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Detection of selected corticosteroids and anabolic steroids in calf milk replacers by liquid chromatographyeelectrospray ionisation e Tandem mass spectrometry

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A B S T R A C T

The use of corticosteroids and anabolic steroids in food producing animals is regulated or banned in the European Union (EU). However, their use as growth promoters cannot be excluded. Milk replacers, considered by EU legislation as feeds, may be a good way of administration of these compounds. In order to improve the control of growth promoter utilization in animal husbandry and preventing possible consequences to animal welfare, we developed a method for multiresidue analysis of prednisolone, prednisone, dexamethasone, cortisone, cortisol, 17a- and 17b-boldenone and their precursor androsta- dienedione (ADD), testosterone, epitestosterone, 17a- and 17b-nandrolone, and trenbolone in powdered milk for calves. All analytes were extracted, after a common deproteinization and defatting sample pre- treatment, by a unique immunoaffinity column and analysed by liquid chromatography tandem mass spectrometry (LCeMS/MS) in both positive and negative electrospray ionization (ESI) modes. The method was validated according to the criteria of the Commission Decision 2002/657/CE. The analytical limits were from 0.39 to 0.73 ng mL^{-1} for the decision limit (CCa) and 0.46e0.99 ng mL^{-1} for detection capability (CCb). The analysis of 50 samples of milk replacers for calves, always revealed the presence of cortisol and cortisone (average concentrations 2.56 and 1.06 ng mL^{-1}, respectively), frequently testos- terone and epitestosterone (1.24 and 0.63 ng mL^{-1}, respectively), occasionally b-nandrolone (0.82 ng mL^{-1}) and prednisolone (0.41 ng mL^{-1}). The other anabolic steroids were never found.

1. Introduction

Successful calf growth depends on a combination of many fac- tors related to health, management and nutrition (Heinrichs, Wells, & Losinger, 1995). From the alimentary aspect, natural milk, as a wholesome food, is the most important source of nutrition for young mammals before they are able to digest other types of food.

Powdered milk is commonly used for the daily feeding of calves, asit is an adequate alternative to the mother's dairy milk and an economically feasible source of all essential nutriments. Feeding with high quality milk replacers allows healthy growth in calves equal to that attainable with whole milk (Jorgensen, Hoffman, & Nytes, 2006). Since manufacturing powdered milk directly from whole milk is an expensive process, the bulk of the constituents of commercial calf milk replacers are either by-products of dairy processing or non-dairy products. Powdered milk replacers aregenerally made up of ingredients such as skim milk powder, vegetable or animal fat, whey protein, soy lecithin and vitamin-mineral premix (Geiger et al., 2014). Protein levels in dry milk re- placers range from 18% to 30% with an average value of approxi- mately 20e22%, preferably of diary origin, but can also include soyprotein, soy flour, wheat proteins, potato and animal plasma pro- tein. Fat levels range from 10% to 28e30%, with 18%e22% being the most common fat levels, mainly added as tallow, lard or coconut oil(Bamn, 2014 and Ontario veal association, 2015).

The inclusion of veterinary drugs in calf milk replacers is a matter of concern, particularly if their administration is not fully regulated and especially when legislation varies across the Coun- tries. For example, in the USA medications (decoquinate, lasalocid, oxytetracycline, chlortetracycline, and neomycin) are approved for inclusion in milk replacers, but the U.S. Food and Drug Adminis- tration (FDA, 2013) recommended a three-year judicious period (starting from December 2013) during which utilisation of antibi- otics in animal husbandry has to be reduced. European legislation does not treat milk replacers individually, but sets out the condi- tions under which medicated animal feeds may be prepared, placedon the market and used within the Community (European Union, 1990 and European Union, 2010 a).

The use of steroids in food-producing animals for therapeutic purposes is regulated (corticosteroids) or banned (anabolic ste- roids) in the European Union; however, their use as growth pro- moters cannot be excluded (Pavlovic et al., 2012). Cortisol, cortisone, testosterone and epitestosterone are endogenous, pred- nisolone (Bertocchi et al., 2013), boldenone (Chiesa et al., 2014) and nandrolone (Glenn Kennedy et al., 2009) are considered pseu- doendogenous steroids while dexamethasone and trenbolone are well-known synthetic steroids. A faster feed conversion rate, improvement of the carcass with improved meat quality, fat reduction, and increase in milk production are some of the notable features that could be achieved by treatment with these sub- stances. Thus, regulations on steroid residues with hormonal ac- tivity in food of animal origin are essential in order to safeguard animal welfare and ascertain any fraud. In the case of therapeutic use of regulated substances, a prescription by a veterinarian is needed and a suspension period has to be respected between the end of treatment and slaughter or milk marketing. The European Commission has established the maximum residue limits (MRLs) for four corticosteroids in several matrices such as muscle, liver, kidney and milk from different animal species (European Union, 2010b).

