards with regards the absence of prohibited substances as prevail
16
17 within the EU i.e. absence of illegal growth promoters. As the number of growth
18
19 promoters likely to be abused in cattle fattening increases the use of multi-residue
20
21 methods that cover a number of classes of compounds becomes increasingly
22
23 important. Methods such as this are employed in the testing of domestic samples of
24
25 urine as part of the national control plan each EU state must carry out. These methods
26
27 usually monitor for a large amount of analytes for example 22 compounds (Hewitt et
28
29 al 2002). Very sensitive analytical techniques for the detection of these substances and
30
31 their metabolites in tissue need be adopted as the levels that accumulate in tissue are
32
33 lower than those found in other matrices (Marchard et al. 2000). Liquid
34
35 chromatography tandem mass spectrometry (LC-MS/MS) has been the determination
36
37 method of choice for the majority of articles published recently concerned with the
38
39 determination of steroidal growth promoters in bovine tissue (Xu et al. 2006, Chursch
40
41 et al 2008, Costain et al. 2008, Malone et al 2009, Schmidt et al 2009, Kaklamanos et
42
43 al 2009, Yang et al 2009). Various types of extraction and purification protocols have
44
45 been adopted in these procedures, the majority of which use solid phase extraction
46
47 (SPE) for sample purification; in some cases a dual SPE step is adopted. A wide range
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49 of SPE sorbents have been utilised for this task; C18 and Oasis MAX (Chursch et al
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51 2008), Oasis HLB and aminopropyl (Schmidt et al 2009), graphitised carbon black and
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3 aminopropyl (Yang et al. 2009) C18 (Xu et al. 2006) and Strata X (Malone et al 2009).
4
5 Prior to extraction and purification a number of methods employ a deconjugation step
6
7 in order to release any glucuronide or sulphate forms of the compounds to their free
8
9 form. This step is required for urine samples as a considerable percentage of these
10
11 compounds are excreted in the glucuronide and sulphate forms in urine (Le Bizec et al
12
13 1992). However in other matrices such as muscle, evidence suggests that this step may
14
15 not be necessary, investigations done by a number of groups found that when
16
17 analysing muscle samples no significant difference in concentrations of analyte
18
19 observed could be found between samples which underwent enzymatic hydrolysis and
20
21 those which did not (Hartmann et al 1997, Marchand et al 2000, Yang et al. 2009).
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27 The main aims of the work in this study were to develop and validate a rapid
28
29 confirmatory method for a wide number of steroidal type growth promoters in bovine
30
31 muscle and apply this method to the analysis of a number of samples of bovine muscle
32
33 imported into Ireland from third countries. Both natural and synthetic compounds were
34
35 investigated. However the presence of any naturally occurring compounds such as
36
37 progesterone, testosterone etc does not necessarily imply misuse.
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43 **Experimental**

44 *Materials and reagents*

45
46 Water, methanol, acetonitrile (HiPerSolv grade) were obtained from BDH (Merck,
47
48 Poole, Dorset, UK). Ammonium acetate and sodium sulphate (ACS grade) were
49
50 obtained from Sigma (St. Louis, MO, USA). d₃ 16-beta-OH Stanozolol, alpha (, beta
51
52 and d₃ beta Trenbolone, alpha Boldenone, d₃ beta Boldenone, alpha Nortestosterone,
53
54 d₃ beta Trenbolone, alpha Boldenone, d₃ beta Boldenone, alpha Nortestosterone,
55
56 d₃ beta Trenbolone, alpha Boldenone, d₃ beta Boldenone, alpha Nortestosterone,
57
58 d₃ beta Trenbolone, alpha Boldenone, d₃ beta Boldenone, alpha Nortestosterone,
59
60 Acetate, d₃ Medroxyprogesterone Acetate, d₃ Methylboldenone, d₃ Megestrol Acetate,

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3 d₃ Melengestrol Acetate, d₄ Zeranol/Taleranol, d₃ Methyltestosterone, d₆
4
5 Diethylstilbestrol, d₉ Progesterone, alpha Testosterone, d₃ beta Testosterone all from
6
7 RIVM (Bilthoven, The Netherlands). Hexestrol, Diethylstilbestrol, Flumethasone,
8
9 Dexamethasone, Progesterone, beta Testosterone, beta Boldenone, beta
10
11 Nortestosterone Medroxyprogesterone Acetate, Megestrol Acetate, Melengestrol
12
13 Acetate, and Fluoxymesterone, Zeranol and Taleranol from Sigma Aldrich (St. Louis,
14
15 MO, USA). 16-beta-OH Stanozolol from LGC Promochem (Teddington, UK) and d₄
16
17 Dexamethasone from QMX Laboratories (Thaxted, UK).

