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Rapid confirmatory method for the determination of sixteen synthetic growth promoters and bisphenol A in bovine milk using dispersive solid-phase extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid liquid chromatographic–tandem mass spectrometric (LC–MS/MS) multi-residue method for the simultaneous quantitation and identification of sixteen synthetic growth promoters and bisphenol A in bovine milk has been developed and validated. Sample preparation was straightforward, efficient and economically advantageous. Milk was extracted with acetonitrile followed by phase separation with NaCl. After centrifugation, the extract was purified by dispersive solid-phase extraction with C18 sorbent material. The compounds were analysed by reversed-phase LC–MS/MS using both positive and negative ionization and operated in multiple reaction monitoring (MRM) mode, acquiring two diagnostic product ions from each of the chosen precursor ions for unambiguous confirmation. Total chromatographic run time was less than 10 min for each sample. The method was validated at a level of 1 $\mu g\,L^{-1}$. A wide variety of deuterated internal standards were used to improve method performance. The accuracy and precision of the method were satisfactory for all analytes. The confirmative quantitative liquid chromatographic tandem mass spectrometric (LC–MS/MS) method was validated according to Commission Decision 2002/657/EC. The decision limit (CCα) and the detection capability (CCβ) were found to be below the chosen validation level of 1 $\mu g\,L^{-1}$ for all compounds.

1. Introduction

Steroid type growth promoters have been extensively used for many decades in animal husbandry with effects such as growth promotion and improvements in feed conversion efficiency. However, because of possible harmful effects on public health [1], their use in food producing animals has been prohibited by the European Union by Council Directive 96/22/EC [2]. A lot of these substances are known endocrine disrupting chemicals and exposure even at low levels presents potential risks to both humans and wildlife. They have been demonstrated to be associated with many diseases such as breast cancer and uterine cancer in humans and hermaphroditism in wildlife [3,4].

Within the EU, monitoring for illicit use of these substances is carried out in accordance with EU Directive 96/23/EC which is implemented through surveillance according to the National Residue Control Plans for each member state [5]. For control at retail level, as well as for imported products however only edible matrices are available for testing such as muscle, fat and milk. There are quite a number of methods detailed in the literature for the analysis of these types of substances in muscle and fat [6–19]. However, as pointed out by Noppe et al. in 2008, it was surprising that very few papers have been published within the framework of residue analysis that address the determination of steroid hormones in milk [20].

Another known endocrine disrupting chemical which is similar in action to the estrogenic steroids and has become a cause of increasing concern in recent times is bisphenol A. It is widely used in the production of numerous resins as well as for the production of polycarbonate plastics and flame retardants [21,22]. Polycarbonate plastics are used in food and drink packaging; resins are used as lacquers to coat metal products such as food cans, bottle tops and milk containers. The migration of BPA from epoxy coated can surfaces, polycarbonate plastics, and PVC products into food has been reported [23–33]. Adverse effects due to exposure to this substance are similar to those detailed earlier for steroid type growth

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promoters. The European Food Safety Authority (EFSA) published its risk assessment for bisphenol A in 2007 and calculated a TDI for BPA of 0.05 mg (kg bodyweight)$^{-1}$ day^{-1} [34]. However further concern has been raised due to the possible adverse effects due to leaching of BPA from milk packaging into children's drinks.

As highlighted previously few methods cover the topic of the analysis steroidal growth promoters in milk [20]. Of the methods that do exist, it seems LC–MS and LC–MS/MS have become the determination methods of choice, due to the wide range of analytes that can be analysed with this technique and also the advantages offered in both selectivity and sensitivity. An article published in 2003 focused on the determination of nine phytoestrogens in bovine milk using LC–MS/MS [35]. Milk samples were extracted with acetone and purified by SPE prior to analysis by LC–MS/MS. The method was capable of reaching levels of 1 μg L^{-1} for each phytoestrogen investigated. In 2005 a method was published for the identification of 11 steroids both natural and synthetic in bovine milk [36]. Samples were hydrolysed overnight prior to extraction with methanol and purification with a hexane wash followed by SPE. The method was capable of reaching levels of determination below 1 μg L^{-1}. In 2006 a paper was published which focused on the determination of three stilbenes compounds in bovine milk using supported liquid membrane as the sample preparation method [37]. Milk samples were extracted with a mixture of methanol and 1% acetic acid before undergoing purification using a supported liquid membrane. The compounds were analysed by LC–MS and the method was able to reach limits of detection of less than 0.05 μg L^{-1}. A method was published in 2009 detailing the analysis of a number of estrogens as well as bisphenol A in bovine milk using an automated online solid-phase extraction followed by LC–MS [38]. The method was capable of detecting 5 estrogens including diethylstilbestrol and ethynylestradiol as well as bisphenol A to levels as low as 0.05 μg L^{-1}. In 2009, Xia et al. published a method for the determination of 6 resorcylic acid lactones in bovine milk using LC–MS/MS [39]. Acetonitrile was added to milk samples to precipitate proteins; the resulting mixture was then applied to an anion exchange SPE cartridge. Analysis was carried out using fast chromatography with tandem mass spectrometry; limits of detection for this method were in the order of 0.05 μg L^{-1}. A comprehensive method for the analysis of 50 anabolic hormones in milk by LC–MS/MS was published in 2009 by Yi Yang et al. [40]. The method involved overnight hydrolysis followed by extraction with methanol before purification by dual SPE. The limits of detection were in the range from 0.04 to 2.0 μg L^{-1}. With regard the analysis of bisphenol A, a number of papers have been published for the determination of this compound in milk using LC–MS/MS. These involved a variety of purification steps such as solid-phase extraction which gave an LOD of 0.7 μg L^{-1} [41] matrix solid-phase dispersive extraction yielding an LOD of 0.1 μg L^{-1} [42]. Other purification steps have also been employed such as molecular imprinted polymers giving an LOD of 0.2 μg L^{-1} [43] and automated on-line solid-phase extraction which gave LOD's of 1.0 μg L^{-1} and 0.3 μg L^{-1} respectively for BPA [44,45]. Also as mentioned earlier a method was published for the detection of bisphenol A along with a number of estrogens by LC–MS/MS using online solid-phase extraction, this gave an LOD of 0.2 μg L^{-1} for BPA [38].

