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Simultaneous determination of clenbuterol, salbutamol and ractopamine in milk by reversed-phase liquid chromatography tandem mass spectrometry with isotope dilution

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ABSTRACT

A simple, sensitive and reliable analytical method was developed for the simultaneous determination of clenbuterol (CLB), salbutamol (SAL) and ractopamine (RAC) in milk by ultra high performance liquid chromatography–positive electrospray ionization tandem mass spectrometry (UHPLC–ESI-MS/MS) with isotope dilution. Samples were directly purified through HLB cartridge. Then the eluate was dried under nitrogen and residues were redissolved in mobile phase. Samples were analyzed by LC–MS/MS on an Acquity UPLC® BEH C_{18} column with gradient elution. The samples were quantified using clenbuterol-D_9, salbutamol-D_3 and ractopamine-D_6 as internal standards. The proposed method was validated according to the European Commission Decision 2002/657/EC determining specificity, decision limit (CCα), detection capability (CCβ), recovery, precision, linearity, robustness and stability. CCα values were 0.054, 0.006 and 0.008 μg/kg for CLB, SAL and RAC, respectively. CCβ values were 0.058, 0.007 and 0.009 μg/kg for CLB, SAL and RAC, respectively. The mean recoveries, repeatability (expressed as coefficient of variation, CV_r), and reproducibility (CV_R) varied from 95.8 to 106.2%, from 3.60 to 6.44% (CVr), and from 4.77 to 7.53% (CV_R), respectively. The method is demonstrated to be suitable for the determination of clenbuterol, salbutamol and ractopamine in milk. The total time required for the analysis of one sample, including sample preparation, was about 45 min.

1. Introduction

Clenbuterol (CLB), salbutamol (SAL) and ractopamine (RAC) are β_2-agonists, originally used in the therapeutic treatment of asthma and preterm labor in humans [1]. However, these compounds are also misused as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues [2]. This misuse had caused some severe accidental poisonings in humans [3,4]. So, all β_2-agonists are banned for growth promotion in animal production in China and EU [5,6]. In order to protect consumers, the EU has established maximum residue limits (MRLs) of 0.10 μg/kg in muscle of bovine and equidae, 0.50 μg/kg in liver and kidney of bovine and equidae, and 0.05 μg/kg in bovine milk for CLB [7]. Therefore, specific and sensitive methods for the identification and quantification of these compounds in milk are required.

To identify β_2-agonists in biological samples, gas chromatography–mass spectrometry (GC–MS) [8–16] and liquid chromatography–mass spectrometry (LC–MS) [16–26] methods have been developed. However, GC–MS (MS/MS) requires time-consuming derivatization steps to enhance the volatility of analytes, which might produce interferences and consequently make more difficult quantitation. LC–MS/MS has become the main analytical technique for determining β_2-agonists due to its shorter chromatographic run time and without time-consuming derivatization procedures. Ultra high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry is one of the most efficient methods, because of the high resolution and rapid separation of UHPLC.

Although many LC–MS/MS methods have been developed for identification of β_2-agonists in biological samples, there is little literature on identification of β_2-agonists in milk. An HPLC–ESI-MS/MS method for determining 10 β_2-agonists in milk has been developed by Wang et al. but the limits of quantitation (LOQs) (0.068–13.20 μg/kg) were above MRL (0.05 μg/kg) of CLB [25] and the deproteinization step was labor-intensive. Ortelli et al. have described a fast LC–MS/MS method for determining 150 veterinary drugs in milk including 6 β_2-agonists, but decision limit (CCα) and detection capability (CCβ) for CLB were 0.30 μg/L and 0.50 μg/L [26], respectively. These values were far higher than MRL of CLB.

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Table 1
Retention time and LC–ESI-MS/MS parameters for CLB, SAL, RAC, CLB-D_9, SAL-D_3 and RAC-D_6.

Analyte	Retention time (min)	Parent ion (*m/z*)	Daughter ion (*m/z*)	Dwell time (s)	Collision energy (eV)	Cone voltage (V)
CLB	3.87	277.0	202.8[a],131.7	0.10	16, 28	22
SAL	2.81	240.0	147.8[a],165.9	0.10	18, 14	24
RAC	3.70	302.1	163.9[a],120.8	0.10	16, 22	22
CLB-D_9	3.86	286.0	204.0	0.10	16	22
SAL-D_3	2.79	243.0	151.0	0.10	18	24
RAC-D_6	3.68	308.0	168.0	0.10	16	22

[a] Ion for quantification.