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22 Primary stock standard solutions of each analyte were prepared in methanol at a
23
24 concentration of 100 µg mL⁻¹. Intermediate single standards solutions each analyte
25
26 were prepared in methanol at a concentration of 10 µg mL⁻¹. A mixed standard
27
28 fortification solution which included each analyte was prepared in methanol at a
29
30 concentration of 100 ng mL⁻¹. A mixed standard fortification solution including each
31
32 internal standard was prepared in methanol at a concentration of 500 ng mL⁻¹. All
33
34 standards were stored at -20 °C. The injection solvent consisted of 0.5mM Ammonium
35
36 Acetate in methanol:water (30:70, v/v).

37 38 39 40 41 42 43 44 *LC conditions*

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46 The LC system was a Shimadzu UFLC-XR equipped with a LC-20AD-XR Binary
47
48 pump, SIL-20AD-XR autosampler and a CTO-20A column oven (Shimadzu, Dublin,
49
50 Ireland). The compounds were chromatographed on a 1.8 µm Agilent Eclipse Plus
51
52 C18 column (2.1 × 50 mm) (Agilent, Dublin, Ireland) and the column temperature was
53
54 maintained at 55 °C. A gradient LC system using 0.5 mM ammonium acetate:
55
56 methanol (70:30, v/v Mobile Phase A) and ammonium acetate: methanol (5:95, v/v
57
58 Mobile phase B) at a flow of 0.5 mL min⁻¹ was operated. The gradient profile began at
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3 90% A held for 3 minutes before changing to 50% A 2 minutes later, held for 1 minute
4
5 and changing to 15% A, 1.5 minutes later and held for 1.5 minutes before returning to
6
7 90% A after 1.5 minutes and left to equilibrate for 2.5 minutes. The total runtime of
8
9 the method was 13 minutes.
10
11

12 13 14 15 *MS/MS parameters*

16
17 The mass spectrometer system used was a 5500 triple quadrupole instrument (Applied
18 Biosystems/ MDS Sciex, Canada). The analysis was performed using both positive and
19
20 negative ion electrospray (ESI) and the MS was operated in multiple reaction
21
22 monitoring (MRM) mode. Two transitions per analyte were monitored whereas only
23
24 one transition was monitored for each deuterated internal standard. The precursor
25
26 product transitions for each analyte as well as their corresponding collision energies
27
28 are shown in Table 1. The MS/MS detector conditions were as follows: Ion spray
29
30 voltage 1500V; Ion Source Gas 1 50 psi; Ion Source Gas 2 60 psi; Temperature 650
31
32 °C; Curtain Gas 15 psi; Collision Gas Pressure 8 (arbitrary units); Entrance Potential
33
34 10V. Data acquisition and integration were performed using Analyst software version
35
36 1.5 (Applied Biosystems/ MDS Sciex, Canada).
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46 *Bovine tissue samples*

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48 Bovine tissue samples were obtained and stored at -20 °C. Samples of this tissue were
49
50 analysed and those found to contain no detectable residues of the analytes of interest
51
52 were used as negative controls to carry out development and validation.
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58 *Sample extraction and clean-up*

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3 Tissue samples (approximately 50 g) were sliced into very fine pieces and mixed
4
5 together. 3 grams of the resulting mixture is weighed into Foodcon7™ polypropylene
6
7 tubes. 30 µL of the mixed internal standard was added at this time, corresponding to a
8
9 concentration of 5 µg kg⁻¹. Samples used to calculate the precision and recovery of the
10
11 method were fortified at levels corresponding to 1, 1.5 and 2 µg kg⁻¹ for each analyte
12
13 by adding 30, 45 and 60 µL portions of the 100 ng mL⁻¹ mixed standard solution. After
14
15 fortification, samples were held for 15 min prior to extraction. Acetonitrile (10 mL)
16
17 was added to the samples followed by sodium sulphate 2.5 grams. The samples were
18
19 then vigorously shaken (30 seconds) followed by homogenisation using a
20
21 MediFASTH₂™ automated homogeniser. The resulting mixture was then transferred to
22
23 50 mL polypropylene tubes and centrifuged (15 mins, 4000 g, 4 °C). The supernatant
24
25 was then transferred to a clean polypropylene tube and dried under nitrogen with heat
26
27 (55 °C) to an approximate volume of 1 mL. The extract is then transferred to 1.5 mL
28
29 ultracentrifuge tubes and centrifuged at 14,000 g for 30 mins. The supernatant (200 µl)
30
31 is then transferred directly to autosampler vials and 15 µl is injected onto the LC-
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33 MS/MS system.
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44 *Matrix-Extracted Calibration*

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46 Matrix extracted calibration curves were prepared and used for quantification. Control
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48 bovine muscle previously tested and found to contain no detectable residues was
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50 prepared as above. One muscle sample was used for each calibration standard level.
51
52 Samples were fortified at levels corresponding to 0, 1, 1.5, 2, 5 and 10 µg kg⁻¹ by
53
54 adding appropriate portions of the 100 ng mL⁻¹. After fortification, samples were held
55
56 for 15 min prior to the extraction procedure as described above. Calibration curves
57
58 were prepared by plotting the response factor (area of the analyte/area of internal
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3 standard) as a function of analyte concentration (0 to 10 $\mu\text{g kg}^{-1}$) and these were used
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5 to quantify samples.
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8 9 10 *Method validation*

