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Extraction, clean-up and gas chromatography–mass spectrometry characterization of zilpaterol as feed additive in fattening cattle

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Abstract

Zilpaterol is an adrenergic drug currently licensed in Mexico and South Africa as a feed additive for cattle close to consignment. In this study an analytical method to detect zilpaterol in commercial feeds was set up. The influence of extraction solvent and matrix was evaluated. The drug as a trimethylsilyl derivative was characterized by GC–MS, on a quadrupole detector, in the electron impact mode. Acidic extraction, solid-phase extraction C_{18} non-endcapped clean-up and mass characterization on ions *m/z* 308, 291, 405, 390 provided zilpaterol recoveries >75.3% and repeatability <3.3% in feeds spiked in the range 30.0–120.0 ng/g. The limits of detection and quantification were 7.5 and 25.0 ng/g, respectively. Such limits are well below the dose of 5.0–20.0 μg/g proposed as effective.

Keywords: Zilpaterol

1. Introduction

The increasing demand from consumers and retailers for production of lean meats, and the economic advantage in mass gain of feedlot steers, has progressively forwarded the use of β_2 agonists at doses above therapeutic ones as feed additives for farm animals [1–3].

On the other hand, the illicit use of drugs like clenbuterol as growth promoters in the European Union (EU), has led to several cases of intoxication outbreaks in humans [4]. On the basis of such epidemiological evidence, the use of adrenergic agonists for therapeutic purposes has been forbidden within the EU, in fattening animals since 1997. In contrast, the USA, Mexico and South Africa licensed molecules, such as zilpaterol and ractopamine, as feed additives for cattle close to consignment and for finishing swine, respectively [5,6]. Recent studies report on supplementation with zilpaterol and ractopamine, both belonging to the class of compounds known as phenethanolamines; improved mass gain, carcass leanness and feed efficiency were recorded, due to an enhanced activity of those enzymes able to shift nutrients toward lean growth [7,8]. For zilpaterol and ractopamine the approved doses as additive in feeds range from 5.0 to 20.0 mg/kg.

In the scientific literature, few papers deal with phenethanolamines in feeds and the molecules analyzed, such as clenbuterol and salbutamol, may not

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be in current use [9,10], due to a probable turnover with a new panel of β-agonists able to elude official controls [11,12].

The present study considered, as a first step, the set-up of an extraction and purification procedure suitable for zilpaterol and ractopamine, in commercial feeds: pH influence on extraction efficiency and matrix effects arising from four different feeds were evaluated. Subsequently, the characterization of zilpaterol as a trimethylsilyl (TMS) derivative by means of gas chromatography–mass spectrometry (GC–MS) on a quadrupole detector in the electron impact (EI) ionization mode, and its quantification, using ractopamine as internal standard, was carried out, for the validation of the method.

2. Experimental

2.1. Materials and reagents

Samples of corn silage, milk replacers, soya meal and feed with mineral supplements were drawn at feed mills.

Zilpaterol standard as hydrochloride salt was provided by The European Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany). Ractopamine standard as hydrochloride salt was a gift from Elanco Animal Health, a Division of Eli Lilly (Windlesham, UK).

Stock standard solutions of zilpaterol and ractopamine at 1 mg/ml in methanol were prepared monthly and stored at −20 °C. Spiking solutions were daily prepared in aqueous solutions of sodium acetate by appropriate dilution. For GC–MS calibration, working solutions were daily prepared in methanol.

Methanol and acetonitrile (HPLC grade), sodium acetate, hydrochloric acid, *n*-hexane, diethylamine and triethylamine (reagent grade) were supplied by Merck (Darmstadt, Germany). Ultra-pure water was obtained from a Milli-Q RG system from Millipore (Bedford, MA, USA).

The derivatization reagent was *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) from Supelco (Bellefonte, PA, USA), NH_4I and 1,4-dithioerythritol (DTE) from Sigma. Solid-phase extraction (SPE) C_{18} NE (non-endcapped) cartridges (500 mg/3 ml) were purchased from Isolute (IST, Stepbio, Bologna, Italy). Millex HA 0.45-μm filters were obtained from Millipore (Molsheim, France).

