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Determination of Ractopamine Residues in Pigs by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry

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1. Introduction

Ractopamine hydrochloride is a xenobiotic that belongs to a large group of ß$_2$-adrenergic agonist compounds. ß$_2$-Agonists are used in human and veterinary medicine for treatment of lung diseases as bronchodilators, tocolytics and heart tonics (Courtheyn et al., 2002; Malucelli et al., 1994; Meyer & Rinke, 1991). Besides their legal use, these drugs are often misused as growth promoters, to improve carcass composition by decreasing fat to the benefit of muscle mass, gaining higher economic benefit to producers (Anderson et al., 2009; Moody et al., 2000). Ractopamine hydrochloride increases the amount of lean meat and decreases the amount of carcass fat when fed to swine during the last 50 kg of gain, also increasing the rate of weight gain and feed conversion (Anderson et al., 1989; Merkel et al., 1987; Watkins et al., 1990; Williams et al., 1994). The biochemical basis of ractopamine effects is increasing the nitrogen retention, protein synthesis, enhancing lipolysis and suppressing lipogenesis (Apple et al., 2007; Armstrong et al., 2004; Carr et al., 2005; Mills, 2002; Mitchell et al., 1990; Mitchell, 2009).

Illegal use of ß$_2$-agonists in 5- to 10-fold therapeutic doses leads to accumulation of these compounds in animal tissues such as liver, kidney and muscle (Smith, 1998; Smith & Shelver, 2002). High amounts of ß$_2$-agonist residues in meat and meat products led to a number of cases of food poisoning in humans in the last 20 years (Brambilla et al. 1997; Garay et al., 1997; Martinez-Navarro, 1990; Pulce at al., 1991; Ramos et al., 2003), although the Council Directive 96/22/EC banned the use of these substances in the European Union. Consequently, detection of ß$_2$-agonists in biological material from farm animals is a high priority because of the public health concern; relatively large numbers of samples have to be analyzed and more stringent criteria used in view of the serious public health implications of positive results. In order to provide quality assurance for the consumer and to satisfy legal testing obligations, the ability to detect drug residues at low concentrations has become a very important issue.

Although all incidents of poisoning were caused by clenbuterol toxicity, the European Union has placed ban upon the use of all β-agonists, thus requiring strict monitoring for the illegal use of this and other β-agonists. Ractopamine was approved by the U.S. Food and

Drug Administration for use in finishing swine in 1999 (Anderson et al., 2009), whereas in European Union the use of ractopamine like other β-agonists is completely banned.
As β-agonists are classified into group A substances having an anabolic effect and unauthorised substances, confirmation of the target analyte is necessary according to analytical performance criteria of the Commission Decision 2002/657/EC. As a result of the requirement of analytical criteria on confirmatory methods, mass spectrometry techniques are widely used to identify trace levels of organic residues and contaminants (Shao et al., 2009). A number of methods, in most cases gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS), are available for the analysis of ractopamine in a variety of matrices. On this, a minimum of four characteristic ions are required to satisfy the four identification points for the GC-MS or LC-MS techniques and one precursor ion and two daughter ions can provide four identification points for tandem mass spectrometry techniques including triple quadropole mass spectrometry and ion trapping techniques (e.g., LC-MS/MS and GC-MS/MS). Fragmentation pathway of ractopamine is shown in Figure 1. However, GC/MS methods require derivation because of their high polarity and low volatility, which is time-consuming, tedious, laborious and expensive (Shao et al., 2009). Because of its high sensitivity and selectivity, LC-MS/MS is often the method of choice in the analysis of trace levels of polar contaminants (Nielen et al., 2008; Pleadin et al., 2011) and recently many authors demonstrated its superior performance in ractopamine analyses (Blanca et al., 2005; Churchwell et al., 2002). So far, ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) is one of the most efficient methods (Nielen et al., 2008; Shao et al., 2009) because of the high resolution, rapid separation of UPLC, and the selectivity and sensitivity characteristic of MS-MS detection (Dong et al., 2011; Zheng et al., 2010).
Analysis of ractopamine residues is mostly performed in urine and plasma samples taken from living animals as well as in tissue samples after slaughtering (muscle, liver, kidney and fat) (Antignac et al., 2002; Gratacós-Cubarsí et al., 2006; Shao et al., 2009; Thompson et al., 2008). Besides tissues and body fluids, hair has been shown to be an excellent site for accumulation of different drugs including ß-agonists and therefore also an appropriate matrix to monitor the presence of drug residues. Another advantage of hair as a matrix of choice to monitor the presence of ß-agonists is the ability to collect hair samples from living animals. On the other hand, there are little data on ractopamine accumulation in the hair of farm animals. Literature data show that in swine, ractopamine hydrochloride is very rapidly eliminated resulting in very low tissue residues, while major elimination route is *via* conjugation with glucuronic acid and urinary excretion (Dalidowicz, 1992).
Bearing in mind rapid urinary excretion of ractopamine hydrochloride and hair affinity for $ß_2$-agonist accumulation, the aim of this study was to determine ractopamine residues using UPLC-MS/MS and to compare residue levels of ractopamine hydrochloride in swine urine and hair samples after sub-chronic treatment with low ractopamine doses.

2. Materials and methods

2.1 Chemicals and apparatus

Ractopamine hydrochloride (Sigma-Aldrich-Chemie, Steinheim, Germany) was used for animal treatment and method validation. Ractopamine-D5-hydrochloride (RIKILT, Wageningen, The Netherlands) was applied as internal standard. Protease Type XIV, from *Streptomyces grisues* (Sigma-Aldrich-Chemie, Steinheim, Germany) was used for hair sample

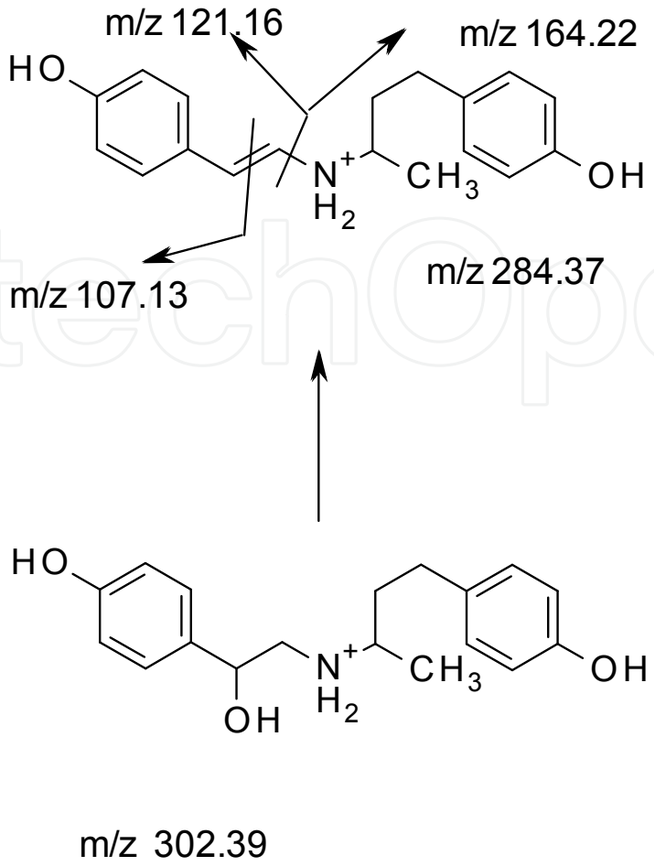


Fig. 1. Fragmentation pathway of ractopamine.

analysis. ß-Glucuronidase/aryl sulfatase (Merck Chemicals, Darmstadt, Germany) was used for urine sample analysis. All solvents used were of HPLC grade. Screen Dau solid phase extraction columns (500 mg, 6 mL) used for clean up were from Amchro (Hattersheim, Germany). The analyses were performed on UPLC/MS/MS Xevo® TQ-S (Waters, En Yvelines Cedex, France). Hair samples were homogenized by means of a MM400 mixer mill (Retsch, Haan, Germany). Filtration and centrifugation of hair samples were performed on Amicon Ultra Centrifugal Filters Ultracell 10 K (Millipore, Carrigtwohill, Ireland). Hair samples were dried in a vacuum drying cabinet VT 6060 M (Heraeus, Hanau, Germany).

2.2 Animals and sampling procedure

The study included 12 male pigs (9 treated and 3 controls), Zegers hybrid type (white hair), 55 kg body weight, farm-bred and kept under same conditions. Animals (n=9) were randomly divided into 3 groups and treated with ractopamine hydrochloride in a dose of 1 mg daily (absolute) *per os* during 28 days (0.51 mg/kg b.w.). Treated animals were orally administered ractopamine hydrochloride in the form of a capsule filled with pure chemical admixed to feed. Three animals served as a control group and were left untreated. On days 1, 3 and 8 after treatment withdrawal, treated animals were sacrificed in groups of 3. Control group animals were sacrificed on day 8 after experimental animal treatment withdrawal. Hair samples were obtained by shaving pigs with a razor blade and stored at room temperature. Urine samples after collection on slaughtering were stored at -20 °C until

analysis. All experiments were performed according to the Croatian Animal Protection Act (Official Gazette of the Republic of Croatia 135/06).

