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# Evaluation of MISPE for the multi-residue extraction of β-agonists from calves urine

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**Abstract**

Methods based on molecular recognition mechanisms for the clean-up of veterinary drugs and their residues, such as immuno-, receptor- and acceptor-affinity and molecularly imprinted polymers (MIPs), have been described as selective tools to improve the selectivity and the reliability of analytical results. In this work, we tested the extraction recovery performances of a MISPE column, designed for multi-residual clean-up of β-agonists. For this purpose, 18 different samples of calf urine were spiked at 0.25, 0.50 and 1.00 ppb with pooled standard solutions of clenbuterol (Clen), tulobuterol (Tolu), isoxsuprine (Isox), brombuterol (Brom), mapenterol (Mape) and ractopamine (Racto) and analysed on two independent analytical sessions, on a LC–MS/MS ion trap detector. Averaged recoveries, constant for each molecule considered, were 64.6% for Racto, 63.0% for Salm, 59.9% for Form, 54.7% for Brom, 52.0% for Clen, 41.8% for Mape, 38.6% for Tolu and 34.5% for Isox, respectively. Reproducibility studies gave a CV $< 11\%$ at the 0.25 ppb level. The decision limit for the identification of the target drugs ranged from 0.01 ppb for mapenterol to 0.19 ppb for salmeterol, when considering one precursor, and two product ions as identification points. Such findings indicate that the choice of the appropriate molecule as template in the MIP preparation is the critical factor to guarantee a reliable analytical multi-residue approach for β-agonists, despite the structural differences among molecules exploiting almost the same pharmacological effect.


*Keywords:* Molecular imprinting; β-Agonists

## 1. Introduction

The alimentary trend to produce lean meat as possible functional food to prevent or limit hypercholesterolemia and obesity in man [1], is pushing the animal production towards the use of tools, able to influence the fat metabolism in farmed animals.

With respect to this, it has recently been proposed that feeds could be supplemented with natural phytosterols from tall oil, able to lower cholesterol absorption in the gut [2]. As an alternative, some repartitioning agents such as the β-agonists zilpaterol (Zilmax®) and ractopamine (Paylean®) have been licensed as feed additives, due to the capability to stimulate lipolysis via β-adrenergic stimulation.

The risk analysis of such a pharmacological modulation, nevertheless, is still in discussion, due to the potential risk for consumers. Several recent reports describe the abuse of the β-agonist clenbuterol in meat production as the main reason for collective intoxication outbreaks in humans, in Portugal [3], China [4] and Mexico [5]. For this reason, EU has officially forbidden the use of adrenergic drugs as growth promoting agents since 1996, while other countries like USA, Mexico and South Africa have licensed some of them at growth promoting doses.

Therefore, monitoring plans have to deal with multi-residual approaches, able to cover a broader panel of molecules sharing the same pharmacological effect. To this respect, the most interesting approaches are based on mixed phase solid-phase extraction (SPE) [6] and molecular recognition mechanisms, such as acceptor affinity [7] and molecularly imprinted polymers (MIPs), with the aim to improve the analytical selectivity of the overall control strategy.

Molecular imprinting is an emerging technology gaining more and more interest in science as well as in industry. With this technology highly cross-linked polymers are

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formed around a template molecule by allowing functional and cross-linking polymers to co-polymerise. The selective recognition sites formed are complementary both in shape and chemical structure to the template molecule and after removal of the template these sites can rebind the template molecule or closely related structural analogues with similar affinities and selectivities as natural antibodies. This was first demonstrated by Vlatakis et al. [8] and recently an increasing number of such comparisons have been made. The advantage of MIPs compared with natural antibodies is the superior stability. These polymers can withstand high temperatures, a large pH range and organic solvents without loosing their recognition properties [9,10]. Furthermore, they are faster and cheaper to produce and no animals are needed.

Due to these properties, they are suited as selective sorbents in solid-phase extraction, allowing selective clean-up of compounds prior to analysis. In recent years, MIPs have been used as sorbents in solid-phase extraction for various compounds such as sameridin [11], nicotine [12], propranolol [13], triazines [14], darifenacin [15] and for the β-agonist clenbuterol [16] to mention some.