The aims of the work in this study were to develop a multi-residue confirmatory method for the detection of a wide range of growth promoters as well as bisphenol A that could be carried out in a time and cost efficient manner and also only require a small initial sample size. Only synthetic growth promoters were considered for investigation in this study due to the problem with discerning whether non-compliant results for naturally occurring steroids arose from exogenous or endogenous sources.

2. Experimental

2.1. Materials and reagents

Water, methanol, and acetonitrile (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Ammonium acetate and sodium chloride (ACS grade) were obtained from Sigma (St. Louis, MO, USA). Bondesil C18, 40 μm, sorbent material was obtained from Varian Inc. (JVA Analytical, Dublin, Ireland). d_3 16-beta-OH Stanozolol, alpha trenbolone, beta trenbolone and d_3 beta trenbolone, medroxyprogesterone acetate, megestrol acetate, melengestrol acetate, methylboldenone, chlormadinone acetate, delamadinone acetate d_3 medroxyprogesterone acetate, d_3 methylboldenone, d_3 megestrol acetate, d_3 melengestrol acetate, d_3 methyltestosterone, d_2 dienestrol, d_4 hexestrol, d_6 diethylstilbestrol, d_3 estradiol all were from RIVM (Bilthoven, The Netherlands). Hexestrol, dienestrol, diethylstilbestrol, flumethasone, bisphenol A, d_{16} bisphenol A (98%), ethynylestradiol and fluoxymesterone were from Sigma–Aldrich (St. Louis, MO, USA). 16-beta-OH Stanozolol was from LGC Promochem (Teddington, UK) and d_4 dexamethasone (97%) was from QMX Laboratories (Thaxted, UK).

Primary stock standard solutions of each analyte were prepared in methanol at a concentration of 100 μg mL^{-1}. Intermediate single standards solutions each analyte were prepared in methanol at a concentration of 10 μg mL^{-1}. A mixed standard fortification solution which included each analyte was prepared in methanol at a concentration of 50 ng mL^{-1}. A mixed standard fortification solution including each internal standard was prepared in methanol at a concentration of 250 ng mL^{-1}. All standards were stored at −20 °C. The injection solvent consisted of 0.5 mM Ammonium Acetate in methanol:water (30:70, v/v).

2.2. LC conditions

The LC system was a Shimadzu UFLC-XR equipped with a LC-20AD-XR Binary pump, SIL-20AD-XR autosampler and a CTO-20A column oven (Shimadzu, Dublin, Ireland). The compounds were chromatographed on a 1.8 μm Agilent Eclipse Plus C18 column (2.1 mm × 50 mm) (Agilent, Dublin, Ireland) and the column temperature was maintained at 55 °C. A gradient LC system using 0.5 mM ammonium acetate:methanol (70:30, v/v Mobile Phase A) and ammonium acetate:methanol (5:95, v/v Mobile Phase B) at a flow of 0.5 mL min^{-1} was operated. The gradient profile began at 75% A held for 1 min before changing to 50% A 2 min later and changing to 25% A and held for 1 min before returning to 75% A after 1 min and left to equilibrate for 2.5 min. The total runtime of the method was 9.5 min. Data acquisition and integration were performed using Analyst software version 1.5 (Applied Biosystems/MDS Sciex, Canada).

2.3. MS/MS parameters

The mass spectrometer system used was a 5500 triple qaudrupole instrument (Applied Biosystems/MDS Sciex, Canada). The analysis was performed using both positive and negative ion electrospray (ESI) and the MS was operated in multiple reaction monitoring (MRM) mode. Two transitions per analyte were monitored whereas only one transition was monitored for each deuterated internal standard. The precursor product transitions for each analyte as well as their corresponding collision energies are shown in Table 1. The MS/MS detector conditions were as follows: ion spray voltage 1500 V; ion source gas 1 50 psi; ion source gas 2 60 psi; temperature 650 °C; curtain gas 15 psi; collision gas pressure 8; entrance potential 10.