2.3 Hair sample preparation and clean up

Hair samples were washed with 2x20 mL of water and dried overnight. Dry hair was homogenized in mixer mill for 2x2 minutes and then mixed "head over head" for 60 minutes. Internal standard of ractopamine-D5-hydrochloride (0.1 ng/µL), 5 mL of tris-buffer (pH=8) and 100 µL of protease solution (50 mg/mL in water) were added to the portion of 500 mg of hair to obtain spiking level of 5 ng/g. The mixtures were incubated overnight at 55 °C in shaker water bath. After incubation, 2 mL of phosphate buffer (pH=6) was added and pH was adjusted to 6. Samples were then shaken in ultrasonic bath at room temperature and centrifuged at 4 °C and 4000 rpm using Amicon filter units. The supernatants were transferred to another tube with addition of 200 µL of methanol, followed by centrifugation at 4 °C and 4000 rpm. The centrifuged extracts were loaded to SPE cartridges conditioned with 2 mL of methanol, 2 mL of water and 2 mL of phosphate buffer pH=6. Cartridges were washed with 1 mL of 1 M acetic acid and evaporated to dryness followed by washing with 2 mL of methanol and evaporating to dryness. The elution was performed with 6 mL of mixture consisting of ethyl acetate and 25% ammonia at a 97:3 ratio. The samples were evaporated to dryness under stream of nitrogen at 35 °C. Residues were then dissolved in 200 µL of HPLC mobile phase consisting of 0.1% formic acid in water (A)/0.1% formic acid in acetonitrile at a 95:5 ratio (B).

2.4 Urine sample preparation and clean up

A portion of 10 mL of urine was spiked with internal standard of ractopamine-D5-hydrochloride (0.1 ng/µL) with addition of 5 mL of sodium acetate buffer (pH=5) and 50 µL of glucuronidase/aryl sulfatase to obtain spiking level of 5 ng/mL. The same steps were performed also without hydrolysis. The samples were shaken and incubated overnight at 37 °C. After cooling at room temperature, 5 mL of phosphate buffer (pH=6) was added. The hydrolyzed solution was centrifuged followed by addition of 200 µL of methanol to obtained supernatants. The supernatants were loaded to SPE cartridges conditioned with 2 mL of methanol, 2 mL of water and 2 mL of phosphate buffer (pH=6). Cartridges were then washed with 1 mL of 1 M acetic acid and evaporated to dryness followed by washing with 2 mL of methanol and evaporating to dryness. The elution was performed with 6 mL of a mixture consisting of ethyl acetate and 25% ammonia at a 97:3 ratio. The samples were evaporated to dryness under stream of nitrogen at 35 °C. Residues were then dissolved in 200 µL of HPLC mobile phase consisting of 0.1% formic acid in water (A)/0.1% formic acid(B) in acetonitrile at a 95:5 ratio.

2.5 Liquid chromatography tandem mass spectrometry conditions

The UPLC separation was performed on Acquity HSS C18 columns (150x2, 1.8 µm particle size) at a flow rate of 0.45 mL/min and temperature 40 °C. The mobile phase consisted of constituent A (0.1% formic acid in water) and constituent B (0.1% formic acid in acetonitrile). A gradient elution program was employed as follows: 0-5 min 95% A, 15 min 50% A, 17 min 50% A, 18 min 10% A, 19 min 10% A, 20 min 95% A and 25 min 95% A. The injection volume was 10 µL. The mass spectrometry conditions were as follows: electrospray ionization, positive polarity, capillary voltage 0.65 kV, source temperature 150 °C, desolvation

temperature 550 °C, cone gas 20 L/h, desolvation gas 1200 L/h, and collision gas 0.1 L/h. The mass spectrometer was operated in multiple reaction monitoring mode, the protonated molecular ion of ractopamine at m/z = 302.2 being the precursor ion. Four product ions at m/z = 284.2, m/z = 164.2, m/z = 121.2 and m/z = 107.1 were monitored. Quantitation was performed with most intensive transition (m/z 302.2 → 164.2) *versus* internal standard monitored (ractopamine-D5, m/z 307.1>167.1) and extrapolation using a six point calibration curves.

2.6 Validation process

Validation was carried out according to Commission Decision 2002/657/EC by an alternative approach of matrix comprehensive in-house validation by means of a factorial design software used for factorial design and calculation was InterVal Plus (quo data, Gesellschaft für Qualitätsmanagement und Statistik GmbH, Dresden, Germany). In validation process, decision limit (CCα), detection capability (CCβ), precision, recovery, repeatability, in-house reproducibility, matrix effects, specificity and ruggedness were studied. Validation process started with factorial design for both matrices. Factors and their levels for hair and urine are presented in Table 1 and Table 3, respectively.

Factor	Level
Species	Calf / Cow
Matrix condition	White / Black
Operator	Analyst 1 / Analyst 2
Storage of extracts (injection solution)	2 days, 4 °C before injection/ without
Instrument	Xevo /Q-TOF
Amount of matrix	200 mg / 500mg

Table 1. Factors of interest and their levels used for validation of ractopamine in hair.

For validation of ractopamine in hair, 16 runs, each for 5 concentration levels, were conducted within 16 days and with different factor combinations. In total, 80 measurements were performed. Table 2 shows experimental design for validation of the method of hair analysis as an example.

Within each run, blank samples were fortified at five concentration levels: 2, 5, 8, 11 and 15 ng/g. In addition, a matrix blank sample and reagent blank sample were included in each run.

Validation for ractopamine in urine was preformed with 32 runs, 8 runs *per* species. Within each run, blank samples were fortified at six concentration levels: 0.125, 0.25, 0.375, 0.50, 0.625 and 0.75 ng/mL. In addition, a matrix blank sample and reagent blank sample were included in each run.

			Factor level combination					
Run	Sample Number	Run name	Species	Matrix cond.	Operator	Storage of extracts	Instrument	Sample amount
Run 15	P100260	AS100502	cow	white	Analyst 1	without	Q-TOF	500 mg
Run 02	P100262	AS100475	cow	white	Analyst 1	2 days 4 °C	Xevo	200 mg
Run 09	P100248	AS100496	calf	black	Analyst 1	without	Xevo	200 mg
Run 04	P100257	AS100477	cow	black	Analyst 1	without	Xevo	500 mg
Run 08	P100246	AS100481	calf	black	Analyst 1	2 days 4 °C	Q-TOF	500 mg
Run 10	P100247	AS100497	calf	black	Analyst 2	without	Q-TOF	500 mg
Run 11	P100250	AS100498	calf	white	Analyst 1	2 days 4 °C	Xevo	500 mg
Run 06	P100253	AS100479	calf	white	Analyst 1	without	Q-TOF	200 mg
Run 05	P100252	AS100478	calf	white	Analyst 2	without	Xevo	500 mg
Run 13	P100259	AS100500	cow	black	Analyst 1	2 days 4 °C	Q-TOF	200 mg
Run 14	P100256	AS100501	cow	black	Analyst 2	2 days 4 °C	Xevo	500 mg
Run 07	P100249	AS100480	calf	black	Analyst 2	2 days 4 °C	Xevo	200 mg
Run 03	P100258	AS100476	cow	black	Analyst 2	without	Q-TOF	200 mg
Run 01	P100261	AS100474	cow	white	Analyst 2	2 days 4 °C	Q-TOF	500 mg
Run 12	P100251	AS100499	calf	white	Analyst 2	2 days 4 °C	Q-TOF	200 mg
Run 16	P100263	AS100503	cow	white	Analyst 2	without	Xevo	200 mg

Table 2. Experimental plan for hair.

Factor	Level
Species	Bull / Calf / Cattle / Pig
Ultrafiltration of injection solution	Without / With
Operator	Analyst 1 / Analyst 2
Storage of matrix before analysis	1 week at 4 °C / 1 week frozen
SPE cartridges	Small /Large
Injection of final solutions	Immediately / 2 days after finishing sample preparation, if stored at 4 °C
Enzyme	Sigma / Merck
Cartridges	Starta X/ CSD

Table 3. Factors of interest and their levels used for validation of ractopamine in urine.

