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Development and validation of a liquid chromatography tandem mass spectrometry method for the analysis of β-agonists in animal feed and drinking water

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ABSTRACT

A reproducible, sensitive and selective multiresidue analytical method for seven β-agonists: clenbuterol (CBT), clenpenterol (CPT), ractopamine (RTP), brombuterol (BBT), mabuterol (MBT), mapenterol (MPT), and hydroxymethylclenbuterol (HMCBT) was developed and validated by using liquid chromatography tandem mass spectrometry (LC–MS/MS) in feed and drinking water samples. The validation was achieved according to the criteria laid down in the Commission Decision 2002/657/EC, however it was necessary to use minimum required performance limits (MRPLs) proposed by the Community Reference Laboratories (CRLs) due to the lack of maximum residue limits (MRLs) for β-agonists. By setting up these MRPLs, allows controlling their use in safe mode, since β-agonists are commonly used in veterinary medicine sometime in a fraudulent manner, for increasing the weigh of animals. Values set for both matrices studied are 50 μg/kg for animal feed, and a range from 0.2 to 10 μg/L for drinking water. CCα values calculated were under the MRPLs suggested; for drinking water the lowest value obtained was 0.12 μg/L, and for animal feed 0.87 μg/kg. Values for CCβ were ranged from 0.08 to 0.13 μg/L in drinking water and from 0.5 to 0.92 μg/kg in animal feed samples. The excellence values obtained, allowed us to conclude that the proposed analytical method is capable to control the β-agonists studied in both matrices and that it can be successfully applied and used as a routine method in laboratories of residue analysis of veterinary food control.

1. Introduction

β-Agonists or β-adrenergic agonists are synthetic phenethanolamine compounds used as bronchodilatory and tocolytic agents for therapeutic purposes. They are similar in structure to the naturally occurring catecholamines dopamine, norepinephrine, and epinephrine involved in increasing protein accretion, improving growth performance, and decreasing adipose tissue deposition in livestock. Nonetheless, exists well-documented adverse effects on human health, such as food poisoning associated with presence of residues in liver [1,2], cardiovascular and central nervous diseases [3]... and the reason is an inappropriate and sometimes illicitly use as growth promoters especially in many European Union (EU) countries. This conflictive situation has given rise to ban them, as it is manifested in Council Directive 96/22/EC [4]. At the same time, in the actually legislation Commission Regulation (EU) N° 37/2010 [5] and in previous Council Regulation (EEC) N° 2377/90 [6] maximum residue limits (MRLs) are fixed only for one β-agonist: clenbuterol (CBT) for specific animal species and tissues as muscle, kidney and liver of equidae and bovine, and in bovine milk. CBT is exclusively used for tocolysis in parturient cows and the treatment of respiratory disorder of equidae.

The "common" fraudulent use of β-agonists in animals, has been highly reported in the literature in the last decade [7–10], and the lack of validated analytical methods to detect these compounds with high structural diversity, led us to focus this job in two objectives: (1) develop and validate a multiresidue analytical method for quantification and confirmation of β-agonists and (2) apply this method to studying two common matrices used in special cases as vehicles of administration of drugs in animals: animal feed and drinking water, with the intention of providing surveillance of food processes and food animal origin.

Any unauthorized and forbidden substance has been established that need to be detected and confirmed according to

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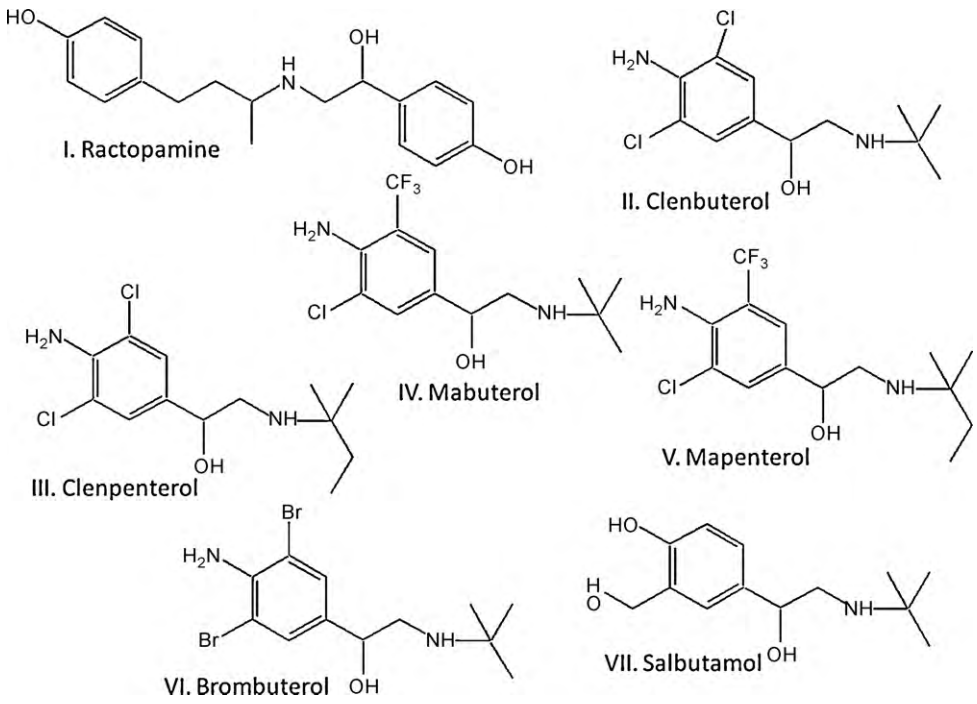


Fig. 1. Structures of β-agonists studied.

analytical performance criteria detailed in the Commission Decision 2002/657/EC [11]. In our case, for β-agonists, other techniques as radioimmunoassay [12] and enzyme immunoassay [13–15] has been used for screening purposes, due to their high sensitivity and high throughput, but these methods are not suitable for confirmation.

