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UPLC–MS/MS determination of ractopamine residues in retinal tissue of treated food-producing pigs

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ABSTRACT

Ractopamine is a β_2-adrenergic agonist, which reduces fat deposition and promotes muscle growth in animals for meat production. In the European Union countries, systematic monitoring and control of this contaminant residue is regularly performed by use of validated analytical methods of detection in different biological materials. The aim of the present study was to assess persistence of ractopamine in retina as a pigmented tissue by determination of its residues using UPLC–MS/MS as a quantitative confirmatory method after pig exposure to a ractopamine dose of 0.51 mg/kg b.w. Experimental group ($n = 9$) of pigs were orally administered ractopamine for 28 days and then randomly sacrificed ($n = 3$) on days 1, 3 and 8 of treatment discontinuation, whereas control animals ($n = 3$) were left untreated. Study results showed mean ractopamine residue concentrations of 110.36 μg/kg, 67.11 μg/kg and 89.93 μg/kg on days 1, 3 and 8 after withdrawal, respectively, indicating high accumulation of ractopamine in retina despite a low dose applied. These data pointed to high affinity of ractopamine for binding to the pigmented segment of the eye, thus supporting the use of pigmented tissues as matrices in the regulatory monitoring of this β_2-adrenergic agonist.

1. Introduction

Ractopamine is a phenethanolamine with β_2-adrenergic agonist properties. β_2-agonists were originally designed and used for smooth muscle relaxation, i.e. as bronchodilators in the treatment of pulmonary conditions, and as tocolytics. Meanwhile, ractopamine was shown to reduce fat deposition through a direct action on adipose tissue, causing an increase in the rate of lipolysis and a decrease in lipid synthesis [1] and thus promoting muscle growth in swine [2]. Literature data show that ractopamine feed concentrations of 10–20 mg/kg of feed and feeding duration of about 30 days result in significant improvements in carcass characteristics [3–6].

Ractopamine was approved by the U.S. Food and Drug Administration for use in finishing swine in 1999. Due to a number food poisoning cases caused by clenbuterol, the European Union banned the use of ractopamine as well as of other β-agonists [7]. Therefore, systematic monitoring and control of this contaminant residue throughout the manufacture of foods of animal origin in accordance with regulations and using validated analytical methods of detection are necessary.

While screening of livestock tissue residues can be done inexpensively using rapid immunochemical tests, accurate quantification and confirmation require the sensitivity and specificity of mass spectrometry coupled with a chromatographic procedure [4]. Confirmatory methods for β-agonists have been performed by gas chromatography–mass spectrometry (GC–MS) or tandem mass spectrometry (GC–MS/MS) and liquid chromatography–mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) [8]. Ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) is one of the most efficient methods in determination of ractopamine [9,10], because of the high resolution and rapid separation of UPLC and the selectivity and sensitivity of MS/MS detection [11]. Fragmentation pathway of ractopamine is shown in Fig. 1.

Literature data show that generally β-agonists accumulate in high concentrations in pigmented tissues such as retina, and that their accumulation in retinal tissue is particularly useful in identifying residues at long withdrawal times (weeks to months) after administration of the drug [12–15]. Previous research in the field by many investigators refers mainly to clenbuterol and there is not enough data about the accumulation of ractopamine residues in retina of farm animals in comparison with other matrices such as liver, kidney, muscle and fat [10,16–18,29]. There are only few study reports on ractopamine determination in retina [4,19–23].

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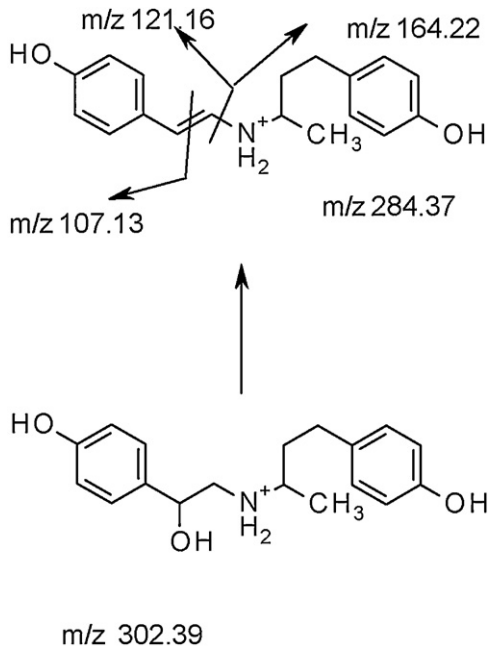

Fig. 1. Fragmentation pathway of ractopamine.