The aim of this work was to verify the extraction performance of the use of a new MIP column for β-agonists, designed to cover a broad spectrum of potential analytes, not limited only to clenbuterol-like compounds (Fig. 1). We chose as default a minimum performance required limit (MPRL) of 0.25 ppb for each of the eight analytes considered in this work. Agonists were chosen as representative of the most abused growth promoters. The MPRL is consistent with the pharmacokinetics of β-agonists, such as clenbuterol [17] and ractopamine [18] in urine of farmed cattle, according to the proposed doses in feeds.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals

Clenbuterol hydrochloride (Clen), tulobuterol hydrochloride (Tolu), isoxsuprine hydrochloride (Isox) were all purchased from Sigma Italia (Milan, Italy) and the purity was at least 95%. Brombuterol, free base (Brom), was manufactured by Synthelec (Lund, Sweden) and the purity was at least 95%. Mapenterol (Map) and ractopamine (Racto) were purchased from RIVM (Bilthoven, The Netherlands). Salmeterol hydroxynaphthoate (Salm) was a kind gift from GlaxoSmithKline (Mölndal, Sweden) and formoterol fumarate dehydrate (Form) was a kind gift from AstraZeneca (Lund, Sweden). Acetonitrile and methanol HPLC grade came from Riedel-de-Haën. Acetic acid p.a. was purchased from Fluka Italia (Milan, Italy) and sodium acetate p.a. was from Merck (Darmstadt, Germany). All water used was distilled and then purified using an ultra pure water system from Elga (Partlille, Sweden).

#### 2.1.2. MISPE columns

MIP4SPE® columns β-agonist, 25 mg, 10 ml, internal diameter 5 mm, average particle size 56 μm, pore size unknown was from MIP Technologies (Lund, Sweden). The polymer was of acrylic type containing a monomer with an acidic functional group, p$K_a$ 4.4.

#### 2.1.3. Biological samples

Calf urine from 20 different subjects around 220 kg body weight fed on 20% fat milk replacer and dry corn silage were sampled at slaughter directly from the bladder. The density ($1.020 \pm 005$) and pH ($5.4 \pm 0.3$) were measured and samples stored at $-20\,^{\circ}C$ until analysed.

### 2.2. Apparatus

An analytical set-up consisting on a HPLC 1100 coupled with a LC/MSD TRAP SL, and with an autosampler (Agilent Technologies Italia, Milan, Italy) were used to determine the recoveries. Nitrogen was supplied by a Parker Balston (Milan, Italy) model 75-72 generating system.

### 2.3. Methods

#### 2.3.1. MIP preparation

The template, acidic monomer (acrylic, p$K_a$ 4.4), difunctional acrylic cross-linker, initiator and porogen were mixed together. The template used contained the common structure of β-agonists (Fig. 2). The polymer solution was purged with nitrogen and polymerised by radical polymerisation. Thereafter the polymer was milled, sieved and washed extensively in several steps to minimise bleeding of the template. The selective cavities formed in the polymer contain acidic groups that interact with the –OH and –NH groups of the different β-agonists, by forming hydrogen bonds (Fig. 2).

#### 2.3.2. MISPE recoveries

To calculate the extraction recoveries, urine samples from 20 different veal calves were analysed as blanks, and spiked at 0.25, 0.50 and 1.00 ng/ml, respectively, with 400 μl of a pooled standard working solution (0.1N acetic acid). In total, six independent replicates for each compound at the three different concentration levels were analysed. Analyses were carried out on two different days to calculate repeatability and reproducibility. An aliquot of 4 ml of calf urine was drawn, centrifuged at $3000 \times g$ for 10 min and mixed with 4 ml water to reach a final volume of 8.4 ml. After the addition of 50 μl arylsulphatase/β-glucuronidase, the urine sample was let to stay at RT for 4 h before extraction. The blanks ($N = 20$) and spiked samples ($N = 18$) were then applied on conditioned MISPE β-agonist columns, according to the procedure earlier described [16], but with minor modifications. A VacMaster Sample Processing Station from International Sorbent Technologies (Mid Glamorgan, UK) was used during extraction. The columns were conditioned prior to 8.4 ml sample application by the