Moreover, limits of detection (LODs) of some screening methods for determination of β_2-agonists in milk were between 0.05 μg/L and 250 μg/L [27–29]. So far, a confirmatory method for simultaneously determining CLB, SAL and RAC in milk by LC–MS/MS with good sensitivity (LOQ below 0.05 μg/kg) has not been developed.

In this paper, we describe a simple and sensitive LC–MS/MS method for simultaneous determination of CLB, SAL and RAC in milk with isotope dilution. Solid phase extraction (SPE) is used for sample preparation without complex deproteinization step. Validation parameters tested were specificity, CCα, CCβ, recovery, precision, linearity, robustness and stability.

2. Materials and methods

2.1. Materials and reagents

Methanol and formic acid were LC grade. Hexane was analytical grade. Clenbuterol, salbutamol, ractopamine, clenbuterol-D_9 (CLB-D_9) (100 μg/mL in acetone) and salbutamol-D_3 (SAL-D_3) (100 μg/mL in acetone) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ractopamine-D_6 (RAC-D_6) was purchased from Toronto Research Chemicals Inc. (North York, Canada). The OASIS® HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters (Milford, MA, USA). Water was purified with a Milli-Q reverse osmosis system (Millipore, Milford, MA, USA).

2.2. Standard solutions

Individual stock solutions of CLB, SAL, RAC and RAC-D_9 (100 μg/mL) were prepared in methanol. Three fortifying mixed standard solutions of CLB, SAL and RAC (2.5, 5.0 and 7.5 μg/L) and one mixed fortifying standard solution for CCβ experiment (5.4 μg/L for CLB, 0.60 μg/L for SAL and 0.80 μg/L for RAC) were prepared by diluting stock standard solution with methanol. An internal working standard solution of CLB-D_9, SAL-D_3 and RAC-D_6 (20 μg/L) was prepared in methanol. Six individual working standard solutions (500 μg/L for each compound) for MS–MS optimization were prepared by diluting each stock solution (100 μg/mL for each compound) with 0.1% formic acid solution/methanol (95:5, v/v). Six mixed working standard solutions (0.1–50 μg/L) were prepared by diluting and mixing working standard solutions (500 μg/L for each compound) with 0.1% formic acid solution/methanol (95:5, v/v).

2.3. Chromatographic conditions

A Waters Acquity UPLC instrument (Milford, MA, USA) was used in the present study. Separation was carried out on an Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm) maintained at 30 °C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (methanol). Initial gradient conditions were set to 5% B and held for 1.5 min before incorporating a linear gradient increasing to 55% B at 4.0 min. At 4.1 min the gradient was programmed to initial conditions to reequilibrate the column for 1.9 min (total run time 6 min). The flow rate was 0.3 mL/min. The injection volume was 10 μL in full loop injection mode.

2.4. Mass spectrometry conditions

Detection was carried out by a Waters Xevo™ TQ triple-quadrupole MS fitted with electrospray ionization (ESI) probe operated in the positive ion mode. The following parameters were optimal: capillary voltage, 3000 V; ion source temperature, 150 °C; desolvation gas temperature, 500 °C; desolvation gas flow rate, 1000 L/h. Detection was carried out in multiple reaction monitoring (MRM) mode. Argon was used as the collision gas, and the collision cell pressure was 4 mBar. Other parameters are shown in Table 1.

2.5. Sample preparation

After addition of 50 μL of 20 μg/L internal standard solution in 10 g milk, the sample was applied to OASIS® HLB SPE cartridge which was activated with 5 mL of methanol followed by 5 mL of water. The cartridge was washed with 5 mL of water, 5 mL methanol–water (1:9, v/v) and dried with strong vacuum for 5 min, then washed with 5 mL of hexane. The analytes were eluted with 2 mL of methanol. The eluate was evaporated until dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 500 μL of 0.1% formic acid in water/methanol (95:5, v/v). The resulting solution was filtered through 0.22 μm filter and 10 μL of the filtrate was injected into the LC.

2.6. Method validation

The evaluation of the suitability of the method for the determination of CLB, SAL and RAC in milk was carried out according to the European Commission Decision 2002/657/EC [30].