3. Results and discussion

3.1 Applicability of study results

There are literature reports on ractopamine determination using different techniques (Shelver & Smith, 2003; Smith et al., 1993; Thompson et al., 2008; Turberg et al., 1995). Studies suggest the use of LC/MS/MS systems as probably the best methods to improve sensitivity in determination of β-adrenergic agonists, while retaining excellent selectivity (Smith & Shelver, 2002), pointing to UPLC-MS/MS as one of the most efficient methods.

As the analysis of ractopamine in samples from all stages of production is important for monitoring illegal use in European Union, development of sensitive and selective methodologies in different matrices is required. Control and monitoring programs mandated by government have also necessitated implementation of assays for determination of ractopamine accumulation and excretion from tissues and body fluids in farm animals.

Studies of ractopamine residue detection after different treatment schedules in different animal species have been reported by several authors (Elliot et al., 1998; Qiang et al., 2007; Smith & Shelver, 2002; Thompson et al., 2008). Published studies report on tissue residues of ractopamine and its urinary excretion (Antignac et al., 2002; Blanca et al., 2005; Dickson et al., 2005; Moragues & Igualada, 2009; Nielen et al., 2008; Thompson et al., 2008; Van Hoof et al., 2005) and ractopamine residues in hair (Nielen et al., 2008). However, there are little data on the accumulation of ractopamine in pig hair (white or black) as a novel matrix for the control of ractopamine illegal use.

The aim of our study was to determine residue levels in swine urine (without and with sample hydrolysis) and hair samples after sub-chronic treatment of animals with ractopamine hydrochloride, using UPLC/MS/MS as a sensitive and reliable analytical method for determination of low ractopamine concentrations. Our study provided additional data on the ractopamine residue excretion and accumulation in swine.

3.2 Method validation

No interference on ractopamine identification was found owing to the highly specific MRM acquisition method and the use of an appropriate deuterated internal standard. The validation results for both analytical matrices are shown in Table 4 and Table 5. It is concluded that the methods showed relevant decision limit (CCα) and detection capability (CCβ), with values 0.37 and 0.51 ng/g for urine and 2.53 and 2.98 ng/g for hair, respectively. The mean recoveries ranged from 93.9% to 94.6% for urine and from 103.0% to 103.6% for hair. Also, the methods showed good repeatability, in-house reproducibility and linearity, and met the validation criteria set for quantitative residue analysis methods according to Decision. Successful validation of the method according to the European Union requirements and its application to real samples demonstrated its efficiency for veterinary control of ractopamine as a β-agonist in hair and urine.

Matrix	Urine	Hair
CCα [ng/g]	0.37	2.53
CCβ [ng/g]	0.51	2.98
Specificity	passed	passed
Ruggedness	passed	passed

Table 4. Validation results for ractopamine in urine and hair.

As part of the whole validation process the short time stability of ractopamine in urine and moreover the long-term stability was investigated. For that purpose a isochronic approach were applied (Lamberty 1998). The results are summarized in Figure 2.

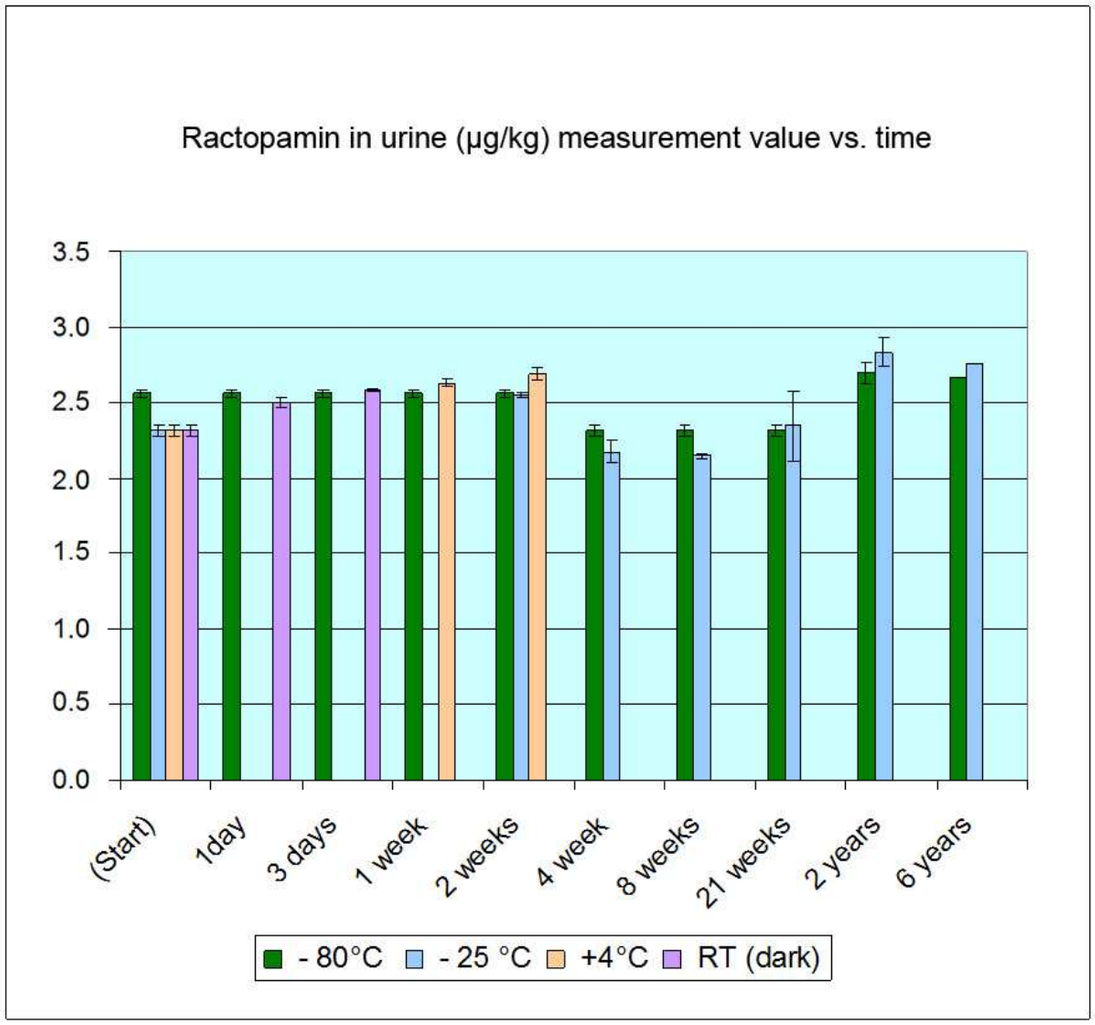


Fig. 2. Short time and long-term stability of ractopamine in urine.

Matrix	Spiked concentration (ng/mL)	s_r (ng/mL)	RSD (%)	s_{WR} (ng/mL)	RSD (%)	Recovery (%)
Urine	0.125	0.008	6.1	0.045	35.8	93.9
	0.250	0.008	3.1	0.045	17.9	94.3
	0.375	0.011	3.0	0.062	16.6	94.5
	0.500	0.015	3.0	0.081	16.2	94.5
	0.625	0.019	3.0	0.100	16.0	94.6
	0.750	0.023	3.0	0.119	15.9	94.6
Hair	2.000	0.076	3.8	0.184	9.2	103.0
	5.000	0.190	3.8	0.374	7.5	103.4
	8.000	0.304	3.8	0.600	7.5	103.5
	11.00	0.418	3.8	0.837	7.6	103.5
	15.00	0.570	3.8	1.155	7.7	103.6

Table 5. Repeatability, in-house reproducibility and recovery.

3.3 Concentration of ractopamine residues

3.3.1 Urine residues

Ractopamine concentrations were determined in urine on days 1, 3 and 8 after 28 days of continuous treatment of pigs. The mean (±SD) ractopamine concentrations in urine samples without and with enzyme hydrolysis on days after treatment discontinuation in the experimental group of animals are shown in Table 6.

