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Multiclass screening method to detect more than fifty banned substances in bovine bile and urine

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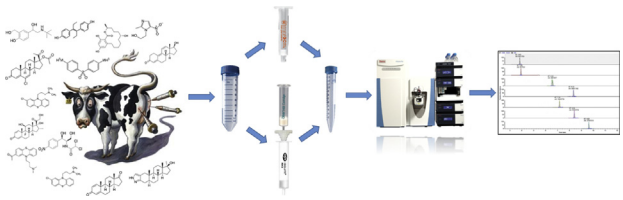
HIGHLIGHTS

- A screening method for banned substances was developed in bovine urine and bile.
- First method fully validated for the simultaneous detection of 53 substances.
- LC-Q-Orbitrap was applied with parallel reaction monitoring acquisition.
- A novel sample clean-up was described to include this wide range of analytes.
- The CCβs were assured irrespective to sample-to-sample variability.

GRAPHICAL ABSTRACT



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ABSTRACT

A multiclass screening method to detect fifty-three forbidden substances by liquid-chromatography coupled to hybrid high-resolution mass spectrometry (LC-Q-Orbitrap) was developed and validated in bovine bile and urine. Eight classes of compounds were included in the method's scope (β-agonists, corticosteroids, nitroimidazoles, progestins, resorcylic acid lactones (RALs), sedatives, steroids and stilbenes) plus chloramphenicol and dapsone. After hydrolysis, the sample was divided in two aliquots, which followed two parallel purification steps. The reunified extracts were injected and two chromatographic runs performed in positive and negative ionization mode, respectively. The validation data (60 different samples per matrix) proved that the method was fit for purpose with detection capabilities lower than 1 $\mu g\,L^{-1}$ in both matrices. The combined application of accurate mass acquisition and two-stage mass spectrometry (parallel reaction monitoring) was crucial to achieve suitable selectivity, which is the most critical parameter mainly for urines. Finally, the long-standing problem of the high rate of false positive results for RALs, due to the natural ingestion of mycotoxin, zearalenone, was taken on including all their labelled standards. That allowed a very satisfactory management of this screening test.

1. Introduction

Treatments in farm with growth-promoting agents has been prohibited in European Union for over 25 years [1,2], although the various commercial controversies and debates, like the so-called

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"beef war" or "beef hormone dispute". The traditional targeted methods have played a fundamental role in the evaluation of human exposure to these substances, as well as to sustain the legislative progress, but they are more and more criticised for the low (unrealistic?) percentages of non-compliant results found against the high number of official controls annually performed all along the European territory. According to the last available EFSA Report, in view of 441677 targeted samples analysed in European Union as part of National Residue Plans (NRPs) for prohibited substances (groups A1 to A6), only 0.11% of non-compliant results were found [3]. Although that can be due to many causes, it is well known that residues in urine are generally no longer detectable after two-five days from the last administration [4,5]. Moreover, the illegal practices in farm evolved over time with the administration of synthetic design steroids, endogenous hormones or cocktails containing more compounds at low levels and hence the analytical strategies need to evolve as well. To fight animal doping, novel approaches have been developed, such as the "untargeted" strategies involving "omics" technologies (proteomics, metabolomics, lipidomics and transcriptomics). These techniques are based on a different principle, i.e. the measure of the effect of a certain drug rather than its residues and they can be helpful as screening methods in order to direct controls towards suspect farms without any knowledge about the administered substances. However, omics approaches are quite complex and still far to be applied in a regulated context, which requires a high standardization level [6]. Less sophisticated strategies such as the analysis of alternative matrices able to bioconcentrate the administered drugs (and/or their metabolites) for a longer period than the "traditional" ones, may be efficient and feasible, as successfully experienced in the past. For instance, traces of the beta-agonist, clenbuterol, can be detected in pig retina 45 days after the administration, as demonstrated by Pleadin and coworkers [7]. Another example is the capability of hair in accumulating beta-agonists, which can be detected even 70 days following the last administration dose [8,9]. Finally, many studies demonstrated the bioaccumulation of some steroids and resorcylic acid lactones (RALs) in bile with the possibility of detecting them in this fluid for several weeks [10–13]. Another way to improve the efficiency of official controls there is the increase of the number of analytes simultaneously tested through multiclass screening methods based on liquid-chromatography coupled to mass spectrometry detection (LC-MS). In fact, frequently, the screening assays are still performed searching for one molecule at a time or, at most, looking for a single class of compounds (β-agonists, corticosteroids etc.) applying immunoenzymatic techniques. Obviously, this common practice restricts the possibility to discover unknown illicit treatments if substances different from those searched have been administered. In addition, the official laboratories are forced to apply dozens of different screening methods in order to detect the whole list of banned substances to be monitored, i.e. substances belonging to groups A1-A6 [14]. Nowadays, the technological advancements, especially in mass spectrometry, made targeted multiclass procedures possible to be developed and daily applied. However, while many papers describing methods for the simultaneous determination of various classes of contaminants in food such as pesticides or permitted veterinary drugs have been published [15], few examples can be found for banned substances [16–18]. The main reason is the need of reaching concentrations about ten-twenty folds lower that those required for the abovementioned groups, besides the remarkable complexity of the matrices involved, above all urine.