To verify the absence of interfering substances around the retention time of analytes, 20 blank milk samples were analyzed.

Calibration curves were constructed using mixed working standard solutions by plotting the peak area ratio of quantitative ion pair of each standard to internal standard at concentrations of 0.1, 0.5, 1.0, 5.0, 10 and 50 μg/L. CLB, SAL and RAC were quantified with CLB-d_9, SAL-D_3 and RAC-D_6, respectively. The concentration of internal standard was 2.0 μg/L.

For CLB, the CCα was established by analyzing 24 blank materials per matrix fortified with CLB at 0.05 μg/kg. The CCβ was established by analyzing 20 blank materials per matrix fortified with CLB at their CCα.

For SAL and RAC, the CCα was established by analyzing 20 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio (qualitative ion pair) was used as CCα. The CCβ was established by analyzing 20 blank materials per matrix fortified with the analyte at their CCα.

Recovery of CLB, SAL and RAC was measured in blank milk that was fortified at MRL level of CLB (0.05 μg/kg), at half of the MRL level of CLB (0.025 μg/kg) and one and a half of the MRL level of CLB (0.075 μg/kg). The fortified samples were analyzed and

the recoveries were calculated by comparing the measured concentration to the fortified concentrations. The repeatability and within-laboratory reproducibility were measured on the same 54 fortified blank milk samples ($n = 6$ replicates per concentration level and analyzed in three independent analytical runs) and expressed by coefficient of variation (CV_r and CV_R, respectively).

Robustness only was established by introducing small changes in the chromatographic system, like flow rate (0.25, 0.30 and 0.35 mL/min), column temperature (25, 30 and 35 °C) and the concentration of formic acid in solvent A (0.05, 0.10 and 0.15%). Robustness of the method was assessed by injecting one matrix standard solution (1.0 μg/L for CLB, SAL and RAC; 2.0 μg/L for CLB-D_9, SAL-D_3 and RAC-D_6) six times under each above conditions.

The stability was determined in two different ways: (a) in solvent (stock solutions) and (b) in matrix (fortified milk at 0.05 μg/kg).

3. Results and discussion

3.1. Sample preparation

For the determination of veterinary drug residue in milk, the sample pretreatment procedures usually include labor-intensive deproteinization procedure [25,31,32]. However, the liquid milk samples may be directly purified through SPE column, and thus make the sample preparation easier and faster. Moreover, the sample preparation without deproteinization procedure was feasible based on method validation experiments.

Cation exchange SPE cartridge was often used to purify β_2-agonists because of its good cleanup efficiency. However, the sample of milk was difficult to pass through the cartridge under gravity when SCX or Oasis® MCX column be used, and the speed of the milk sample through the cartridge was often less than 0.5 mL/min even if strong vacuum be used. So the Oasis® HLB column was chosen in this study because of its good cleanup efficiency, good recovery and good SPE processing speed.

3.2. MS–MS optimization

Working solutions of 500 μg/L were infused to optimize the MS–MS parameters of CLB, SAL, RAC, CLB-d_9, SAL-D_3 and RAC-d_6 and to select the appropriate diagnostic ions. The infusion process was carried out with the same chromatographic conditions as those used during analysis. The ESI^+ was selected due to its sensitivity, ruggedness and easy handling and maintenance.

For β_2-agonists were included in group A of Annex, Council Directive 96/23/EC [33], a minimum of four identification points are required. In this experiment, four identification points were obtained by monitoring one parent ion (1 point) and two transitions (each 1.5 points). The selected transitions for the β_2-agonists and the optimal MS–MS conditions are shown in Table 1.

3.3. Method validation

3.3.1. Linearity

The calibration graphs were obtained by plotting the peak area ratio of the quantitative ion pair of each standard to internal standard versus drug concentration in 0.1–50 μg/L. The linear equations were $Y = 0.5737X + 0.0294$, $Y = 0.6243X - 0.0326$ and $Y = 0.2601X - 0.0105$ for CLB, SAL and RAC, respectively. The correlation coefficients (R^2) of the calibration curves were 0.9998, 0.9999 and 0.9999 for CLB, SAL and RAC, respectively. Using these curves, recoveries can be calculated at each fortification level. The MRM chromatograms of standard solution are shown in Fig. 1.