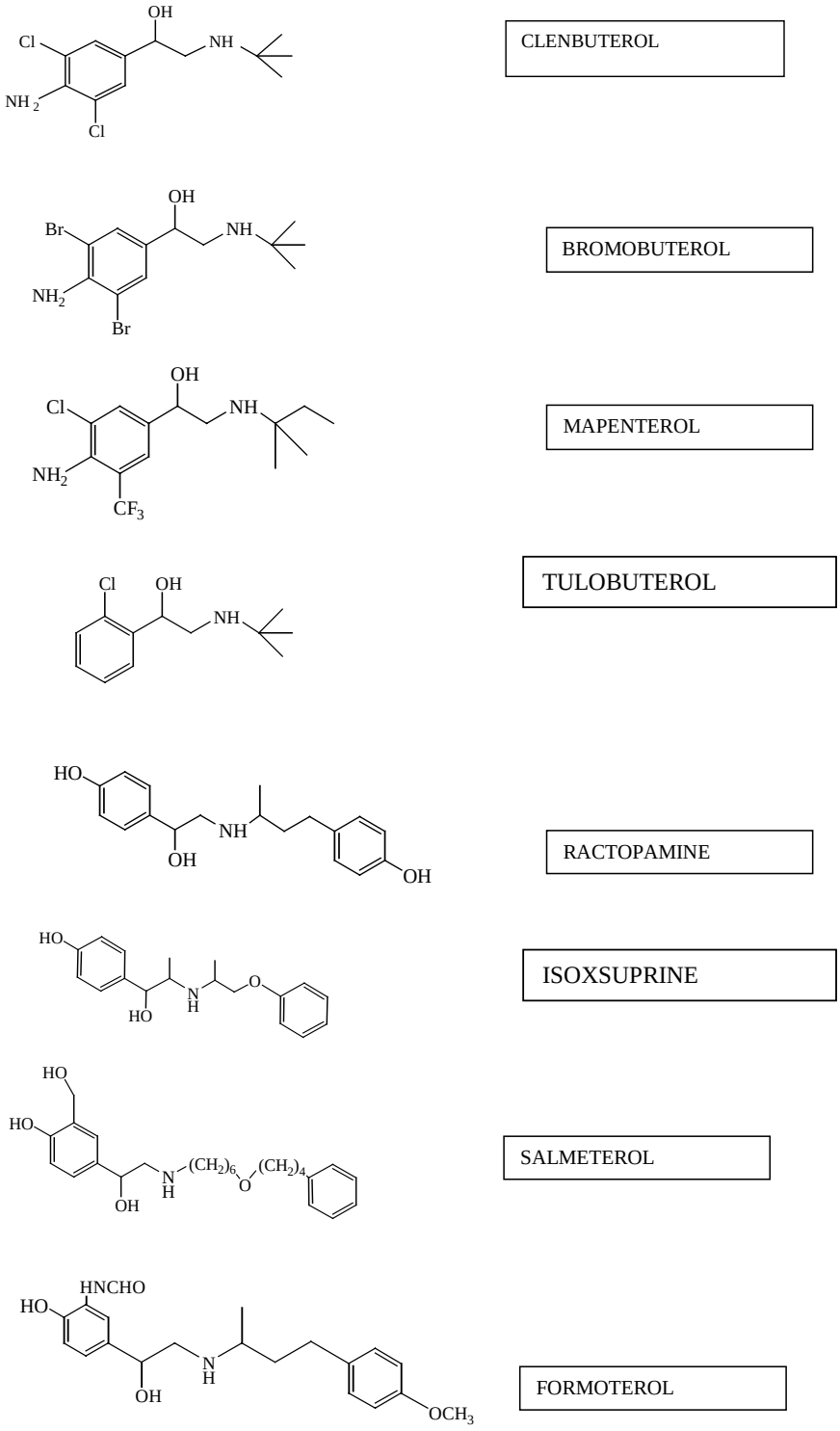


Fig. 1. Structures of β-agonists considered.