On the basis of the regular protocol applied, there are a few principal techniques by which medication can be introduced into an animal: oral administration, intramuscular, subcutaneous and intravenous injection or implantation under the skin (Courtheyn et al., 2002). Unfortunately, some illegal growth-promoting agents are suspected of being administered with feed, despite the fact that they are not licenced as additives (Courtheyn et al., 1993 and European Union, 2004). Therefore, in order to achieve comprehensive surveillance and have insight into how a medica- tion was delivered to an animal, analysis of the feed for the pres- ence of steroids should be included as well. It should be emphasized that the presence of steroid hormones in feedstuffs can be also unintentional, due to cross-contamination or owing to the appearance of pseudo-endogenous substances such as predniso- lone (Chiesa et al., 2014). Among feedstuffs used in animal hus- bandry, powdered milk replacers are perhaps most suitable for illegal tampering as drug distribution via this route is very simple: during the reconstitution of milk replacers, immediately before feeding. As hormones and steroids migrate to milk from the cow bloodstream, we need additional information about their physio- logical levels in milk related to milk replacers (Jouan, Sylvie, Gauthier, & Laforest, 2006). To the best of our knowledge, there has been neither a preliminary assessment of the status of endogenous or exogenous steroids, nor a fully validated method for their screening in powdered milk used in calf breeding.

Taking into account all the above mentioned factors, with the intention of improving residue control and preventing possible consequences for animal welfare and the consumer, our aim was to develop a liquid chromatographyetandem mass spectrometry (LC- MS/MS) analytical method for

evaluating selected glucocorticos- teroids and anabolic steroids in milk replacers used as dairy feed replacement in calf rearing.

Nowadays, LC-MS/MS is the most suitable technique for detecting veterinary drugs in feedstuffs because it provides un- ambiguous identification and a reliable confirmation. On the other hand, milk replacers are complex matrices, containing many sol- utes with different physico-chemical properties: fatty acids, pro- teins, neutral lipids (glycerides, phospholipids and sterols), glycides, vitamins and minerals, which may interfere with analyses.

The removal of these compounds is necessary in LC-MS/MS methods, especially if low ng mL^{-1} of steroid levels are to be screened for. Applying adequate and efficient purification, ion suppression can be successfully avoided together with improve- ments in overall method performances such as the detection limit
and reproducibility.

There have been just a few studies in the literature on powdered milk e infant formulae for human use (Romero-González, Aguilera- Luiz, Plaza-Bolaños, Frenich, & Vidal, 2011 and Zhan et al., 2013) and only one that described a multi-residue method for detecting
17 selected veterinary hormones in six different powdered in- gredients derived from bovine milk used modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation (Ehling & Reddy, 2013). Other researchers devised a method for the detection of eight corticosteroids in milk replacers, through C18 SPE, but with relatively high detection limits (Fiori, Pierdominici, Longo, & Brambilla, 1998). Immunology-based pre-treatment techniques have been introduced recently, but have not yet been used in powdered milk analysis. For other matrices (urine, bile) this kind of purification in general has exhibited better selectivity than those obtained with common procedures (Chiesa et al., 2014, 2015). This is the reason we decided to take advantage of an immu- noaffinity sample cleaning approach in the multi-drug method presented in this paper.

To this end, the main objective of this study was the establish-
ment of a LC-MS/MS method able to identify steroids such as cor- ticosteroids (prednisolone, prednisone, dexamethasone, cortisone and cortisol) and anabolic steroids (17a- and 17b-boldenone, their precursor androstadienedione (ADD), testosterone, epitestoster- one, 17a- and 17b-nandrolone and trenbolone) in calf milk powder. All analytes were investigated after a common pretreatment step of deproteinization and defatting followed by immunoaffinity column (IAC) clean-up and LCeMS/MS analysis, validated according to Commission Decision 2002/657/EC (European Union, 2002). Finally, we applied the validated method to the analysis of 50 samples of commercially available powdered bovine milk.

2. Chemicals and reagents

All solvents were of HPLC or analytical grade and were pur- chased from Fluka (SigmaeAldrich, St. Louis, MO, USA). Formic acid 98e100% was obtained from Riedel-de Haën.

(SigmaeAldrich, St. Louis, MO, USA). Water was purified by a Milli-Q System. The IACs were provided by Randox (DM 2185, Randox Laboratories, Antrim, UK). Concentrated wash and storage buffers, diluted following the manufacturer's instructions before use, were supplied with the columns. ADD and b-boldenone were purchased from Fluka (SigmaeAldrich, St. Louis, MO, USA); a-bol- denone was obtained from LGC Standards (Teddington, UK). Their internal standard was b-boldenone-d3 (LGC Standards, Teddington, UK). Cortisol, prednisone, prednisolone and dexamethasone were purchased from Fluka (SigmaeAldrich, St. Louis, MO, USA) and their internal standard, prednisolone-d6, was obtained from C/D/N Iso- topes Inc. (Pointe-Claire, Quebec, Canada). Testosterone, epi- testosterone, 17a- and 17b- nandrolone, trenbolone and their internal standard 17b-nandrolone-d3 were purchased from Fluka (SigmaeAldrich, St. Louis, MO, USA).

