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Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography–positive electrospray tandem mass spectrometry

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Abstract

Beta-agonist compounds are widely used in human therapeutics because of bronchodilator or heart tonic properties; they are also used as growth promoters in food-producing animals. Ractopamine is a forbidden molecule in the EU, but is registered as an additive in other countries such as in the USA for pigs. Consequently, efficient analytical methods were developed to survey residues in edible tissue and urine samples. This paper describes a protocol based on a powerful extraction and purification process and a liquid chromatography–positive electrospray mass spectrometry identification method. A validation was performed according to the ''DG SANCO 1805/2000'' European decision. The obtained decision limit (CCα) and detection capability (CCβ) were as low as 10 and 30 ng/kg (ppt), respectively. This method appeared very efficient on incurred samples, including porcine edible tissues (meat, liver, kidney), tissues enriched in β-agonist receptors (lung, retina), and finally bovine urine samples.

Keywords: Ractopamine

1. Introduction

1.1. Illegal use of ractopamine as growth promoter

Beta-agonist compounds, characterised by structural and pharmacological properties very close to those of catecholamines, are widely used as bronchodilators, tocolytics or heart tonics in human and veterinary medicine. On the other hand, several studies focused during the past 10 years on the effect of such synthetic molecules on growth rates and performances in calves, pigs and poultry, particularly when administered per os within feedingstuffs [1,2]. The impact of ractopamine on growth performances and carcass merit was clearly demonstrated in pig, mainly in terms of increased weight gain and lean tissue accretion, as well as improved feed conversion ratio [3–6]. More precisely, it was shown that the ractopamine growth promoting effect is improved when associated with high dietary protein level and that this effect can be linked to a myofibrillar protein

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synthesis stimulation. Because of this growth promoting effect, ractopamine is authorised in pigs in several countries but remains strictly forbidden in the EU. For the survey of the illegal use of ractopamine within the EU and for the control of meat products imported from third countries, non-ambiguous sensitive methods needed to be developed.

1.2. Present analytical state

Several authors proposed screening methods for ractopamine in calf urine using EIA or ELISA immunoassay [7–9], with detection limits in the 1–50 ng/ml (ppb) range. The cross-reactivity of the currently used polyclonal or monoclonal antibodies for other β-agonists is generally less than 0.5% [10,11], but disturbing matrix effects were often mentioned with such immuno-techniques. Other authors presented screening methods dedicated to poultry plasma, serum or tissue samples using HPLC with electrochemical detection, detection limits being around 0.5 ppb [12,13]. Very few studies were devoted to confirmatory analysis, even whether GC–MS [7] or LC–MS–MS [8] have already been used. As far as the extraction–purification process is concerned, the existing methods used minimal sample treatment, i.e. direct analysis for urine samples and only a liquid–liquid extraction with methanol or ethyl acetate for tissue samples. Moreover, it is now well known that beta-agonist compounds are extensively metabolised and lead to a large proportion of glucuronide and/or sulfate conjugated forms [14]. Consequently there is the need for a preliminary deconjugation step. Finally, because the body distribution of ractopamine and metabolites have not been widely studied, we decided to investigate the tissue repartition of the consecutive residues in pig.

1.3. Present work objectives

In this context, the purpose of this paper was first to develop a highly improved process for the extraction and purification of ractopamine residues from tissue and urine samples. Then, a sensitive and specific confirmation method was proposed, based on LC–MS–MS, permitting to fulfil the identification criteria fixed by the ''DG SANCO 1805/2000'' European decision. Target concentration range was fixed to 10–100-fold below existing methods, i.e. less than 50 ng/kg (ppt). Secondly, the proposed methodology was validated according to the decision guideline. In a last step, the described method was applied to the identification of residues in different edible tissues (liver, kidney, meat), tissues enriched in beta-agonist receptors (lung, retina) of pigs treated with ractopamine, as well as in urine samples.