following sequence: 1 ml methanol, 1 ml water and 1 ml 25 mM sodium acetate, pH 6.7. The flow rate by gravity was chosen (around 0.5 ml/min), facilitating column dripping by applying a slight vacuum, if necessary. Thereafter, the columns were washed with 1 ml water, followed by 2 min of vacuum (−0.7 bar), to allow the columns to semi-dry. Another wash with 1 ml acetonitrile containing 1% acetic acid was then applied, followed by a short vacuum prior to elution of the β-agonists two times with 1 ml methanol, containing 10% acetic acid. A slight vacuum was applied between the two elution aliquots. The columns were used only once and then discarded. The extracts were evaporated

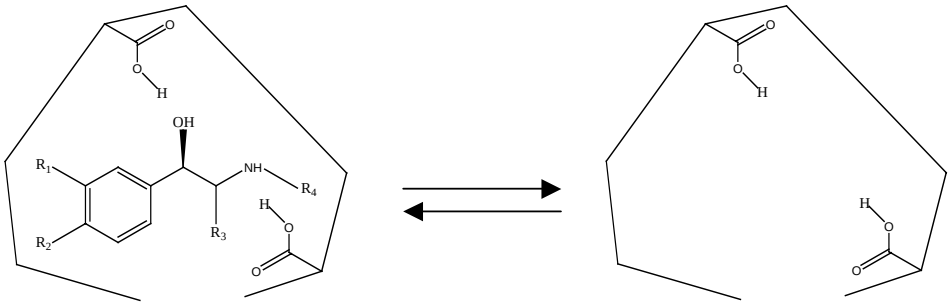


Fig. 2. Proposed interactions between β-agonists shared functional groups and the MIP carboxylic moieties.

with $N_{2(g)}$ at 60 °C, and reconstituted in 50 μl MeOH before LC–MS/MS analysis. To evaluate possible ionisation suppression phenomena of the analytes induced by the matrix, blank extracts were re-suspended with pooled standard methanolic solutions, to a final concentration of 1.00, 2.00 and 4.00 ng in a 50 μl volume.

### 2.4. LC–MS/MS analysis

A 10 μl fraction of the extracts was injected into the LC/MSD TRAP SL system, with electrospray interfaces, under the following conditions: column MS C8 X terra Waters, 2.1 mm × 150 mm, 5 μm; mobile phase: (A) 1% acetic acid, (B) methanol; chromatographic run: flow 0.35 ml/min, from 3% B (hold 3 min), then a linear gradient to 30% B in 12 min, then to 100% B in 3 min, hold for 3 min, then back to 3% B in 1 min; nebuliser set at 30 psi; nitrogen as dry gas at 8 l/min; temperature at +350 °C. Acquisition performed in MRM positive ions mode, by selecting the precursor ion and monitoring two product ions specific for each drug, as reported in Table 1. The ratio of relative abundances of the two product ions has also been taken into account, according to EU's recent criteria about proper identification of the compounds.

### 2.5. Data treatments

The calculation of CCα as the decision limit for the identification (the minimum amount of analyte eventually present in the sample that allows to conclude that the sample is not compliant, with a 99% level of probability) was according to the EU Commission Decision 2002/657/EC. The calculation was done by building up the calibration curves in matrix, with the sum of the area of product ions of each drug plotted against its nominal concentration; the values of the intercept of the curves on the *y*-axis + 2.33S.D. of the within lab reproducibility corresponded to the decision limit. Recovery rates for each drug ($N = 18$) were tested for significance with ANOVA test, with a $P < 0.05$.

## 3. Results and discussion

Repeatability and reproducibility studies and overall mean recovery rates, are reported in Tables 2 and 3, respectively. Among recovery rates, significant differences ($P < 0.05$) were found between Salm and Racto versus Brom, Clen, Mape, Tolu and Isox; and between Salm, Racto, Form, Brom and Clen versus Tolu and Isox. Within such a frame it is worthy to note that Salm recoveries were not significant with respect to Racto and Clen ones, whereas Mape recoveries were significant with respect to those of Clen and Tolu, respectively.

The decision limits for the proper identification, expressed as CCα, are summarised in Table 4. In all spiked urine samples, the ratio between the relative abundances of the selected product ions for each drug considered fell within the tolerances of 10%, thus fulfilling the criteria of EU legislation for their identification.

