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Bovine teeth as a novel matrix for the control of the food chain: liquid chromatography–tandem mass spectrometry detection of treatments with prednisolone, dexamethasone, estradiol, nandrolone and seven β_2-agonists

Luca Maria Chiesa[a], Maria Nobile[a], Sara Panseri[a], Bartolomeo Biolatti[b], Francesca Tiziana Cannizzo[b], Radmila Pavlovic[a] and Francesco Arioli[a]

[a]Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy; [b]Department of Veterinary Science, University of Turin, Grugliasco (Turin), Italy

ABSTRACT

Veterinary drugs usually have rapid clearance rates in the liver and kidney, hampering their detection in conventional matrices such as the liver or urine. Pharmacological principles such as esterification may be applied to facilitate the administration of veterinary drugs and increase drug half-life. Prednisolone, whose therapeutic administration is regulated for food producing animals in the EU, is available in its acetate form as well as nandrolone, a banned anabolic steroid, which may be obtained as nandrolone phenylpropionate and estradiol as a benzoyl ester. While the distribution and accumulation of lipophilic and hydrophilic substances in human teeth have been well documented, studies on residues in bovine teeth are lacking. We hypothesised that analysis of bovine teeth could be used to detect both regulated and banned veterinary drugs. Steroids may be illegally used as growth promoters in food producing animals, alone or combined with β2-agonists; therefore, we developed, and validated, in accordancewith the Commission Decision 2002/657/EC, two analytical confirmatory LC-MS/MS methods to detect these classes of com- pounds following a unique liquid extraction procedure. Finally, we analysed teeth from three male Friesian veal calves treated with intramuscular estradiol benzoate, oral prednisolone acetate or intramuscular nandrolone phenylpropionate in combination with oral ractopamine, respec- tively, and from seven bovines from the food chain. Teeth from treated animals were positive for their respective drugs, with the exception of nandrolone phenylpropionate. One sample from a food chain bovine was positive for isoxsuprine, one of the seven β2-agonists studied. Non- esterified forms of the steroids were not found. These results demonstrate that bovine teeth are a suitable matrix for the determination of pseudoendogenous substances or illicit adminis- tration of veterinary drugs.

Introduction

The illicit administration of veterinary drugs for growth promoting purposes in cattle breeding has been banned in the EU since 1988 (European Union 2003), and detailed in Council Directives 96/22/EC and 96/23/EC (European Union 1996a, 1996b). Veterinary drugs generally show high clearance rates in conventional biological matrices such as urine, blood, liver and muscle, hampering the detection of many active compounds. This is true for the active compounds of synthetic, natural or pseudoendogenous origin depend- ing on the commercial formulation.

β_2-Adrenoceptor agonists have powerful bronchodi- lator and tocolytic actions, but may also be administered as growth promoters to improve the production of lean meat by lowering fat levels through increased lipolytic activity. Although the EU, China and other Asian countries have banned the use of β_2-agonists for growth promoting purposes, the USA authorised rac- topamine as a feed additive for swine in 1999, cattle in 2003 and turkey in 2008 (Flynn 2014). The EU has set the maximum residue levels (MRL) for clenbuterol in muscle, liver and kidney of bovine and horses as well as in cow milk (European Union 2010). Its only approved usage is as a tocolytic to parturient cows (EMEA 2000), and cannot be used under any circumstances on other categories of cattle. Isoxsuprine administration is simi- larly regulated, but MRLs are not indicated (European Union 2010) due to its infrequent use; moreover it is rapidly absorbed, distributed and excreted, making it unlikely the animal will be sent for slaughter during or immediately after the treatment (EMEA 1996). The

metabolic cycle of ractopamine is also very short and its degradation pathway varies among animals, making it hard to test for ractopamine during the withdrawal period (Wu et al. 2014).

Estradiol benzoate is often used in combination with a progestin to induce oestrus, ovulation and increase the number of embryos in domestic livestock. Estradiol also stimulates the somatotropic axis to produce growth hormone and increase carcass weight and feed efficiency. As it is a potential carcinogen, its use in food producing animals has been banned since 2008 in the EU (European Union 1999, 2008). The possibility of widespread abuse of hormonal substances by unscru- pulous farmers and veterinary professionals in some parts of Europe has been reported, due to the economic benefits that these illicit substances provide in animal husbandry and the possibility to obtain the substances in non-European countries where they are authorised (Stephany 2001).

Nandrolone phenylpropionate can be utilised in medical veterinary practices to slow degenerative pro- cesses and to promote tissue repair. Its use in cattle breeding is prohibited in the EU (European Union 1996a), yet it is one of the

most frequently applied illegal anabolic steroids. The controls by the official organisms are complicated by the pseudoendogenous nature of nandrolone in bovine. The presence of its metabolites in untreated, injured males and pregnant females is detectable (Kennedy et al. 2009).