Other techniques have been used for β-agonists determination, however each one has its peculiarity. On one side liquid chromatography–mass spectrometry (LC–MS) has been widely used to identify trace levels of organic residues and contaminants [16–20]. Nonetheless when MS/MS techniques are used, and mass fragments are obtained, the system of identification points (IP) has to be used to confirm the data obtained according to EU criteria, even for the analysis of veterinary drug residues as β-agonists. IP system is detailed in the Commission Decision 2002/657/EC [11] for any tandem MS technique, either triple quadrupole mass spectrometry and/or ion trapping techniques [21,22]. A minimum of 4 IP for the confirmation of substances listed in Group A (β-agonists) of Annex I of Directive 96/23/EC [4] are required. Using the LC–MS/MS for β-agonists detection, 4 IP can be achieved with one precursor ion and two product ions.

On the other side, GC–MS methods have been also used for analysis of β-agonists residues in various biological samples [23–25]; however, GC–MS methods for β-agonists require sample derivation, because of their high polarity and low volatility, which is a time-consuming, tediousness, laborious and expensive process. Also, quantitative results are significantly affected by sample purity when GC–MS is used for β-agonists analysis. In these cases sample preparation becomes the most important part of the analysis method [26]. SFE is an effective technique for this purpose, being necessary two SPE columns connected in series for some methods. This cause difficulty in the optimization procedures, and recoveries and reproducibility for β-agonists are poor which makes GC–MS application more limited [27,28].

It has to be pointed that when GC–MS has been used for these compounds, complications to obtain enough characteristic ions in some β-agonists has been obtained, which implies the scarceness confirmation of their presence, as Hernández-Carrasquilla [29] have reported for CBT.

There are several papers published about the concern of the MS–MS application for detecting β-agonists residues [30–35]. More recently, Nielen et al. [17] and Shao et al. [36] reported a method for multiresidue analysis of β-agonists, in bovine and porcine urine, feed and hair using LC–MS/MS and in pig liver, kidney and muscle with UPLC–MS/MS, respectively, but there is no validation of the method with the recent guidelines and matrices presented in these paper are different from our samples.

This report is presented as the first in which 7 β-agonists: clenbuterol (CBT), clenpenterol (CPT), ractopamine (RTP), brombuterol (BBT), mabuterol (MBT), mapenterol (MPT) and hydroxymethylclenbuterol (HMCBT), at trace levels were detected all together with a developed and validated LC–MS/MS method by triple MS. It is applied to animal drinking water and animal feed, both matrices used in special cases as vehicle of drugs administration by farmers and veterinarian.

2. Experimental

2.1. Chemicals and standards

β-Agonists compounds used in this study were purchased from different suppliers: CBT and RTP were purchased from Sigma-Aldrich® and CPT, BBT, MBT, MPT and HMCBT from Witega®. The structure of these compounds is shown in Fig. 1. As internal standards (IS) ractopamine-d5 (RTP-d5) and clenbuterol-d9 (CBT-d9) were used and were purchased from RIVM and Fluka®, respectively. Ultra pure water was obtained by using a Milli-Q Ultra pure System (Millipore®). Organic solvents used were *n*-hexane, ter-butylmethylether, and acetic acid and were purchased from Scharlau®. Methanol and acetonitrile were supplied from Merck®, and hydrochloric acid, sodium chloride and ammonium hydroxide from Panreac®. All solvents used in sample preparation were HPLC grade and chromatographic separations LC–MS–MS grade. Extraction cartridges C_{18} (1 g, 6 mL) and Bond Elut Plexa™ PCX (150 mg, 6 mL) were purchased from Waters® Co. and Varian®, respectively.

2.2. Preparation of standard solutions

Standard stock solutions of individual β-agonists were prepared in methanol at the following concentration: CBT and RTP at 1 mg mL^{-1}, CPT, BBT, MBT, MPT, and HMCBT at 0.5 mg/mL. These solutions were stored in dark glass bottles at −10 °C. A standard stock mixture solution of β-agonists (10 μg/mL) was prepared also in methanol and it was stored at 8 °C. Working standard solutions of β-agonists (100 and 10 μg/L) were prepared daily with the above mentioned solution and held at 8 °C before of being added to samples or being injected into the LC–MS/MS equipment.

Stock solutions of individual IS were prepared in methanol at concentrations of 200 μg/mL for CBT-d9 and 10 μg/mL for RTP-d5. These solutions were stored in dark glass bottles at −10 °C. A standard mixture of IS at 1 μg/mL were prepared also in methanol and stored at 8 °C and stable for at least 1 year. Working standard solution of IS at 10 ng/mL was prepared weekly in methanol with the previous solution and held at 8 °C before addition to samples.

2.3. Sample preparation

Samples received from farmers and veterinaries from Valencia Community (Spain) during a period of 12 months, identified, and labeled were received and stored appropriately in our lab. Previous to the extraction of β-agonists compounds, samples were prepared as follows: for animal feed samples, 100 g was blended and a portion of 2.5 g powdery feed was weighed and accurately introduced into a centrifuge tube. For drinking water samples, they were previously shaken and 5 mL were transferred into 15 mL polypropylene centrifuge tube.

2.4. Extraction procedures

Due to the study of two different matrices, two extraction procedures were carried out.