2. Experimental

2.1. Reagents and chemicals

Methanol (analytical and HPLC grade), hexane, diethylether, ethyl acetate, glacial acetic acid and 32% ammonium hydroxide were provided by Solvents Documentation Syntheses (SDS, Peypin, France). Sodium acetate and sodium phosphate were purchased by Merck (Darmstadt, Germany). Standard reference ractopamine and isoxsuprine (internal standard) were from Eli Lily (Indianapolis, IN, USA) and Sigma (St. Louis, MO, USA), respectively. Standard solutions were prepared at 1 mg/ml in methanol and stored in darkness at −20 °C. Concerning the internal standard, our choice was mainly motivated by two reasons. On the one hand, the high structural similarity of isoxsuprine with the target analyte ractopamine ensure both a good specificity during the purification process and an improved sensitivity because of some common MRM transitions in MS–MS detection. Moreover, such a closely analogous internal standard is generally preferable in terms of quantitative analysis. On the other hand, even if isoxsuprine is also a beta-agonistic compound potentially used as growth promoter, this risk was taken into account. But to our knowledge no case has been reported regarding its field utilization, and we believe that the known amount of isoxsuprine introduced in the samples (not so far from the noise level) could reveal such misuse.

2.2. Liquid chromatography

An Alliance® 2690 HPLC pump was used (Waters®, Milford, MA, USA). Reversed-phase liquid chromatography was performed on octadecyl

grafted silica Nucleosil® C_{18}AB (50×2 mm, 5 μm) stationary phase (Macherey-Nagel®, Düren, Germany) with a guard column (Nucleosil® C_{18}AB, 10×2 mm, 5 μm). Elution solvents were methanol (A) and 0.5% (v/v) acetic acid in water (B). Mobile phase composition (A:B; v/v) was 95:5 at 0 min, 50:50 at 10 min, and 10:90 at 20 min and 95:5 from 30 to 40 min. Flow rate was 220 μl/min and injected volume was 10 μl.

2.3. Mass spectrometry

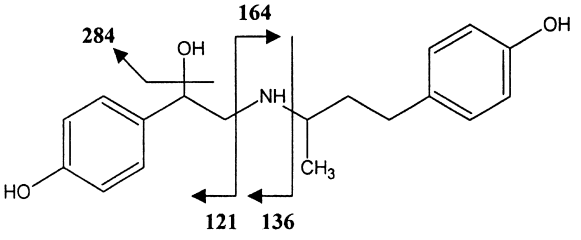
Data were acquired in the positive electrospray mode using a QuattroLC® triple quadrupole analyser (Micromass®, Manchester, UK). Nitrogen was used as nebulisation and desolvation gas, at flow-rates of 90 and 600 l/h, respectively. Source and desolvation temperatures were 120 and 350 °C, respectively. Potentials applied on the capillary and on the sampling cone were 3.5 kV and 25 V, respectively. MS–MS experiments were performed using argon as collision gas at a pressure of 4.0×10^{-4} mbar and a collision energy varying from 15 to 25 V. The signal acquisition was performed on the multiple reaction monitoring mode (MRM). Both for ractopamine and isoxsuprine (internal standard), the precursor ion was the pseudo-molecular ion ($m/z = 302$). For isoxsuprine, the two diagnostic product ions were $m/z = 284$ and 150. For ractopamine, the six diagnostic product ions were $m/z = 284$, 164, 136, 121, 107, and 91 (Table 1).

2.4. Extraction/purification

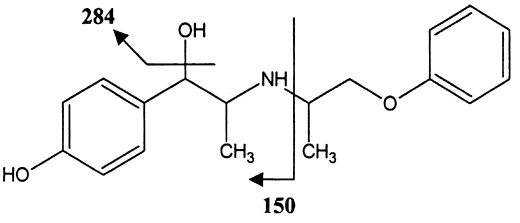
Fresh tissue samples (15 g of liver, kidney, meat, lung or 200–500 mg of retina) were freeze-dried and groud. Twelve ml methanol and 15 ml acetate buffer (2 *M*, pH 5.2) were added. After stirring (30 min) and centrifugation (2000 *g*, 15 min), supernatant was collected and methanol was evaporated. Four hundred μl of a purified *Helix pomatia* preparation containing 25 units/μl (Sigma) were added, and the hydrolysis of conjugated metabolites was carried out at 60 °C for 15 h. After centrifugation, supernatant was applied to ChromP© SPE columns (Supelco, St Quentin Fallavier, France), previously activated with 6 ml ethyl acetate, 6 ml methanol and 6 ml water. After washing with 5 ml hexane and 12 ml hexane–diethyl ether (70:30, v/v), analytes were eluted with 24 ml diethyl ether. After evaporation to dryness, the extract was reconstituted in 6 ml phosphate buffer (0.1 *M*, pH 6). It was then applied to Screen DAU SPE columns (Supelco) previously activated with 2 ml methanol, 2 ml water and 2 ml phosphate buffer. After washing with 1 ml acetic acid 1 *M* in water and 6 ml methanol, analytes were eluted with 6 ml ethyl acetate–32% ammonium hydroxide (97:3, v/v). After evaporation to dryness, the extract was reconstituted in 50 μl 0.5% acetic acid in water–methanol (95:5, v/v) and 10 μl were injected in LC–MS–MS. For urine samples (10 ml), 2 ml acetate buffer (2 *M*, pH 5.2) were added and samples were hydrolysed and purified according to the same procedure.