2.1. Powdered milk replacer used for method validation andapplication

For the method validation we used a commercially available powdered calf milk. It contained partially skimmed milk whey, whey protein, fat, wheat gluten, vegetable oil, pregelatinized wheat flour, pea fibre and potato protein. The analytical constituents were: crude protein 22.50%, oils and fats 22.50%, crude fibre 0.30%, crude ash 8%, calcium 0.65%, sodium 0.50% and phosphorus 0.65%. Vitamin A, vitamin D_3 and vitamin E were present as additives in all
complementary milk (25,000 UI kg^{-1}, 3700 UI kg^{-1} and 75 mg kg^{-1},
respectively). The formulation also contained the following quan- tities per kg: choline 5 mg, copper pentahydrate sulphate 5 mg, manganese sulphate 45 mg, zinc sulphate 135 mg, potassium io- dide 1 mg and sodium selenite 0.32 mg. All the information about the feedstuff composition came from the manufacturer's certificates.

With the aim to check the method effectiveness, we used 50 samples of powdered calf milk, collected in farms from North Italy. No information about their composition was available.

2.2. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared inmethanol and kept at 40 °C. Working solutions containing each of the studied analytes at concentrations of 10 and 100 ng mL^{-1} wereprepared daily. Each working solution was maintained at 4 °C during the method validation procedures.

3. Sample extraction

The sample was initially deproteinized and defatted following the Wang et al. protocol (Wang, Zhou, & Jiang, 2011) slightly modified as regard the relative amounts of matrix and reagents, and acetonitrile substituted by methanol. Briefly, samples of powdered milk (1 g) were
spiked with the internal standards to a final concentration of 2 ng mL^{-1} and reconstituted in water (10 mL).
The mixture was vortexed and then sonicated for 10 min, followed
sequentially by through the addition of methanol (10 mL), 2 min of shaking, and 10 min of sonication. After the addition of sodium chloride (2 g), 2 min of shaking, and 10 min of centrifugation 4500 g were carried out. The supernatant was transferred into a 50 mL polytetrafluoroethylene centrifuge tube and defatted with
2 × mL of n-hexane extraction. Each time, after centrifugation at 2500 g, the n-hexane layer was removed. The methanol/water layer was evaporated and reconstituted in 5 mL of water for furtherpurification and extraction by using the IAC. The column was pre-
viously washed with 5 mL ethanol:water (70:30 v/v) and equili- brated with 3 5 mL wash buffer (flow rate 3 mL min^{-1}, i.e. about one drop per second). The samples were loaded by gravity flow. Wash buffer (2 5 mL) and water (1 5 mL) were used to wash the column. The elution of the bound analytes was then performed by

the application of 4 mL ethanol:water (70:30 v/v) (flow rate 3 mL min^{-1}), which was collected in a 15 mL polypropylene tube. The IAC could be used again, starting from the equilibration described above, after a washing step with 2 × 5 mL ethanol:water (70:30 v/v). We also checked the number of runs sustainable by a column and the results were similar to the ones already shown for urine (Chiesa et al., 2015): using a column for 10 cycles before discarding it is advisable. The eluate was evaporated in a rotary vacuum evaporator. The dried extract was reconstituted in 200 μL of methanol:water (50:50 v/v) and transferred into an auto-sampler vial. The injection volume was 10 μL.

4. LC-MS/MS analyses

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) consisting of a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20 μL loop. Chromatographic separation was achieved using a Synergi Hydro RP reverse-phase HPLC column (150 × 2.0 mm, 4 μm internal diameter) with a C18 (4 × 3.0 mm) guard column (Phenomenex, Torrance, CA, USA), which was kept at 30 °C. The mobile phase consisted of methanol (solvent A) and 0.1% aqueous formic acid (solvent B). The gradient program began at 60% A for 1 min, changing to 95% A in 10 min, which was then held for 2 min. Then it returned to 60% A in 2 min and equilibrated for another 7 min.

The flow rate was 200 μL min^{-1} and the overall run time was 22 min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in both positive (ESI) and negative (ESI) electrospray ionization modes. Acquisition parameters were optimized in the ion-spray mode by direct continuous pump- syringe infusion of standard solutions of the analytes at a concentration of 1 μg mL^{-1}, a flow rate of 20 μL min^{-1} and a MS pump rate of 100 μL min^{-1}. The following conditions were used: capillary voltage 3.5 kV, ion-transfer capillary temperature 340 °C; nitrogen as sheath and auxiliary gas at 30 and 10 arbitrary units, respec- tively, argon as the collision gas at 1.5 mTorr and peak resolution 0.70 Da at full width half maximum (FWHM). The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. Three diagnostic product ions were chosen for each ana- lyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM). The selected diagnostic ions, one of which was chosen for quantification, and the collision energies are reported in Table 1. Data were acquired and elaborated using Xca- libur™ software from Thermo Fisher.