At 2.5 g of powdered animal feed samples previously weighted, 20 mL of 0.1 N HCl was added and the mixture was sonicated for 10 min and centrifuged at 11,000 rpm for 10 min at 10 °C. The supernatant was transferred into a glass, and subjected to solid phase extraction (SPE) by a polymeric cation-exchange resin using Bond Elut Plexa™ PCX cartridge (150 mg, 6 mL) previously conditioned with 3 mL of methanol and 3 mL of water, at a flow rate of 1–2 mL min^{-1}. Once the supernatant was passed all through, the cartridge was washed twice: first with 3 mL of water and 3 mL of methanol to eliminate interferences retained in the column, and secondly with 3 mL of *n*-hexane to eliminate fatty proportion. After, air stream of nitrogen was passed through the cartridge to dryness. Elution step of β-agonists was done with 5 mL of methanol:ammonium hydroxide (95:5, v/v). The eluate was dried by using TurboVap® (Zymark) under a gentle nitrogen stream at 45 °C, and reconstituted with 2 mL of water. These 2 mL were subjected to liquid–liquid extraction process twice as follows: 2 mL of ter-butylmethylether and 1 g of sodium chloride were added, shaked vigorously and led it until two phases were separated. The supernatant was collected into a polypropylene tube, and the residue subjected once more time to the same liquid–liquid extraction. Finally the collected volume of ter-butylmethylether (4 mL approximately as a final volume) was dried under a gentle nitrogen stream at 45 °C and reconstituted to 0.4 mL with acetonitrile:water (25:75, v/v) with 10 mM of acetic acid, being ready for the analysis.

In drinking water samples, at 5 mL of shaked water, 2 mL of 0.1 M KH_2PO_4 (pH 9) were added and mixed. The mixture was subjected to SPE with a C_{18} cartridge (1 g, 6 mL), previous treatment of the SPE cartridge. First, it was conditioned with 5 mL water and 5 mL methanol at a flow rate of 1–2 mL min^{-1}. Secondly, the cartridge was washed with 5 mL water and 5 mL of methanol:water (50:50, v/v), washed with 5 mL of *n*-hexane, between each wash the column was dried with stream nitrogen air. And finally, the compounds were eluted with 3 mL of methanol. The eluate was dried under a gentle nitrogen stream at 45 °C, and reconstituted to 0.2 mL with acetonitrile:water (25:75, v/v) containing 10 mM of acetic acid.

2.5. Calibration

For quantitation of the seven β-agonists in both matrices, solutions of each one by separate and matrix-fortified calibration curves using different blank matrices were prepared. A multi-component standard solution was set by spiking standard mixture and the calibration curve-solution was built at the following concentration 0, 0.8, 1.6, 4, and 12 μg/kg for animal feed blank, and at 0, 0.1, 0.4, 1.0, and 3.0 μg/L for drinking water blank. These solutions were injected directly in the LC–MS/MS and analyzed in the mode of multiple reaction monitoring (MRM) transitions. Calibration curves were plotted with the highest intense signal area *vs* concentration. All samples, blanks and controls were spiked with the IS, CBT-d9 and RCP-d5, at 0.2 μg/L in drinking water and 1.6 ng/g in animal feed.

2.6. Liquid chromatography

Chromatographic separation was carried out on a Finnigan® Surveyor Autosampler coupled to a Finnigan® Surveyor LC Pump and to a ThermoFinnigan® TSQ Quantum Ultra AM triple quadrupole detector (Milford MA, USA). The separation was done on a Luna PFP Phenomenex® column (100 mm × 2.0 mm, 3 μm particle size) in combination with a guard column with same characteristics as Luna PFP (4.0 × 2.0 mm). The mobile phase consisted in water containing 10 mM acetic acid (A) and acetonitrile (B), and the flow rate was set at 0.3 mL min^{-1}. The initial conditions were 20% A and 80% B. A gradient elution was performed as follows: A phase was increased linearly to 90% in 13.0 min, and then increased to 100% in 0.2 min. It was kept for 5 min and finally returned to the initial conditions in 0.1 min. The volume of sample injection was 25 μL and between injections, the column was maintained under the last detailed setting for 5 min to ensure the return to initial conditions. In order to maintain constant the column conditions during the injection, the column support temperature was set at 40 °C.

2.7. Mass spectrometry

Mass spectrometric acquisition was carried out on a triple quadrupole tandem mass spectrometer, ThermoFinnigan TSQ Quantum Ultra AM MS (Milford MA, USA) using the electrospray ionisation (ESI) ion source in positive mode. The instrument conditions were set at: 4500 V the spray voltage, sheath gas pressure 35, auxiliary gas pressure 10, capillary temperature 300 °C, and capillary offset 35 V. The gas used for the nebulizer, dissolve, and cone, was nitrogen. Ultra high purity argon gas was used as collision gas at 1.5 mTorr.

2.8. Method validation

The validation process was carried out according to the criteria of the Commission Decision 2002/657/EC [11] for forbidden compounds, which specify to use calibration curves at five concentrations levels including blank level. Each injection of sample contained an IS (CBT-d9 or RCP-d5). After that linearity, accuracy, precision and reproducibility were studied.

According to Commission Decision 2002/657/EC [11], for those non-permitted substances, the decision limit (CCα) and the capacity of decision (CCβ) has to be determined as follows: $CC\alpha = 3S/N_{(20\ \text{representative blank samples})}$; and $CC\beta = CC\alpha + 1.64$

$SD_{(20\ representative\ samples\ spiked\ at\ CC\alpha\ level)}$. In this study we analyzed twenty controlled blank drinking water and twenty animal feed samples (among porcine, bovine, ovine, caprine, equine, rabbit, and broiler) in order to determine the specificity of the method by looking for interfering peaks within a 2.5% margin of the relative retention time of each compound.