2.2. Apparatus

The solid-phase cartridge extraction system was a Vacuum Manifold Visiprep from Supelco. The thermostated incubator shaker was a Thermoshake from C. Gerhardt, Fabrik und Lager Chemischer Apparate (Bonn, Germany). The centrifuge was a Sorvall Superspeed RC-2 from Kendro Laboratory Products (Hanau, Germany).

The ultrasonic water bath was a Bransonic Model 321 from Branson Ultrasonic (Danbury, CT, USA).

Evaporation under a nitrogen stream and TMS derivatization of the extracts were carried out on an Heat Block (Pierce, Rockford, IL, USA).

2.3. HPLC instrument and conditions

HPLC analyses were carried out on a System Gold apparatus equipped with a pump Model 126, a UV/DAD detector Model 168, and an autosampler Model 501 from Beckmann Analytical (S. Ramon, CA, USA). Chromatographic conditions were as follows: LiChrosphere RP C_{18} column with guard column, 250×4 mm I.D., 5 μm, from Merck; mobile phase (A) 0.017 *M* phosphoric acid brought to pH 2.8 with diethylamine, (B) acetonitrile (ACN)–water (80:20, v/v); flow-rate 1 ml/min; linear gradient 10% B hold for 5 min, to 100% B in 15 min, 100% B hold for 4 min, to 10% B in 1 min, 10% B hold for 5 min; loop of 100 μl, detector set at 280 nm, 4 nm bandwidth, spectra recorded in the range 220–350 nm.

2.4. GC–MS instrument and conditions

Spectrometric analyses were performed on a GC–MS 5973 Network mass quadrupole selective detector equipped with a HP1 fused-silica capillary column 18 m×0.20 mm I.D., 0.11 μm film thickness (Hewlett-Packard, Palo Alto, CA, USA). The injector temperature port was set at 270 °C; injection was made in the pulsed splitless mode keeping a pressure of 54 p.s.i. for 1 min (1 p.s.i.=6894.76 Pa). A constant velocity of carrier gas (He) was maintained

at 33 cm/s. The oven temperature program was as follows: initial 120 °C for 1 min, rate at 20 °C/min to 180 °C holding this temperature for 1 min, then 3 °C/min to 230 °C at a rate of 30 °C/min to 300 °C, holding this temperature for 3 min. Ionization was in the EI mode, 70 eV. The full scan (FS) acquisition was performed in the mass range *m/z* 70–650; selected ion monitoring (SIM) acquisition parameters were as follows: segment 1 started at 11.0 min and the ions *m/z* 308, 291, 405, 390 were monitored for zilpaterol; segment 2 started at 20.0 min and the ions *m/z* 250, 267, 179 were monitored for ractopamine.

2.5. Extraction procedure

According to the p*K* of the functional groups present in the molecules studied, different aqueous solvents were tested for zilpaterol and ractopamine extraction yield: 0.15 *M* sodium acetate solution brought to pH 7.4, to pH 5.0 and to pH 2.8 with 2 *M* HCl. Corn silage, milk replacers, soya meals and feed with mineral supplements were used to reproduce various field conditions. Five samples for each kind of feed were tested.

Aliquots of 1 g of feed were weighed in polypropylene tubes and fortified at 50 μg/g with zilpaterol and ractopamine (100 μl of the standard pool solution at 500 ng/μl) and allowed to stand at room temperature for 60 min to allow drug–matrix interaction. After addition of 10 ml of the extracting solvent, the sample was placed in an ultrasonic bath for 10 min and then gently shaken for 60 min at room temperature. After centrifugation at 4000 *g* for 15 min, an aliquot of 1 ml of the supernatant was filtered and 100 μl was injected into the HPLC system to evaluate the extraction efficiency.