5. Method validation

Validation was performed according to the criteria and recom- mendations of European Commission Decision 2002/657/EC (European Union, 2002). After a preliminary screening of some samples of reconstituted milk as described in the 'Sample extrac- tion' section, it was observed that all of them contained cortisol, cortisone, testosterone and epitestosterone.

We therefore made preliminary trials using a milk replacer batch, containing the lowest amount of these analytes, diluted 1:10 with water: no analyte was so detected. Then we compared the 2 six-point standard curves (0.0, 0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1}) performed both in milk replacer reconstituted following the manufacturer indication and in the diluted one. The slopes and the Y-intercepts for each analyte of each curve were then compared with the unpaired t-test. No significant difference was observed in slopes of all analytes. The Y-intercepts of cortisol, cortisone, testosterone and epitestosterone were slightly different ($P < 0.05$) in the two standard curves, due to the presence of these endoge- nous hormones in milk. Therefore, the validation was performed on diluted 1:10 calf milk replacer.

For each analyte, the method's performance was assessed

Table 1
MS/MS conditions for MRM acquisitions of all analytes and relative internal standards. Ions for quantification are in bold characters. CE: collision energy, expressed in Volts.

Analyte	Precursor ion [M—H]$^-$ or [M—H]$^+$ (m/z)	Product ions$_{CE}$ (m/z)	ESI
Cortisol	407	**282_{37}**,297_{33}, 331_{20}	(—)
Cortisone	405	**301_{21}**, 329_{20}, 359_{12}	(—)
Prednisolone	405	**187_{30}**,280_{35}, 329_{19}	(—)
Prednisone	403	**299_{21}**, 327_{19}, 357_{12}	(—)
Dexamethasone	437	**307_{33}**,361_{20}, 391_{14}	(—)
Prednisolone-d6	411	**284_{37}**, 299_{32},333_{19}	(—)
Testosterone and epitestosterone	289	**97_{21}**, 109_{23}, 253_{16}	(+)
ADD	285	**121_{22}**, 151_{14}, 267_{11}	(+)
α- and β-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone d3	290	**121_{27}**, 138_{14}, 272_{10}	(+)
α- and β-nandrolone	275	**91_{40}**, 109_{27}, 239_{16}	(—)
Trenbolone	271	**165_{56}**, 199_{23}, 253_{19}	(+)
β-nandroloned3	278	**109_{19}**, 242_{16}, 260_{15}	(+)

through its qualitative parameters [molecular identification in terms of retention time (RT) and transition ion ratios that charac- terize selectivity and specificity], through its quantitative parame- ters (linearity, recovery, accuracy in terms of trueness and precision expressed as intra- and inter-day repeatability) and through the analytical limits [decision limit (CCα) and detection capability (CCβ)]. At the end, we also tested the robustness of the method and the stability of the standard solutions in the solvent and in the matrix.

Specificity, for analytical methods, is the power to discriminate between the analyte and closely related substances (isomers, me- tabolites, degradation products, endogenous substances, matrix constituents, etc.). Therefore, potentially interfering substances should be chosen and relevant blank samples should be analysed to detect the presence of possible interference and estimate the impact of the interference. We analysed an appropriate number of representative blank samples (n 20) and checked for any inter- ference (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute. Selectivity was evaluated in the same samples. Selectivity requires compliance with the relative retention times for each analyte. Moreover, three transi- tions from the analyte molecular peak were monitored with a signal-to-noise ratio greater than 3.

Instrumental linearity was evaluated by drawing five-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng mL^{-1} each) with analyte concentrations corresponding to

0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1}. Matrix validation curves were obtained by spiking milk samples with each of the analytes, resulting in three analytical series; each series comprised of six replicates for three concentration levels (0.3, 0.6 and

0.9 ng mL^{-1} for all analytes except for 17a- and 17b-nandrolone,positivized at 0.5, 1.0 and 1.5 ng mL^{-1}). Trueness was assessed through recovery and was evaluated using the matrix curve results from the three analytical series, expressed in terms of the per- centage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability was evaluated by calculating the relative standard deviation of the re- sults obtained for six replicates of each analyte at three concen- tration levels of the three analytical series. The data from the matrix validation curves were used to calculate the decision limit (CCa) using parallel extrapolation to the x-axis at the lowest experi- mental concentration and detection capability (CCb) according to the guideline described in Commission Decision 2002/657/EC (European Union, 2002) and clarified in document SANCO/2004/ 2726 revision 4 (European Union, 2008). Experiments to evaluate matrix effects corresponded to Matuszewski's strategy (Matuszewski, Constanzer, & Chavez-Eng, 2003), that requires sample extracts with the analyte of interest added postextraction compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest. The difference in response between the postextraction sample and the pure solution divided by the pure solution response determines the extent of thematrix effect occurring for the analyte in question under chro-matographic conditions.