Table 1
MS/MS parameters for all investigated analytes.

Compound	Transition	Polarity	Collision energy (eV)	Retention time (min)	Internal standard
Medroxyprogesterone acetate	387 > 123[a] 387 > 327	ESI+	39 20	5.27	d_3 MPA
Melengestrol acetate	397 > 279[a] 397 > 337	ESI+	28 20	5.34	d_3 MLA
Megestrol acetate	385 > 267[a] 385 > 224	ESI+	26 38	5.15	d_3 MGA
Delmadinone acetate	403 > 205[a] 403 > 181	ESI+	27 29	4.89	d_3 MLA
Chlormadinone acetate	405 > 345[a] 405 > 309	ESI+	18 21	5.18	d_3 MLA
Methylboldenone	301 > 149[a] 301 > 121	ESI+	21 31	4.03	d_3 MBOLD
Methyltestosterone	303 > 97[a] 303 > 109	ESI+	35 28	4.61	d_3 MTEST
Fluoxymesterone	337 > 281[a] 337 > 241	ESI+	30 33	3.67	d_3 MTEST
Alpha trenbolone	271 > 253[a] 271 > 199	ESI+	27 32	3.77	d_3 BTREN
Beta trenbolone	271 > 253[a] 271 > 199	ESI+	27 32	3.43	d_3 BTREN
16 Beta hydroxy Stanozolol	345 > 95[a] 345 > 81	ESI+	53 76	4.18	d_3 16STAN
Dienestrol	265 > 235[a] 265 > 93	ESI−	30 34	4.23	d_2 DIEN
Hexestrol	269 > 119[a] 269 > 134	ESI−	55 20	4.37	d_4 HEX
Diethylstilbestrol	267 > 237[a] 267 > 222	ESI−	38 44	4.04	d_6 DES
Ethynylestradiol	295 > 143[a] 295 > 145	ESI−	64 46	3.97	d_3 EthEs
Flumethasone	379 > 305[a] 379 > 325	ESI−	31 24	3.00	d_4 DEXA
Bisphenol A	227 > 133[a] 227 > 212	ESI−	32 24	3.05	d_{16} BPA
d_3 Medroxyprogesterone acetate	390 > 330	ESI+	20	5.26	NA
d_3 Melengestrol acetate	400 > 340	ESI+	20	5.33	NA
d_3 Megestrol acetate	388 > 270	ESI+	26	5.14	NA
d_3 Methylboldenone	304 > 121	ESI+	31	4.01	NA
d_3 Methyltestosterone	306 > 97	ESI+	40	4.59	NA
d_3 Beta trenbolone	274 > 256	ESI+	28	3.40	NA
d_3 16 beta hydroxy Stanozolol	348 > 81	ESI+	84	4.17	NA
d_2 Dienestrol	267 > 237	ESI−	38	4.22	NA
d_4 Hexestrol	273 > 121	ESI−	55	4.35	NA
d_6 Diethylstilbestrol	273 > 237	ESI−	38	4.02	NA
d_3 Estradiol	274 > 145	ESI−	50	3.86	NA
d_4 Dexamethasone	363 > 309	ESI−	31	3.22	NA
d_{16} Bisphenol A	241 > 142	ESI−	34	2.96	NA

[a] Indicates the most abundant transition; which is used for quantitation.

2.4. Negative control milk

Bovine milk was obtained and stored at −20 °C in 50 mL polypropylene centrifuge tubes. Samples of this milk were analysed and those found to contain no detectable residues of the analytes of interest were used as negative controls to carry out development and validation.

2.5. Sample extraction and purification

Milk samples (1 mL) were weighed into 15 mL polypropylene tubes. Samples were fortified with mixed internal standard at a level corresponding to 5 μg L^{-1} by adding 20 μL portions of the mixed internal standard fortification solution. Samples were fortified at levels corresponding to 1, 1.5 and 2.0 times the chosen validation level of 1 μg L^{-1} for each of the investigated analytes by adding appropriate amounts of the mixed standard fortification solution. After fortification, samples were held for 10 min. Acetonitrile (2 mL) was added to each tube followed by sodium chloride (0.5 g). The samples were then shaken vigorously for 30 s and centrifuged (4000 × *g*, 15 min, 12 °C). The acetonitrile (top layer) was then removed and transferred to 2.5 mL microcentrifuge tubes. To these tubes C18 sorbent material (50 mg) was then added and the tubes were then vortexed vigorously for 30 s, followed by centrifugation (16,000 × *g*, 20 min, 12 °C). The extracts were then centrifuged (16,000 × *g*, 10 min, 12 °C) and the supernatant

removed and evaporated to dryness (50 °C) under nitrogen. The samples were finally reconstituted in water: methanol (70:30, v/v) (150 μL) and transferred to LC vials for LC–MS/MS analysis. An aliquot (15 μL) was injected into the LC–MS/MS system.