3.3.2. Specificity

The specificity was evaluated by analyzing 20 blank milk samples. Fig. 2a and b indicates that there were no interfering peaks from endogenous compounds at the retention times of CLB, SAL and RAC.

3.3.3. Recovery and precision

CLB, SAL and RAC were spiked into blank milk samples at three different concentrations (0.025, 0.050 and 0.075 μg/kg). The results are shown in Table 2. The mean recoveries, repeatability, and reproducibility varied from 95.8 to 106.2%, from 3.60 to 6.44% (CV_r), and from 4.77 to 7.53% (CV_R), respectively. These recoveries and coefficients of variation (CVs) for CLB, SAL and RAC are better than many previously developed methods for milk and other biological samples by LC–MS/MS [16–18,21,23–25], possibly due to the use of isotope compounds as internal standard for quantification in the present study.

3.3.4. CCα and CCβ

According to the concept of the European Commission Decision 2002/657/EC, the CCα (decision limit) and CCβ (detection limit) have been estimated. The results of the CCα were 0.054 μg/kg, 0.006 μg/kg and 0.008 μg/kg for CLB, SAL and RAC, respectively. The results of the CCβ were 0.058 μg/kg, 0.007 μg/kg and 0.009 μg/kg for CLB, SAL and RAC, respectively. Additionally, LODs were 0.006 μg/kg, 0.006 μg/kg and 0.008 μg/kg for CLB, SAL and RAC, respectively, based on three times signal to noise ratio. LOQs were 0.02 μg/kg, 0.02 μg/kg and 0.027 μg/kg for CLB, SAL and RAC, respectively, based on 10 times signal to noise ratio. The LOD of this method is better than those of previously published MS methods [8–14,16–26] for β_2-agonists in biological samples except one GC–MS method (LOD was 0.003 μg/L when the volume of urine was 50 mL) [15].

3.3.5. Robustness

The Waters Xevo™ TQ MS is a robust platform for quantitative LC–MS/MS. Compared with conventional LC–MS/MS, many more MRM transitions can be acquired with higher sensitivity in a single analysis, especially combined with ultra high performance LC systems.

The analytical results of the matrix standard solution were quantified with a standard solution (1.0 μg/L for CLB, SAL and RAC; 2.0 μg/L for CLB-D_9, SAL-D_3 and RAC-D_6). The concentrations of the matrix standard solution were 0.989–1.008 μg/L, 0.992–1.010 μg/L and 0.987–1.009 μg/L for CLB, SAL and RAC, respectively. The CVs ranged from 0.36 to 0.74%. These results demonstrate that changes of chromatographic conditions did not influence significantly the analytical results.

3.3.6. Stability

The stock standard solutions in methanol were stored for at least 6 months at −20 °C. The stock solutions were analyzed every month and the instrumental responses were compared with the peak areas obtained at the moment of solution preparation ($t = 0$). The acceptance criterion was a response comprised between 95 and 105% of the initial one [34]. Fortified milk samples of three β_2-agonists at 0.05 μg/kg stored at −20 °C were analyzed after 3, 7 and 14 days. It was found that the recoveries of three β_2-agonists had no obvious change.

3.4. Applications of the method

Twenty milk samples commercially available from the local market were analyzed for three β_2-agonists using the above method. No β_2-agonists were found in these samples. Unusan had reported 68.3% milk samples were contaminated with CLB