The aim of the present study was to assess persistence of ractopamine residues in retina as a pigmented tissue using UPLC–MS/MS as a quantitative confirmatory method after pig exposure to a low ractopamine dose.

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 used as internal standard. Protease Type XIV from *Streptomyces griseus* (Sigma–Aldrich-Chemie, Steinheim, Germany) was used for sample analysis. All solvents were of HPLC grade. Screen Dau solid phase extraction columns (500 mg, 6 mL) used for clean up were from Amchro (Hattersheim, Germany). Filtration and centrifugation of samples were performed on Amicon Ultra Centrifugal Filters Ultracell 10 K (Millipore, Carrigtwohill, Ireland). The analyses were performed on UPLC/MS/MS Xevo® TQ-S (Waters, En Yvelines Cedex, France).

2.2. Animals and sampling procedure

The study was performed on 12 male pigs (9 treated and 3 controls), Zegers hybrid type, 55 kg body weight, farm-bred and kept under the same conditions. Experimental group animals ($n = 9$) were randomly divided into 3 groups and treated with ractopamine hydrochloride in a dose of 1 mg daily (absolute) *per os* for 28 days (0.51 mg/kg b.w.). Capsules filled with pure ractopamine hydrochloride standard admixed to feed were daily administered orally to experimental animals. Three animals were left untreated and served as a control group. After 28 days of treatment, treated animals were placed on control diet (without ractopamine) and on days 1, 3 and 8 of treatment withdrawal they were sacrificed in groups of 3. Control animals were sacrificed on day 8 after experimental animal treatment withdrawal. Eyeballs of treated and control animals were collected after slaughtering and stored at −25 °C prior to analysis for ractopamine residues. All experiments were performed according to the Croatian Animal Protection Act (Official Gazette of the Republic of Croatia 135/06) [24].

2.3. Sample preparation and clean up

After dissecting the retina from the eye, the retinal tissue was weighed in ground centrifuge tube. Then, ten times retina's weight of TRIS-buffer (pH 8) was added to the tube and homogenized with the potter. After homogenization, 1 g of retina suspension was weighed, 10 mL of TRIS-buffer and internal standard were added and the mixture was homogenized again with ultrasonic homogenizer. Subsequently, 100 μL of protease solution (50 mg/mL in water) was added; the samples were vortexed and incubated overnight at 50–60 °C. The next day the samples were centrifuged for 10 min at 4 °C and 3000–4000 rpm. The upper layer was taken to a new tube and the centrifugation residue was extracted two times with 1 mL phosphate buffer (pH 6), whereas centrifugation was repeated each time. The supernatants were combined and pH was adjusted at 6. The samples were then loaded to SPE cartridges conditioned with 2 mL of methanol, 2 mL of water and 2 mL of phosphate buffer pH 6, respectively. 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 mixture consisting of ethyl acetate and ammonia at a 97:3 ratio. The samples were evaporated to dryness under stream of nitrogen at 35 °C. The residues were then dissolved in 200 μL of HPLC mobile phase consisting of 0.1% formic acid in water/0.1% formic acid in acetonitrile at a 95:5 ratio.

2.4. Liquid chromatography tandem mass spectrometry conditions

The UPLC separation was performed on Acquity HSS C18 columns (150 × 2, 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). The 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 was performed on Xevo® TQ-S with conditions 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 $m/z = 302.2$ (cone 20 V) being the precursor ion. Four product ions at $m/z = 284.2$ (collision energy 12 V), $m/z = 164.2$ (collision energy 14 V), $m/z = 121.2$ (collision energy 22 V), and $m/z = 107.1$ (collision energy 32 V) were monitored. Ractopamine-d5 as internal standard was measured by recording of two transitions: 307.1 > 167.2 (cone 20 V, collision energy 18 V) and 307.1 > 289.3 (collision energy 12 V). Although only two product ions are needed for ractopamine quantification and confirmation four product ions were monitored to obtain more identification points. 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.