Table 1
Structure and MS–MS product ions of ractopamine and isoxsuprine

Ractopamine
$C_{18}H_{23}NO_3$
$M_w = 301.39$



Isoxsuprine (I.S.)
$C_{18}H_{23}NO_3$
$M_w = 301.39$



3. Results and discussion

3.1. Results on spiked samples

Fig. 1 shows the observed ion chromatograms for a blank and a 50 ng/kg (ppt) spiked porcine liver sample. The high specificity both of the purification and of the detection methods led to profiles without any interfering compounds and facilitated interpretation and conclusions. Moreover, signals were found very sensitive, with a non-ambiguous identification based on two MRM transitions even at the ppt level. Fig. 2 shows six MRM transitions for a blank and a 500 ppt spiked bovine urine sample. This profile confirmed the high specificity of the method with signal-to-noise ratios clearly higher than 10 even for the worst MRM transition. This corresponded to 10 identification points (one point for the precursor ion and 1.5 for each product ion) according to the DG SANCO 1805/2000 decision, for which four points are enough for non-ambiguous identification.

3.2. Validation process and method performances

The method validation process consisted first in the analysis of 20 blank porcine edible tissue samples from different origins, in order to verify the absence of target analyte and potential interfering compounds. Specificity was found very good, particularly for the ''284>164'' MRM transition completely free of interferences. Then, these blank samples were pooled, and a calibration curve was built using spiked samples at 25, 50, 100 and 500 ng/kg (ppt). The coefficient of determination (R^2) of the calculated regression curve was 0.9975. In the case of unauthorized substances, the decision limit