Withdrawal time (days)	Urine ractopamine concentrations (mean±SD, ng/mL)	
	Without hydrolisis	With hydrolisis
1	1.52±0.17	7.63±0.19
	0.29±0.03	4.47±0.04
	0.76±0.04	8.15±0.10
3	0.15±0.01	2.40±0.08
	0.47±0.08	6.32±0.15
	[a]	[a]
8	0.38±0.01	6.53±0.12
	0.22±0.00	4.94±0.01
	[a]	[a]

[a] Insufficient urine obtained for analysis

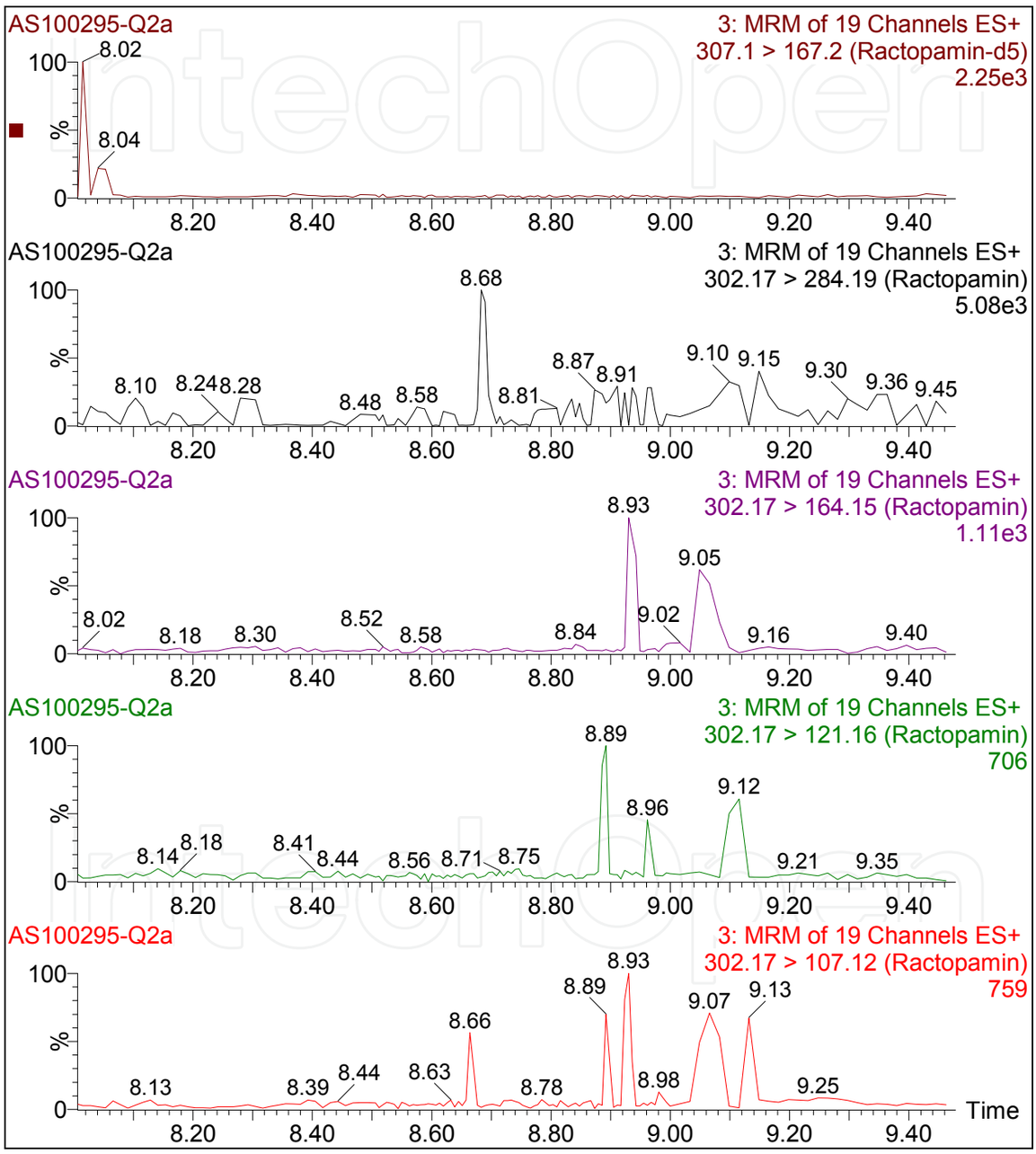
Table 6. Concentrations of ractopamine detected in urine by UPLC-MS/MS on days after withdrawal.

As ß$_2$-adrenergic agonist compounds are extensively metabolized to β-glucuronide and/or sulfate conjugates in humans and animals, in the present study deconjugation was used as a second step on urine sample preparation. The ractopamine concentrations determined in

swine urine samples were much greater after β-glucuronidase hydrolysis than those determined without this analytical step (Table 6). Figure 3 shows the UPLC-MRM chromatograms of confirmatory analysis of ractopamine in pig urine on days after withdrawal determined with hydrolysis. The concentration of ractopamine in urine samples processed with enzyme hydrolysis was almost 10-fold that recorded in urine samples analyzed without enzyme hydrolysis. Deconjugation step confirmed ractopamine to be excreted mainly in the form of glucuronide metabolites, as reported previously (Qiang et al., 2007; Shelver & Smith, 2003). Therefore, further analyses (hair) were performed exclusively with sample hydrolysis and values obtained with sample enzyme hydrolysis were used on interpretation of the urine ractopamine concentrations.

Earlier investigations performed in pigs with the use of a higher ractopamine anabolic dose (18 mg ractopamine hydrochloride/kg of feed) showed significantly higher concentrations of ractopamine in urine, with mostly twofold concentrations determined with sample enzyme hydrolysis (Qiang et al., 2007).

Studies carried out in cattle and sheep report on detectable ractopamine residues in urine 5 to 7 days after the last exposure to dietary ractopamine, pointing that hydrolysis of ractopamine metabolites may extend the period in which it is detected in cattle (Smith & Shelver, 2002). In their study, Thompson et al. (2008) fed pigs a feed containing 18 mg/kg ractopamine once daily for 10 days (180 mg of ractopamine in total). Ractopamine residues in pig urine were detectable by both screening and confirmatory methods until day 21 of treatment withdrawal. Urine sample analyses showed high concentrations of ractopamine ranging from 473.6 ng/mL to 1131.6 ng/mL on day 1 of withdrawal period. After seven days, the concentration of ractopamine was considerably lower, ranging from 3.4 ng/mL to 6.2 ng/mL. In our study, which was also performed in pigs but with a dose approximately 6 times lower (28 mg of ractopamine in total), the mean ractopamine residues in urine ranged from 6.7±1.8 ng/mL on day 1 of withdrawal to 5.7±0.9 ng/mL on the last day of withdrawal. Our study indicated that in spite of rapid urinary excretion, ractopamine residues can be detected in urine samples eight days after treatment cessation. Elliott et al. (1998) report ractopamine depletion in calves; their study showed the high concentration of ractopamine detected on day 1 of withdrawal (280 ng/mL) to be followed by a decline to the level of 18 ng/mL (day 3 of withdrawal), and no ractopamine residue detectable on day 14. In calf, the ractopamine residue concentrations found after drug withdrawal were also substantially lower than during the medication period, and were only detectable in one animal 2 weeks after removal of medication from the diet (Elliot et al., 1998). In their study on cattle treated with 20 mg/kg dietary ractopamine for seven days, Smith and Shelver (2002) obtained similar results concerning rapid urinary excretion of ractopamine. Ractopamine residues were 2523±1367 ng/mL on day 1 of withdrawal and on day 6 residue levels were below the limit of quantification. This study was simultaneously, in the same conditions and treatment schedule, conducted in sheep. Results obtained by analyzing sheep urine samples were rather different. The concentration of ractopamine in urine samples after 7 days of withdrawal was still detectable, 178±78 ng/mL (Smith & Shelver, 2002). These results indicate that urinary excretion of ractopamine is species dependent, meaning that even with similar doses, different animal species excrete ractopamine in different time frames. Literature data indicate that swine eliminate nearly 85% of the ractopamine administered during the first day, resulting in relatively low tissue residues (Dalidowicz et al, 1992). In comparison with some literature data (Qiang et al., 2007; Smith & Shelver, 2002), low concentrations of ractopamine urinary residues determined in our study could be explained by low exposure of animals to ractopamine.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8.02
8.04
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302.17 > 284.19 (Ractopamin)
5.08e3
8.68
8.10
8.24
8.28
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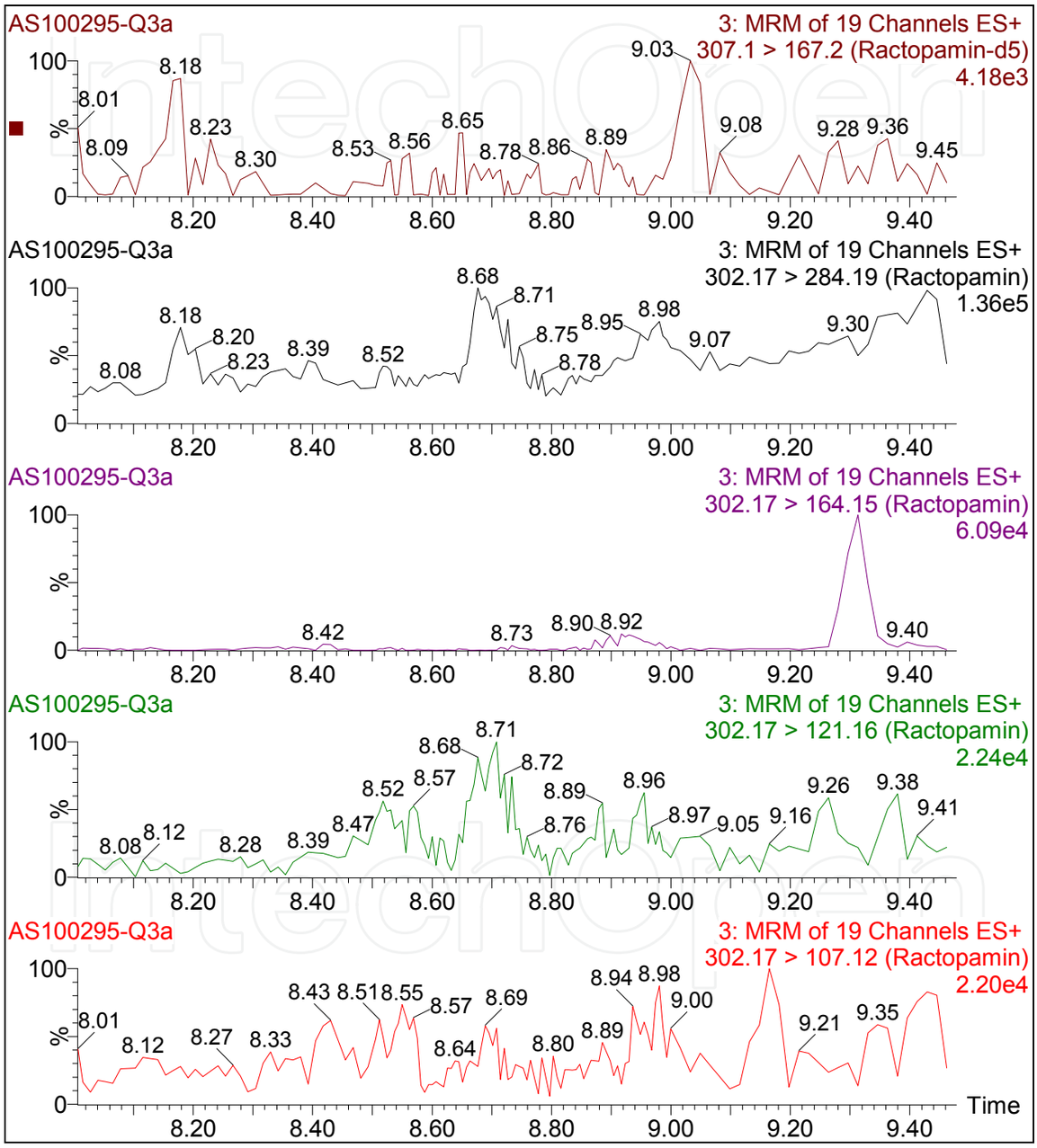
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Time