2.5. Validation process

Validation was carried out according to the Commission Decision 2002/657/EC [25] by an alternative approach of matrix comprehensive in-house validation by means of a factorial design. The software used for factorial design and calculation was InterVal Plus (quo data, Gesellschaft für Qualitätsmanagement und Statistik

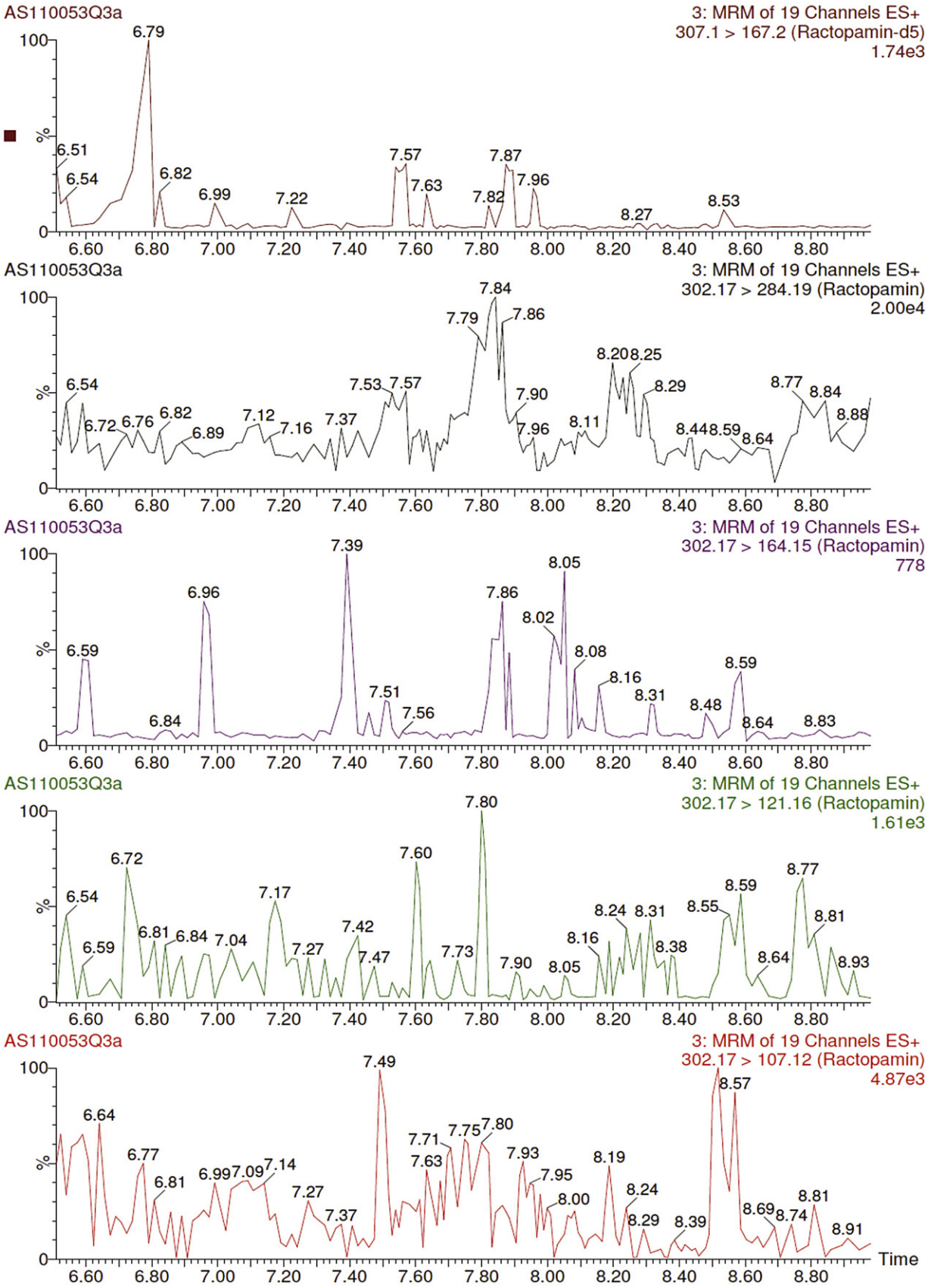

Fig. 2. Typical UPLC–MS/MS-MRM-chromatogram of blank retina sample.