Stability was evaluated by testing spiked samples and standard

solutions over time from one week to one month under defined storage conditions (20 °C), and quantitation of components was determined by comparison to freshly prepared standards. If the concentration variations were lower than 2% the stability was considered acceptable.

Finally, we evaluated robustness using the approach of Youden (European Union, 2002), a fractional factorial design. Eight exper- iments were carried out, fortifying eight samples at the lowest validation level, changing the nominal values reported in the 'Sample extraction' section slightly (±10%) of seven factors that may influence the outcome of the analysis. The seven factors were: the initial reconstitution volume in water, the volume of methanol during the deproteinization step, the sonication time, the volume of exane during the defatting step, the percentage ethanol in the elution solution of the IAC column, the elution volume of the IAC column and the resuspension volume of the dry extract. Finally, the eight samples were quantified using a calibration curve constructed in conjunction with the Youden experiment.

6. Results and discussion

6.1. Method development

During method development, different options were evaluated in order to optimize chromatographic separation and detection parameters as well as sample extraction.

6.2. Optimization of LC–MS/MS conditions

An LC–MS/MS method was developed to perform analyses of selected synthetic and natural corticosteroids and anabolic steroids in calf milk powder. Initially, in order to achieve high sensitivity of the target analytes, optimisation of the liquid chromatography and mass spectrometry conditions was performed by injecting standard solutions of a mixture of all the analytes. After preliminary trials, in full-scan mode from 50 to 500 m/z, the three product ions with the highest signal-to-noise ratio (s/n) for each analyte and the internal standards were chosen for identification. The collision energy (CE) and de-clustering potential (DP) for each transition were adjusted in multiple reaction monitoring (MRM) mode in order to reach the highest sensitivity. For a method to be deemed confirmatory under Commission Decision 2002/657/CE, (European Union, 2002) it must

yield a minimum of four identification points (IPs) for Group A (forbidden substances) of Directive 1996/23/CE or three IPs for substances listed in Group B (permitted substances) (EuropeanUnion, 1996). Each one of the three product ions is equal to
1.5 IPs, making a total of 4.5 IPs. The three diagnostic product ions, among which is the ion chosen for quantification, their CEs are reported in Table 1.

Special attention was paid to the separation of stereoisomeric compounds (a-boldenone from b-boldenone, a-nandrolone from b- nandrolone) (Fig. 2) that exhibited the same (or similar) fragmen- tation pattern. Additionally, the composition of the mobile phase was shown to be adequate for both types of ionisation (positive and negative), further promoting the remarkable sensitivity to the target compounds. Under our LC–MS/MS conditions, it was possible to individuate all compounds of interest at concentration levels suit- able for research aimed for control purposes (Figs. 1 and 2).

6.3. Optimization of sample purification and IAC extraction

The critical step in the method setup is the sample preparation procedure, owing to the high percentages of proteins and lipids and

the overall complexity of the matrix. There are a number of po- tential pitfalls associated with corticosteroid and anabolic steroid analysis of milk samples. A major problem with some currently available methods is interference with steroid determination by a significant number of other lipids. The purification procedure using an immunoaffinity approach could eliminate most of these in- terferences. Additionally, in our previous studies we demonstrated the high efficiency of IAC purification of bile (Chiesa et al., 2014) and urine samples (Chiesa et al., 2015). IAC also turned out to be a good strategy in this study, as selected analytes were retained and extracted with satisfactory efficiency: overall method recoveries ranged between 99.6 and 105.4% for all analytes investigated (Table 2).

6.4. Performance characteristics of the methods

The curves prepared to check instrumental linearity showed correlation coefficients greater than 0.99 for all compounds, indi- cating a good fit.

Selectivity showed compliance with the relative retention timesfor each analyte, which in our case were found to be within a