2.6. Matrix-matched calibration

Matrix matched calibration curves were prepared and used for quantification. One milk sample was used for each calibration standard level. Samples were fortified at levels corresponding to 0, 1, 2, 5, 7.5 and 10 μg L^{-1} by adding 0, 20, 40, 100, 150 and 200 μL aliquots of a 50 ng mL^{-1} standard solution. After fortification, samples were held for 10 min prior to extraction as above (2.5).

Calibration curves were prepared by plotting the response factor (peak area analyte/internal standard peak area) as a function of analyte concentration (0–10 μg L^{-1}).

2.7. Method validation

For estimation of accuracy, blank bovine milk samples were fortified with each analyte at 1, 1.5 and 2.0 μg L^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. On a fourth occasion in order to determine any effects caused by different types of milk, samples from a variety of sources were analysed. On the fourth occasion the samples were analysed in duplicate, initially only fortified with internal standard, and then fortified with both internal standard and the analytes of interest at a concentration equivalent to 1 μg L^{-1}. For the estimation of the precision of the method, intra-assay and inter-assay repeatability was calculated. The decision limit (CCα) of the method were calculated according to the calibration curve procedure using the intercept (value of the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα.

3. Results and discussion

3.1. Preliminary experiments

The MS/MS method was developed to provide confirmatory data for the analysis of bisphenol A and sixteen different synthetic growth promoters from a variety of classes including androgens, estrogens, stilbenes, gestagens and corticosteroids. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound. For a method to be deemed confirmatory under CD 2002/657/EC [46] it must yield 4 identification points. In this method a precursor ion (parent mass) and two fragments (corresponding to strong and weak ion) are monitored for each analyte (Table 1). This yields 4 identification points (1 for the precursor ion and 1.5 for each fragment ion), hence it can be deemed a confirmatory method. LC–MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times and ion ratios. In all instances these were within the tolerances set out laid in Commission Decision 2002/657/EC.

3.1.1. Extraction and purification

Deconjugation of milk samples by overnight hydrolysis using an enzyme such as helix pomatia was not used for this method. Previously this step has been shown to be unnecessary; as the majority of the steroids in milk exist in the free form [38,40,47,48]. In order to make the method more time and more cost efficient a sample size of only 1 mL of milk was used. This volume is much lower than volumes used in previously reported methods in this area where volumes of 5 or 10 times this amount are traditionally used [36,37,40] resulting in a lot more organic solvents being required for extraction and more time being consumed for sample transfer and concentration steps.

Table 2
Comparison of intensities observed for each compound analysed with and without the use of dispersive SPE purification step.

Compound	Intensity observed (area of peak)		Difference (%)
	No C18 SPE	With C18 SPE	
Medroxyprogesterone Acetate	694,800	2,812,000	+304
Melengestrol Acetate	922,800	6,346,000	+588
Megestrol Acetate	1,382,600	5,329,000	+285
Delmadinone Acetate	834,800	3,241,000	+288
Chlormadinone Acetate	1,083,100	3,457,000	+219
Methylboldenone[a]	NA	NA	NA
Methyltestosterone	3,355,000	5,828,000	+73
Fluoxymesterone	3,128,000	2,986,000	−5
alpha Trenbolone	5,898,000	7,563,000	+28
beta Trenbolone	1,813,500	2,871,000	+58
16 beta hydroxy Stanozolol	1,895,000	1,815,600	−4
Dienestrol	354,000	2,540,000	+618
Hexestrol	349,830	1,176,500	+236
Diethylstilbestrol	254,800	1,827,900	+617
Ethynylestradiol[a]	NA	NA	NA
Flumethasone	6,575,000	6,149,000	−6
Bisphenol A	607,700	1,254,900	+106

[a] These compounds were not included at this stage of the study.

Due to the wide variety of compounds being investigated and the differences in extraction profile for each of these, a very simple extraction and purification procedure was necessary as most complex purification techniques would inevitably lead to losses of analyte. Initially a procedure which had previously been developed in our laboratory for the analysis of both non-steroidal anti-inflammatory drugs as well as some corticosteroids in milk was investigated [49]. This involved extraction of milk with acetonitrile followed by phase separation using sodium chloride. This method appeared to extract all the analytes of interest; however some interferences were noted in the transitions for some of the analytes such as methyltestosterone. Also significant ion suppression was noted for particular analytes. An approach taken by Kinsella et al. to purification of both milk and liver for the analysis of anthelmintics was investigated [50]. Their method involved extraction of milk (5 mL) with acetonitrile followed by partitioning with both sodium chloride and magnesium sulphate. Their extract then underwent dispersive solid-phase extraction using 200 mg of sorbent material before analysis by LC–MS/MS. This approach was modified in our laboratory by omitting the addition of magnesium sulphate and also using a smaller amount of C18 sorbent material (50 mg), as the initial sample of milk we used was much smaller (1 g). A lot of the interferences observed in milk samples extracted without C18 purification were removed by the use of this dispersive SPE step. This is illustrated for the case of methyltestosterone (Fig. 1) where chromatograms are shown for a milk sample fortified at a level of 2 μg L^{-1} and extracted with and without the use of the dispersive SPE step. The chromatograms shown for milk samples purified with C18 material show the absence of a lot matrix peaks which are evident in milk samples extracted without the use of C18 material. Also as shown in Table 2, significant improvement can be seen in the signal intensity observed for the majority of analytes when comparing intensities observed for samples purified with and without C18 sorbent material. This is most probably due to the removal of matrix components which may have been causing ion suppression.