The same applies to prednisolone (Bertocchi et al. 2013), a corticosteroid commercially available as an acetate that is allowed for therapeutic purposes as an anti-inflammatory drug or for treatment of ketosis (McSherry et al. 1960; Aiello 2014). Apart from its legal usage, prednisolone could also be administered at low doses, either alone or in combination with other steroids or β_2-agonists, to promote growth.

The natural or pseudoendogenous origin, rapid excretion and low dosages (especially if cocktails are used) of illegally applied anabolic substances make it challenging to concretely verify their use. Thus, it is necessary to identify a matrix in which these drugs accumulate and persist in their administered chemical form to undoubtedly demonstrate that treatment occurred, discriminate between exogenous or endogen- ous origin and improve the framework of controls.

In recent years, many studies have proposed hair analysis as a useful strategy to detect drug residues in food producing animals (Gaillard et al. 1999; Rambaud et al. 2005; Nielen et al. 2006) Although hair provides a long retention window, it is limited to a monthly time scale (Hinners et al. 2012); in teeth, exposure or admin- istration markers remain stable enabling a longer detection window (Gulson et al. 1997). Human teeth have been used to assess exposure to inorganic chemi- cals since the 1960s (Altshuller et al. 1962) and to determine prenatal exposure to environmental organic chemicals in the early 2000s (Andra et al. 2015). In particular, ^{14}C-labeled substances have been shown to penetrate into the calcified tissues and pulp of decid- uous and permanent teeth in humans, rendering this biological matrix a potentially important deposit of exogenous substances (Haustein et al. 1994). The stu- dies that to date use teeth as a matrix, however, are almost exclusively limited to human teeth and have been used to detect drugs of abuse (opiates, cocaine, nicotine, etc.) or environmental contaminants. To our knowledge, only Spinner et al. (2014) used artificially loaded bovine dentine as an experimental model to demonstrate the possibility to detect common drugs of abuse for toxicological-forensic purposes. However, bovine teeth have not yet been demonstrated as a useful matrix to determine illicit anabolic treatment in veterinary medicine.

We propose that the analysis of bovine teeth, a non-disruptive matrix when collected at the slaughterhouse, is a powerful strategy to demonstrate the administra- tion of growth promoters in livestock. To test whether teeth could be used for detection of drugs in food producing animals, we developed a simple and unique liquid extraction procedure from teeth followed by two LC-MS/MS analyses. The first one dealt with seven β_2- agonists (cimaterol, clenbuterol, isoxsuprine, mabu- terol, ractopamine and terbutaline) and the second with selected steroids (prednisolone acetate, predniso- lone, dexamethasone, estradiol benzoate, nandrolone phenylpropionate and nandrolone). Their structures are shown in Figure 1. The developed methods were validated according to Commission Decision 657/2002/ CE (European Union 2002) to demonstrate the power of this novel, unconventional matrix to uncover illicit administration of these drugs. Finally, these methods were used to analyse teeth from experimentally treated veal calves collected at the slaughterhouse and from bovines from the food chain.

Materials and methods

Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid 98–100% was obtained from Riedel- de Haën (Sigma-Aldrich, St. Louis, MO, USA). Water was purified by a Milli-Q System (Millipore, Merck KGaA, Darmstadt, Germany). Ractopamine, isoxsuprine, clenbuterol, salbutamol, terbutaline, mabuterol, cima- terol, estradiol benzoate, prednisolone acetate, predniso- lone, dexamethasone and nandrolone were purchased from Sigma-Aldrich (St. Louis, MO, USA). The veterin- ary medicament Nandrosol (AST Farma BV, Oudewater, the Netherlands) consisting of nandrolone phenylpropio- nate 50 mg mL^{-1} was used. The internal standards were ractopamine-d6 and testosterone benzoate-d3 (RIKILT Laboratory, Wageningen, the Netherlands), predniso- lone-d6 (C/D/N Isotopes Inc, Pointe-Claire, Quebec, Canada) and testosterone-d3 (LGC Standards, Teddington, UK).