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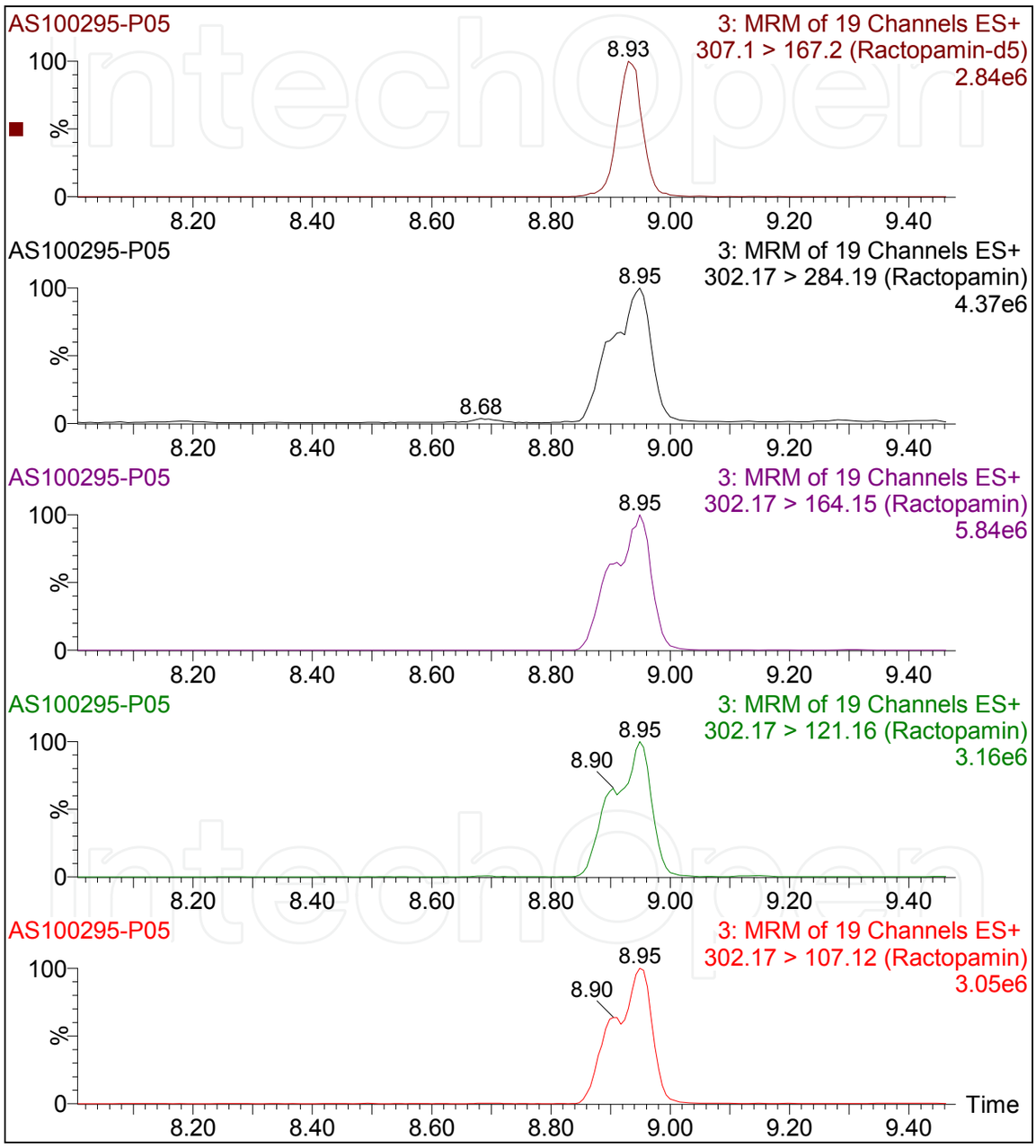
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4.37e6
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AS100295-P05
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302.17 > 164.15 (Ractopamin)
5.84e6
8.95
AS100295-P05
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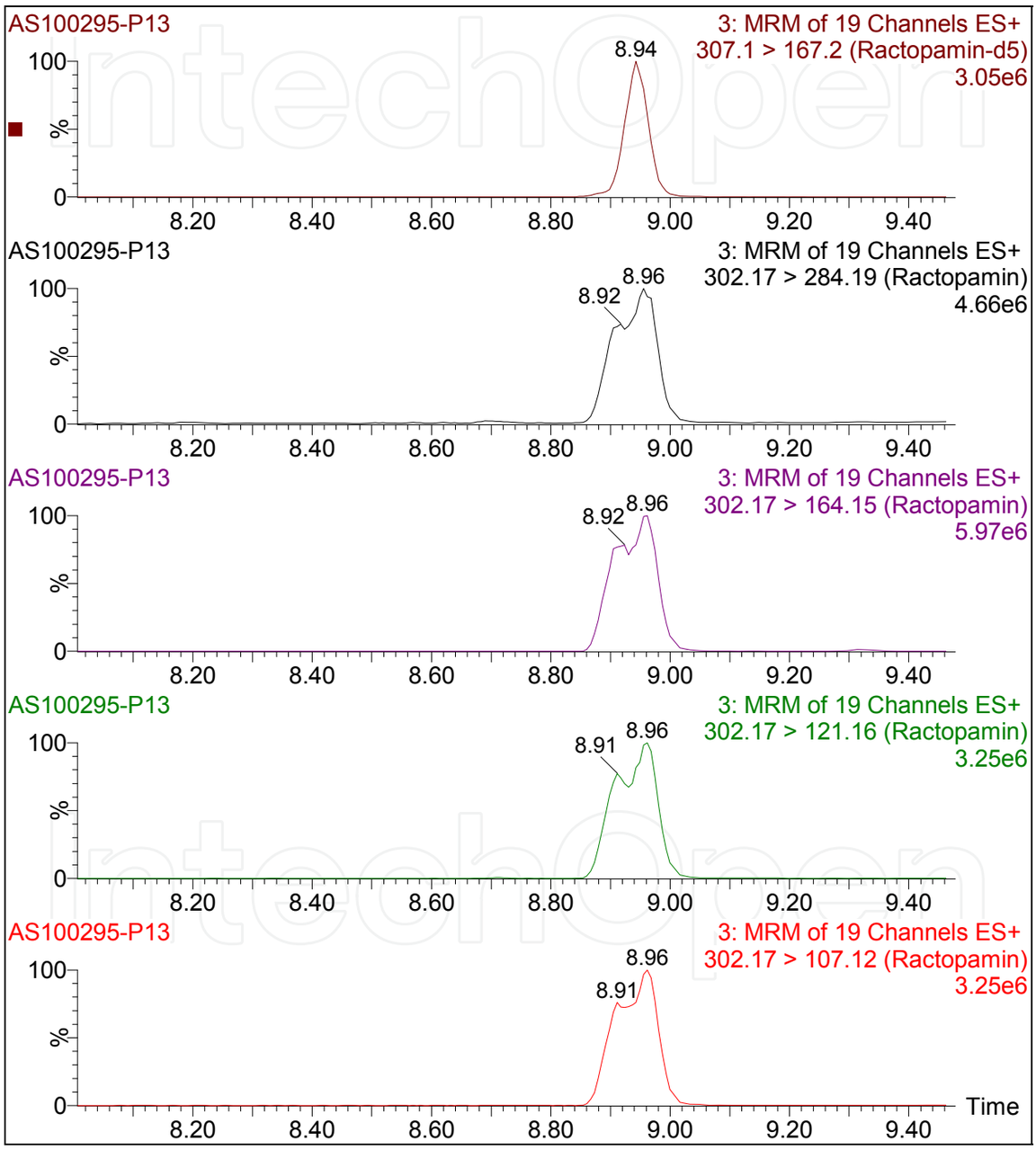
Time