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12 The LC-MS/MS method was validated according to 2002/657/EC guidelines. LC-
13 MS/MS identification criteria were verified throughout the validation study by
14
15 monitoring relative retention times (see Table 1 for information with regard which
16
17 internal standards are used with which analytes), signal to noise ratios (S/N) and ion
18
19 ratios. Several method validation parameters were determined including linearity,
20
21 specificity, recovery, precision (repeatability and within-laboratory reproducibility),
22
23 decision limit $CC\alpha$, and detection capability $CC\beta$ and measurement of uncertainty.
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25 The decision limit ($CC\alpha$) of the method was calculated according to the calibration
26
27 curve procedure using the intercept (value of the response factor, y , where the
28
29 concentration, x is equal to zero) and 2.33 times the standard error of the intercept for
30
31 a set of data with 6 replicates at 3 levels. The detection capability ($CC\beta$) was
32
33 calculated by adding 1.64 times the standard error to the $CC\alpha$. The specificity of the
34
35 method was verified through the analysis of 20 blank muscle samples obtained from
36
37 different sources. To test the linearity of the method, matrix extracted calibration
38
39 curves were prepared on each of the three days to give 6-point calibration curves
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41 ranging from 0 to 10 $\mu\text{g kg}^{-1}$ for each compound.
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50 Since no certified reference materials were available for the analytes and matrix of
51
52 interest, accuracy was determined from fortified negative samples as an alternative to
53
54 trueness. The accuracy and precision were determined through the analysis of blank
55
56 muscle samples fortified in 6 replicates at 1, 1.5, and 2 $\mu\text{g kg}^{-1}$ on three separate days.
57
58 To evaluate the precision of the method, repeatability and within-laboratory
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3 reproducibility were determined. Repeatability results were obtained at the three
4
5 validation levels under identical conditions by the same operator on a particular day.
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8 The within-laboratory reproducibility results were obtained at the three validation
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10 levels on three separate days. The measurement of uncertainty was estimated by
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12 taking into account the within laboratory reproducibility over days 1, 2 and 3.
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15 16 17 **Results and Discussion**

18 19 *Preliminary experiments:-*

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24 The LC-MS/MS method was developed to provide confirmatory data for the analysis
25
26 of bovine muscle for a wide number of steroidal type growth promoters. Electrospray
27
28 ionisation (ESI) was chosen as the ionisation method as it provided the best responses
29
30 on average for the wide array of compounds being investigated. Both positive and
31
32 negative polarity ESI were tried for each of the analytes; some responded better in
33
34 ESI- and others with ESI+, the final polarity mode chosen for each analyte can be seen
35
36 in Table 1. The MS/MS fragmentation conditions were investigated and collision
37
38 energies were optimised for each precursor-product ion transition, the fragmentation
39
40 patterns observed were similar to those found in previously published work (Hewitt et
41
42 al). For a method to be deemed confirmatory in accordance with Commission Decision
43
44 2002/657/EC four identification points are required. These identification points are
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46 obtained by monitoring one precursor ion and two transition products (corresponding
47
48 to strong and weak ion transitions). The precursor and product transitions for each of
49
50 the steroidal type compounds assayed for are listed in Table 1 as well as their
51
52 associated collision energy.
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3 Previous work carried out in this laboratory for the analysis of steroids in muscle
4 involved a deconjugation step using *Helix Pomatia*. However the necessity of this step
5
6 has been called into question for the analysis of tissue samples. Work carried out by
7
8 others (Hartmann et al 1997, Marchand et al 2000, Yang et al. 2009) has shown that
9
10 only a small percentage of the steroid exists as the glucuronide or sulphate form in
11
12 muscle. It was decided to omit this step as part of this study so only the unconjugated
13
14 steroid is being measured. Due to the absence of this deconjugation step, there was no
15
16 need for the use of acetate buffers to bring the pH of the samples to approximately 5.2
17
18 as has been seen before in numerous other publications. Extraction with organic
19
20 solvent was then the initial step in this method. Various solvents (acetonitrile,
21
22 methanol, diethylether, ethyl acetate) and combinations of these have been used
23
24 previously for the extraction of steroids from tissue samples. Due to the large number
25
26 of steroids being examined in this study, a generic extraction solvent was required in
27
28 order to minimize losses for some of the compounds. Acetonitrile was chosen initially
29
30 for this purpose and initial studies indicated that the analytical recovery achieved was
31
32 adequate to allow for the determination of these substances at and below $1 \mu\text{g kg}^{-1}$.
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34