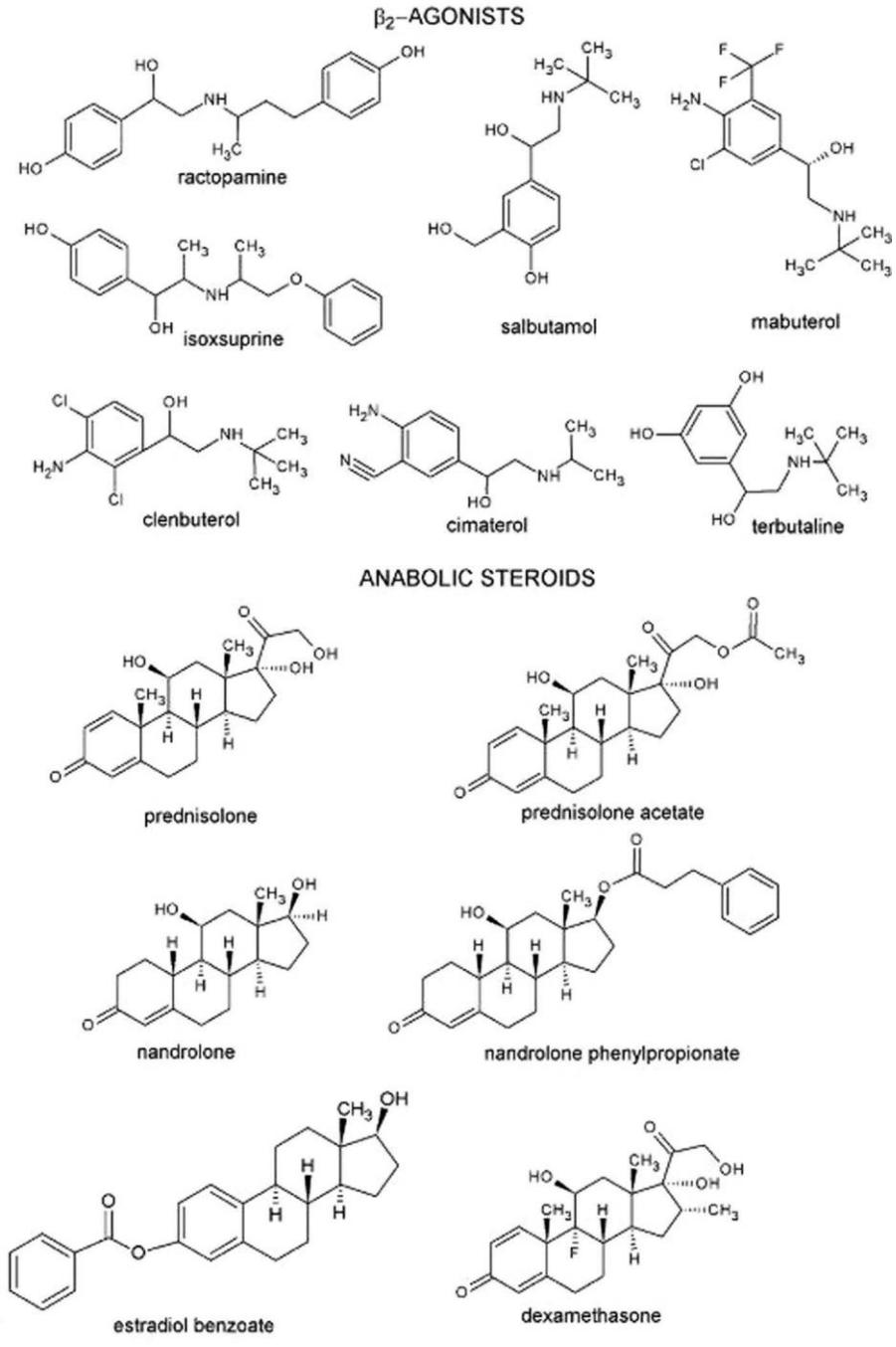

Figure 1. Chemical structures of the seven β_2-agonists and the selected steroids.

Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and stored at −20°C. Working solutions at concentrations of 10 and 100 ng mL^{-1} were prepared daily. Each working solution was maintained at −20°C during the method validation procedure.

Sample collection

The teeth used for the analyses included 20 blank samples used for validation of the methods, taken at the slaughterhouse from the food chain bovines, gath- ered in a pool (two molars or premolars and two incisors for each animal), and teeth samples used for application of the methods. In the last case, the samples (two molars or premolars and two incisors) were col- lected from seven bovines from the food chain and from three male Friesian veal calves aged 3 months with known treatments. The first treated animal was intramuscularly given once a week 5 mg estradiol benzoate for 6 weeks and slaughtered 1 week after the last treatment. The second calf was orally treated with 15 mg day^{-1} prednisolone acetate for 32 days and slaughtered 3 days after the last treatment. The third calf was intramuscularly treated with 150 mg of nan- drolone phenylpropionate every 2 weeks for 6 weeks in combination with 80 mg day^{-1} oral ractopamine, start- ing from the 21st day for 32 days, and slaughtered 4 days after the last treatment with ractopamine. All teeth were collected at the slaughterhouse and stored at −20°C until the analysis was performed.