Table 1
Recovery, repeatability and in-house reproducibility in determination of ractopamine retinal residues.

Spiked ractopamine concentration (μg/kg)	Recovery (%)	Obtained concentration (μg/kg)	s_r[a] (μg/kg)	RSD (%)	s_{WR}[b] (μg/kg)	RSD (%)
2.0	92.7	1.854	0.087	4.3	0.185	9.3
2.5	93.5	2.337	0.109	4.3	0.209	8.4
3.0	94.0	2.820	0.130	4.3	0.235	7.8
3.5	94.3	3.301	0.152	4.3	0.262	7.5
4.0	94.6	3.784	0.174	4.3	0.292	7.3
5.0	95.0	4.750	0.217	4.3	0.363	7.3
6.0	95.3	5.718	0.261	4.3	0.435	7.3
7.0	95.5	6.685	0.304	4.3	0.508	7.3
8.0	95.6	7.648	0.348	4.3	0.581	7.3
10.0	95.8	9.580	0.435	4.3	0.727	7.3
12.0	95.9	11.508	0.522	4.3	0.874	7.3
16.0	96.1	15.376	0.696	4.3	1.168	7.3
20.0	96.2	19.240	0.870	4.3	1.463	7.3

[a] s_r: repeatability standard deviation.
[b] s_{WR}: in-house reproducibility standard deviation.

GmbH, Dresden, Germany). In validation process, decision limit (CCα), detection capability (CCβ), precision, recovery, repeatability, in-house reproducibility, matrix effect, specificity and ruggedness were studied.

3. Results and discussion

3.1. Method validation

In validation process, no interference on the ractopamine identification was found owing to the highly specific MRM acquisition method and the use of an appropriate deuterated internal standard of ractopamine. Fig. 2 shows typical MRM chromatogram of a blank retina sample.

The results of method repeatability, in-house reproducibility and recovery are shown in Table 1.

The average recoveries ranged from 92.7% to 96.2%, showing good repeatability and in-house reproducibility. The RSD values were below 10%, with repeatability determination value of 4.3% and in-house reproducibility determination values ranging from 7.3% to 9.3%.

The method described resulted in relevant decision limit (CCα) and detection capability (CCβ), with values of 2.576 and 3.027 μg/kg, respectively. According to all study parameters, the method met the validation criteria set for quantitative residue analysis methods in the European Union [25]. The successful validation of this method and its application to real samples demonstrated its efficiency for veterinary control of ractopamine as a β-agonist in retina.

Table 2
Concentrations of ractopamine determined in retina by UPLC–MS/MS on days after withdrawal.

Group	Withdrawal time (days)	Animal mark	Ractopamine residues[b] (μg/kg)
Treated	1	3T	55.84 ± 0.25
		4T	132.35 ± 1.20
		6T	142.88 ± 0.84
	3	2T	96.15 ± 2.05
		5T	86.64 ± 3.10
		9T	18.55 ± 0.21
	8	1T	89.40 ± 1.56
		7T	[a]
		8T	90.45 ± 1.20
Control		1C	n.d.[c]
		2C	n.d.
		3C	n.d.

[a] Sample destroyed on processing.
[b] Repeatability standard deviations calculated on the basis of two parallel analyses.
[c] Not detected.

3.2. Ractopamine retinal concentrations

In the literature, accumulation of β-agonists in pigmented tissues is explained by the action of electrostatic forces that are formed between the positive-charged drug molecules and negative groups of melanin polymers as well as by Van der Waals' forces on the aromatic ring conjugates and melanin aromatic indol nucleus. Furthermore, some studies suggest that the interaction between drug and melanin may also be based on charge transfer from drug as a potent electron donor to melanin [26]. Earlier studies with clenbuterol showed delayed elimination of residues from retina [13,27,28] and report on the usefulness of retinal tissue in the control of β-agonist illegal use in farm animals.