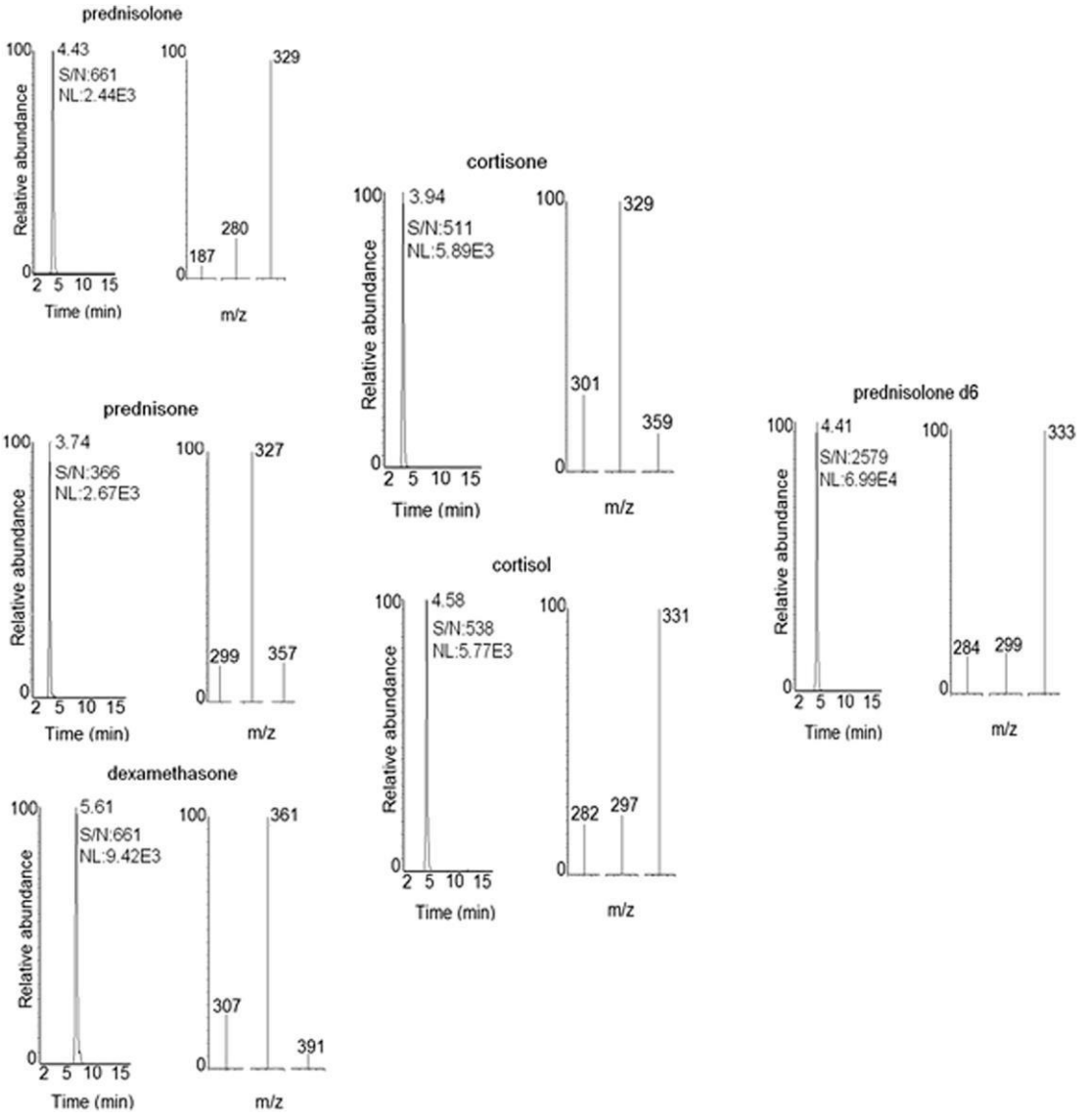

Fig. 1. LCeMS/MS chromatograms and related MS spectra of the five corticosteroids in a powdered milk sample spiked at the lowest concentration level for validation. Right-hand side: internal standard (concentration ¼ 2 ng mL^{-1}).