C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302.17 > 284.19 (Ractopamin)
4.66e6
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8.96
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302.17 > 164.15 (Ractopamin)
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8.96
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302.17 > 107.12 (Ractopamin)
3.25e6
8.91
8.96
100
%
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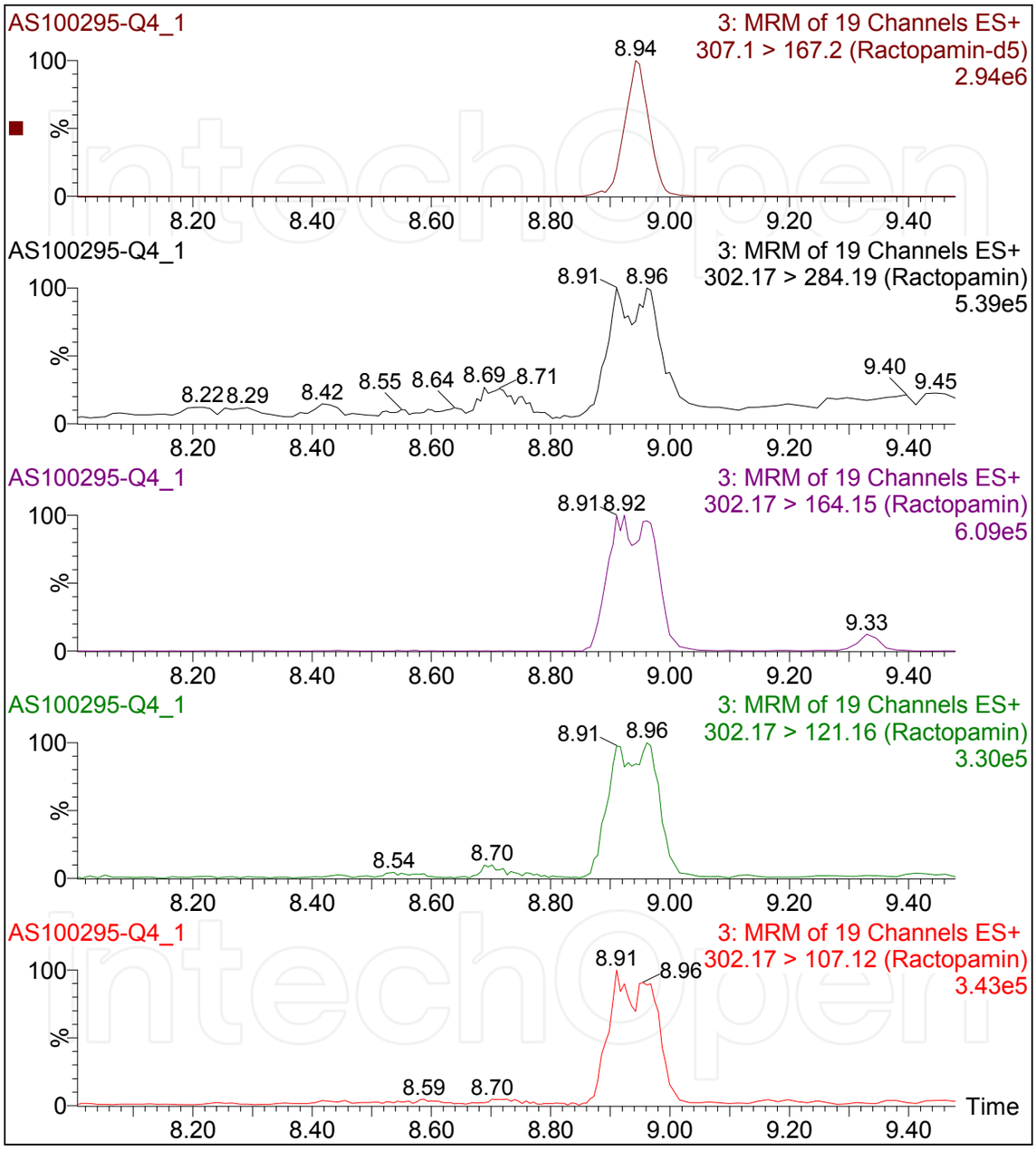
8.60
8.80
9.00
9.20
9.40
Time

D



E

Fig. 3. UPLC-MRM chromatograms of confirmatory analysis of ractopamine in pig urine: (A) urine reagent blank; (B) urine matrix blank; (C) urine sample of treated animal on day 1 after withdrawal; (D) urine sample of treated animal on day 8 after withdrawal; (E) blank urine sample spiked with 0.1 μg/kg ractopamine and 0.5 μg/kg ractopamine-d5.