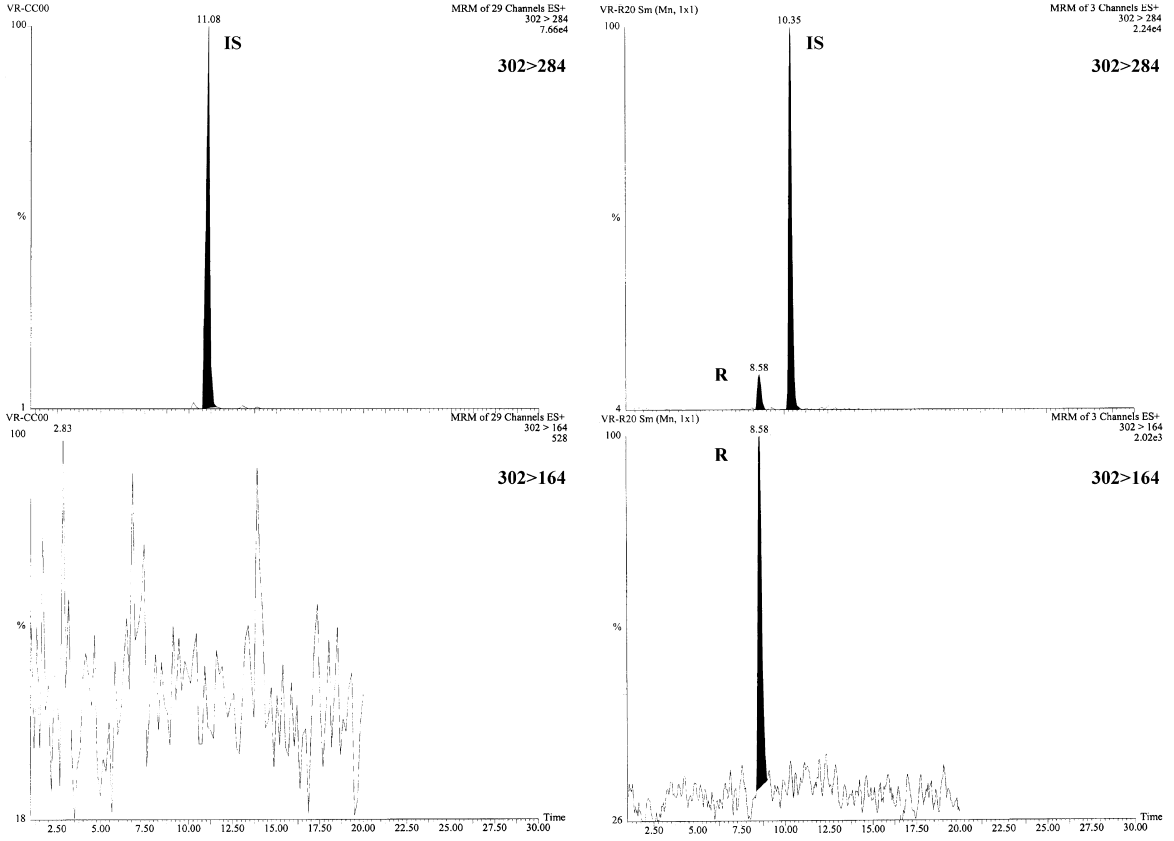


Fig. 1. LC–ESI+–MS–MS (MRM) diagnostic ion chromatograms for a blank (left) and a 50 ppt spiked porcine liver sample (right). I.S., internal standard isoxsuprine; R, ractopamine.