Sample extraction

Prior to analysis, teeth samples were cleaned by immer- sing them in distilled boiling water for 10 minutes to remove residual blood and, subsequently, the adherent tissue was removed with a scalpel. Once dry, teeth were cleaved and reduced in size with a hammer. After scrap- ing out the pulp, teeth were pulverised by a ball mill (30 freq sec^{-1}, 40 sec). One gram of teeth was spiked with internal standards at a concentration of 2 ng g^{-1} and, after the addition of 3 mL of ethyl acetate: *tert*-butyl methyl ether (4:1, v/v) mixture, was sonicated for 1 hour. After centrifugation at room temperature at 2500 × g for 5 min, the supernatant was collected and evaporated in a rotary vacuum evaporator at 37°C. The dried extract was reconstituted in 200 µL of methanol: aqueous formic acid 0.1% (50:50 v/v) and then transferred to an auto-sampler vial. The injection volume was 10 µL.

LC-MS/MS analyses

4 µm) and a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of a binary mixture of solvents A (aqueous formic acid 0.1%), and B (methanol). The following gradient programme was used for β2-agonists: solvent A was decreased from 95% to 45% over 10 min, decreased to 10% over 1 min, held for 6 min at 10%, increased to 95% over 5 min and equilibrated for another 8 min. The flow rate was 0.2 mL min^{-1} and the overall run time was 30 min. Anabolic steroids were separated with the following gradient programme: solvent A was maintained at 60% for 1 min, decreased to 40% over 1 min, decreased to 5% over 11 min, held at 5% for 1 min, increased to 40% over 2 min, increased 60% over 5 min and equilibrated for another 8 min. The flow rate was 0.2 mL min^{-1} and the overall run time was 29 min.

The mass spectrometer was a triple-quadrupole TSQ Quantum mass spectrometer (MS) (Thermo Fisher Scientific) equipped with an electrospray ionisation (ESI) interface that was set in both positive (ESI+) and negative (ESI-) mode. Acquisition parameters were optimised in the electrospray mode by direct continuous pump-syringe infusion of standard 1 µg mL^{-1} solutions of analytes at 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; nitro- gen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full-width half-maximum (FWHM). Three diagnostic product ions were chosen for each analyte and internal stan- dard. The acquisition was made in multiple reaction- monitoring (MRM) mode. The selected diagnostic ions (one of which was chosen for the quantification), their relative intensities and the collision energies are reported in Tables 1 and 2 for β2-agonists and anabolic steroids, respectively. Acquisition data were recorded

Table 1. MS/MS conditions for the MRM acquisitions of the seven β_2-agonists, as well as for the internal standard. Ions used for quantification are in bold. The values in parentheses represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

Analyte	Precursor ion (*m/z*)	Product ions(%)$_{CE}$ (*m/z*)	ESI
cimaterol	220	143(55)$_{23}$, **160(60)$_{16}$**, 202(100)$_{7}$	(+)
terbutaline	226	125(30)$_{24}$, **152(100)$_{16}$**, 170(20)$_{11}$	(+)
salbutamol	240	130(35)$_{29}$, **148(50)$_{18}$**, 222(100)$_{10}$	(+)
clenbuterol	277	158(18)$_{29}$, **203(50)$_{18}$**, 259(100)$_{10}$	(+)
ractopamine	302	107(53)$_{30}$, 121(62)$_{22}$, **164(100)$_{15}$**	(+)
isoxsuprine	302	107(25)$_{29}$, 150(20)$_{21}$, **284(100)$_{14}$**	(+)
mabuterol	311	217(50)$_{26}$, **237(100)$_{17}$**, 293(45)$_{11}$	(+)
ractopamine-d6	308	121(58)$_{23}$, 168(95)$_{16}$, 290(100)$_{12}$	(+)

Chromatographic separations were carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) made up of a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven kept at 30°C and a Rheodyne valve with a 20 µL loop equipped with a Synergi Hydro RP reverse- phase HPLC column (150 × 2.0 mm, internal diameter and elaborated using Xcalibur software from Thermo Fisher Scientific.