In our study, ractopamine residues were determined in retina samples from animals slaughtered on days 1, 3 and 8 after 28 days of continuous ractopamine treatment. The mean (±SD) ractopamine concentrations in retina on days after treatment discontinuation are shown in Table 2.

After seven-day ractopamine treatment with 20 mg/kg ractopamine in a diet, Churchwell et al. [21] determined low levels of ractopamine in a range of 0.5–3 ng/g and found the concentrations of unconjugated ractopamine in ovine and bovine retina to be slightly increased over 7-day withdrawal period. The authors conclude that retina can serve as a longer-lived marker of ractopamine exposure in livestock (relative to serum, urine, or liver) [21]. Treatment of pigs with 10 mg of ractopamine *per* day for 10 days resulted in a high amount of ractopamine residues detected in retina (19,480 ng/g), indicating high affinity for the analyte due to the large number of β-agonist receptors in this tissue [19]. Another study assessing ractopamine accumulation in ocular tissue was conducted on cattle and turkeys [23]. After seven days of ruminant treatment with [^{14}C] ractopamine in a dose of 0.90 mg/kg, there was no detectable ractopamine residue in whole eye homogenates obtained on days 2, 4 and 6 of withdrawal. On the other hand, studies in turkeys fed a diet containing 7.5, 22.5 and 30 mg/kg of [^{14}C] ractopamine for seven days revealed that ractopamine residues could be detected in retina/choroid/sclera and cornea/iris tissue, while there were no detectable residues in aqueous humor. The highest mean concentrations of ractopamine were found in retina/choroid/sclera tissue from animals fed a diet containing 30 mg/kg [^{14}C] ractopamine, i.e. 251 ± 51 ng/g for male and 255 ± 60 ng/g for female animals.

Our study also showed high mean ractopamine residue concentrations of 110.36 μg/kg, 67.11 μg/kg and 89.93 μg/kg on days 1, 3 and 8 of withdrawal, respectively, indicating high accumulation