2.6. Clean-up procedure

Feeds (1 g) spiked with 100 μl of the zilpaterol and ractopamine pool solution at 1.25, 2.5, 5.0 ng/μl (three replicates for each fortification level per four kinds of feed) were extracted with 10 ml of acetate solution, pH 2.8 (the extraction solvent of choice). Supernatants (5 ml) recovered by centrifugation were loaded onto SPE C_{18} NE cartridges previously conditioned with 6 ml of methanol and 3 ml of the acetate solution, pH 2.8, at a flow-rate of 1 ml/min. SPE columns were then subsequently washed with 5 ml of water (flow-rate of 1.5 ml/min), 2 ml of methanol (1% 1 *M* HCl) (flow-rate of 0.5 ml/min), 1 ml of *n*-hexane (flow-rate of 1.5 ml/min). The elution was carried out with 3 ml of methanol (1% triethylamine) at flow-rate of 1 ml/min. Each organic fraction was collected, brought to dryness under a gentle stream of nitrogen and re-suspended in 100 μl of mobile phase and analyzed by HPLC–UV/DAD. Recovery was calculated by the external standard method, using calibration curves in the range 30.0–1000 ng of standards injected.

2.7. GC–MS characterization

Data acquisition was performed preliminarily on pooled zilpaterol and ractopamine standards, in the scan mode, mass range *m/z* 70–650, EI ionisation. Then, after the fragmentation studies, for identification purposes the analytes, both on standards and on samples, were identified in the SIM mode, by monitoring four diagnostic ions for zilpaterol, *m/z* 308, 291, 405, 390. For ractopamine, selected as internal standard (I.S.), the ions *m/z* 250, 267, 179 were chosen for SIM experiments. For quantification purposes, the area of the most abundant ions, *m/z* 308 for zilpaterol and *m/z* 250 for ractopamine, were taken into consideration. To determine the linearity of the GC–MS method, calibration curves were prepared on feeds fortified in the concentration range of 30.0–250.0 ng/g. Graphs were calculated by fitting the peak area ratio of the analyte to I.S. (100.0 ng/g) versus zilpaterol concentration.

The limit of detection (LOD) was calculated on 20 blanks (five blanks for each kind of feed considered) on the basis of a signal-to-noise ratio of 3:1 at the analyte retention time on the ion *m/z* 390 that resulted to be the ion with the lowest relative abundance. The limit of quantification (LOQ) was estimated for a signal-to-noise ratio of 10:1.

2.8. GC–MS accuracy and precision

As a consequence of the results obtained from the extraction and purification study, the GC–MS method validation was carried out with the following analytical procedure.

Aliquots of 1 g of homogenized feed were weighed and fortified with zilpaterol in the range 30.0–120.0 ng/g and ractopamine as I.S. at 100.0 ng/g. A 10-ml volume of sodium acetate, pH 2.8 was added and extraction performed as described in Section 2.5, clean-up of 5 ml of the acid solution on SPE C_{18} NE columns as in Section 2.6, evaporation of the eluate to dryness under N_2 stream and derivatization with 30 μl MSTFA–NH_4I–DTE (1000:40:4) for 20 min at 70 °C, and finally, injection of 1 μl into the GC–MS system. Each derivatized solution was injected three times.

Method accuracy and precision were obtained by analyzing three replicates for each spiking level and by repeating the overall analytical procedure in two different analytical sessions. The accuracy was expressed as the agreement between measured and nominal concentrations and the precision as the RSDs for the repeated measurements.

3. Results

3.1. Extraction and clean-up

The results of the extraction study on 20 feeds (five samples per four kinds of feed) by HPLC–UV/DAD analysis are reported in Table 1. The HPLC–UV/DAD recoveries from clean-up procedure, after strong acid extraction, on 60 samples (15 samples for each kind of feed, three samples for each spiking level) are reported in Table 2.

3.2. GC–MS analysis

Fig. 1 shows GC–MS spectra, in the EI mode of zilpaterol (scan acquisition). Its fragmentation pattern is reported in Fig. 2. The chromatograms of the extracted ions of a blank soya feed with I.S. at 100.0 ng/g, and of a soya feed with zilpaterol at 25.0 ng/g (LOQ value) and I.S. at 100.0 ng/g are illustrated in Fig. 3 on the left and on the right side, respectively.

Zilpaterol retention time and its diagnostic ions with their relative abundance (in parentheses), the accuracy and precision of the overall analytical procedure for each tested concentration, are summarised in Table 3.

The zilpaterol minimum amount detectable (LOD) was 7.5 ng/g and the LOQ was equal to 25.0 ng/g in feed. Correlation coefficients of calibration curves in matrix were equal to or higher than 0.9960, by GC–MS analysis.