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36 In relation to the purification step, again this needed to be quite generic in order to
37
38 avoid analytical losses for some of the analytes. The possibility of using dispersive
39
40 SPE with C18 sorbent material was investigated, however for some of the compounds
41
42 examined; the analytical losses were too high to allow for determination at the level of
43
44 interest. The addition of both sodium chloride and sodium sulphate during the initial
45
46 extraction was also examined. Promising results were observed from the addition of
47
48 sodium sulphate so an extraction procedure involving the addition of acetonitrile and
49
50 sodium sulphate was chosen. A high speed centrifugation step was also implemented
51
52 prior to LC-MS/MS analysis in order to aid the removal of particulates. The method
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3 proved to be quite robust with no deterioration in method performance being observed
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5 for all validation batches and survey samples.
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8 The compounds were chromatographed on an Agilent Eclipse C₁₈, 50 x 3mm, 1.8 µm
9
10 column, the gradient profile was developed to permit rapid analysis but also to allow
11
12 for the separation of the various isomers being examined which could not be separated
13
14 by their m/z ratio; so needed to be separated chromatographically. Deuterated
15
16 analogues of a number of the analytes were used as internal standards.
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22 *Validation study*

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24 Validation of the method was according to procedures described in Commission
25
26 Decision 2002/657/EC covering specificity, calibration curve linearity, recovery
27
28 (accuracy), precision, decision limit (CC α) and detection capability (CC β).
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34 *Liquid Chromatography Tandem Mass Spectrometry*

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36 The criteria for relative retention times and ion ratios were examined for all samples
37
38 and standards used for the validation study. The values for these were in agreement
39
40 with the EU requirements for all the analytes investigated in this study. In terms of
41
42 relative retention time, the analyte peaks in samples were found to be within 2.5%
43
44 tolerance when compared with the matrix extracted standards. Furthermore two
45
46 transition ions were monitored for each of the analytes. The most intense ion was used
47
48 for quantitation. All ion ratios of samples were within the required tolerances as
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50 required by EU criteria when compared with standards used during the validation
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52 study.
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Specificity

The technique of LC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, a variety of muscle samples from different sources were fortified with the analytes of interest and their non-fortified equivalent samples were also analysed. No interfering peaks were observed at the retention time for any of the analytes.

Linearity of the response

The linearity of the chromatographic response was tested with matrix extracted curves using 6 calibration points in the concentration range of 0 to 10 $\mu\text{g kg}^{-1}$ for each analyte. The regression coefficients (r^2) for all the calibration curves used in this study were ≥ 0.980 .

Recovery

The recovery of the method was determined using muscle samples fortified at 1, 1.5 and 2 $\mu\text{g kg}^{-1}$. The resulting values were calculated from matrix extracted calibration curves. The mean corrected recovery ($n = 18$) for each of the analytes, determined over three separate assays is shown in Table 3 and ranged between 94.6 and 102.9 %.

Precision

The usefulness of suitable isotopically labelled internal standards is demonstrated in the excellent repeatability and within-laboratory reproducibility obtained for the compounds which have a corresponding deuterated analogue. No deuterated analogues were available for a number of these compounds and the precision values estimated for these were in the majority of cases worse than those observed for analytes with

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3 deuterated analogues. The precision of the method, expressed as RSD values for the
4 within-lab reproducibility at the three levels of fortification can be seen in Table 2.
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10 *CC α and CC β*

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12 The decision limit is defined as the limit above which it can be concluded with an
13 error probability of α , that a sample contains the analyte. In general, for prohibited
14 substances an α value equal to 1 % is applied. The detection capability is the smallest
15 content of the substance that may be detected, identified and quantified in a sample,
16 with a statistical certainty of $1-\beta$, where $\beta = 5\%$. $CC\alpha$ and $CC\beta$ were calculated using
17 the intercept (value of the signal, y , where the concentration, x is equal to zero) and the
18 standard error of the intercept for a set of data with 6 replicates at 3 levels on three
19 separate days. Blank muscle was fortified at 1, 1.5 and 2 $\mu\text{g kg}^{-1}$. $CC\alpha$ is the
20 concentration corresponding to the intercept + 2.33 times the standard error of the
21 intercept. $CC\beta$ is the concentration corresponding to the signal at $CC\alpha$ + 1.64 times
22 the standard error of the intercept (i.e. the intercept + 3.97 times that standard error of
23 the intercept). The values determined for both $CC\alpha$ and $CC\beta$ can be seen in Table 2.
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45 *Measurement Uncertainty*