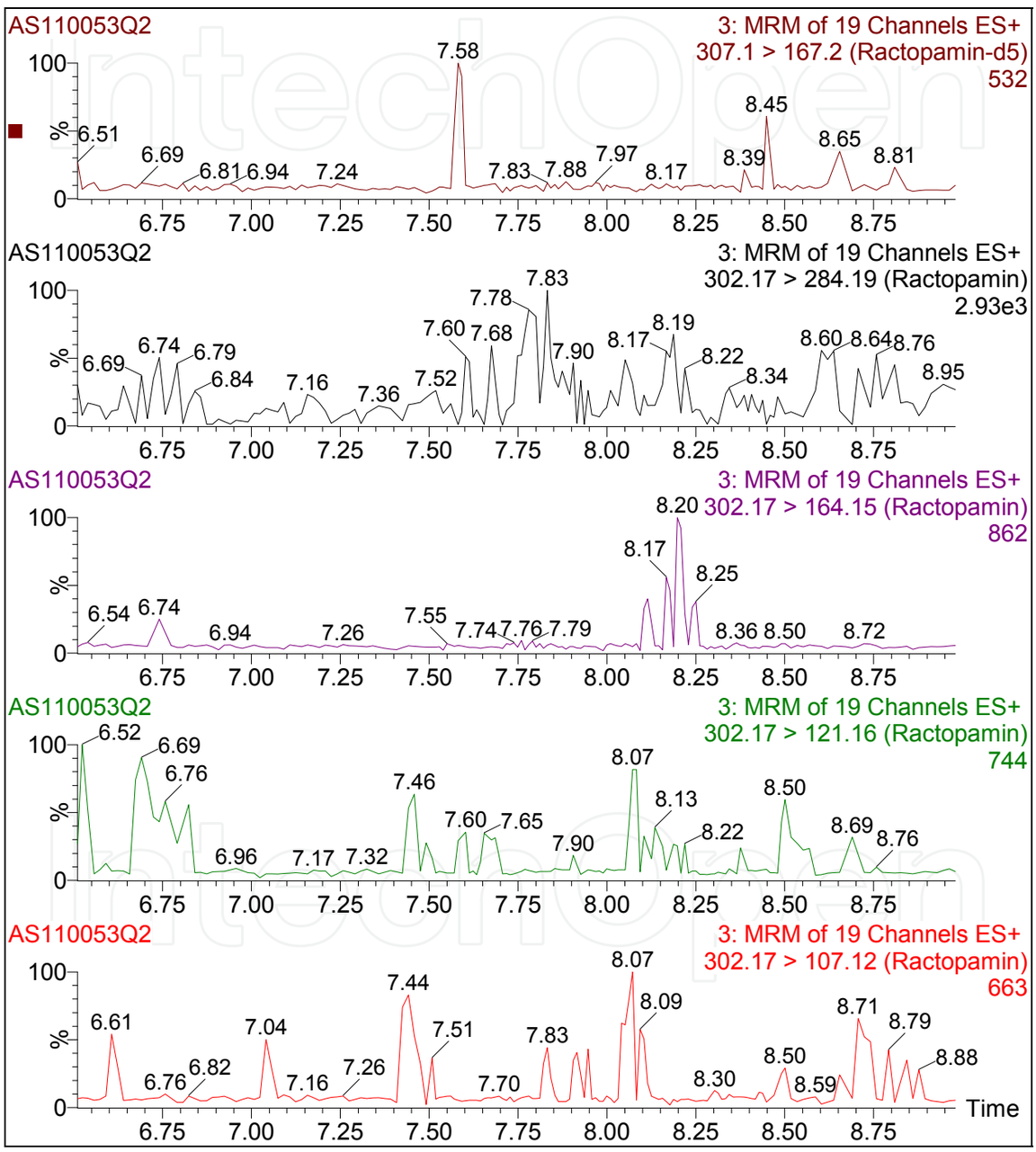
3.3.2 Hair residues

On days 1, 3 and 8 after 28 days of continuous treatment of pigs, ractopamine concentrations were determined in hair (white) samples. The mean (±SD) ractopamine concentrations in hair on days after treatment discontinuation in the experimental group of animals are shown in Table 7. In spite of the low ractopamine dose administered to pigs in our study, residues were determined in hair with the UPLC-MS/MS method, showing its selectivity and sensitivity, i.e. applicability in the control of ractopamine illegal use using hair as a matrix. Chromatograms of the UPLC-MS/MS method of confirmatory analysis of ractopamine in pig hair on days after withdrawal are shown in Figure 4.

Withdrawal time (days)	Hair ractopamine concentrations (mean±SD, ng/g)
	9.34±1.58
1	13.31±0.16
	13.72±0.68
	11.06±0.21
3	13.7±0.45
	9.8±0.41
	9.95±0.05
8	8.65±0.95
	7.70±0.10

Table 7. Concentrations of ractopamine detected in hair by UPLC-MS/MS on days after withdrawal.

In our study, the mean concentrations of ractopamine determined in hair samples were 12.12±2.42 ng/g on day 1 after withdrawal, 11.52±1.99 ng/g on day 3 and 8.77±1.13 ng/g on the last day after withdrawal. Analyses of hair samples showed the concentrations of ractopamine on the same days of withdrawal to be significantly higher in hair samples than in urine. The hair/urine concentration ratios on days 1, 3 and 8 of withdrawal were 1.8, 2.6 and 1.5, respectively. Radeck and Gowik (2010) conducted a study on non-lactating cows treated with 6 different ß$_2$-agonists, including ractopamine in a dose of 1500 mg overall. That study revealed the concentration of ractopamine on days after withdrawal to be higher in urine samples than in hair samples, both white and black. Results of our study performed on pigs indicated the concentration of ractopamine on days after withdrawal to be higher in white hair samples than in urine samples. This discordance of results can be interpreted with regard to different experimental animal species, the dose of ractopamine used and different ractopamine application schemes employed. Bearing in mind the scarce data on ractopamine residues in swine hair, both black and white, there is a need to perform another study that would include swine with black hair in order to yield data on ractopamine residues in both hair types. Also, a long-term depletion study of ractopamine on swine would be useful to determine maximum withdrawal time with detectable residues in hair samples.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862
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302.17 > 121.16 (Ractopamin)
744
AS110053Q2
3: MRM of 19 Channels ES+
302.17 > 107.12 (Ractopamin)
663
%
Time
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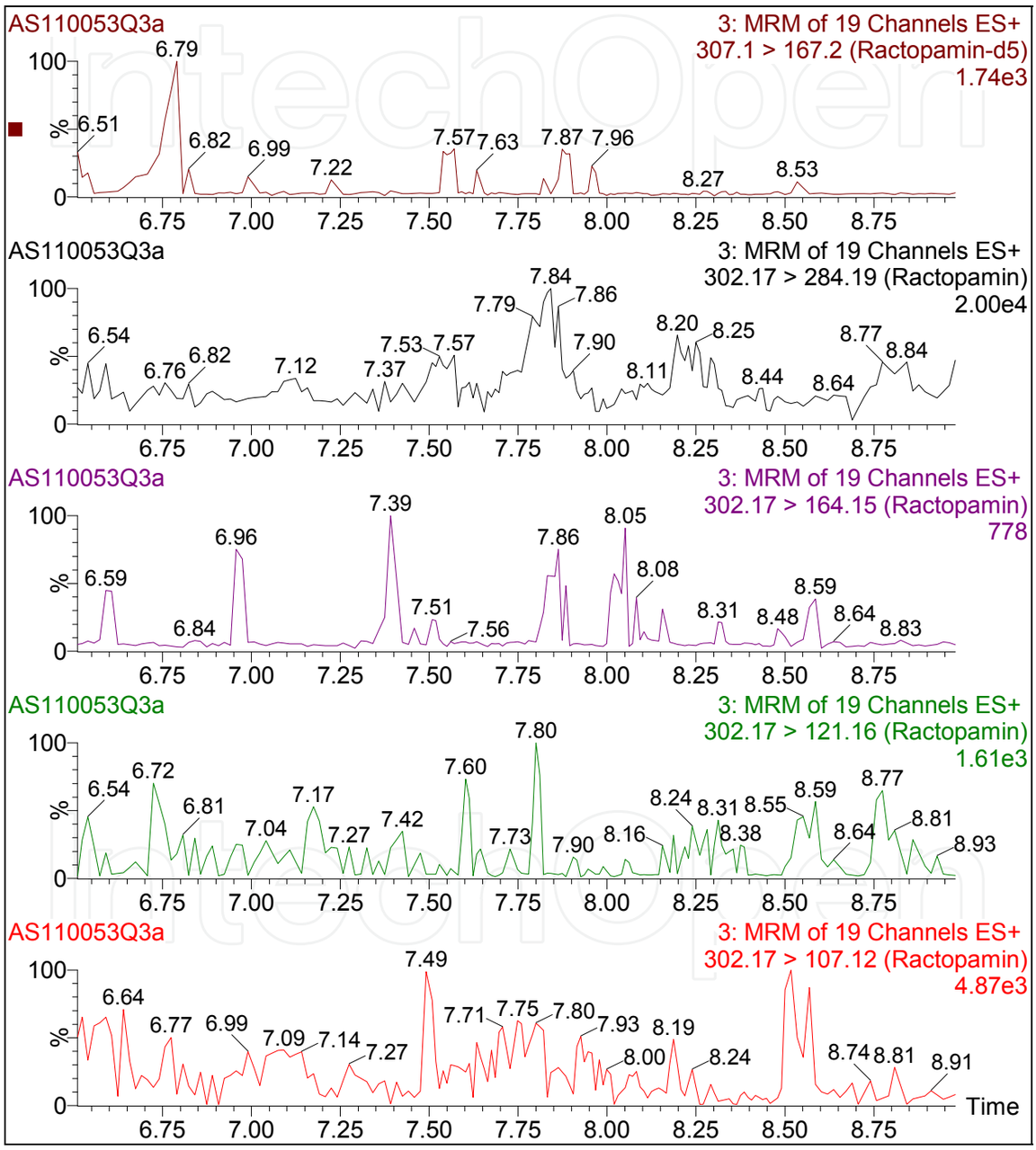
A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302.17 > 164.15 (Ractopamin)
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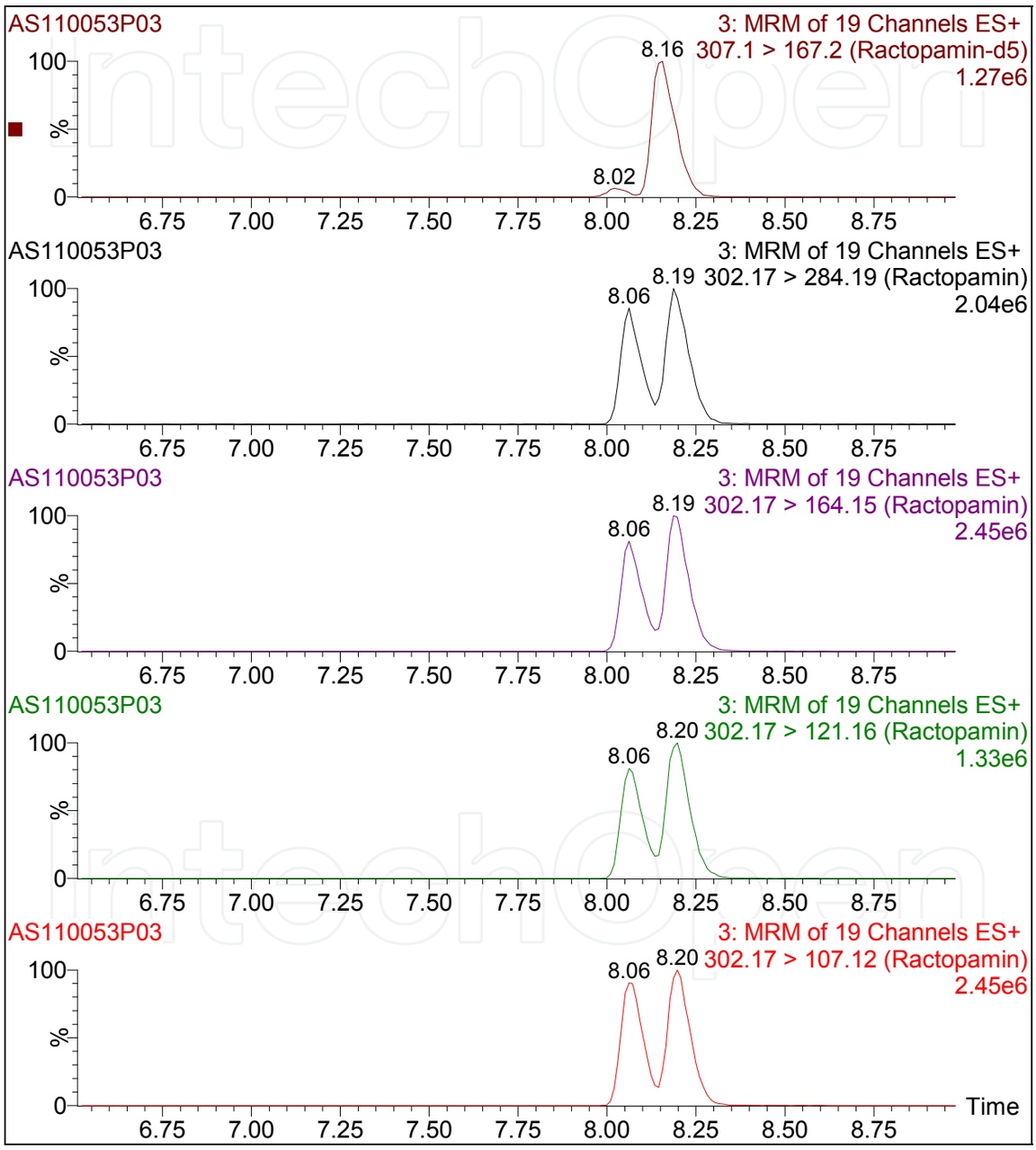
Time