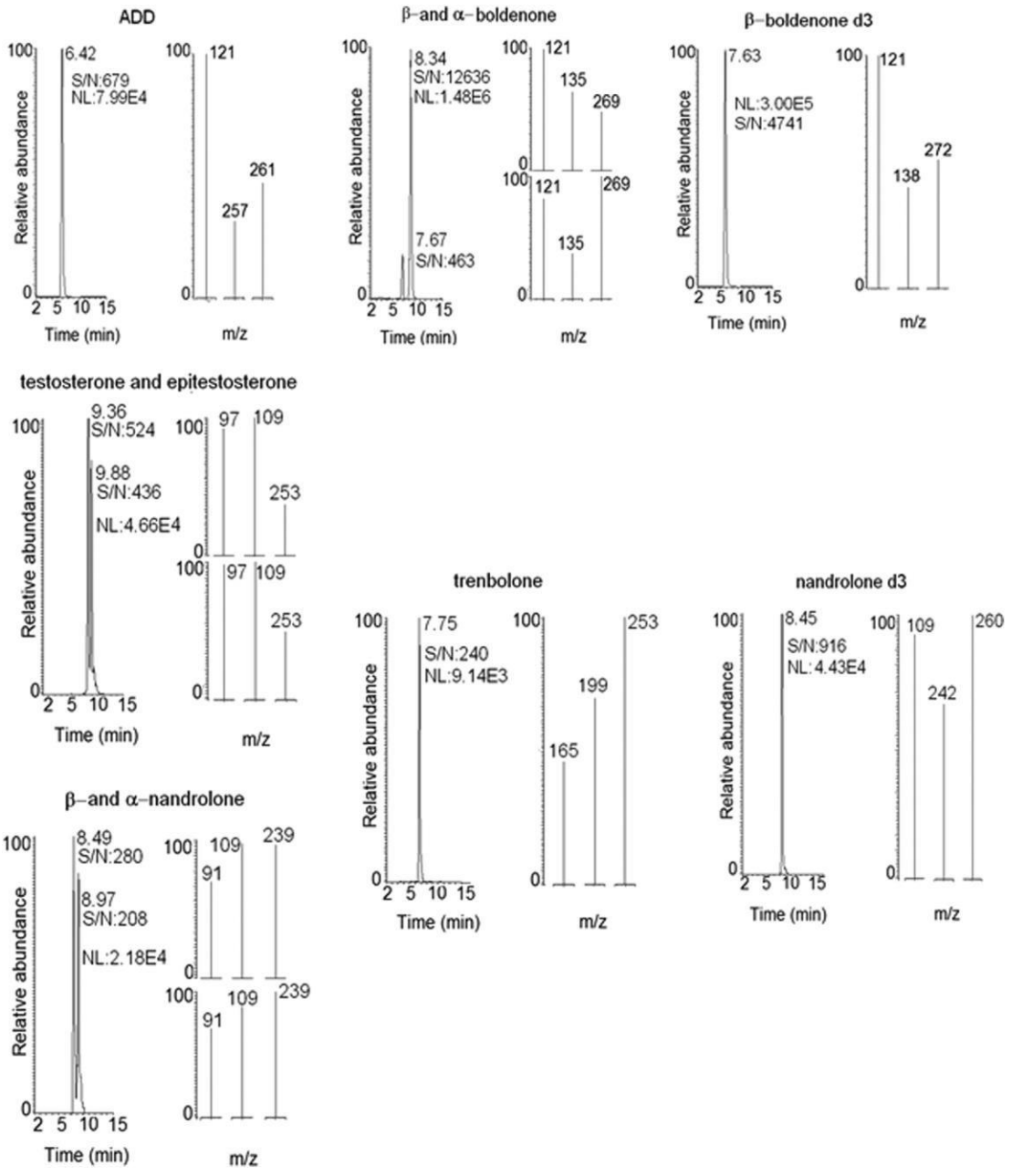

Fig. 2. LCeMS/MS chromatograms and related MS spectra of the selected anabolic steroids in a powdered milk sample spiked at the lowest concentration level for validation. Right- hand side: internal standards (concentration ¼ 2 ng mL^{-1}). Chromatographic peaks and ion spectra of stereoisomers are in the sequence listed in their respective headings.

tolerance of 2.5% when compared with standards. Moreover all ion ratios of analytes in the samples were within recommended tol- erances as required by Commission Decision 2002/657/EC (European Union, 2002) when compared with standards.

Blank and spiked samples were analysed and did not show any interference (signals, peaks, ion traces) in the region of interest where the target analytes were expected to be (European Union, 2002).

The matrix validation curves were demonstrated to be linear in the range 0.3e0.9 ng mL^{-1} for all analytes except 17a- and 17b- nandrolone, which spiked in the range between 0.5 and

1.5 ng mL^{-1}. A linear regression, obtained using the least-square method, demonstrated a good fit for all analytes with a correla- tion coefficient >0.99.

The matrix effect value calculated according to Matuszewski et al., (2003) was approximately 100% for each compound, indi- cating the absence of ion suppression. It is likely the clean up performed by IAC tends to eliminate the matrix effect.

The precision, calculated by applying one-way analysis of vari- ance (ANOVA), was expressed as the coefficient of variability (CV) in terms of intra- and inter-day repeatability. The reported results showed that the intra- and inter-day repeatability for all analytes was less than 16.2 and 20.9%, respectively. These CVs were lower than 22%, as proposed by Thompson (Thompson, 2000), repre- senting good method repeatability. CCa and CCb were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008) using parallel extrapolation to the *x*-axis at the lowest experimental concentration (Table 2). Standard solutions and samples showed an acceptable stability with less than 2% change after one month.

Finally, according to Youden's experiment (European Union, 2002), none of the seven changed factors showed significant vari- ation in the concentration measurements, confirming the good robustness of the method.

Table 2
Validation parameters for all analytes.