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47 The measurement of uncertainty was estimated by taking into account the within
48 laboratory reproducibility over days 1, 2 and 3. The within laboratory reproducibility
49 was multiplied by a coverage factor of three to give an overall figure for the
50 uncertainty of the measurement. This approach of using the within laboratory
51 reproducibility as a good estimator of measurement of uncertainty is taken from the
52 SANCO/2004/2726rev1 document (SANCO 2004/2726rev1 2004). It recommends
53 using the within laboratory reproducibility and using a coverage factor of 2.33 to
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3 estimate expanded uncertainty, however as it was felt that not all the environmental
4 and other factors that could be varied over the course of the validation were examined,
5
6 it was felt that a coverage factor of 2.33 may underestimate the true uncertainty of the
7
8 method. So a value of 3 was chosen instead to give a more realistic value for the true
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10 uncertainty.
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17 ***Surveillance of synthetic and natural growth promoters in imported bovine muscle***
18
19 ***samples***
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22 ***Criteria for confirmation of residue identity***
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26
27 The analytical method was designed to be used for both the screening and
28 confirmation for 24 different natural and synthetic growth promoters. Several criteria
29 based on those laid out by the European Union (2002/657/EC), all of which must be
30 simultaneously satisfied were established for the confirmation of identity of a
31 suspected residue. The first criterion is that both precursor-product ion transitions
32 corresponding to the quantification and confirmation MRM transitions must contain
33 peaks with a relative retention time (RRT) within 2.5% of the average RRT for the
34 calibration standards under identical instrumental conditions. The second criterion is
35 that the relative ratio of the peak areas in the precursor-product ion transitions
36 corresponding to the quantification and confirmation MRM transitions must fall within
37 an acceptable range of the average ratio observed for the calibration standards
38 analysed with that batch of samples, the ranges for ion ratio deemed acceptable in
39 accordance with 2002/657/EC are shown in Table 3. The calculated concentration of
40 any analyte found in the survey samples must also be above the $CC\alpha$ determined for
41 that compound for positive identification also.
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Survey Results

A total of 102 bovine muscle samples imported from third countries into Ireland were collected over a period of three years (2007-2010). No information was available on the breed, sex, age or species of the animals from which the muscle came from. Levels of progesterone were found in a number of samples at concentrations ranging between $0.28 \mu\text{g kg}^{-1}$ and $30.30 \mu\text{g kg}^{-1}$. Levels of alpha and beta testosterone were also found in a number of samples at concentrations ranging between 0.22 to $8.63 \mu\text{g kg}^{-1}$.and 0.16 to $2.08 \mu\text{g kg}^{-1}$ respectively. Table 4 presents a breakdown of the number of samples found to contain these three substances and also presents the concentration ranges found. There were a number of other samples where the criteria for positive identification were met for progesterone, alpha testosterone and beta testosterone, however the concentrations observed were less than the determined $\text{CC}\alpha$ values in each case. There were no observed levels above $\text{CC}\alpha$ for any of the synthetic steroidal growth promoters investigated as part of this study. However in one sample; the criteria for confirmation were met with respect to dexamethasone but the calculated concentration was well below the $\text{CC}\alpha$ value determined for this compound. Figure 3 shows chromatograms of one of the survey samples which was found to contain alpha testosterone at a concentration of $0.22 \mu\text{g kg}^{-1}$ and beta testosterone at a concentration of $0.37 \mu\text{g kg}^{-1}$. The presence of progesterone, alpha testosterone and beta testosterone in tissue samples does not necessarily imply misuse of these substances, however one sample for contained a level of alpha testosterone approximately four times higher than the levels observed for the other 101 samples. The levels of progesterone found in muscle are in line with previous studies carried out in bovines with levels as high as $43.7 \mu\text{g kg}^{-1}$ being reported previously in bovine females (Hartwig et al 1997, Tsujioka

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3 et al 1992). With regard beta testosterone; the highest concentration recorded in this
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5 study was $2.08 \mu\text{g kg}^{-1}$ unfortunately no data is available on the sex of the animals in
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7 this study but levels of beta testosterone in the range of $0.2\text{-}2.8 \mu\text{g kg}^{-1}$ have been
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9 observed previously for intact males (Hartwig et al 1997) All natural levels measured
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11 were below the tolerance value of $3.2 \mu\text{g kg}^{-1}$ for free testosterone in the muscle of
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13 fully grown cattle set by the Scientific Veterinary Committee (Scientific Veterinary
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15 Committee 1989). In relation to alpha testosterone; the highest level found in this
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17 study was $8.63 \mu\text{g kg}^{-1}$, this is much higher than previously reported levels which
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19 ranged between $0.2\text{-}0.6 \mu\text{g kg}^{-1}$ (Hartwig et al 1997).
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26 **Conclusions**