2.9. Matrix effects study

Ionization, suppression or enhancement, is an additional parameter which was evaluated during the method development, since it has demonstrated to interfere in the ion identification during the analysis of many compounds [37,38]. We carried out this study by observing the instrumental responses in post-cleanup spiked sample, and comparison with the compound in the mobile phase solution, taking into account the background contribution. It was also examined the pre-extraction spiked samples, which were analyzed to evaluate efficiency of the total process. By the *n*-hexane washing step and organic liquid–liquid extraction ion suppression was reduced.

3. Results and discussion

3.1. Extraction and cleanup

Ion suppression is an additional parameter which was evaluated during the method development, since it has demonstrated to interfere in the ion identification during the analysis of many compounds [37]. We carried out this study by observing the instrumental responses in post-cleanup spiked sample, and comparison with the compound in the mobile phase solution, taking into account the background contribution. It was also examined the pre-extraction spiked samples, which were analyzed to evaluate efficiency of the total process. By the *n*-hexane washing step and organic liquid–liquid extraction ion suppression was reduced.

Nonetheless, since it is a non-desirable phenomenon, we included a selective cleanup step during the extraction procedure in order to avoid its effect and decrease the matrix interference. Our purpose was hidden the ion suppression effect as much as possible, showing the success of the measures adopted.

The matrix interference was defined as the ratio in between the slope of matrix-matched standard curves and the slope of standard solution curves. By applying this in our study, the results indicated that the ion suppression decreased significantly for most compounds after passing the extract through C_{18} cartridges, when drinking water samples were analyzed, and through Bond Elut Plexa™ PCX for animal feed samples.

Both drinking water and animal feed were analyzed with C_{18} cartridges and tested two washed solvent step (methanol:water and hexane) and tree different proportion of methanol:water (30:70, 50:50 and 80:20). The results indicated that two washed step with methanol:water in proportion of 50:50 (v/v), and hexane the recoveries were higher for both matrices (70–82% in drinking water and 56–68% in animal feed), but in animal feed, it was observed that the ion suppression was not resolved, and recoveries values ranged between 43% and 25% by following this procedure, so that, β-agonists' extracts from animal feed samples required more attention (Table 1).

In animal feed the extract obtained was in acidic aqueous solution, since 20 mL of HCl was added to the amount of powdered sample. β-Agonists in acidic medium are protonated, and difficulties in being retained on reversed phase SPE sorbents as C_{18} occurs and in consequence, a diminishing of recoveries is obtained. With the objective of resolve this inconvenient, the pH of the acid extract was adjusted to basic conditions; however, when pH was adjusted the recoveries still remained below 90% and time-consuming was increased, which it is an important point that has to be taken in consideration. Since C_{18} SPE was not adequate for extracting our analytes in animal feed samples, we studied different columns, and Bond Elut Plexa™ PCX provided the highest recoveries and any pH adjustment was necessary to carry out. The distinctiveness of this column is that it contains a polymeric sorbent which combines reversed phase with strong cation-exchange functionalities and allows to bind basic analytes under acidic conditions. Bond Elut Plexa™ PCX proportionate a reduction of ion suppression phenomenon in the analyzed feed samples, due to its high polarity and because hydroxylated polymer surface was entirely amide-free and it did not provide binding sites for macromolecules, which are commonly presented in this complex matrices.

Table 1
Ion suppression of target compounds at levels of 0.4 μg/L in drinking water and at 1.6 μg/kg in animal feed with C_{18} cartridges ($n = 6$).

Compound	Ion suppression (% ± SD)	
	Drinking water	Animal feed
RTP	29 ± 5	41 ± 9
HMCBT	25 ± 3	40 ± 11
CBT	31 ± 4	43 ± 8
BBT	28 ± 5	37 ± 12
CPT	15 ± 6	26 ± 13
MBT	26 ± 4	33 ± 8
MPT	17 ± 7	25 ± 9

RTP: ractopamine; HMCBT: hydroxymethylclenbuterol; CBT: clenbuterol; BBT: brombuterol; CPT: clenpenterol; MBT: mabuterol; MPT: mapenterol.

Due to the structure similarity in between β-agonists and natural agents, previously mentioned, particular attention has to be taken since the matrix interference effect can produce ionization suppression or enhancement. Even though SPE particle surface of the columns used, allowed to minimize possible protein and lipid bindings.

Despite the selection of an adequate column for β-agonists extraction, in animal feed samples, an additional cleanup step was required. This step consisted in washing the SPE by adding extra *n*-hexane to eliminate fatty acids, and including an organic liquid–liquid extraction to the whole process (ter-butylmethylether and water), as it is detailed in Section 2.4. After that, ion suppression phenomenon was evaluated and its values of target compounds at levels of 1.6 μg/kg in animal feed with Bond Elut Plexa™ PCX ($n = 6$) followed of a cleanup step with *n*-hexane and liquid–liquid extraction with ter-butylmethylether and water, were ranged between 9% and 18%.

Ion suppression of target compounds at levels of 0.4 μg/L in drinking water and at 1.6 μg/kg in animal feed with C_{18} cartridges are summarized in Table 1. Both matrices are vehicles of drug administration and exposed to the animals in same conditions, but water samples were cleanest and easiest to manipulate than the feed ones, hence less interference components were found in water samples than in feed samples. As reflection of this fact, lower ion suppression values for drinking water samples than for animal feed ones were observed, being below 43% in animal feed and below 31% in drinking water.