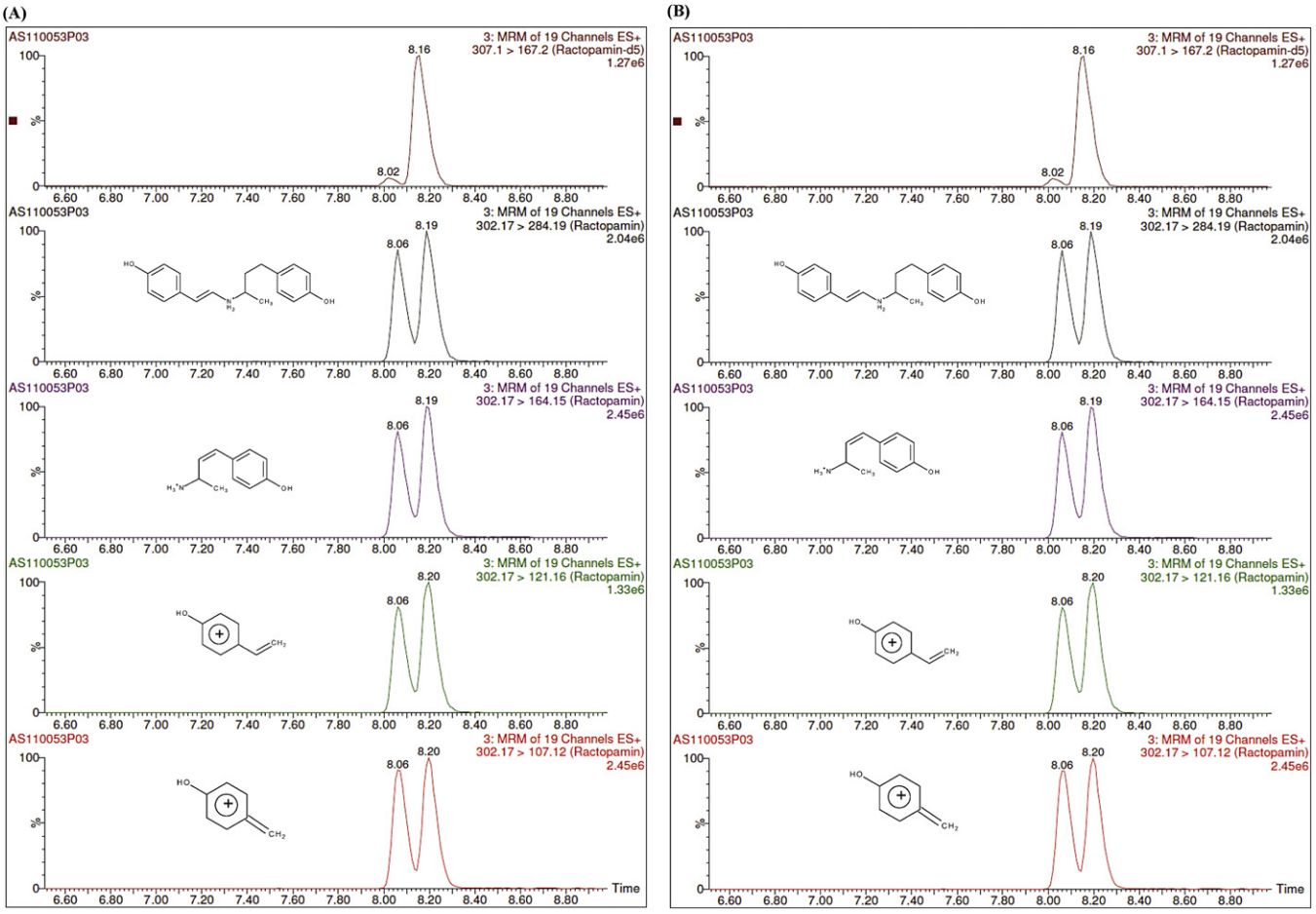

Fig. 3. Typical UPLC–MS/MS-MRM-chromatograms of retina sample on day 1 (A) and day 8 of withdrawal (B).

of ractopamine despite a low dose applied (0.51 mg/kg b.w.). The highest ractopamine concentration of 142.88 ng/g was determined in retina sample from day 1 after withdrawal, while the lowest concentration of 18.55 ng/g was determined in retina sample from day 3 after withdrawal. In the control group of animals ractopamine was not detected.

Study results revealed a large concentration variation on the same day after withdrawal *per* animal, with the ractopamine residues on day 8 after withdrawal to be still significant. Other studies also pointed to large variation in ractopamine concentration in different matrices, e.g. lung and liver tissues [18]. Fig. 3 presents UPLC–MS/MS-MRM-chromatograms of confirmatory analysis of ractopamine in pig retina samples on days after withdrawal.

Our future studies will be focused on the assessment of ractopamine accumulation in pig retina over a longer period after treatment, i.e. attempting to identify the period following administration of the usual anabolic dose of ractopamine when it cannot be detected in this matrix anymore.

4. Conclusion

Our study results revealed high residual ractopamine levels in retinal tissue of treated pigs, pointing to its high binding affinity in the pigmented segment of the eye and supporting the use of pigmented tissues as matrices in the regulatory monitoring of the β_2-adrenergic agonist ractopamine misuse. Our results also showed ractopamine residues to persist in high concentration in retinal tissue 8 days of withdrawal, suggesting that this matrix could be used in the control of this agent misuse at a longer period after treatment.