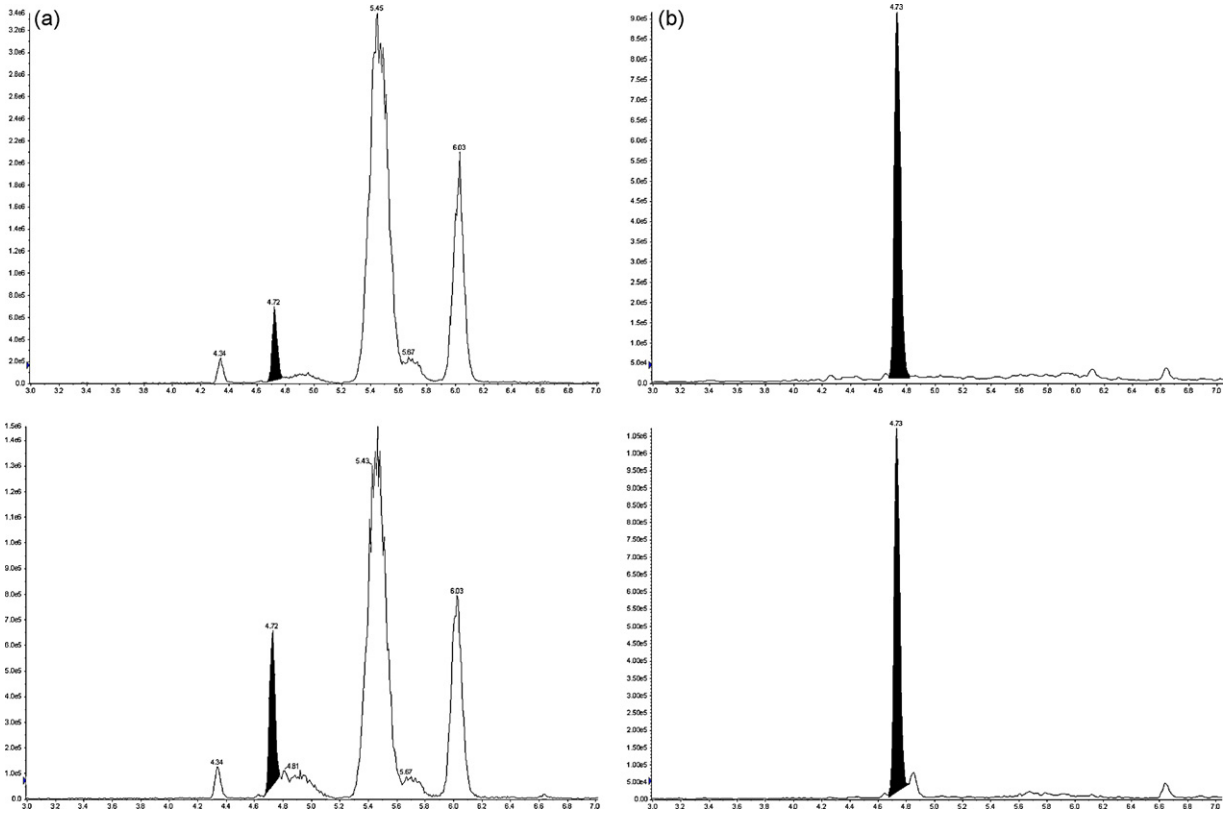

Fig. 1. Chromatograms showing methyltestosterone transitions (strong and weak) in a milk sample fortified at a level of 2 μg L^{-1} and (a) analysed without dispersive SPE purification step and (b) with the use of dispersive SPE purification step.

3.1.2. Optimisation of LC and MS parameters

As efficiency was one of the primary objectives in developing the method it was necessary to optimise the speed of the determination step. Ultra high pressure chromatography was used which resulted in shorter chromatographic run times. Run times of less than 5 min were possible; however this led to co-elution of some peaks which shared common transitions. This problem was evident for both alpha and beta trenbolone and especially diethylstilbestrol and d_2 dienestrol. Hence the LC conditions were altered to allow for baseline resolution of these peaks but still maintaining a runtime of less than 10 min. Another feature which can reduce the efficiency of the method is the necessity of making two injections for each sample [13]. This may be necessary in the situation where some analytes are ionized in positive polarity and others in negative polarity but due to the narrow peak widths and slow positive–negative switching times of the available instrument insufficient data points are generated for reliable quantitation. However, for this method an instrument was chosen which could rapidly switch between positive and negative polarities (50 ms switching time) allowing for sufficient data points to be generated for all of the peaks of interest.