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28 The aim of this work was to develop and validate a rapid multi-residue LC-MS/MS
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30 confirmatory method that can simultaneously identify and quantify and quantify a
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32 large number of natural and prohibited steroidal type growth promoters in bovine
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34 muscle samples. This goal was successfully achieved with satisfactory results in terms
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36 of sensitivity, accuracy, linearity and repeatability being observed for all of the 24
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38 analytes under investigation. The calculated $CC\alpha$ values were less than $0.28 \mu\text{g kg}^{-1}$ in
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40 all cases except for hexestrol where the calculated $CC\alpha$ value was $0.46 \mu\text{g kg}^{-1}$.
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45 The developed method was successfully used to carry out a small surveillance study
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47 into the presence of these 24 analytes in 102 samples of bovine muscle imported into
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49 Ireland from third countries over a period of three years. Application of the method
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51 demonstrated that it was capable of detecting the presence at very low levels of the
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53 naturally occurring steroids; progesterone, alpha testosterone and beta testosterone in a
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55 variety of these samples.
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3 Figure 1 LC-MS/MS chromatograms (weak transition) of a blank muscle sample
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5 fortified with each of the analytes which ionize in ESI - at a level of $1 \mu\text{g kg}^{-1}$.
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10 Figure 2 LC-MS/MS chromatograms (weak transition) of a blank muscle sample
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12 fortified with each of the analytes which ionize in ESI + at a level of $1 \mu\text{g kg}^{-1}$.
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17 Figure 3 LC-MS/MS chromatograms of one of the survey samples which was found to
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19 contain alpha testosterone at a concentration of $0.22 \mu\text{g kg}^{-1}$ and beta testosterone at a
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21 concentration of $0.37 \mu\text{g kg}^{-1}$.
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Compound	Transition	Polarity	Collision Energy (eV)	Retention Time (min)	Internal Standard
Medroxyprogesterone Acetate	387>123*	ESI+	39	7.78	d ₃ MPA
	387>327		20		
Melengestrol Acetate	397>279*	ESI+	28	7.85	d ₃ MLA
	397>337		20		
Megestrol Acetate	385>267*	ESI+	26	7.67	d ₃ MGA
	385>224		38		
Delmadinone Acetate	403>205*	ESI+	27	7.42	d ₃ MLA
	403>181		29		
Chlormadinone Acetate	405>345*	ESI+	18	7.70	d ₃ MLA
	405>309		21		
Methylboldenone	301>149*	ESI+	21	6.35	d ₃ MBOLD
	301>121		31		
Methyltestosterone	303>97*	ESI+	35	7.06	d ₃ MTEST
	303>109		28		
Fluoxymesterone	337>281*	ESI+	30	6.05	d ₃ MTEST
	337>241		33		
alpha Trenbolone	271>253*	ESI+	27	6.14	d ₃ BTREN
	271>199		32		
beta Trenbolone	271>253*	ESI+	27	5.87	d ₃ BTREN
	271>199		32		
16 beta hydroxy Stanozolol	345>95*	ESI+	53	6.5	d ₃ 16STAN
	345>81		76		
Progesterone	315>109*	ESI+	27	7.80	d ₉ PROG
	315>97		31		
alpha Testosterone	289>109*	ESI+	39	7.43	d ₃ BTEST
	289>97		33		
beta Testosterone	289>109*	ESI+	39	6.61	d ₃ BTEST
	289>97		33		
alpha Boldenone	287>121*	ESI+	64	6.71	d ₃ BBOLD
	287>135		46		
beta Boldenone	287>121*	ESI+	30	6.06	d ₃ BBOLD
	287>135		20		
alpha Nortestosterone	275>109*	ESI+	39	6.83	d ₃ BNORT
	275>239		25		
beta Nortestosterone	275>109	ESI+	39	6.18	d ₃ BNORT
	275>239		25		
Taleranol	321>277*	ESI-	28	5.67	d ₄ TAL
	321>259		30		
Hexestrol	269>119*	ESI-	55	6.74	d ₆ DES
	269>134		20		
Diethylstilbestrol	267>237*	ESI-	38	4.22	d ₆ DES
	267>222		44		
Flumethasone	379>305*	ESI-	31	5.58	d ₄ DEXA
	379>325		24		
Dexamethasone	361>292*	ESI-	29	5.76	d ₄ DEXA
	363>307		25		
Zeranol	321>277*	ESI-	28	6.19	d ₄ ZER
	321>259		30		

* Indicates the most abundant transition; which is used for quantitation

Table 1: MS/MS Parameters for all investigated analytes.

Compound	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	R.S.D. (%)	M.U. (%)	Accuracy (%)
Medroxyprogesterone Acetate	0.12	0.20	4.4	13.2	101.7
Melengestrol Acetate	0.13	0.22	4.8	14.4	100.1
Megestrol Acetate	0.13	0.22	5.3	15.9	100.4
Delmadinone Acetate	0.17	0.29	6.5	19.5	98.3
Chlormadinone Acetate	0.17	0.30	6.4	19.2	99.5
Methylboldenone	0.16	0.28	6.3	18.9	100.7
Methyltestosterone	0.11	0.19	4.0	12	98.7
Fluoxymesterone	0.20	0.34	7.4	22.2	101.7
alpha Trenbolone	0.16	0.27	6.1	18.3	100.1
beta Trenbolone	0.13	0.22	4.7	14.1	101.5
16 beta hydroxy Stanozolol	0.17	0.29	7.3	21.9	99.3
Progesterone	0.23	0.39	8.8	26.4	96.8
alpha Testosterone	0.20	0.34	7.6	22.8	102.6
beta Testosterone	0.16	0.27	5.7	17.1	99.7
alpha Boldenone	0.15	0.25	6.6	19.8	100.6
beta Boldenone	0.11	0.19	4.5	13.5	100.0
alpha Nortestosterone	0.17	0.29	6.0	18.0	100.7
beta Nortestosterone	0.16	0.28	6.2	18.6	102.2
Taleranol	0.12	0.21	5.8	17.4	99.8
Hexestrol	0.46	0.79	11.8	35.4	94.6
Diethylstilbestrol	0.28	0.48	9.0	27.0	102.9
Flumethasone	0.17	0.29	6.0	18	101.8
Dexamethasone	0.13	0.21	4.8	14.4	100.1
Zeranol	0.14	0.23	5.5	16.5	99.1