3.2. Optimization of LC–MS/MS

β-Agonists' elutes obtained in the extraction procedure, were assessed with different ratio of acetonitrile–water 10 mM acetic acid as a reconstituted solvent for being injected into the LC–MS/MS. The results suggested that when acetonitrile–water containing 10 mM acetic acid (75:25, v/v) was used, satisfactory peaks and good sensitivities were achieved. In fact, under these conditions, an excellent resolution for the seven β-agonists and the two IS compounds were achieved.

Table 2
LC–MS/MS acquisition parameters of seven studied β-agonists.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Tube lens (V)
RTP	302	106.8	20	125
		120.7	30	
		163.7[a]	15	
HMCBT	293	131.8	30	125
		275.0	15	
		202.7[a]	20	
CBT	277	131.8	30	125
		259.0	15	
		202.7[a]	20	
BBT	367	211.7	30	125
		292.6[a]	20	
		348.6	15	
CPT	291	131.7[a]	30	125
		167.6	25	
		202.5	20	
MBT	311	216.7	30	125
		236.7[a]	20	
		293.0	10	
MPT	325	216.7[a]	30	125
		236.6	20	
		306.7	15	
CBT-d9[b]	286	203.7[a]	20	125
RTP-d5[b]	307	167.0[a]	15	125

[a] Product ion used for quantification.
[b] Internal standard; (compound abbreviation identification as in Table 1).

During the optimization of LC–MS/MS, ion suppression phenomenon was also taken into account and studied, since difficulties for quantitative analysis are usually present. Some authors propose to use the isotopic dilution technique, which is advantageous to compensate for signal irreproducibility, matrix interference and loss of recovery. For this purpose and to compensate the variation of the volumes of the final extracts and to check the instrumental performance, isotopic labeled IS were used. Among the IS studied, only two were chosen according to retention time in chromatograms and structural similarities with the β-agonists studied: CBT-d9 and RTP-d5.

IS deuterated CBT, with nine deuteriums (CBT-d9) was used as IS for calibration curves of CBT, CPT, BBT, MBT, MPT and HMCBT; and deuterated RTP with five deuteriums (RTP-d5) was selected for calibration curves of RTP.

Other parameters in MS/MS for the analysis as spray voltage, gas pressure, capillary offset, tube lens, and collision energy, were optimized; as well as the perfect transitions for each β-agonists analyzed. The best conditions were chosen according to the best recoveries obtained and these are mentioned above in Section 2.7 and collected in Table 2.

The confirmation procedure by the Commission Decision 2002/657/EC [11] for banned substances, establish that by using one precursor ion and two transitions with two different product ions the confirmation is achieved since four IP are almost reached. In our method, at least three product ions were detected and one of them used for quantification which allowed us to reach those IP.

Table 4
CCα and CCβ for the drinking water and animal feed samples ($n = 20$).

Compound	Drinking water (μg/L)		Animal feed (μg/kg)	
	CCα	CCβ	CCα	CCβ
RTP	0.10	0.12	0.87	0.91
HMCBT	0.06	0.08	0.46	0.50
CBT	0.10	0.12	0.82	0.85
BBT	0.12	0.13	0.78	0.80
CPT	0.11	0.12	0.87	0.91
MBT	0.10	0.12	0.87	0.92
MPT	0.10	0.12	0.84	0.88

$CC\alpha = 3S/N_{(20\ \text{representative blank samples})}$; $CC\beta = CC\alpha + 1.64\ SD_{(20\ \text{representative samples spiked at CC}\alpha\ \text{level})}$ (compound abbreviation identification as in Table 1).

3.3. Method validation

Analytical features of the method validation are shown in Tables 3–5 and are based in linearity, CCα and CCβ, accuracy and precision as it is established in the Commission Decision 2002/657/EC [11].

3.3.1. Linearity, CCα and CCβ

The calibration curves were obtained by performing a linear regression analysis of the spiking experiment using the area against concentrations of the analytes, ranged from 0 to 12.0 μg/kg to animal feed and 0–3 μg/L to drinking water (see Table 3). When these curves were plotted, by plotting the β-agonist versus IS ratio obtained against the concentration of each β-agonist, an excellent linearity was obtained for all analytes, with correlation coefficients always (R^2) > 0.99 for all both distinct matrices studied.

CCα and CCβ of the analytes upon the method were determined as described in Section 2.6. In drinking water these values ranged from 0.06 to 0.12 μg/L for CCα, and from 0.08 to 0.13 μg/L for CCβ, being BBT the highest value in both cases. Regarding the animal feed matrix, CCα ranged from 0.46 to 0.87 μg/kg and from 0.50 to 0.92 μg/kg for CCβ being the highest value for the β-agonist studied in both parameters RTP, CPT, and MBT (Table 4).

Results summarized in Table 4 for CCα and CCβ of HMCBT were much lower than those obtained for the other analyzed compounds in both matrices. The reason of this value is because of the high sensitivity of this compound upon LC–MS/MS. Comparing between matrices, drinking water samples presented lower values of CCα and CCβ, than in animal feed samples. This could be attributed to the high complexity of animal feed matrix, which makes the blank samples with high interferences and in consequence high peaks

Table 3
Linearity equation's parameters of fortified samples in relation to CBT-d9 (CBT, CPT, BBT, MBT, MPT and HMCBT) and RTP-d5 (RTP).