3.2. Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [46] covering specificity, calibration curve linearity, recovery (accuracy), repeatability, decision limit (CCα) and detection capability (CCβ), measurement of uncertainty was also examined.

3.2.1. Specificity

The technique of LC–MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, milk samples were fortified with the sixteen analytes and the internal standards and non-fortified samples were also analysed. On each of Days 1, 2 and 3; milk samples from 3 different sources were examined and on Day 4, a variety of milk samples from 10 different sources were examined. No interfering peaks were observed at the retention time for any of the transitions. Traces of bisphenol A were seen in one of the milk samples analysed on Day 4 but the calculated concentration was considerably less than the 1 μg L^{-1}. Figs. 2 and 3 show chromatograms of each analyte fortified at a level of 1 μg L^{-1}.

3.2.2. Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using 6 calibration points in the concentration range of 0 to 10 μg L^{-1}. The regression coefficients (r^2) for all the calibration curves used in this study were ≥0.990.

3.2.3. Accuracy

The accuracy (trueness) of the method was determined using bovine milk samples fortified at 1.0, 1.5 and 2 μg L^{-1} for each analyte. Mean corrected recovery (n = 18) of the analytes, determined in four separate assays is shown in Table 3. The values ranged between 95 and 106% for the seventeen analytes. As the method is performed utilising internal standards no absolute recovery values were required for quantitation, as each sample was individually corrected.

3.2.4. Repeatability

The values attained for the inter-assay precision are shown in Table 3. Relatively low repeatability estimates were achieved for the majority of compounds investigated. The main reason for this can be attributed to the availability of thirteen deuterated analogues of the compounds being examined. For those

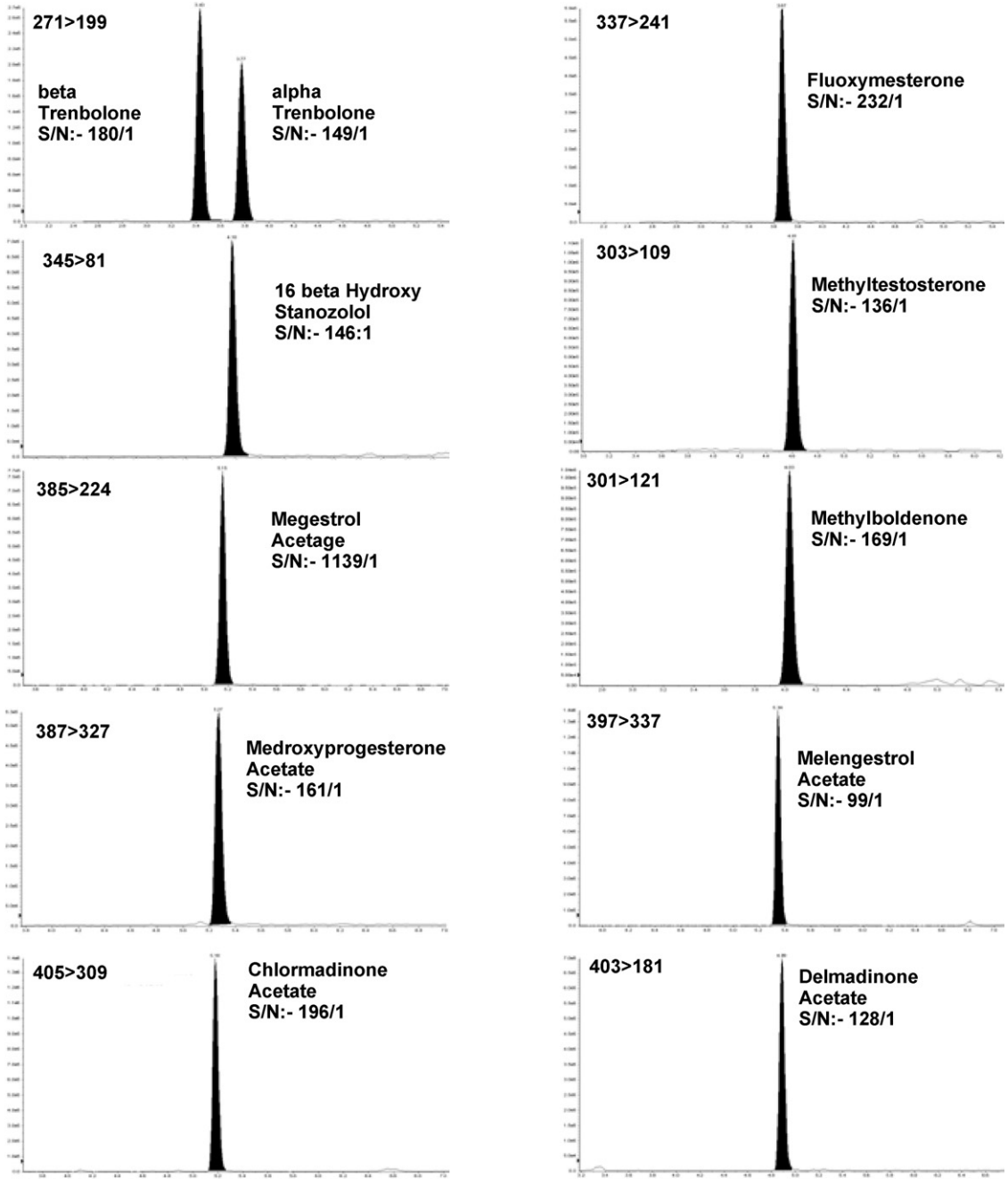

Fig. 2. LC–MS/MS chromatograms (weak transition) of a blank milk sample fortified with each of the analytes which ionize in ESI+ at a level of 1 $\mu g\,L^{-1}$.