Table 2. MS/MS conditions for the MRM acquisitions of the selected steroids, as well as for the internal standards. Ions used for quantification are in bold. The values in parentheses represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

Analyte	Precursor ion (*m/z*)	Product ions(%)$_{CE}$ (*m/z*)	ESI
prednisolone acetate	403	**307(78)**$_{13}$, 325(40)$_{11}$, 385(100)$_{9}$	(+)
prednisolone	405	187(7)$_{30}$, **280(18)**$_{35}$, 329(100)$_{19}$	(–)
dexamethasone	437	307(24)$_{33}$, **361(100)**$_{20}$, 391(7)$_{14}$	(−)
prednisolone-d6	411	284(20)$_{37}$, **299(18)**$_{32}$, 333(100)$_{19}$	(−)
estradiol benzoate	377	105(100)$_{26}$, 135(20)$_{15}$, 359(32)$_{11}$	(+)
testosterone benzoate-d3	396	105(100)$_{25}$, 256(36)$_{20}$, 274(40)$_{16}$	(+)
nandrolone phenylpropionate	407	105(100)$_{31}$, 239(82)$_{18}$, 257(94)$_{16}$	(+)
nandrolone	275	91(75)$_{40}$, **109(98)**$_{27}$, 239(100)$_{16}$	(−)
testosterone-d3	292	109(100)$_{25}$, 123(20)$_{27}$, 256(19)$_{18}$	(+)

Method validation

The unique pre-treatment method followed by two different LC-MS/MS analyses was fully validated for all analytes according to the criteria of Commission Decision 657/2002/CE (European Union 2002). The following performance parameters were assessed for each analyte: specificity, selectivity, linearity, trueness, recovery, precision, decision limit (CCα), detection capability (CCβ), ruggedness and matrix effect.

To confirm specificity of these methods, 20 bovine tooth blank samples previously checked for the absence of the analytes were tested to ensure the absence of possible interferences at the retention time where the target analyte was expected to elute.

Selectivity was tested by verifying a signal-to-noise ratio greater than three at the expected retention time of the analyte and the ion abundance ratio for all MRM transitions.

Matrix validation curves were performed by spiking the pooled blank samples with known concentrations of each analyte resulting in three analytical series. Each series had six replicates for three concentration levels, C_0, 2 × C_0 and 3 × C_0, where C_0 was the minimum concentration detectable with our instrumentation. The β_2-agonists, estradiol benzoate, nandrolone, nan- drolone phenylpropionate and dexamethasone were tested at 0.1, 0.2 and 0.3 ng g^{-1}. Prednisolone was tested at 0.2, 0.4, 0.6 ng g^{-1} and prednisolone acetate was tested at 0.5, 1.0, 1.5 ng g^{-1}. The chromatograms and MS/MS spectra of the β_2-agonists and of the ster- oids in the matrix spiked with each analyte at the low- est validation level (C_0) are shown with their related internal standards (2 ng g^{-1}) in Figures 2 and 3, respectively.

Instrumental linearity was evaluated on pure standard solutions at six concentration levels in two replicates, from the minimum concentration detectable with our instrumentation up to 15 ng mL^{-1}, in order to estimate if the method's quantification range overlaid the instrumental linear range. From these data, slope and intercept were determined by the least squares regression method and the linear fit was verified using squared correlation coefficient (R^2).

Matrix calibration curves, applied for the quantita- tion of the real samples, were similarly built by analys- ing two replicates of blank tooth samples spiked with working solutions at the same concentration range used to evaluate instrumental linearity. The following deuterated standards were used for quantitation: racto- pamine-d6 for β_2-agonists; testosterone benzoate-d3 for estradiol benzoate; prednisolone-d6 for predniso- lone acetate, prednisolone and dexamethasone; testos- terone-d3 for nandrolone phenylpropionate and nandrolone.

The trueness estimated through recovery was eval- uated using the data from the validation points of the three analytical series and expressed in terms of the percentage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability, estimated as the percentage coefficient of variation (CV%), was evaluated by calculating the relative standard deviation of the results obtained from the 54 validation replicates and applying analysis of variance test (ANOVA).

CCα and CCβ values were calculated from the vali- dation curves using the x-axis extrapolation method as clarified in the document SANCO/2004/2726 revision 4 (European Union 2008b).

Ruggedness was evaluated using the fractional fac- torial design of Youden as described in the Commission Decision 2002/657/EC (European Union 2002). This test was conducted by introducing slight variations (±10%) to seven potentially critical analytical parameters in eight different trials by fortifying eight blank teeth samples at the lowest validation concentration. The parameters selected included the frequency of the ball mill during pulverisation, the time of pulverisation, the extraction mixture volume, the extraction mixture percentage composition, the sonication time,

the centrifugation time and the eva- poration temperature of the extract. The Fisher test was applied to compare the standard deviation of the dif- ferences obtained from the high- and the low-value setting for each experimental parameter with the stan- dard deviation of the method carried out under within- laboratory reproducibility condition.