4. Discussion

The continuous turnover of adrenergic drugs as feed additives for the production of lean meat makes mandatory the use of analytical approaches based on hyphenated techniques to screen for the presence of molecules structurally different but sharing almost the same pharmacological activity. In fact, at present, commercially available immunoassay kits cannot cover all the range of molecules potentially administered to feedlots and the combination of different

Table 1
Recovery rates as mean percentage±SD of the extraction procedure at different pH on corn silages (CS), milk replacers (MR), soya meals (S) and mineral supplements (MS) for zilpaterol and ractopamine (spiking level 50 μg/g in feed)

Feed	Number of samples (*n*)	pH 2.8	pH 5.0	pH 7.4
Zilpaterol				
S	5	89.0±1.6	71.2±2.9	55.3±4.3
MR	5	90.3±1.7	74.5±2.5	41.6±2.3
CS	5	87.1±1.4	66.1±1.3	33.7±2.0
MS	5	90.8±1.0	73.3±1.1	51.3±1.1
Ractopamine				
S	5	88.7±1.4	75.7±2.3	54.8±3.1
MR	5	89.3±1.5	63.9±1.6	40.8±2.7
CS	5	90.3±0.9	71.5±1.8	50.6±1.6
MS	5	89.8±1.0	58.2±1.3	41.1±1.1

HPLC–UV/DAD analysis, λ=280 nm.

Table 2
Recovery rates of zilpaterol and ractopamine on SPE C_{18} light load columns after extraction in pH 2.8 media

Feed	*n*	Nominal level (ng applied on columns)	Zilpaterol		Ractopamine	
			Found level (mean±SD, ng)	RSD (%)	Found level (mean±SD, ng)	RSD (%)
Soya	5	62.5	50.4±0.3	0.6	48.3±0.3	0.6
	5	125.0	107.8±0.9	0.8	100.1±2.3	2.3
	5	250.0	199.1±1.8	0.9	210.3±4.3	2.0
MR	5	62.5	45.2±1.0	2.2	50.2±0.9	1.8
	5	125.0	106.5±1.5	1.4	103.5±1.3	1.3
	5	250.0	189.3±1.9	1.0	185.0±0.8	0.4
CS	5	62.5	47.3±0.8	1.7	49.6±1.0	2.0
	5	125.0	106.3±1.1	1.0	102.6±0.5	0.5
	5	250.0	200.9±2.0	1.0	225.0±2.0	0.9
MS	5	62.5	50.5±0.8	1.6	55.1±1.0	1.8
	5	125.0	107.9±1.9	1.8	109.8±1.2	1.1
	5	250.0	198.4±2.3	1.2	210.2±2.2	1.0

HPLC–UV/DAD analysis, λ=280 nm.

immunotests is obviously affected by a lack of specificity and a higher cost per analysis [13].

In this work we focused our attention on zilpaterol recently licensed in Mexico and South Africa.

The first effort was to set-up an extraction procedure effective for zilpaterol and ractopamine, despite the differences in their chemical structure, in order to achieve a multi drug approach. The pH 2.8