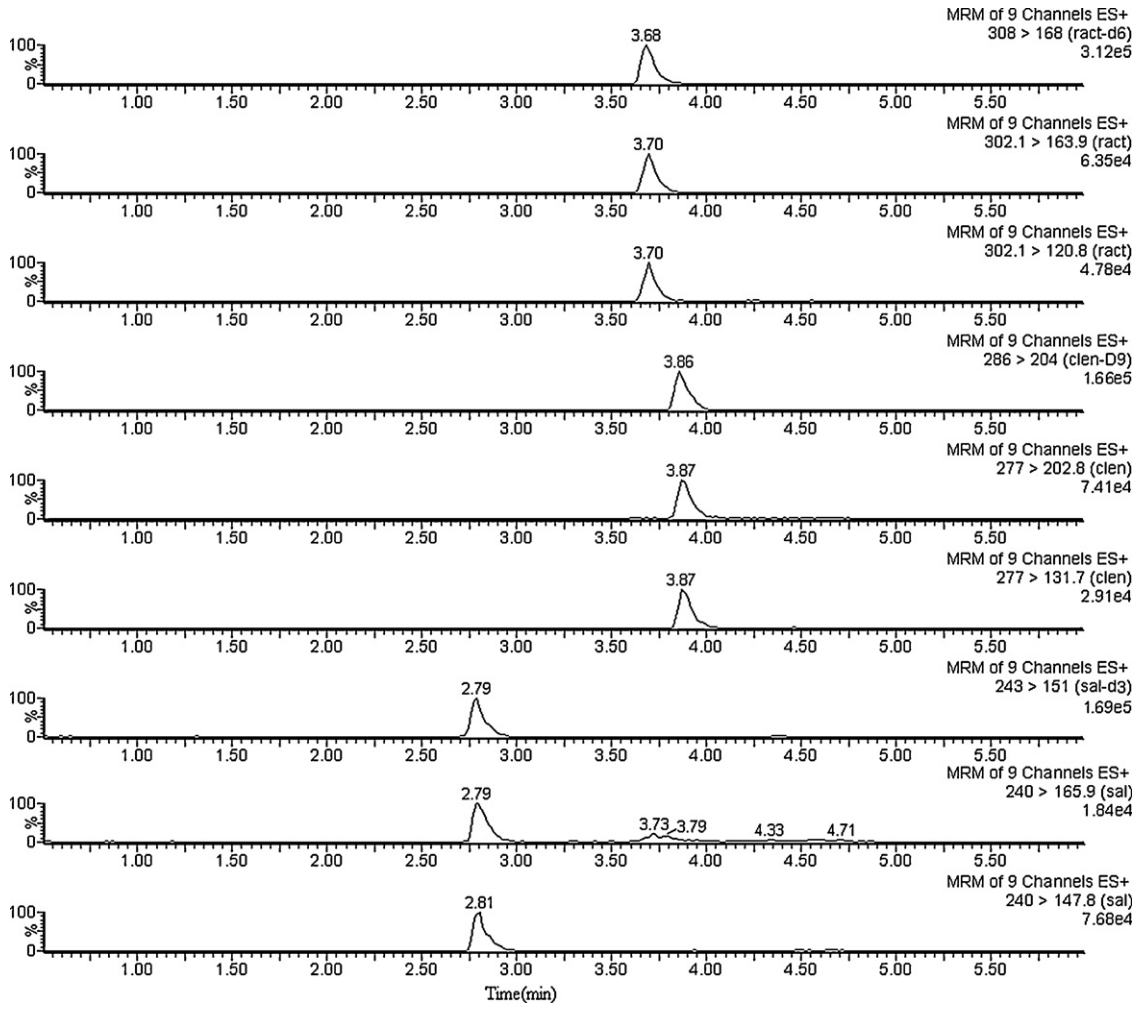

Fig. 1. The MRM chromatogram of CLB, SAL, RAC, CLB-D_9, SAL-D_3 and RAC-D_6 in standard solution (1.0 μg/L for CLB, SAL and RAC; 2.0 μg/L for CLB-D_9, SAL-D_3 and RAC-D_6). The MRM transitions from top to bottom correspond to RAC-D_6 (308 > 168), RAC (302.1 > 163.9 and 302.1 > 120.8), CLB-D_9 (286 > 204), CLB (277 > 202.8 and 277 > 131.7), SAL-D_3 (243 > 151) and SAL (240 > 165.9 and 240 > 147.8).