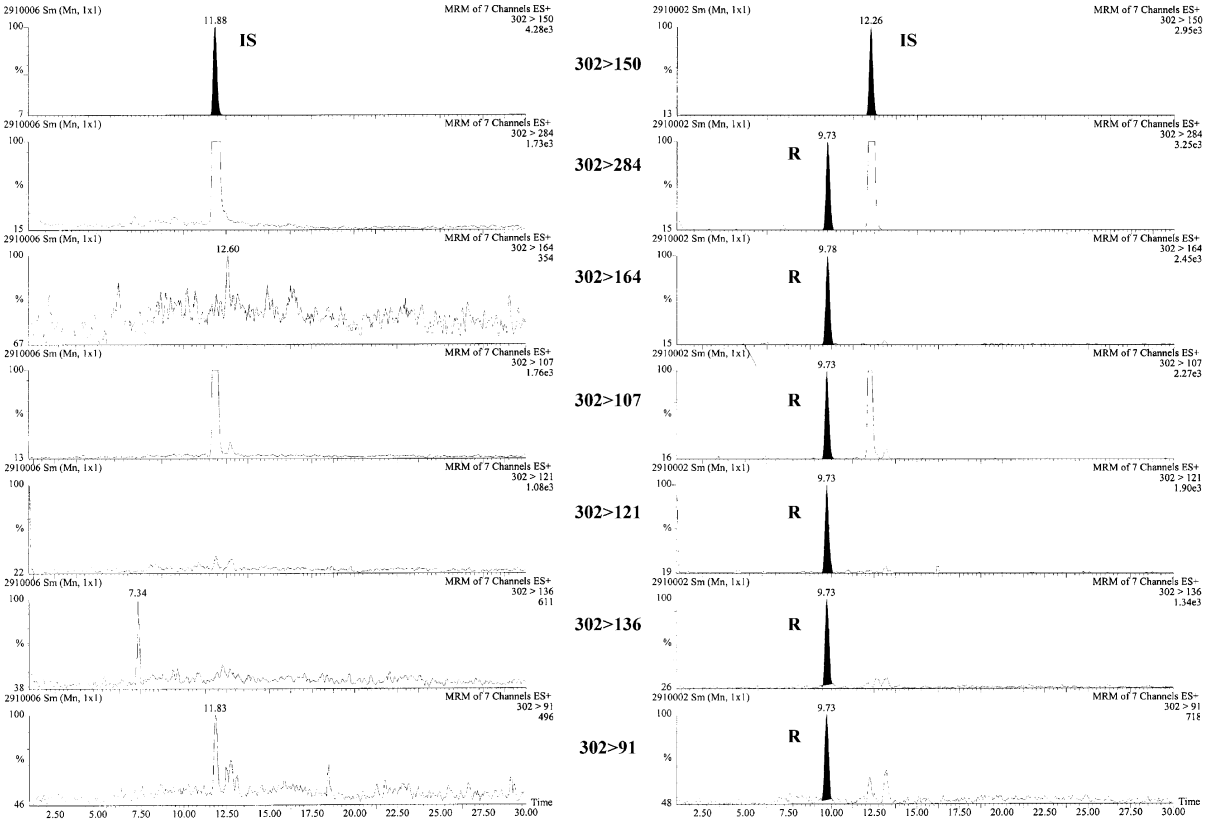


Fig. 2. LC–ESI+–MS–MS (MRM) diagnostic ion chromatograms for a blank (left) and a 500 ppt spiked bovine urine sample (right). I.S., internal standard isoxsuprine; R, ractopamine.

(CCα) is defined in the DG SANCO decision as ''the lowest concentration level at which a method can discriminate with a statistical certainty of $1-\alpha$ whether the identified analyte is present''. For the present application, our proposed interpretation of this text (with the discussion still running between the different CRLs and NRLs) was to define CCα as the minimal concentration permitting to declare the sample different than a blank sample, with a confidence level of 99%. In practice, CCα is the concentration inducing a signal intensity $I_{CC\alpha}$ given by Eq. (1), where μ_B and σ_B are, respectively, the mean and the standard deviation of the noise amplitude, estimated on the basis of the 20 blank samples. These values were calculated on the basis of the most intense transition, i.e. the one permitting the more sensitive detection. The calibration curve resulting in Eq. (2), where a is the slope of the fitting curve, CCα was finally calculated using Eq. (3), leading to a value of: CCα = 9 ppt

$$I_{CC\alpha} = \mu_B + 2.33\sigma_B \quad (1)$$

$$I_{CC\alpha} = \mu_B + a\,CC\alpha \quad (2)$$

$$CC\alpha = 2.33\sigma_B / a \quad (3)$$

In a third step, the 20 blank samples were spiked at 50 ppt, in order to determine the repeatability as well as the detection capability (CCβ). The repeatability of the relative retention time (2.1%), of the relative signal intensities for the two more intense MRM transitions (17.3% and 18.1%) and of the ion ratio between these two signals (7.2%) appeared acceptable. The detection capability (CCβ) is defined in the DG SANCO decision as ''the lowest

concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1-\beta$''. For the present application, our proposed interpretation of this text was to define CCβ as the lowest concentration permitting to identify unambiguously the analyte in the sample with a confidence level of 99%. In practice, CCβ is the concentration inducing a signal intensity $I_{CC\beta}$ given by Eq. (4), where μ'_B and σ'_B are respectively the mean and the standard deviation of the noise amplitude (estimated on 20 blank samples) and σ_S the standard deviation of the signal observed for the 20 spiked samples. These values were calculated on the basis of the less intense transition, i.e. the one permitting the unambiguous identification. The previous calibration curve resulting in Eq. (5), where a' is the slope of the fitting curve, CCβ was finally calculated using Eq. (6), leading to the value of:

$$CC\beta = 28 \text{ ppt}$$

$$I_{CC\beta} = \mu'_B + 2.33\sigma'_B + 1.64\sigma_S \quad (4)$$

$$I_{CC\beta} = \mu'_B + a'CC\beta \quad (5)$$

$$CC\beta = (2.33\sigma'_B + 1.64\sigma_S)/a' \quad (6)$$

Finally, three spiked samples at 50 ppt were analysed in order to calculate the recovery which was estimated to 18%. This relative low value was nevertheless judged satisfying regarding the improved sensitivity and the aspect of the ion chromatograms. In conclusion, the method performances were found very satisfying, particularly in terms of specificity and sensitivity, with detection limits between 5 and 10 times better than values found in the literature, and at least fit for the purpose.