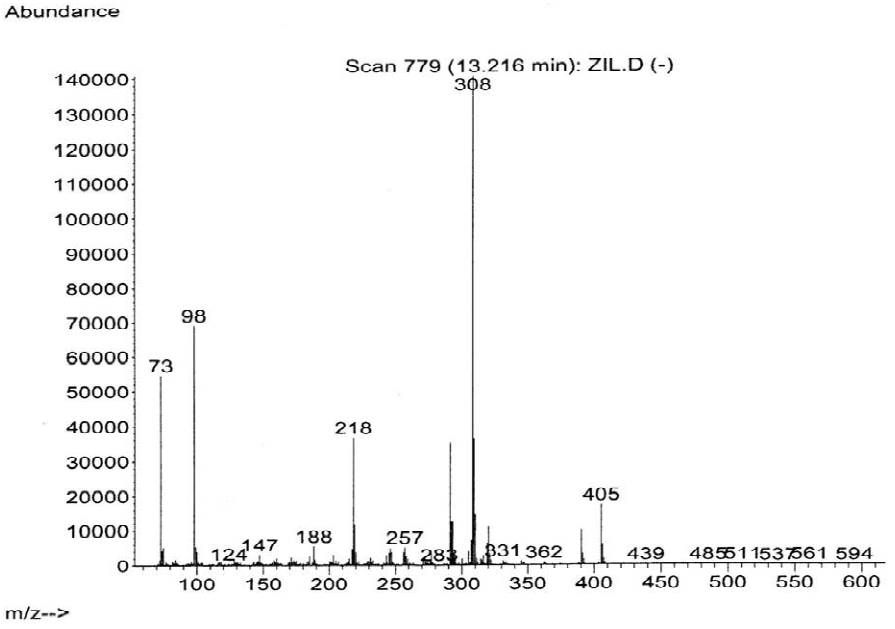

Fig. 1. MS spectra of zilpaterol, FS acquisition, EI mode.

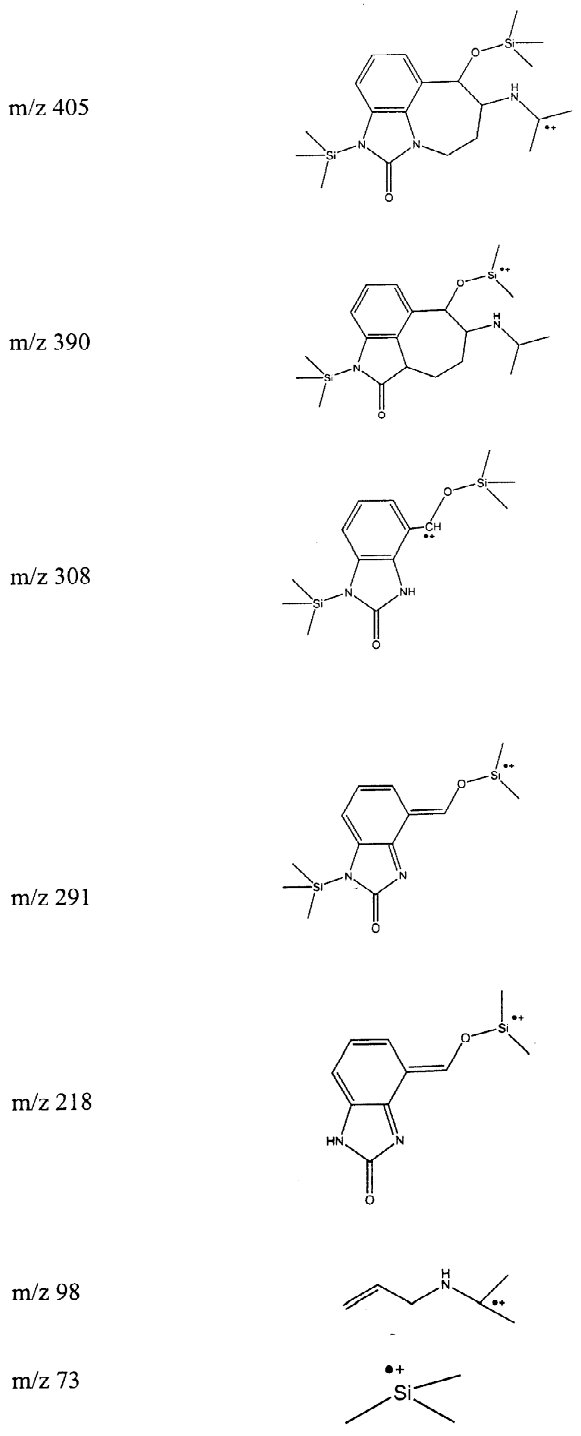

Fig. 2. Proposed fragmentation pattern of zilpaterol.

of the extraction solvent creates a positive net charge on amino groups, thus improving the extraction in aqueous solutions. Moreover, the acid pH determines a reliable inactivation of carboxylic groups, present especially in soya meal, responsible for the formation of ionic bonds with amino groups. Last but not least, pH 2.8 causes the denaturation of proteins in feeds improving the cleanness of the extract, most evident for milk replacers. Zilpaterol and ractopamine mean recoveries from strong acid extraction were 89.3 and 89.5%, respectively.