Compound	Drinking water			Animal feed		
	Range (0–3.0 ng/mL)			Range (0–12 ng/g)		
	Slope ($a \pm$ SD)	Intercept ($b \pm$ SD)	Coefficient correlation ($R^2 \pm$ SD)	Slope ($a \pm$ SD)	Intercept ($b \pm$ SD)	Coefficient correlation ($R^2 \pm$ SD)
RTP	0.4522 ± 0.159	0.1099 ± 0.078	0.9963 ± 0.009	−1.1028 ± 0.093	0.2706 ± 0.078	0.9997 ± 0.017
HMCBT	0.3707 ± 0.034	0.1181 ± 0.023	0.9956 ± 0.007	−0.0189 ± 0.145	0.1078 ± 0.004	0.9954 ± 0.011
CBT	0.3499 ± 0.092	0.1171 ± 0.025	0.9998 ± 0.005	0.3118 ± 0.056	0.1969 ± 0.004	0.9972 ± 0.001
BBT	0.3920 ± 0.166	0.0882 ± 0.040	0.9994 ± 0.001	0.2183 ± 0.006	0.0866 ± 0.005	0.9973 ± 0.001
CPT	0.2030 ± 0.059	0.0484 ± 0.015	0.9999 ± 0.007	0.0295 ± 0.014	0.0334 ± 0.002	0.9969 ± 0.001
MBT	0.1386 ± 0.096	0.2146 ± 0.052	0.9908 ± 0.005	0.6187 ± 0.013	0.2894 ± 0.016	0.9964 ± 0.002
MPT	0.2393 ± 0.041	0.1215 ± 0.016	0.9993 ± 0.004	0.1474 ± 0.038	0.0616 ± 0.002	0.9940 ± 0.004

(Compound abbreviation identification as in Table 1).

Table 5
Accuracy (*R*) and precision within-day and between-day (SD) of seven compounds in spiked drinking water and animal feed samples ($n = 6$).

Compound	Within-day ($n = 6$)												Between-day ($n = 6$)									
	Drinking water %						Animal feed %						Drinking water %						Animal feed %			
	0.1 μg/L		0.4 μg/L		1.0 μg/L		0.8 μg/kg		1.6 μg/kg		3.2 μg/kg		0.1 μg/L		0.4 μg/L		1.0 μg/L		0.8 μg/kg		1.6 μg/kg		3.2 μg/kg	
	R	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD	*R*	SD
RTP	84	10	87	7	93	7	82	12	98	10	99	7	77	15	81	12	89	7	80	19	85	15	89	9
HMCBT	77	11	97	6	105	6	96	11	87	9	98	9	67	16	87	11	99	6	80	17	87	14	92	10
CBT	101	5	106	6	110	9	85	10	89	8	95	10	98	10	96	11	91	9	81	15	89	13	90	10
BBT	81	13	86	9	91	11	90	10	92	9	105	8	78	18	82	14	85	11	75	11	82	10	86	9
CPT	77	6	82	5	94	9	86	9	93	8	96	9	67	11	82	10	83	9	66	12	73	10	86	9
MBT	105	9	106	8	108	7	93	11	99	9	110	7	90	9	96	8	98	7	82	10	89	9	92	7
MPT	99	9	101	8	107	7	92	10	98	9	107	7	89	9	87	8	90	7	78	10	81	9	89	8

R: recovery; SD: standard deviation; (compound abbreviation identification as in Table 1).

Table 6
Data regarding of β-agonists occurrence in the analyzed samples.

Compound	Occurrence			
	Drinking water ($n = 66$)		Animal feed ($n = 42$)	
	Level (μg/L)	CCβ (μg/L)	Level (μg/kg)	CCβ (μg/kg)
RTP	0.67–81.50	0.12	4.34–4.62	0.91
HMCBT	0.07–6.07	0.08	0.29–0.92	0.50
CBT	N.D.	0.12	N.D.	0.85
BBT	0.12	0.13	N.D.	0.80
CPT	N.D.	0.12	N.D.	0.91
MBT	N.D.	0.12	N.D.	0.92
MPT	N.D.	0.12	N.D.	0.88

(Compound abbreviation identification as in Table 1) N.D.: No detected.

are observed, and this issue contained in the calculation of both factors is increased. Also, difference of values for CCα and CCβ in both matrices can be attributed to the pH extract obtained from the feed matrix, which depending on the nutrient proportion (proteins, carbohydrates, lipids, vitamins, minerals, etc) due that CCα and CCβ values can increase.

The sensitivity of the described assay is lower than for several other forms of screening procedures described for β-agonists in animal feed and drinking water [17,39]. Boyd et al. [39] have been validated a bioassay of clenbuterol, cimaterol, mabuterol, mapenterol, ractopamine, salmeterol and zilpaterol in animal feed and the limit of detection are ranging between 50 and 500 μg/kg and the CCβ values are ranging between 250 and 1000 μg/kg, all of them are one thousand times higher than our CCα and CCβ values obtained. In the same way, our results obtained of CCβ in animal feed have been lower than the reported by Nielen et al. [17] which are performed as a multiresidue analysis of 21 β-agonists in feed using LC–MS/MS, and their values of CCβ for animal feed were less than 10 μg/kg, except for CBT which is 5 μg/kg (see Table 4).

3.3.2. Accuracy and precision

The accuracy was evaluated by compound individually, calculating the recoveries obtained by this procedure in spiked blank samples at concentrations of 0.1, 0.4 and 1.0 μg/L for drinking water, and at concentrations 0.8, 1.6 and 3.2 μg/kg for animal feed. Results are shown in Table 5, and they were obtained after six replicates of each condition. The average recoveries of each compound ranged in drinking water from 67% to 110%, and in animal feed 66–110%. Fig. 2 contains a chromatogram of a spiked drinking water sample and spiked animal feed sample at 1.0 μg/L and 0.8 μg/kg, respectively (other concentrations not shown).