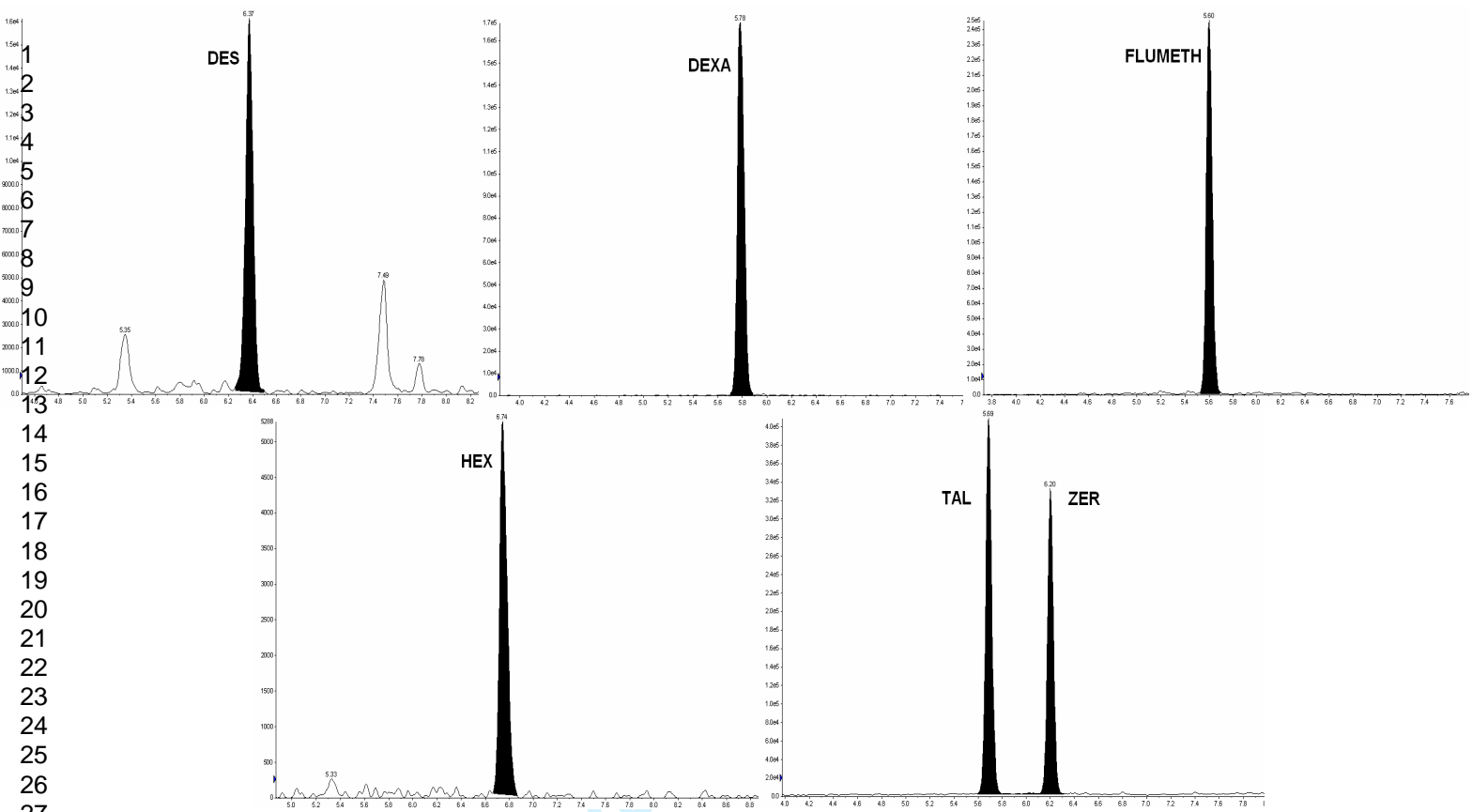
Table 2: CC α , CC β , Inter-assay RSD (%), Measurement of Uncertainty (%) and Accuracy (%) values for each of the analytes investigated.

Relative Intensity (% of base peak)	Relative Maximum Tolerance (%)
	Technique: LC-MS/MS
>50	±20
20-50	±25
10-20	±30
≤10	±50

Table 3: Maximum permitted tolerances for ion ratio values for LC-MS/MS analysis according to Commission Decision 2002/657/EC.