Table 1
LC–MS/MS acquisition parameters of the selected β-agonists

| Drug | $t_R$ | Precursor ion (amu) | Collision energy (V) | Product ions (amu) | Relative abundance of product ions (%) |
|---|---|---|---|---|---|
| Ractopamine | 8.7 | 302 | 1 | 284, 164 | 100, 70 |
| Clenbuterol | 9.1 | 277 | 1 | 259, 203 | 100, 20 |
| Tulobuterol | 9.9 | 228 | 1 | 154, 172 | 100, 71 |
| Brombuterol | 10.5 | 367 | 1 | 349, 293 | 100, 73 |
| Formoterol | 10.6 | 345 | 1 | 327, 149 | 100, 37 |
| Isoxsuprine | 11.6 | 302 | 1 | 284, 150 | 100, 10 |
| Mapenterol | 13.5 | 325 | 1 | 307, 237 | 100, 83 |
| Salmeterol | 19.9 | 416 | 1 | 398, 380 | 100, 56 |

The retention time ($t_R$) is referred to that of the precursor ion.

Table 2
Repeatability of recovery rates on urine of veal calves (8.4 ml final volume applicated), spiked at three different levels (six independent replicates for each level)

| Drug | Recovery (%) | | | S.D.$_r$ | | | CV (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.25[a] | 0.50 | 1.00 | 0.25 | 0.50 | 1.00 | 0.25 | 0.50 | 1.00 |
| Ractopamine | 57.9 | 66.3 | 64.6 | 5.4 | 0.6 | 2.7 | 9.4 | 1.0 | 4.1 |
| Clenbuterol | 55.2 | 51.2 | 49.6 | 2.9 | 4.3 | 1.9 | 5.2 | 8.4 | 3.9 |
| Tulobuterol | 33.8 | 42.3 | 39.6 | 2.0 | 2.2 | 5.2 | 6.0 | 2.6 | 6.5 |
| Brombuterol | 57.4 | 53.1 | 53.6 | 4.7 | 3.5 | 1.7 | 8.2 | 6.6 | 3.2 |
| Formoterol | 42.8 | 63.7 | 65.5 | 3.7 | 3.5 | 2.1 | 8.6 | 5.5 | 3.3 |
| Isoxsuprine | 37.6 | 33.8 | 32.1 | 3.2 | 2.5 | 1.01 | 8.5 | 7.5 | 3.2 |
| Salmeterol | 54.0 | 61.8 | 63.8 | 6.3 | 3.3 | 2.1 | 11.7 | 5.3 | 3.2 |
| Mapenterol | 43.7 | 40.2 | 41.6 | 3.6 | 1.4 | 1.0 | 8.1 | 3.4 | 2.5 |

[a] Fortification level in μg/kg.

Table 3
Reproducibility of recovery rates on urine of veal calves, spiked at three different levels on two independent analytical sessions

| Drug | S.D.$_{wlR}$ | | |
|---|---|---|---|
| | 0.25[a] | 0.50 | 1.00 |
| Ractopamine | 7.2 | 1.1 | 3.3 |
| Clenbuterol | 3.8 | 6.7 | 3.0 |
| Tulobuterol | 2.3 | 3.5 | 3.0 |
| Brombuterol | 7.5 | 4.5 | 2.3 |
| Formoterol | 4.7 | 5.6 | 2.6 |
| Isoxsuprine | 3.9 | 3.3 | 1.2 |
| Salmeterol | 10.5 | 3.6 | 2.9 |
| Mapenterol | 5.9 | 1.8 | 1.6 |

[a] Fortification level in μg/kg.

An example of multi-residue analysis of urine spiked at 0.25 ng/ml with pooled standards and a blank urine is displayed in Fig. 3. The large number of molecules sharing the same adrenergic effects on adipocytes, but differing in the chemical structure, has progressively prompted the development of multi-residue methods, with the aim to limit as much as possible the occurrence of false negative results. Immuno-affinity columns have been firstly developed with polyclonal sera produced in laboratory animals immunised against target molecules. Nevertheless, this approach is affected by the limited loading capacities of the columns (in the order of a few hundred nanograms per analyte), the need to use lab animals in large quantities, to produce enough antibodies, and the need to immunise animals against different target molecules, in order to broaden the selectivity. The latter limitation is mainly determined by the coupling of the target drug with a high molecular weight immunogen carrier (such as bovine serum albumin), thereby hindering the immune systems to react against some functional groups of the drug [7,19].