Analyte	Concentration level (ng mL^{-1})	Recovery % (n ¼ 18)	Repeatability %		CCα (ng mL^{-1})	CCβ (ng mL^{-1})
			Intra-day (CV; n ¼ 6)	Inter-day (CV; n ¼ 18)		
Cortisol	0.3	95.1	10.2	20.1		
	0.6	105.0	10.0	20.0	0.51	0.73
	0.9	98.4	9.1	17.3		
Cortisone	0.3	95.0	14.8	16.0		
	0.6	105.0	10.0	11.8	0.41	0.51
	0.9	98.3	7.7	7.9		
Prednisolone	0.3	101.5	15.3	16.1		
	0.6	98.5	13.1	14.0	0.41	0.51
	0.9	100.5	9.0	9.2		
Prednisone	0.3	98.1	12.2	13.1		
	0.6	101.9	7.7	8.9	0.39	0.46
	0.9	99.4	10.8	10.9		
Dexamethasone	0.3	105.4	10.8	14.7		
	0.6	94.6	7.6	12.6	0.40	0.49
	0.9	101.8	9.5	10.1		
Testosterone	0.3	98.9	7.9	13.0		
	0.6	101.1	10.7	14.9	0.39	0.48
	0.9	99.6	9.1	9.7		
Epitestosterone	0.3	97.0	15.4	19.2		
	0.6	99.0	10.0	10.2	0.43	0.54
	0.9	102.9	8.0	9.8		
ADD	0.3	100.2	14.8	15.4		
	0.6	99.8	6.8	8.1	0.41	0.49
	0.9	100.0	9.2	9.3		
α-boldenone	0.3	101.3	13.2	14.4		
	0.6	99.0	9.4	11.0	0.40	0.49
	0.9	100.4	8.1	8.4		
β-boldenone	0.3	102.7	9.7	18.6		
	0.6	97.3	15.1	20.9	0.43	0.57
	0.9	101.0	8.0	9.6		
α-nandrolone	0.5	102.4	12.8	13.6		
	1.0	97.6	8.9	9.5	0.65	0.78
	1.5	100.8	13.9	14.0		
β-nandrolone	0.5	96.2	12.2	20.1		
	1.0	103.7	14.2	20.2	0.73	0.99
	1.5	98.8	10.6	11.9		
Trenbolone	0.3	96.7	15.4	20.3		
	0.6	103.3	16.2	20.2	0.45	0.61
	0.9	98.8	12.0	12.3		

6.5. Application of the method

In order to monitor selected corticosteroids and anabolic steroid residues in bovine powdered milk, 50 samples were subjected to analysis. All samples analysed revealed the presence of cortisol and cortisone; testosterone was found in 45, epitestosterone in 34, prednisolone in 2 and b-nandrolone in 7 samples (Table 3), quan- tified by extrapolation from calibration curves which were built specifically of 6 points: 0, 0.5, 1.0, 2.0, 5.0, 10.0 ng mL^{-1} for all analytes. Very good, satisfactory linearity was obtained for all curves ($R^2 > 0.99$).

The average concentration is expressed as ng mL^{-1} of recon- stituted milk (1 g/10 mL, considering that the dilution generally recommended by the manufacturers is approximately 100 g ofpowdered milk in 1 L of water).

The physiological concentrations of cortisol and cortisone in milk vary from 0 to 50 ng mL^{-1} (Jouan et al., 2006), a range that includes the concentrations shown in Table 3. As regards testos- terone and epitestosterone in milk, very high variability is reported in the literature. The concentration of testosterone increases pro- portionally during pregnancy from 20 to 120 pg mL^{-1} (Gaiani, Chiesa, Mattioli, Nannetti, & Galeati, 1984), varying from unde- tectable to 50 pg mL^{-1} in milk at oestrus and 150 pg mL^{-1} during the luteal phase (Hoffman & Rattenberger, 1977). These steroids are probably found in lower concentrations or are even absent after the process of milk skimming, but they are present in tallow and lard (Ontario veal association, 2015), often added to raise the lipid concentrations of milk replacers. However, in bovine fat and boar backfat, testosterone is present at concentrations of up to 10.95 ± 8.68 mg kg^{-1} and 20.34 mg kg^{-1}, respectively (Hartmann, Lacorn, & Steinhart, 1998). In light of the above, available infor- mation does not allow defining the "natural" values of testosterone and epitestosterone concentrations in powdered milk. We occa- sionally detected in the milk replacers the pseudoendogenous steroids prednisolone and b-nandrolone. To our knowledge, no data are available in the scientific literature about their presence in cow milk so we are not able to hypothesize if their origin is endogenousor due to administration.

7. Conclusions

The validation parameters of this method demonstrate its effectiveness for the analysis of selected corticosteroids and anabolic steroids in milk replacers. As already stated, this feed could be the vehicle for the administration of regulated or forbidden substances. Moreover, no studies are available on the presence in cow milk of substances with a pseudoendogenous origin. This method could therefore be a useful tool both for research purposes aimed to the improvement of control offeedstuff.

Table 3
Survey of the steroids detected in the 50 samples of reconstituted calf milk replacers (1 g/10 mL). Concentration is expressed in ng mL^{-1}.

	Cortisol	Cortisone	Prednisolone	Testosterone	Epitestosterone	b-nandrolone
Positives	50	50	2	45	34	7
Concentration mean ± SD	2.56 ± 0.89	1.06 ± 0.37	0.41 ± 0.00	1.24 ± 0.68	0.63 ± 0.23	0.82 ± 0.13
Maximum concentration	3.81	1.64	0.41	3.72	1.33	1.03
Minimum concentration	0.76	0.42	0.41	0.48	0.43	0.73

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2015.09.028.

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