The within-day and between-day reproducibility were evaluated by spiked blank samples at three levels of the compounds, in six replicates within a day and over the course of five consecutive days, respectively (Table 5). The within-day reproducibility ranged from 5.0% to 13.0% in drinking water and 7–12% in animal feed, and the between-day reproducibility ranged from 6% to 18.0% in drinking water and 7–19% in animal feed. It should be noticed that within-day shown lower values for animal feed in comparison to drinking water, which might be due to the higher complexity of matrix.

4. Application to real samples

The method developed and validated was applied for a total of 108 samples, in between animal feed and drinking water samples, for a total period of time of 12 months.

The method developed and validated was applied for a total of 108 samples, in between animal feed ($n = 66$) and drinking water ($n = 42$) samples, for a total period of time of 12 months (Table 6).

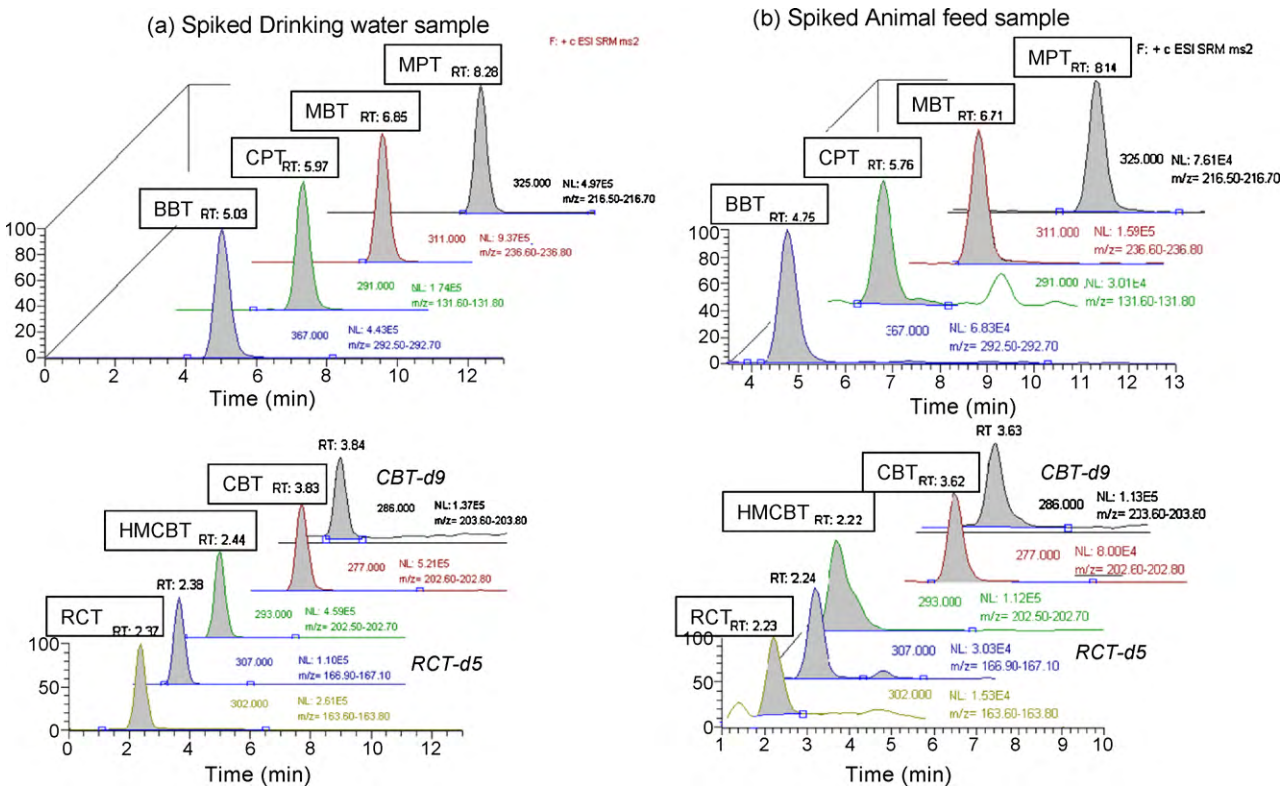
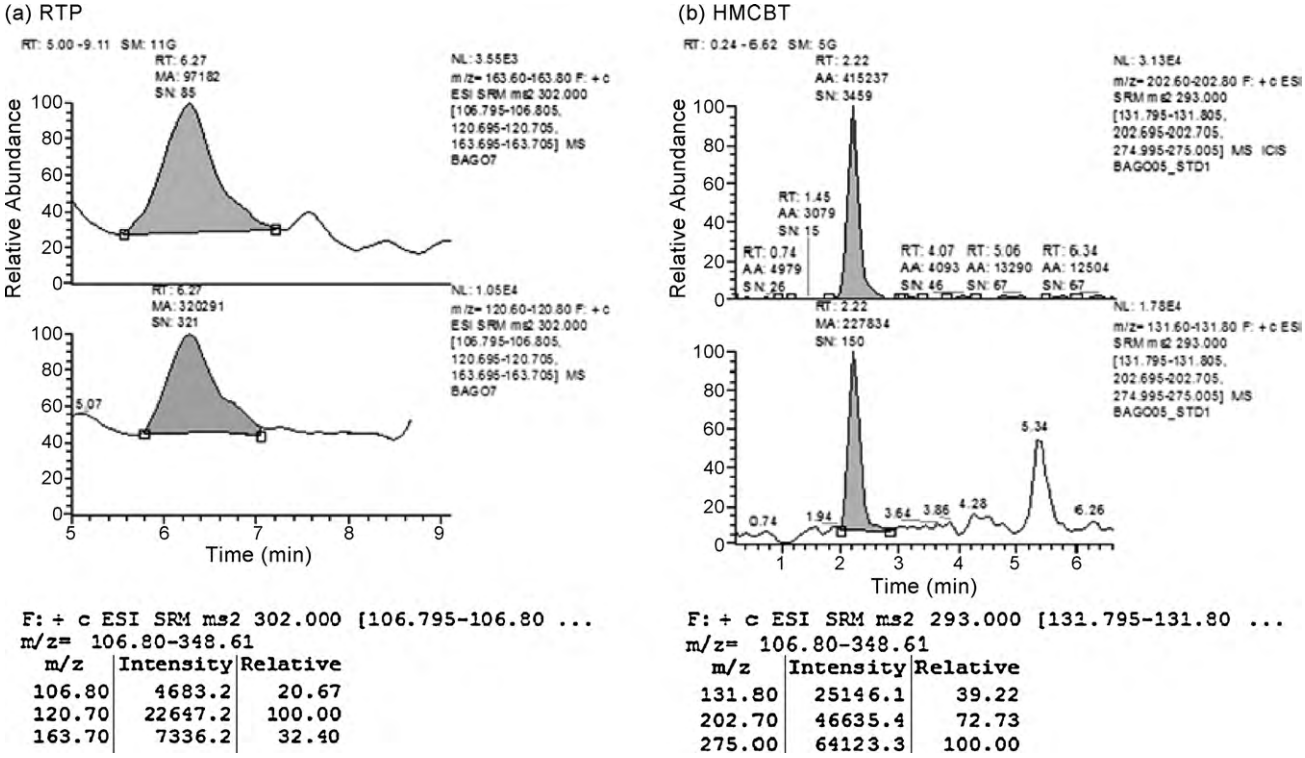