Table 4
Limit of decision, expressed as CCα, for the identification of each compound considered

| Compound | CCα (ppb) |
|---|---|
| Racto | 0.03 |
| Clen | 0.03 |
| Tolu | 0.05 |
| Brom | 0.05 |
| Form | 0.07 |
| Isox | 0.10 |
| Salm | 0.19 |
| Mape | 0.01 |

On the other hand, SPE procedures based on mixed phase columns have revealed to be selective not only for β-agonists, but also for other basic drugs, such as tranquillisers and hypnotic sedatives. As a matter of fact, in this case the hydrophobic and the ion exchange interactions between the analyte and the sorbent do not occur at the same time, as in MISPE, thus leading to a larger use of solvents and a more time-consuming extraction procedure [20].

To overcome such critical factors, we focused our attention on MIP technology. The β-agonists share a common structure (shown in Fig. 1) where the –OH and –NH groups hydrogen bond with acidic functional monomers in the MIP (Fig. 2). The importance when choosing the template is thus that the template contains these groups at the same positions.

The recovery rates, accounting to the significant differences among the panel of agonists, can be considered constant for each compound in the concentration range chosen (Tables 2 and 3). These data support the claim of our MISPE selective recognition mechanism of the analytes considered. In a previous experiment made with a non-imprinted polymer all the analytes were eluted within the application and washing steps described in this paper [21], thus supporting that selective binding only occurs when drugs are able to access to the imprinted sites, in presence of sterically-oriented carboxylic moieties (Fig. 2). According to the p$K_a$ of such functional group, it is possible to modulate the acetic acid concentration in the washing (1%, v/v) and the elution (10%, v/v) solvents, respectively. The selective interaction occurs in acetonitrile allowing strong hydrogen bonding between the β-agonists and the polymer. By adding small amounts of acetic acid to acetonitrile, non-selective binding is restrained. The eventual non-selective hydrophobic interactions between the polymer and the analytes can be controlled by the eluotropic force of 1 ml acetonitrile used