B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8.02
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302.17 > 284.19 (Ractopamin)
2.04e6
8.19
8.06
AS110053P03
3: MRM of 19 Channels ES+
302.17 > 164.15 (Ractopamin)
2.45e6
8.19
8.06
AS110053P03
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302.17 > 121.16 (Ractopamin)
1.33e6
8.20
8.06
AS110053P03
3: MRM of 19 Channels ES+
302.17 > 107.12 (Ractopamin)
2.45e6
8.20
8.06
100
%
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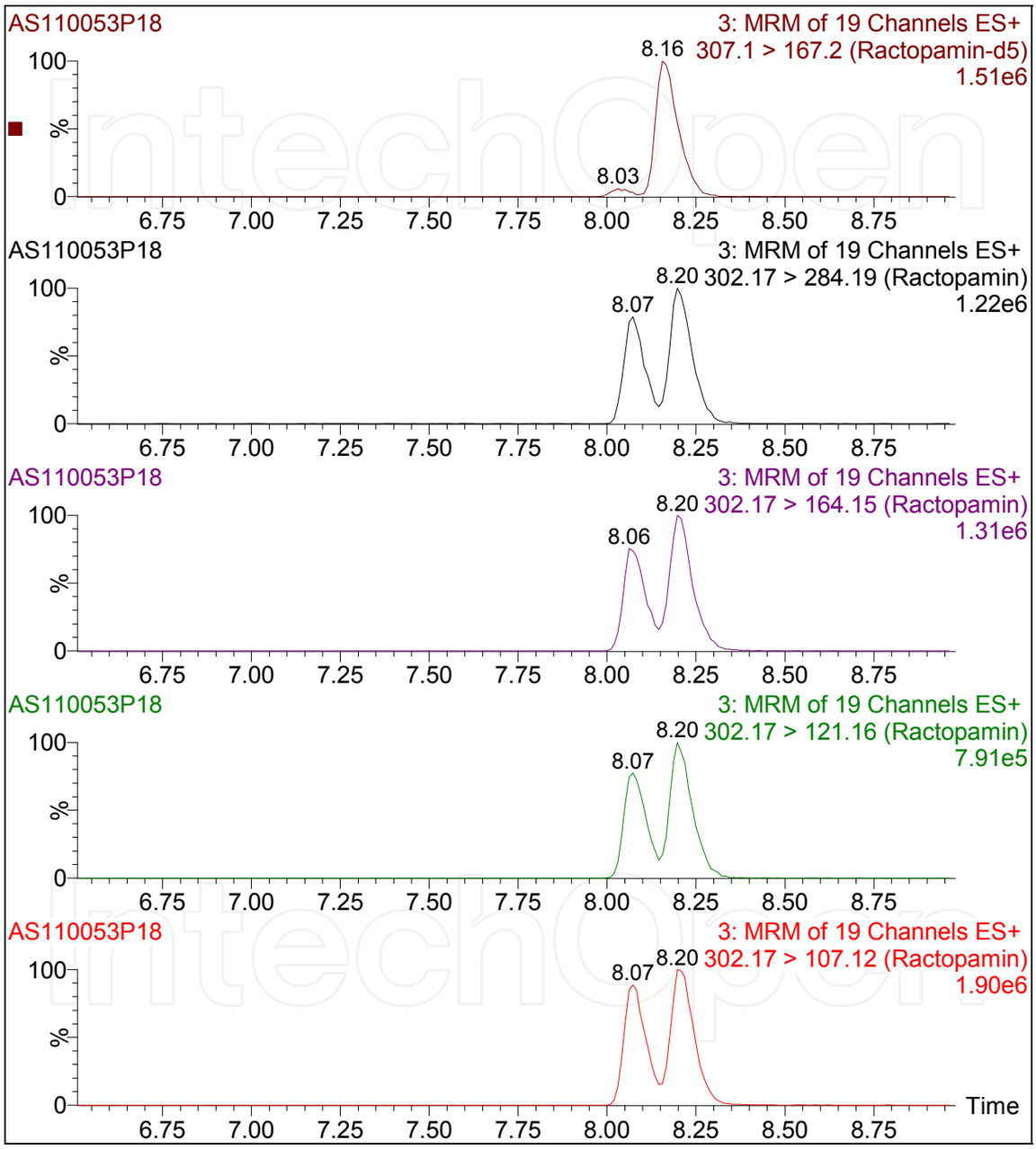
8.00
8.25
8.50
8.75
Time

C



D

Fig. 4. UPLC-MRM chromatograms of confirmatory analysis of ractopamine in pig hair: (A) hair reagent blank; (B) hair matrix blank; (C) hair sample of treated animal on day 1 after withdrawal; (D) hair sample of treated animal on day 8 after withdrawal.

4. Conclusion

A validated UPLC-MS/MS method was employed for determination of ractopamine in pig urine and hair at trace levels. The method features were found to be fit-for-purpose, with successful method validation according to the European Union requirements and its suitability for determination of low ractopamine residues in real samples. Study results indicated that the excretion of ractopamine in pig urine and accumulation in hair could clearly point to its abuse in pigs as food producing animals, in particular when using sample hydrolysis with β-glucuronidase on ractopamine determination, which extended the period in which ractopamine could be detected. Results of our study indicated the concentration of ractopamine on days after withdrawal to be higher in white hair samples than in urine samples.

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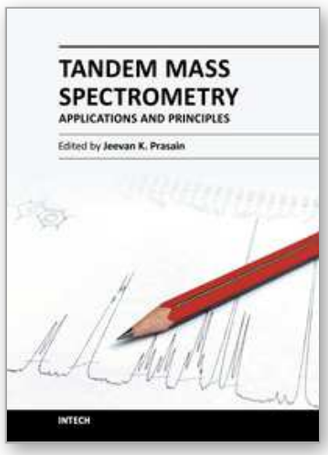

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