As regards the clean-up step, the advantages in using a solid-phase approach are multiple: rapid and simple operations, reduced volumes of organic solvents. In our case, the selectivity of the clean-up procedure consists of the use of SPE C_{18} NE columns as mixed-phase columns, taking advantage of both hydrophobic (primary) and ion-exchange (secondary) interactions and, a higher load with respect to mixed-phase columns for complex matrices, such as feeding stuffs, a candidate for multi-drug purposes [14]. In fact, during the application step drugs are efficiently retained by the alkylic chains. In the washing step, the methanol removes lipophilic interferences without drug losses due to polar interactions between drugs and free silanols. The acidification of the washing solvent ensured the analyte is maintains charge during the rinse step and is transfered to ion-exchange sites. The addition of a counter ion (due to the triethylamine in the elution solvent) guarantees quantitative recoveries in the elution step. The clean-up proves to be effective in removing possible interferences from different kinds of matrices. In a typical chromatogram trace of a blank sample (soya feed) (Fig. 3b) no interferences are present on zilpaterol retention time. After purification by SPE, zilpaterol and ractopamine mean recoveries at the lowest fortification level were 77.4 and 81.3%, respectively.

Because, no significant differences were noted between zilpaterol and ractopamine, the latter was chosen as internal standard to demonstrate the suitability of such a procedure for a broad beta agonists spectrum analysis. Although the two substances are meant to be administered to different species, zilpaterol to cattle and ractopamine to pigs, it could be not excluded in the near future a possible extension of their use to other species. Owing to the