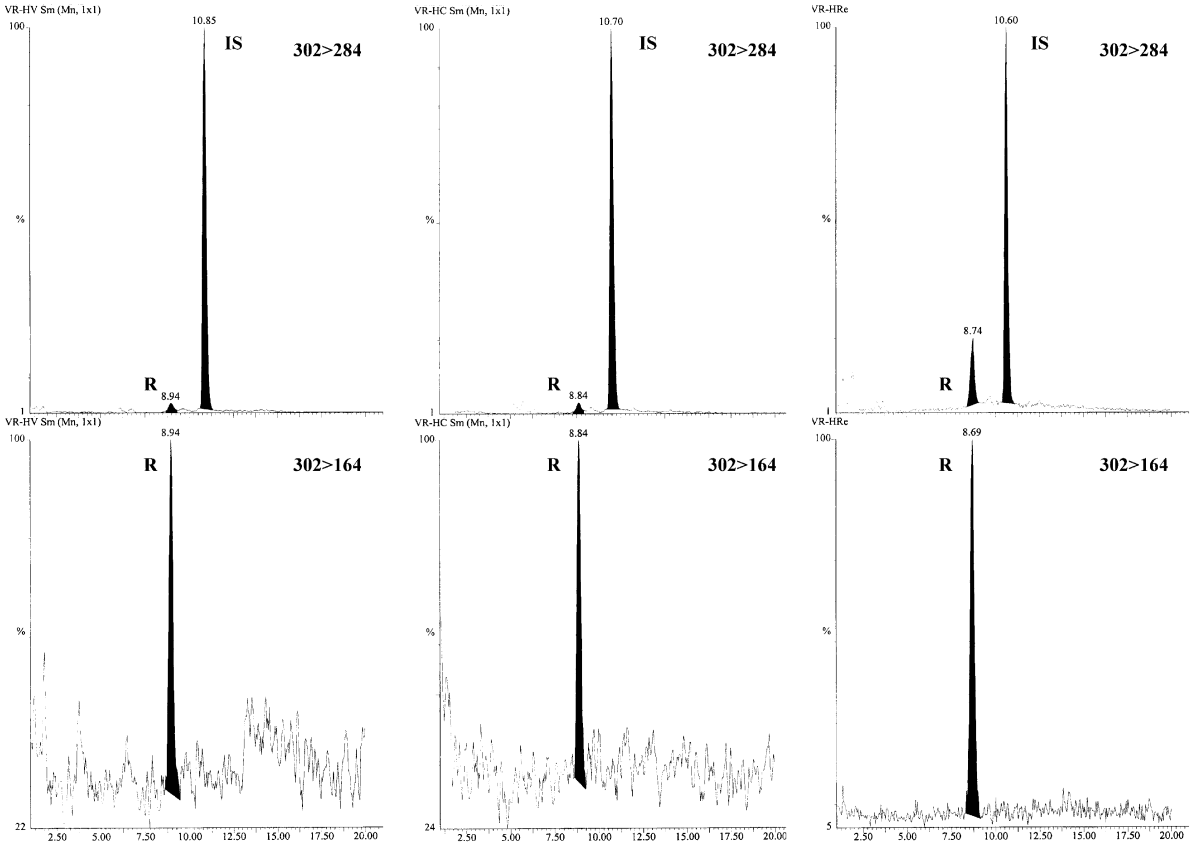


Fig. 3. LC–ESI+–MS–MS (MRM) diagnostic ion chromatograms for incurred edible tissue samples collected from a treated pig: meat (left), heart (center) and kidney (right). I.S., internal standard isoxsuprine; R, ractopamine.

3.3. Results on incurred samples

Two pigs (European race, 3 months old and 60 kg) were treated with dietary ractopamine for 10 days (10 mg/day). They were slaughtered 5 days after the last administration and the different organs and tissues were collected and frozen. Samples were extracted and purified as previously described. Fig. 3 shows the diagnostic ion chromatograms obtained for edible tissues, i.e. muscle, heart and kidney samples. Ractopamine was detected in muscle and heart at a concentration estimated to 10 and 15 ppt, respectively, and clearly identified in kidney at a concentration estimated to 95 ppt. Fig. 4 shows the ion chromatograms obtained after extraction of lung and retina samples. In these samples, the high amount detected (concentration estimated to 740 ppt in lung and 19,480 ppb in retina) demonstrated the high affinity for the analyte due to the large number of β-agonist receptors in these tissues. The observed distribution of ractopamine should be also explained by the administration mode. Indeed, it can be assumed that dietary intake increases oral bioavailability and local effect (for instance in lung) compared to intra-venous or intra-muscular injection that induces a more systemic effect and distribution in edible tissues. These results demonstrated first the efficiency of the proposed analytical methodology for ractopamine residue identification in various tissues, and secondly the suitability of particular and unusual matrices for the control of the illegal use of this compound. Indeed, the developed method permitted to demonstrate the administration of ractopamine even after its persistent delay in conventional tissues.