Fig. 2. Chromatograms of two different type spiked samples analyzed with the optimized method and by LC–MS/MS in ESI +: (a) spiked drinking water at 1.0 μg/L; (b) spiked animal feed at 0.8 μg/kg (compound abbreviation identification as in Table 1).

The samples were received in our laboratory of veterinary control food, from different farmers and veterinaries to ensure the conditions of their livestock and animals in general, which were feed with the same food and water.

Among the samples studied, animal feed samples presented highest incidence respect drinking water samples and almost all samples in this matrix were detected with two β-agonists simultaneously: RTP and HMCBT. Only one sample was detected with a



F: + c ESI SRM ms2 302.000 [106.795-106.80 ...
m/z= 106.80-348.61

m/z	Intensity	Relative
106.80	4683.2	20.67
120.70	22647.2	100.00
163.70	7336.2	32.40

F: + c ESI SRM ms2 293.000 [131.795-131.80 ...
m/z= 106.80-348.61

m/z	Intensity	Relative
131.80	25146.1	39.22
202.70	46635.4	72.73
275.00	64123.3	100.00

Fig. 3. Chromatograms obtained using the optimized method and determinate by LC–MS/MS in ESI + of two positive samples analyzed: (a) drinking water containing 0.79 μg/L of RTP, (b) animal feed containing at 0.92 μg/kg HMCBT.

single β-agonist: HMCBT at a concentration of 0.92 μg/kg (Fig. 3). The values detected in samples with RTP and HMCBT simultaneously were in all these cases > CCβ calculated (Table 4) RTP ranged from 4.34 to 4.64 μg/kg, and HMCBT ranged between 0.28 and 0.69 μg/kg (Table 6). In animal drinking water three samples resulted to be positive, two for RTP and one for HMCBT with concentrations > CCβ calculated (Fig. 3), nonetheless two samples were detected with RTP only, and one with HMCBT and BBT at values < CCβ calculated.

5. Conclusions

The developed method was sensitive and specific for the confirmation and quantitative analysis of seven β-agonists in drinking water and animal feed samples, and it was validated according the steps described in the Commission Decision 2002/657/EC [11]. The extraction procedure optimization was of high relevance to obtain good results in detection and quantification, to obtain a cleanup sample and to provide ion suppression below (17 ± 5)% in feed samples and below (31 ± 4)% in drinking water.

The method was fully validated at levels below the MRPL. It demonstrated to obtain the required sensitivity for these β-agonists and to reach the suggested MRPLs by the CRLs established, 0.2–10 μg/L in drinking water and 50 μg/kg in animal feed obtaining the lowest value of CCα obtained were 0.12 μg/L and 0.87 μg/kg for drinking water and animal feed, respectively.

The ability of this method to confirm various β-agonists residues in animal feed and drinking water at this level of sensitivity, promise to be useful for regulatory control programs and to monitor these drugs. Also, the method performs very well in terms of accuracy (>66%) and within-day reproducibility (>77%) for all the studied β-agonists in both matrices.

Among all analyzed samples two analytes together were obtained, fact that puts in relevance the possibly of synergism effect. By administrating two drugs at low levels at the same time, a plus effect can be obtained and the final purpose in animals achieved (as weigh increasing) however the human consumption of this food can cause harmful effects of synergism in the population and risk in hypersensibility reactions revealed.

In the future, other existing or emerging β-agonists will be necessary to study for these matrices, to ensure a safe control in veterinary drug residues, an analysis work predicted to be developed in our lab.

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