A strategy developed by Matuszewski et al. (2003) was used to evaluate matrix effects. Sample extracts spiked with the analyte of interest before analysis were compared to pure solutions prepared in the mobile phase containing equivalent amounts of the analyte. The percentage ratio between the correspond- ing peak area for the standard spiked after extraction and the peak area obtained in standard solution was used to determine the extent of the matrix effect occur- ring for the analyte in question under chromatographic conditions.

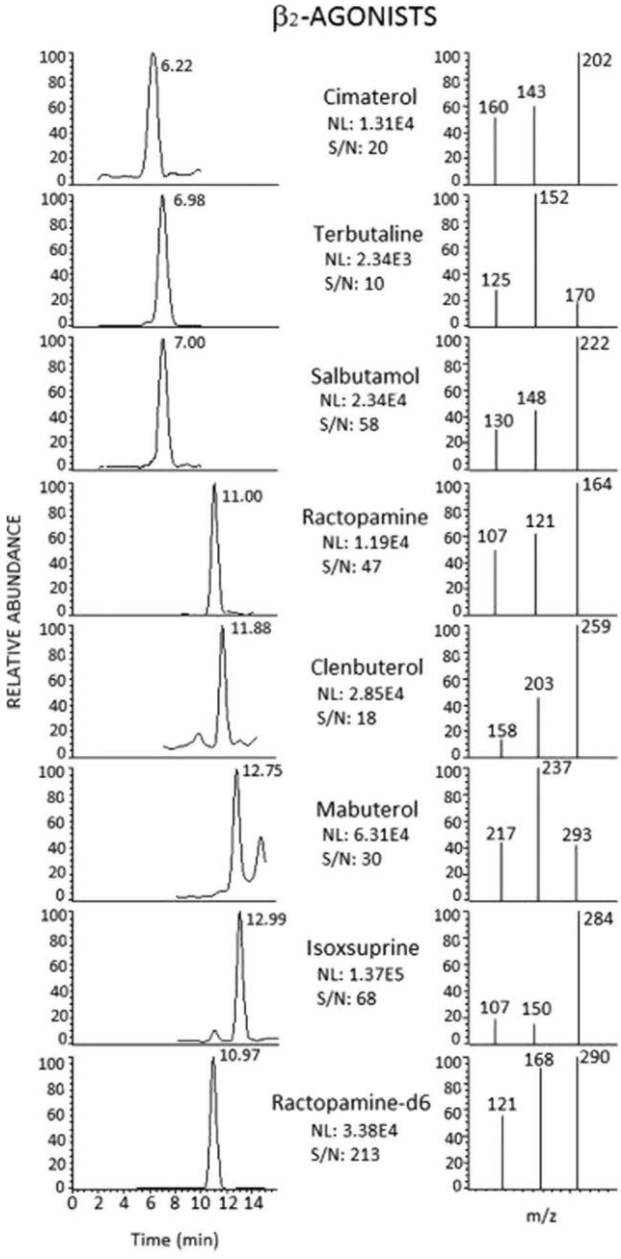

Figure 2. HPLC–MS/MS chromatograms and related MS spectra of the seven β_2-agonists in teeth sample spiked at the lowest validation concentration level (0.1 ng g^{-1}) and the internal standard ractopamine-d6 (2 ng g^{-1}).