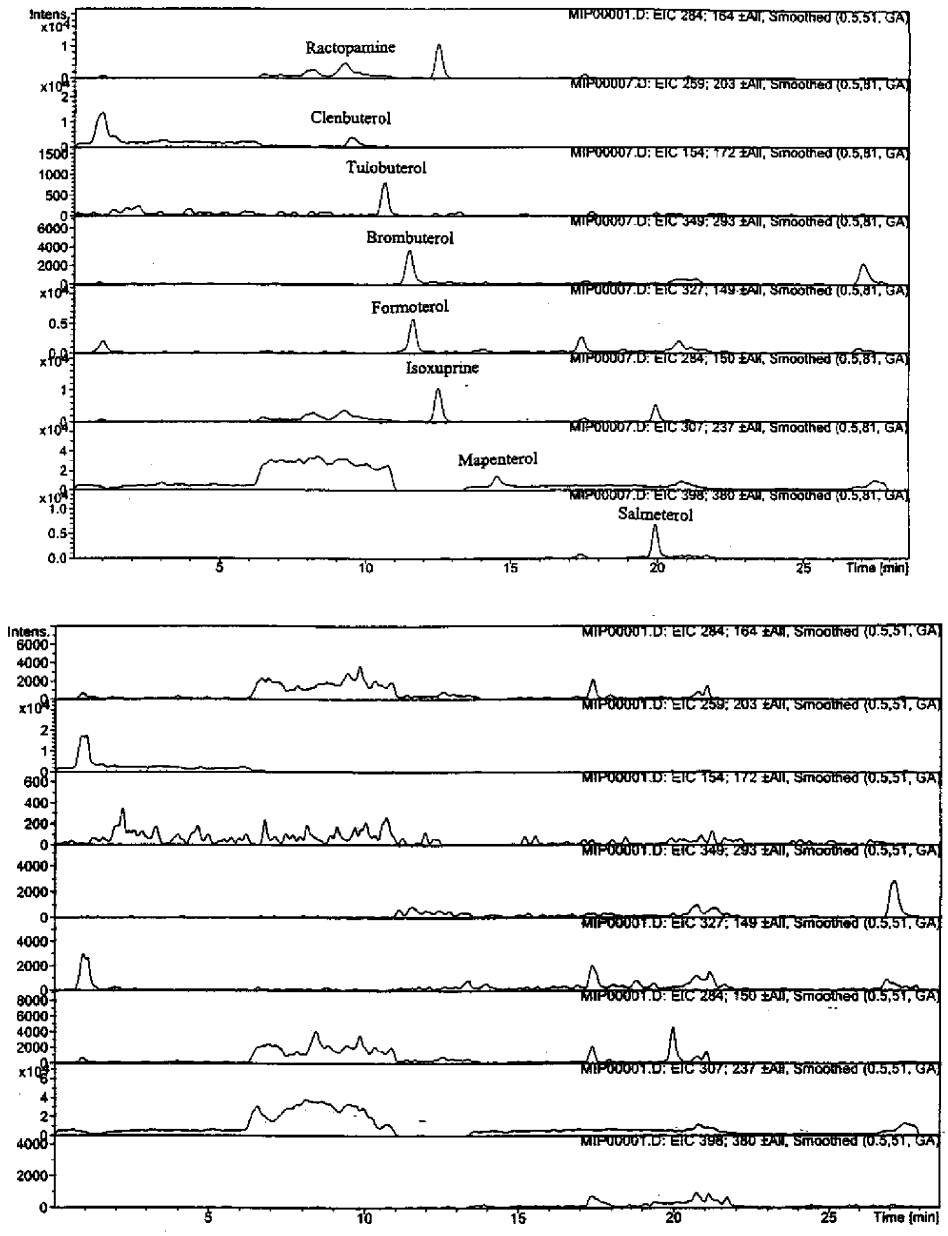


Fig. 3. Example of a LC–MS/MS multi-residue analysis of an urine sample spiked at 0.25 ppb with the pooled standards (upper panel) and of the corresponding blank (lower panel).

in the washing step, with respect to the less than 200 μl dead volume of the MISPE columns. As a consequence, the observed differences in recovery rates can be mainly explained by the different sterical hindrances of the drugs, with respect to that of the template. Additionally, the compounds with the lowest recoveries contain electron-rich groups close to the binding sites, decreasing the strength of the hydrogen bonds between the analyte and polymer.

With respect to previous papers dealing with MIP imprinted with brombuterol and clenbuterol for the SPE of clenbuterol, with reported recoveries above 80% [16,21], it is worth noting that in the present work the choice of the template has been changed, to explore the possibility to use only one template able to embrace the largest number of molecules of the same pharmacological class. Despite that the recoveries are not quantitative, their reproducibility allows the achievement of sharp decision limits consistent with the pharmacokinetics of such drugs in urine after their administration at growth promoting doses in feedlots. Due to this, the use of LC–MS/MS device is mandatory both to reach low detection limits and to confer forensic validity to the results, according to the identification points and to the relative intensities ratio of the product ions (Table 1). The limited background noise at the RT (Fig. 2) of each compound, the maintenance of an appropriate ratio between product ions, the observed small incidence of

matrix induced ion suppression phenomena (less than 10% of variation between the pure standard and the same amount in matrix, simulating the 100% of recovery injected into the LC–MS/MS (data not reported) suggests that this approach could be valuable for its cost/benefits ratio for routine analyses.

The comparison of the recovery rates supported by the analysis of the variance indicates the possible use of shared internal standards for the Racto and Salm group and for the Brom and Clen group, respectively, due to the not significant differences recorded in the recovery study.

Further work is in progress to make imprints with templates expected to be key factors for effective multi-residual analysis, taking into account the described occurrence of new drugs in animal productions [22], with the aim both to improve recovery rates and to better understand the mechanisms affecting molecular recognition.

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