References

[1] T.A. Peterla, C.G. Scanes, J. Anim. Sci. 68 (1990) 1024.
[2] D.E. Moody, D.L. Hancock, D.B. Anderson, in: J.P.F. D'Mello (Ed.), Farm Animal Metabolism and Nutrition, CAB International, New York, 2000, p. 65.
[3] L.E. Watkins, D.J. Jones, H. Mowrey, D.B. Anderson, E.L. Veenhuizen, J. Anim. Sci. 68 (1990) 3588.
[4] N.H. Williams, T.R. Cline, A.P. Schinckel, D.J. Jones, J. Anim. Sci. 72 (1994) 3152.
[5] T.A. Armstrong, D.J. Ivers, J.R. Wagner, D.B. Anderson, W.C. Weldon, E.P. Berg, J. Anim. Sci. 82 (2004) 3245.
[6] M.T. See, T.A. Armstrong, W.C. Weldon, J. Anim. Sci. 82 (2004) 2474.
[7] Commission of the European Communities, Council Directive 96/22/EC on the Prohibition of the Use of Certain Substances Having a Hormonal and Thyreostatic Action and β-Agonists in Animal Husbandry, Official Journal of the European Communities, L 125, 1996.
[8] Z. Qiang, F. Shentu, B. Wang, J. Wang, J. Chang, J. Shen, J. Agric. Food Chem. 55 (2007) 4319.
[9] M.W.F. Nielen, J.J.P. Lasaroms, M.L. Essers, J.E. Oosterink, T. Meijer, M.B. Sanders, T. Zuidema, A.A.M. Stolker, Anal. Bioanal. Chem. 391 (2008) 199.
[10] B. Shao, X. Jia, J. Zhang, J. Meng, Y. Wu, H. Duan, X. Tu, Food Chem. 114 (2009) 1115.
[11] H. Zheng, L.G. Deng, X. Lu, S.C. Zhao, C.Y. Guo, J.S. Mao, Y.T. Wang, G.S. Yang, H.Y. Aboul-Enein, Chromatographia 72 (2010) 79.
[12] C.T. Elliot, C.S. Thompson, C.J.M. Arts, S.R.H. Crooks, M.J. van Baak, E.R. Verheij, G.A. Baxter, Analyst 123 (1998) 1103.
[13] D.R. Doerge, M.I. Churchwell, C.L. Holder, L. Rowe, S. Bajic, Anal. Chem. 68 (1996) 1918.
[14] A. Cristino, F. Ramos, M.I.N. da Silveira, J. Pharm. Biomed. Anal. 32 (2003) 311.
[15] T. Gojmerac, J. Pleadin, I. Bratoš, A. Vulić, N. Vahčić, Meat Sci. 80 (2008) 879.
[16] J.E. Dalidowicz, T.D. Thomson, G.E. Babbitt, in: D.H. Hutson, D.R. Hawkins, G.D. Paulson, C.B. Struble (Eds.), Xenobiotics and Food-Producing Animals, ACS Symposium Series 503, Washington, 1992, p. 234.

[17] J. Blanca, P. Muñoz, M. Morgado, N. Méndez, A. Aranda, T. Reuvers, H. Hooghuis, Anal. Chim. Acta 529 (2005) 199.
[18] Y. Dong, X. Xia, X. Wang, S. Ding, X. Li, S. Zhang, H. Jiang, J. Liu, J. Li, Z. Feng, N. Ye, M. Zhou, J. Shen, Food Chem. 127 (2011) 327.
[19] J.P. Antignac, P. Marchand, B. Le Bizec, F. Andre, J. Chromatogr. B 774 (2002) 59.
[20] B. Bocca, M. Fiori, C. Cartoni, G. Brambilla, J. AOAC Int. 86 (2003) 8.
[21] M.I. Churchwell, C.L. Holder, D. Little, S. Preece, D.J. Smith, D.R. Doerge, D. Rapid, Commun. Mass Spectrom. 16 (2002) 1261.
[22] A.C.E. Fesser, L.C. Dickson, J.D. MacNeil, J.R. Patterson, S. Lee, R. Gedir, J. AOAC Int. 88 (2005) 61.
[23] D.J. Smith, W.L. Shelver, J. Anim. Sci. 80 (2002) 1240.
[24] Act on Animal Welfare No. 135, Official Gazette of the Republic of Croatia, 2006.
[25] Commission of the European Communities, Commission Decision 2002/657/EC of 12th August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results, Official Journal of the European Communities, L 221, 2002.
[26] P. Gowik, B. Jülicher, M. Ladwog, D. Behrendt, Analyst 125 (2000) 1103.
[27] C.T. Elliott, J.D.G. McEvoy, W.J. McCaughey, H.D. Shortt, S.R.H. Crooks, Analyst 118 (1993) 447.
[28] M.J. Sauer, S.P.L. Anderson, Analyst 119 (1994) 2553.
[29] J. Pleadin, N. Perši, A. Vulić, D. Milić, N. Vahčić, Meat Sci. 90 (2012) 755.