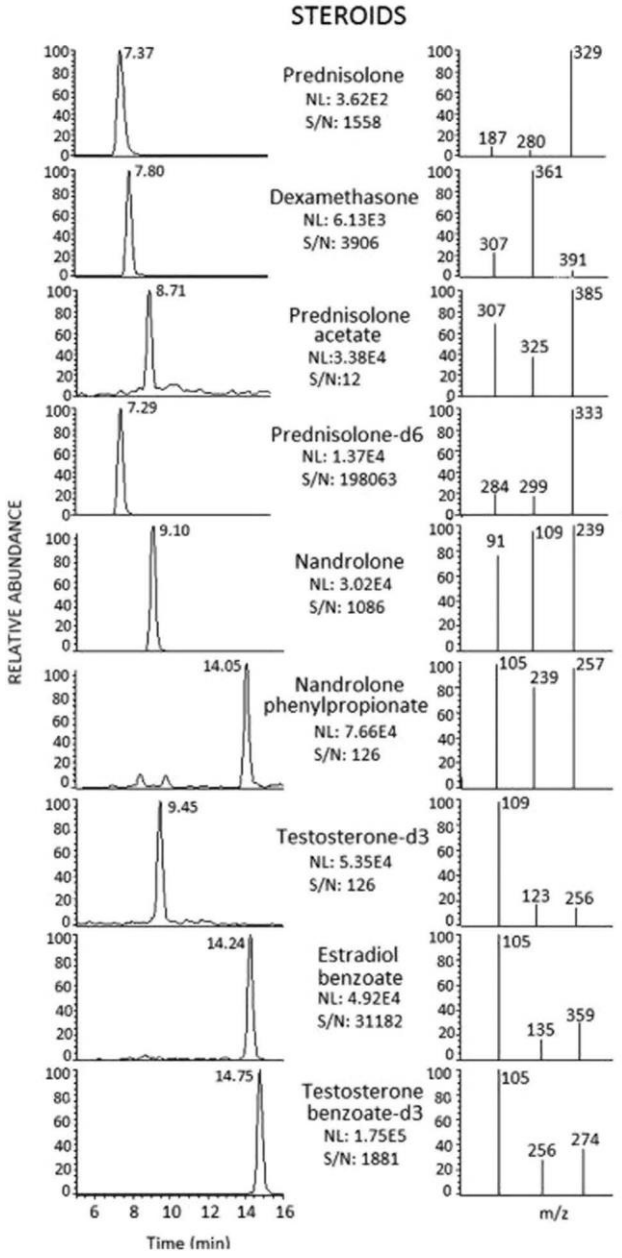

Figure 3. HPLC–MS/MS chromatograms and related MS spectra of the selected steroids in teeth sample spiked at the lowest validation concentration level (0.1 ng g^{-1} for estradiol benzoate, nandrolone, nandrolone phenylpropionate and dexamethasone; 0.2 ng g^{-1} for prednisolone and 0.5 ng g^{-1} for prednisolone acetate). Each group of analytes is followed by the related internal standard (2 ng g^{-1}).

Results and discussion

The proposed analytical protocol was entirely validated as a confirmation method, in agreement with the European guidelines (European Union 2002, 2008b) for all steroids and β_2-agonists tested. Previous trials were made to develop a unique procedure, i.e. one extraction followed by just one analysis for all studied molecules. However, in a unique chromatographic run, the separation of the steroids hampered the retention of some β_2-agonists (cimaterol, terbutaline and salbu- tamol) that instantly eluted, making their detection impossible. As the purpose of this study was to demonstrate the suitability of bovine teeth as a matrix for the detection of veterinary drugs, we preferred to perform two chromatographic analyses having optimal conditions.

The chromatographic profiles obtained from blank tooth samples did not show the presence of any inter- ference signals at the relative retention time expected for our compounds, demonstrating the method speci- ficity. The methods also displayed selectivity, with sig- nal-to-noise ratios greater than three and the expected ion ratio abundances in correspondence of retention time for each analyte.

The least squares regression method was used to confirm instrumental linearity of standard solutions, with a R^2 greater than 0.997. Similarly, the R^2 was greater than 0.991 for the calibration curves for quantitation of the real samples, indicating a good fit of the curves on the experimental points.

The validation parameters presented in Tables 3 and 4 confirm that the extraction method and analyses are compliant with European guidelines. In particular, the recoveries ranged from 95 to 106% for the β_2-agonists and from 94 to 105% for the steroids, demonstrating that a simple liquid extraction without incubation in acidic or alkaline solution, as some authors perform (Andra et al. 2015), was able to extract all analytes from the complex structure and composition of the tooth.

The precision, in terms of intra- and inter-day repeatability, calculated by applying the one-way ana- lysis of variance (ANOVA) and expressed as the per- centage coefficient of variation (CV%), ranged from 10 to 20% for β_2-agonists and from 9 to 21% for steroids. These values were lower than 23%, as proposed by Thompson (2000) and considered satisfactory accord- ing to the international guidelines.

Calculated CCα values ranged from 0.13 to 0.17 ng g^{-1} and from 0.16 to 0.76 ng g^{-1}, for β_2-agonists and steroids, respectively. The CCβ values ranged from 0.16 to 0.21 ng g^{-1} and from 0.23 to 0.87 ng g^{-1} for β_2-agonists and steroids, respectively. These experi- mentally determined levels were slightly higher than the lowest levels of validation chosen through the minimum concentration detectable with our instru- mentation, ensuring compliance with all the identifi- cation criteria.

The sample quantitation planned to carry out the Youden (European Union 2002) approach for rugged- ness evaluation, was interpreted through both Student and Fisher tests. No significant variation was found by these analyses even when slight alterations of the seven potentially critical analytical parameters were intro- duced in the sample preparation and extraction steps. The modest matrix effect gave values ranging from 88 to 106% for the β_2-agonists and from 84 to 109% for the anabolic steroids.

Table 3. Validation parameters for the seven β2-agonists.