Compound	Concentration Range				
	<i>0.2-1 $\mu\text{g kg}^{-1}$</i>	<i>1-5 $\mu\text{g kg}^{-1}$</i>	<i>5-10 $\mu\text{g kg}^{-1}$</i>	<i>10-20 $\mu\text{g kg}^{-1}$</i>	<i>>20 $\mu\text{g kg}^{-1}$</i>
Progesterone	25	4	12	3	4
Alpha Testosterone	29	4	1	0	0
Beta Testosterone	18	4	0	0	0

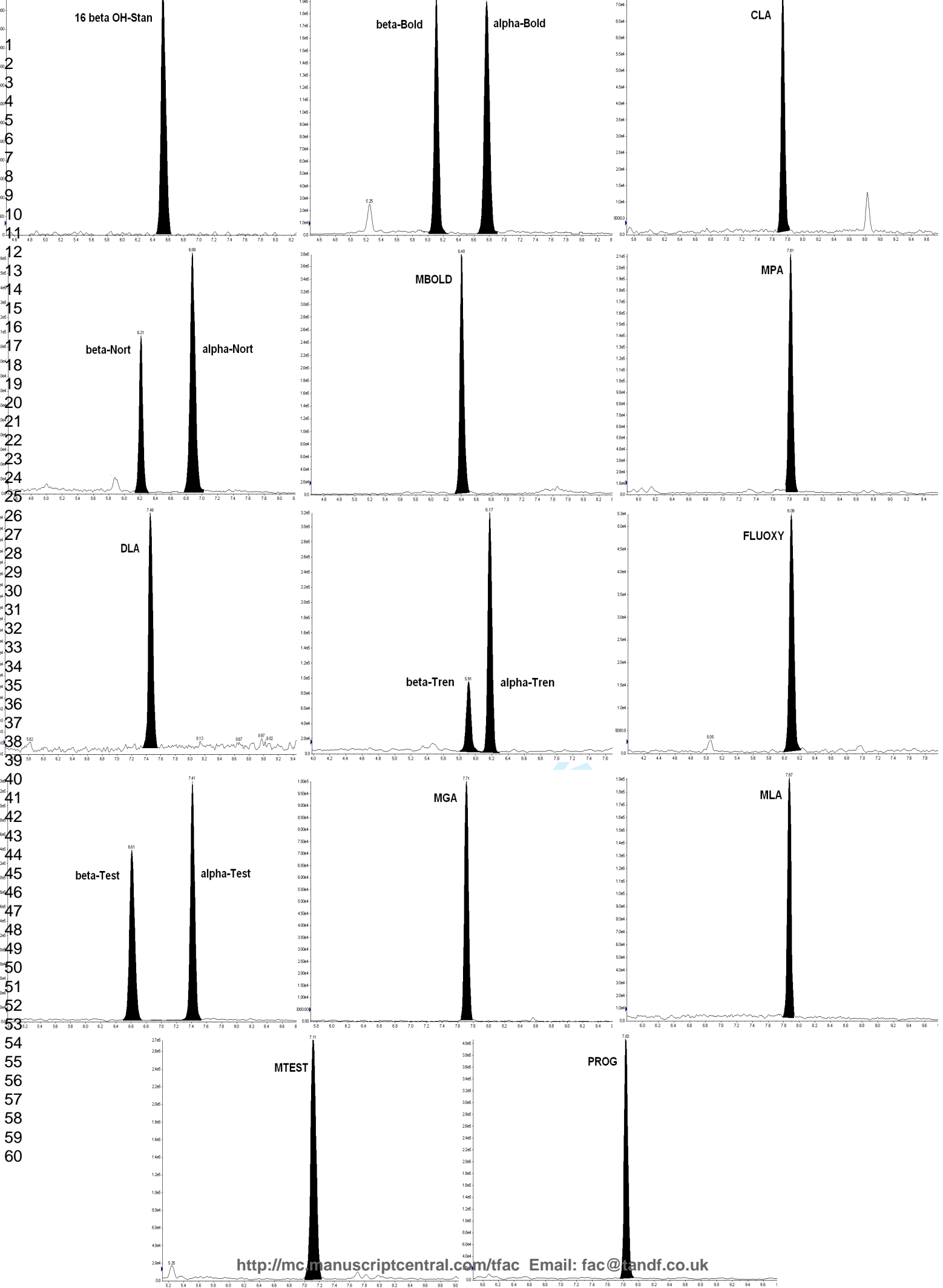
Table 3: Number of survey samples which contained concentrations of Progesterone and alpha and beta Testosterone in the ranges $0.2-1 \mu\text{g kg}^{-1}$, $1-5 \mu\text{g kg}^{-1}$, $5-10 \mu\text{g kg}^{-1}$, $10-20 \mu\text{g kg}^{-1}$ and $>20 \mu\text{g kg}^{-1}$.



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Food Additives and Contaminants



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