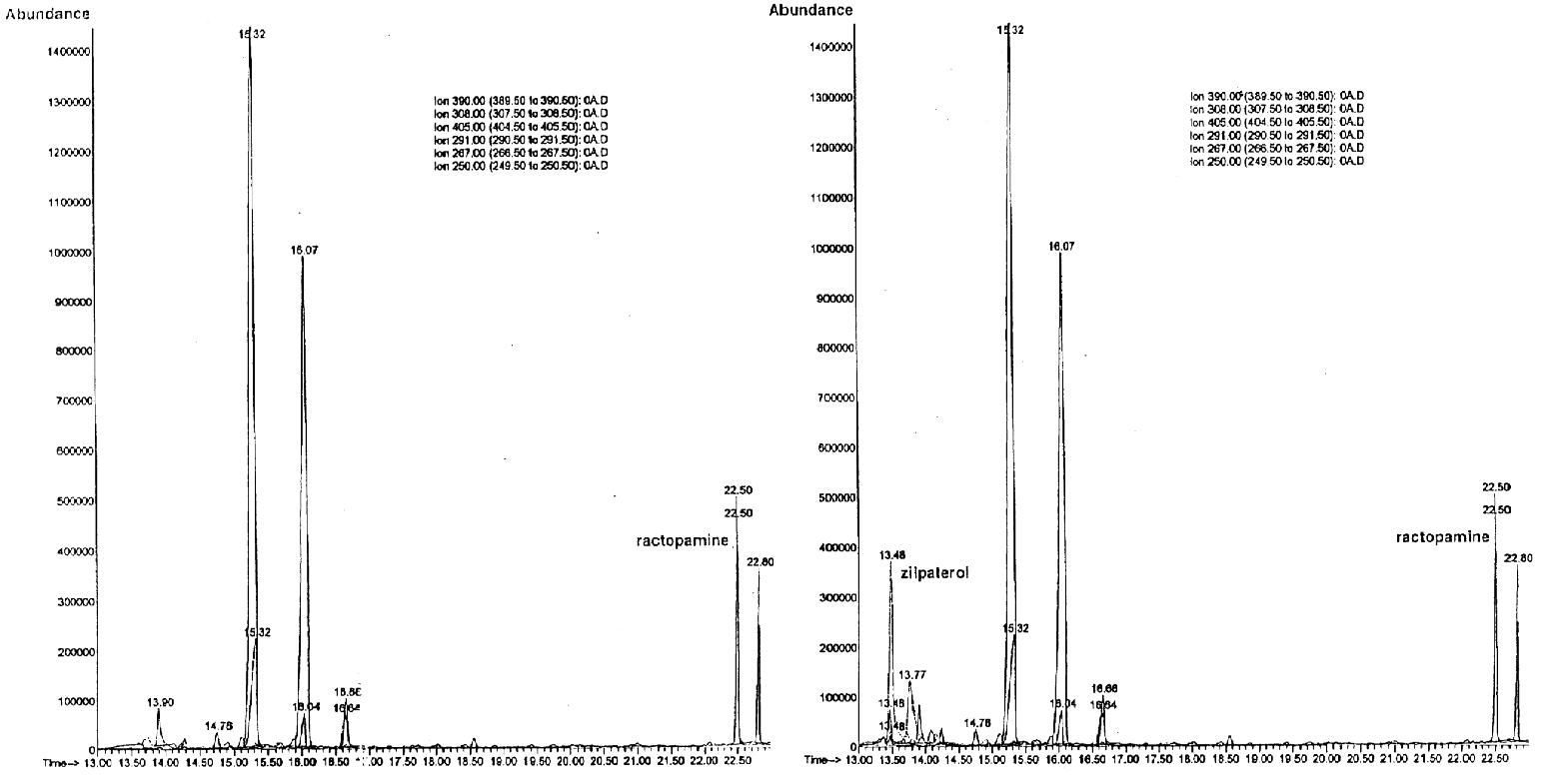

Fig. 3. SIM extract ion chromatograms of a blank soya feed with I.S. at 100.0 ng/g (left), and of a soya feed with zilpaterol at 25.0 ng/g and I.S. at 100.0 ng/g (right).

Table 3
Retention times (t_R), diagnostic ions and their relative abundance (%), accuracy and precision on two different analytical sessions for each spiking level tested

Compound	t_R (min)	RRT	Ions (m/z)	Spiking level (ng/g)	Found level (mean±SD, ng/g) (n=6)	RSD (%)
Zilpaterol	13.48	0.60	308 (100), 291 (24.4), 405 (11.6), 390 (10.1)	30.0	24.1±0.8	3.3
				60.0	48.6±1.1	2.3
				120.0	90.3±1.5	1.7

RRT=Relative retention time.

above, the choice of ractopamine as internal standard should be reconsidered, to avoid masking and or interfering effects in the analytical results. Nevertheless, the multi-drugs features allow the use of all those agonists, that share the ability to form ion-exchange interactions with the free silanols of the SPE column in the clean-up step.

For zilpaterol mass spectrometric identification, four ions with relative intensity more than 10% in the scan spectra, molecular ion included, were selected according to the minimum number of identification points the European Commission intends to propose as revision of Dir 93/256/EC [15] among the criteria to be fulfilled for an unambiguous identification of veterinary drugs residues (Fig. 1, Table 3). In this respect, the relative intensities of the selected ions, expressed as percentage of the intensity of the most intense ion, recorded in spiked feeds correspond to those of the standard, taking account the proposed tolerances of: 10% for relative abundances >50%; 15% for those in the range <50% and >20%; 20% for those <20% and >10%. Lower masses, such as m/z 98, were excluded because chromatogram traces of extracted ions were more influenced by the background noise.

The proposed pattern fragmentation is reported in Fig. 2. Because the bis derivatisation of zilpaterol, it is worth noting that the loss of a methyl group (−15 a.m.u.) can be contributed by either of the two TMS groups.

GC–MS zilpaterol recoveries, applying the overall procedure on commercial feeds and using ractopamine as internal standard, are always above 75.3% and show no great difference among the spiking levels; the repeatibility on two different analytical sessions is less than 3.2%. The application of such a procedure allows the zilpaterol identification at a level of 7.5 ng/g (LOD) and quantification at a level of 25.0 ng/g (LOQ) in feed. Such limits are well below the zilpaterol dose of 5.0 μg/g proposed as effective in feeds.

5. Conclusions

The extraction procedure proposed for zilpaterol and ractopamine was compatible with clean-up and chromatographic end-methods, and led to good acceptable yield and a low number of co-extractives. Moreover, no matrix mismatches were observed between different commercial products. GC–MS quantitative data for zilpaterol, using ractopamine as internal standard, showed good precision and accuracy. The method described for the determination of zilpaterol in animal feeds appeared to be highly specific and sensitive taking into account the doses proposed for feed addition. Results suggested this method could be suitable to detect other phenethanolamines used illegally in animal production, such as ractopamine itself, well below the dose proposed in feeds.

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