Analyte	CCα (ng g^{-1})	CCβ (ng g^{-1})	Concentration level (ng g^{-1})	Recovery % (*n* = 18)	Repeatability intra-day (CV; *n* = 6)	Repeatability inter-day (CV;*n* = 18)
cimaterol	0.16	0.20	0.1	102	18	20
			0.2	97	14	17
			0.3	101	13	14
terbutaline	0.16	0.19	0.1	97	17	19
			0.2	99	13	15
			0.3	100	12	13
salbutamol	0.17	0.21	0.1	104	17	20
			0.2	96	16	18
			0.3	101	13	15
clenbuterol	0.13	0.16	0.1	97	13	14
			0.2	103	13	14
			0.3	99	10	10
ractopamine	0.15	0.19	0.1	106	17	18
			0.2	97	12	14
			0.3	102	12	12
isoxsuprine	0.15	0.19	0.1	97	17	18
			0.2	102	12	15
			0.3	95	10	11
mabuterol	0.14	0.18	0.1	95	16	19
			0.2	104	14	17
			0.3	98	12	12

Table 4. Validation parameters for the selected steroids.

Analyte	CCα (ng g^{-1})	CCβ (ng g^{-1})	Concentration level (ng g^{-1})	Recovery % (*n* = 18)	Repeatability intra-day (CV; *n* = 6)	Repeatability inter-day (CV;*n* = 18)
			0.5	104	18	21
prednisolone	0.76	0.87	1.0	96	16	17
Acetate			1.5	101	13	14
			0.2	101	19	20
prednisolone	0.25	0.37	0.4	96	13	15
			0.6	100	11	13
			0.1	105	16	17
dexamethasone	0.16	0.23	0.2	94	13	15
			0.3	101	11	12
			0.1	99	19	21
estradiol	0.20	0.35	0.2	101	19	20
benzoate			0.3	100	19	20
			0.1	95	14	17
nandrolone	0.25	0.38	0.2	105	15	16
phenylpropionate			0.3	98	11	12
			0.1	103	11	15
nandrolone	0.17	0.25	0.2	100	9	13
			0.3	100	10	11

Application of the methods to real samples

We next applied our method, consisting of a common liquid extraction followed by two analyses, to test our hypothesis of accumulation of veterinary drugs in teeth. Samples from three veal calves subjected to treat- ment and from seven anonymous bovines were ana- lysed. Importantly, all of the analytes from the treatment protocols were detectable in their respective tooth samples (ractopamine 8.90 ng g^{-1}, estradiol benzoate 8.78 ng g^{-1}, prednisolone acetate 2.90 ng g^{-1}) except nandrolone phenylpropionate. A major observa- tion is that we did not find the free form where the hydrophilic pharmaceutical esterified form was detected, likely due to the nature of the matrix, con- stituted for the most part by hydroxyapatite, for which the esterified form should have a much higher affinity. The detection of the pharmaceutical form could there- fore be a valid proof of illegal treatment, particularly in the case of endogenous (estradiol) or pseudoendoge- neous substances (nandrolone and prednisolone) (Kennedy et al. 2009; Bertocchi et al. 2013). The data collected do not allow us to explain the absence of nandrolone ester. Some suggestions could however be given: the gap between the last administration and the slaughtering was 2 weeks long, different from all the time intervals of other drugs. Nandrolone phenylpro- pionate could not accumulate into teeth, as it could

require a longer time to reach the teeth and/or the cocktail with ractopamine could affect the distribution of the steroid.

Finally, we detected isoxsuprine in one of the unknown teeth samples at a concentration of 13.67 ng g^{-1} –further evidence of the effectiveness of this method. None of the other analytes in this study was ever found.

Conclusion

Two LC–MS/MS methods for the analysis of bovine teeth with a common liquid extraction was validated and applied to samples from treated and anonymous bovines. The analytes included seven β_2-agonists (cimaterol, clenbuterol, isoxsuprine, mabuterol, racto- pamine, salbutamol and terbutaline), and four steroids in free or esterified forms (prednisolone acetate, pre- dnisolone, dexamethasone, estradiol benzoate, nandro- lone phenylpropionate and nandrolone). The methods were validated in accordance with the criteria of the European Commission Decision (2002/657/CE) (European Union 2002) and SANCO/2004/2726 revi- sion 4 (European Union 2008b).

The application of the methods to teeth from ani- mals with known anabolic treatment leads to effective detection of ractopamine, prednisolone acetate and estradiol benzoate. However, nandrolone phenylpro- pionate was not found in teeth. The detection of iso- xsuprine in one unknown sample confirms the suitability of this method for the detection of β_2-ago- nists use.

The utilisation of teeth as an accumulation matrix will be the subject of further studies that will deal with different tooth groups and animals of varying ages

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Disclosure statement

No potential conflict of interest was reported by the authors.

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