compounds without a deuterated analogue namely alpha trenbolone, flumethasone, fluoxymesterone; delmadinone acetate, chlormadinone acetate, ethynylestradiol; d_3 beta trenbolone, d_4 dexamethasone, d_3 methyltestosterone, d_3 melengestrol acetate and d_3 estradiol were used respectively and these corrected reasonably well for any analytical losses or matrix suppression of these compounds.

3.2.5. CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. For prohibited substances an α value equal to 1% is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1-\beta$, were $\beta = 5\%$. CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (1, 1.5 and 2 $\mu g\,L^{-1}$). Blank milk was fortified at 1, 1.5 and 2 times the validation level of 1 $\mu g\,L^{-1}$ for each analyte; 1 $\mu g\,L^{-1}$ for each compound has been used for the method validation in this work as this is the level suggested by the Community Reference Laboratory in RIVM for most of these analytes in other matrices. CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CCα values of for all seventeen compounds are listed in Table 3 and are all below 0.3 $\mu g\,L^{-1}$. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times that standard

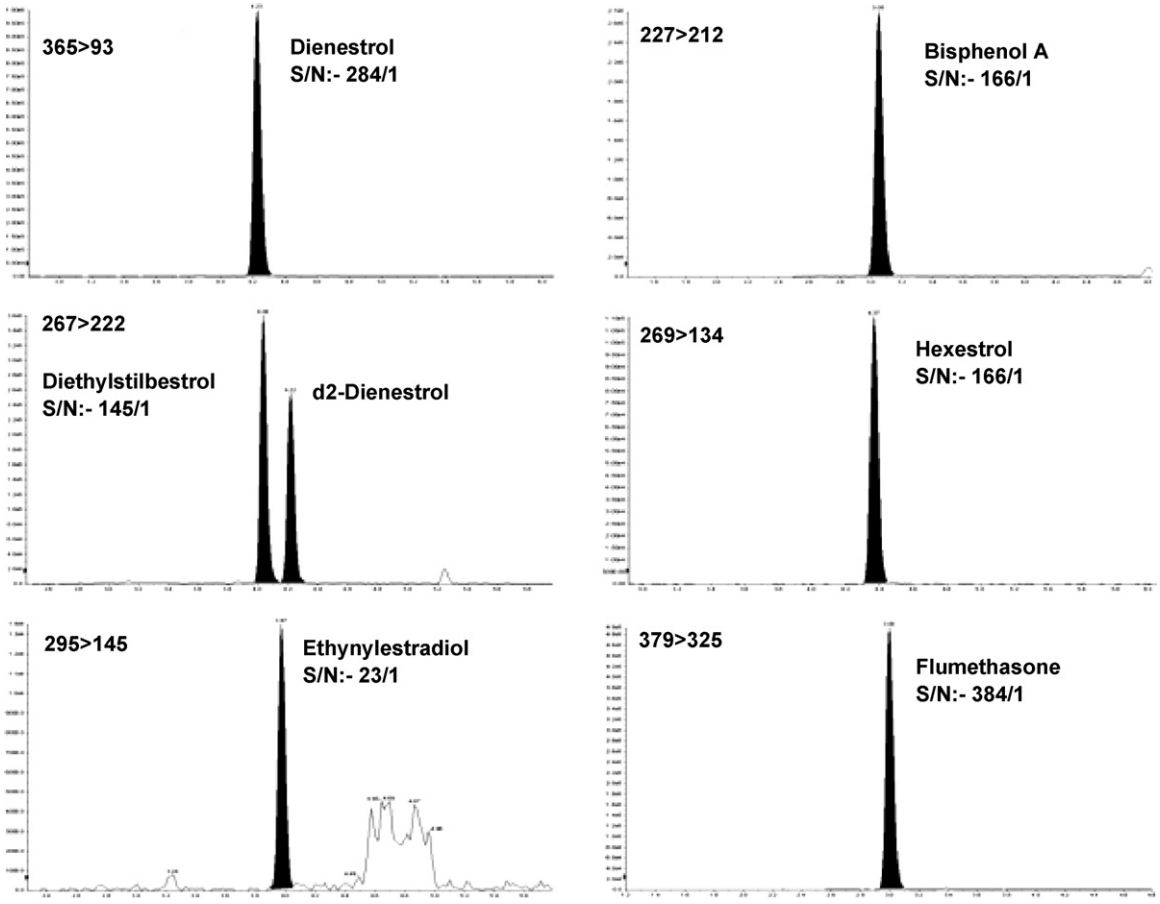

Fig. 3. LC–MS/MS chromatograms (weak transition) of a blank milk sample fortified with each of the analytes which ionize in ESI− at a level of 1 $\mu g\,L^{-1}$.