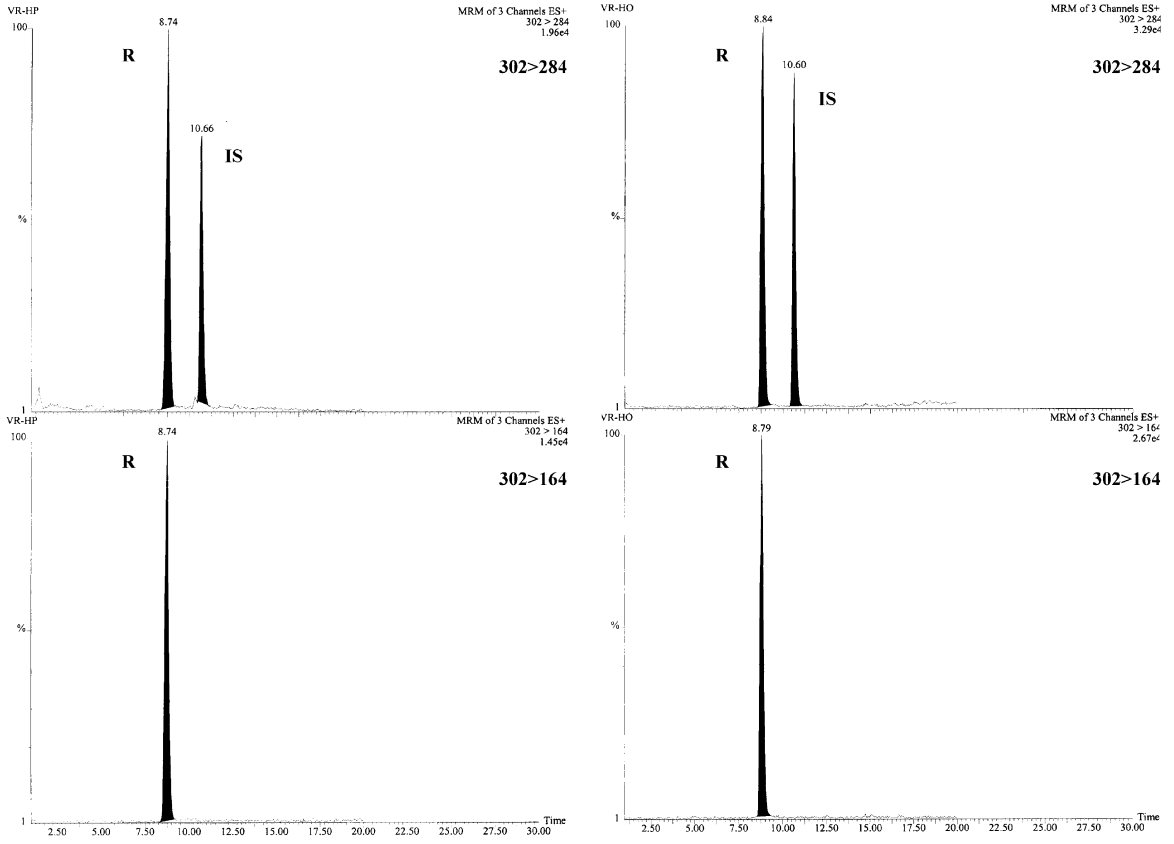


Fig. 4. LC–ESI+–MS–MS (MRM) diagnostic ion chromatograms for incurred lung (left) and retina (right) samples. I.S., internal standard isoxsuprine; R, ractopamine.

4. Conclusion

A method was described for identification of ractopamine residues in different tissues and in urine, based on liquid chromatography–positive electrospray tandem mass spectrometry. The achieved decision limit (10 ppt) and detection capability (30 ppt) proved the efficiency of this methodology for the control of ultra-trace levels of this substance. Moreover, the specificity of the developed purification and MS–MS detection methods led to successful application for identification of ractopamine in various matrices including edible tissues, matrices enriched in β-agonist receptors and urine samples. Due to its capacity to fulfil European analytical criteria, this method should be proposed as a candidate reference confirmatory method to complete the existing screening methods.

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