In this study, we developed and validated a multiclass screening method covering eight classes of compounds (β-agonists, corticosteroids, nitroimidazoles, progestins, resorcylic acid lactones (RALs), sedatives, steroids and stilbenes) plus chloramphenicol and dapsone (Fig. 1) in bovine bile and urine applying liquid-chromatography coupled to hybrid high-resolution mass spectrometry (LC-Q-Orbitrap). In addition, since the presence of zeranol in urine and bile might occur both from an illicit treatment with zeranol or, naturally, from the metabolism of the mycotoxin zearalenone which can contaminate animal feedstuffs [13], practical criteria were given to avoid high rates of false positive results.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile for LC-MS, ethyl acetate reagent grade, ethyl ether, methanol for LC-MS (MeOH), acetic acid for LC-MS, sodium chloride (NaCl), sodium hydroxide (NaOH), and *Helix Pomatia* β-glucuronidase type H-2 containing also aryl sulfatase, code G0876 (used for the enzymatic hydrolysis) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Ammonia solution 30%, Hydrochloride (HCl) solution 37%, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate bibasic (Na_2HPO_4) were from Carlo Erba (Milan, Italy). Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) was supplied from AppliChem GmbH (Darmstadt, Germany). Sodium acetate was obtained from VWR International Srl (MI). Deionized water came from a MilliQ water purification system (Millipore Co, Billerica, MA, USA). SPE Isolute NH_2 (500 mg, 6 mL) cartridges were obtained from Biotage (Uppsala, Sweden), SPE Strata-X-C 33u (200 mg, 6 mL) were from Phenomenex (Torrance, CA, USA) and SPE Oasis HLB (200 mg, 6 mL) from Waters (Milford, MA, USA). Millipore PTFE filters (0.2 μm) were supplied from Merck S.p.a. (Vimodrone, Italy). LC columns Kinetex XB - C18 100A (100 × 3 mm 2.6μ, code 00d-4496-Y0), C6-Phenyl (100 × 2.00 mm 3 μm, code 00D-4443-B0) and Kinetex PFP (100 A 100 × 2.10 mm 2.6 μm, code 00D-4477-AN) were purchased from Phenomenex (Torrance, CA, USA). Hypersil Gold (100 × 2.1 mm 5 μm, code 25005–102130) was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Acetate buffer 0.2 M was prepared by dissolving, in a 1000 mL graduated flask, 16.4 g of sodium acetate in 800 mL of MilliQ water and adjusting pH to 5.2. with acetic acid. Then, solution was made up to the mark with ultrapure water and mixed. Phosphate buffer 0.1 M (pH = 6.0) was prepared by dissolving, in a 1000 mL graduated flask, 2.7 g of sodium phosphate monobasic (NaH_2PO_4), 14.3 g of sodium phosphate bibasic (Na_2HPO_4) and 9 g of sodium chloride (NaCl) in 800 mL of MilliQ water and adjusting the pH with HCl. Then, solution was made up to the mark with ultrapure water and mixed. Buffer TRIS/HCl (pH = 9) was prepared by mixing 250 mL of 0.2 Tris(hydroxymethyl) aminomethane hydrochloride solution (24.23 g L^{-1}) and 53 mL of 0.1 M HCl. Then, pH was adjusted and the solution was made up to 1000 mL with ultrapure water in a graduate flask.

2.2. Reference standards

17-α-methyltestosterone, acepromazine, α-zearalenol, zeranol, azaperol, azaperone, beclomethasone, β-boldenone, betamethasone, β-trenbolone, β-zearalenol, taleranol, carazolol, clenbuterol, clenpenterol, chloramphenicol, chloramphenicol-d5, chlorpromazine, chlormadinone acetate, dapsone, dexamethasone, dienestrol, diethylstilbestrol, dimetridazole, hexestrol, flumethasone, HMMNI, ipronidazole, ipronidazole-OH, isoxsuprine, medroxyprogesterone, megestrol acetate, melengestrol acetate, methylprednisolone, metronidazole, metronidazole-OH, prednisolone, prednisone, promazine, propionyl promazine, ractopamine, ronidazole, salbutamol, terbutaline, triamcinolone, triamcinolone acetonide, zearalanone and zearalenone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone-d5 was supplied by

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