Table 3
CCα, CCβ, Inter-assay RSD (%), measurement of uncertainty (%) and accuracy (%) values for each of the analytes investigated.

Compound	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)	RSD (%)	M.U. (%)	Accuracy (%)
Medroxyprogesterone acetate	0.16	0.28	7.3	35.6	106.0
Melengestrol acetate	0.16	0.28	5.9	34.1	104.3
Megestrol acetate	0.14	0.24	6.9	20.6	101.7
Delmadinone acetate	0.22	0.38	13.9	41.7	104.0
Chlormadinone acetate	0.16	0.28	12.2	36.7	98.3
Methylboldenone	0.16	0.27	5.9	34.7	101.2
Methyltestosterone	0.16	0.27	6.4	19.4	102.1
Fluoxymesterone	0.28	0.48	14.3	72.6	104.1
alpha Trenbolone	0.19	0.32	7.7	23.0	101.4
beta Trenbolone	0.22	0.38	7.5	35.9	104.8
16 beta hydroxy Stanozolol	0.28	0.47	10.2	30.7	95.0
Dienestrol	0.18	0.30	10.6	31.7	103.4
Hexestrol	0.16	0.27	5.1	33.2	103.3
Diethylstilbestrol	0.18	0.31	5.8	29.1	102.9
Ethynylestradiol	0.22	0.38	9.4	29.9	104.2
Flumethasone	0.15	0.26	7.8	37.1	99.9
Bisphenol A	0.26	0.44	9.3	27.9	102.1

error of the intercept). CCβ values of for all seventeen compounds are listed in Table 3 and are all below 0.5 $\mu g\,L^{-1}$.

3.2.6. Measurement of uncertainty

The measurement of uncertainty was estimated by taking into account the within laboratory reproducibility over Days 1, 2 and 3 as well as considering the repeatability on Day 4 due to matrix effects caused by different varieties of milk types. These two variabilities were combined and multiplied by a coverage factor of three to give an overall figure for the uncertainty of the measurement. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev1 document [51]. It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however as it was felt that not all the environmental and other factors that could be varied over the course of the validation were examined, it were felt that a coverage factor of 2.33 may underestimate the true uncertainty of the method. So a value of 3 was chosen instead to give a more realistic value for the true uncertainty, this approach was acceptable to ISO17025 auditors who visited our laboratory as well.

3.3. Analysis of results

The method developed in this paper utilises LC–MS/MS for the determination step, and covers a large number of classes of steroids i.e. gestagens, stilbenes, androgens, estrogens and corticosteroids as well as including bisphenol A. It was validated in accordance with criteria set out in Commission Decision 2002/657/EC [46]. The method can be carried out much quicker than a lot of previously published methods as it utilises small sample and extraction solvent volumes and uses only dispersive SPE as a purification step. The determination step is also rapid with the use of ultra high pressure chromatography and fast positive–negative polarity switching of the mass spectrometer resulting in total run times of less than 10 min for each sample. The method is also very sensitive with all estimated CCα and CCβ values much lower than the provisional validation level which was set at $1\,\mu g\,L^{-1}$. It is a quantitative method and demonstrates good reproducibility and also excellent linearity.

4. Conclusions

A multi-residue LC–MS/MS confirmatory method has been developed that simultaneously identifies and quantifies sixteen synthetic growth promoters as well as bisphenol A in bovine milk. The method can be considered as rapid as it utilises a very simple and straightforward extraction and purification. The developed method also offers the advantage of using a small sample size (1 mL) and uses very little extraction solvent (acetonitrile 2 mL). It also utilises fast chromatography with all analytes eluting within 6 min with a total run time of only 9.5 min. The method however still achieves baseline resolution of the isomers beta and alpha trenbolone and also d_2 dienestrol and diethylstilbestrol which share the same transitions. The method takes advantage of fast polarity switching meaning only one injection is needed to analyse compounds in both positive and negative polarities.

The method includes a number of classes of synthetic growth promoters including stilbenes, androgens, estrogens, gestagens and corticosteroids. No naturally occurring steroids were investigated due to ambiguity in discerning whether their presence was derived from endogenous or exogenous sources. The obtained data fulfils the requirements laid down in Commission Decision 2002/657/EC [46] and allows the calculation of all relevant performance characteristics. This study shows that the developed method easily meets the required sensitivity of $1\,\mu g\,L^{-1}$ which was the chosen validation level. The CCα and CCβ values determined for each analyte are considerably lower than this level. The method performs very well in terms of accuracy and repeatability for each of the analytes due to the utilisation of thirteen different deuterated internal standards. The values achieved for % accuracy, RSD and measurement of uncertainty all fall within acceptable ranges. The applicability of the method for use on different types of milk samples was demonstrated by the satisfactory results obtained from the Day 4 analysis.

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