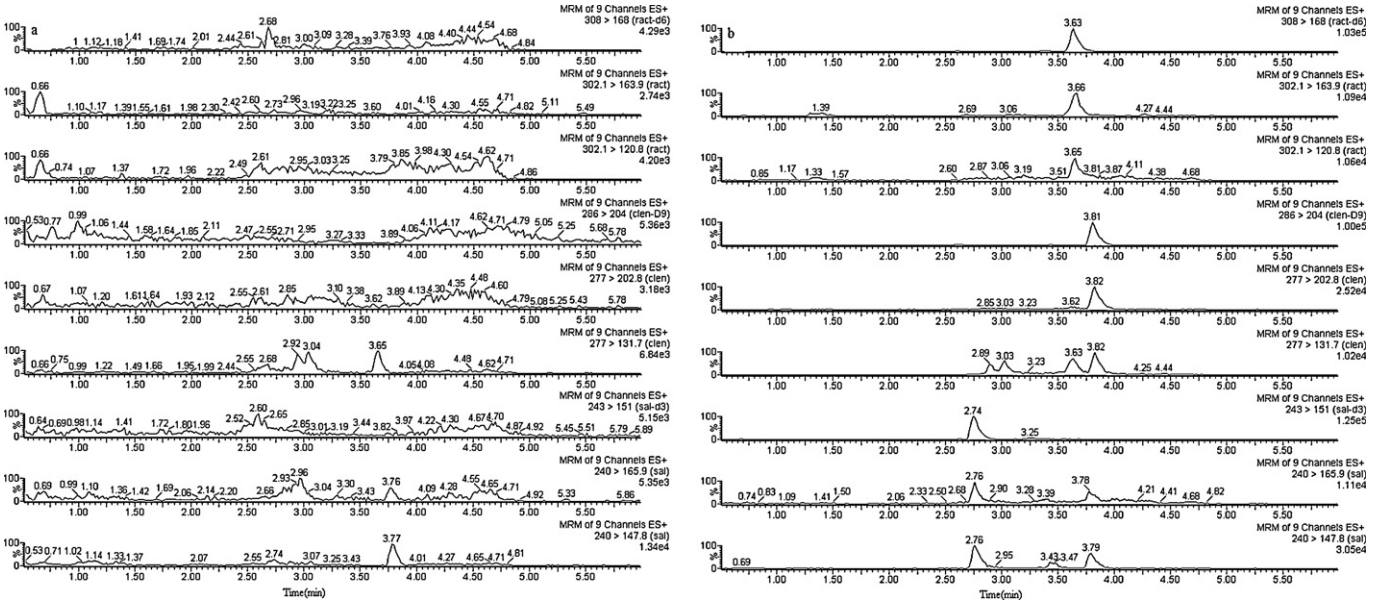

Fig. 2. (a) The MRM chromatogram of blank milk. (b) The MRM chromatogram of blank milk fortified with CLB, SAL and RAC at 0.025 μg/kg and CLB-D_9, SAL-D_3 and RAC-D_6 at 0.10 μg/kg.

Table 2
Mean recoveries and variation coefficients (CV_r and CV_R) of CLB, SAL and RAC from milk by LC–MS/MS.

Drug	Fortified concentration (μg/kg)	Mean recovery (%, $n = 6$)			CV_r (%)	CV_R (%)
CLB	0.025	105.2	102.4	97.5	5.19	7.12
	0.050	104.1	101.5	106.2	4.98	5.30
	0.075	98.4	103.7	102.8	5.55	5.84
SAL	0.025	98.7	96.7	99.3	6.44	7.53
	0.050	97.1	102.4	98.8	5.06	4.77
	0.075	99.3	98.6	98.4	5.19	6.62
RAC	0.025	97.3	95.8	98.7	5.92	5.46
	0.050	98.0	99.2	97.3	4.57	5.93
	0.075	102.1	98.5	100.6	3.60	5.07

in Turkey [27] by ELISA method. However, β_2-agonists are of little interest for the normal milk production scheme because β_2-agonists CLB cannot improve milk production [28]. So, further studies should be conducted about the occurrence of β_2-agonist residues in milk by confirmation method with large number of samples.

4. Conclusion

In the present study, a simple and sensitive method for the simultaneous determination of CLB, SAL and RAC in milk by LC–MS/MS with isotope dilution was developed. This method was validated with fortified milk samples and good recoveries with excellent CVs were obtained. The CCα and CCβ were found to be sufficiently low to determine the residues of CLB, SAL and RAC in milk.

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References

[1] J.G. Hardman, L.E. Limbird (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, New York, 1996.
[2] E. Shishani, S.C. Chai, S. Jamokha, G. Aznar, M.K. Hoffman, Anal. Chim. Acta 483 (2003) 137.
[3] J.F. Martinez-Navarro, Lancet 336 (1990) 1311.
[4] S. Maistro, E. Chiesa, R. Angeletti, G. Brambilla, Lancet 346 (1995) 180.
[5] Ministry of Agriculture of the People's Republic of China, No. 235 Bulletin of the Ministry of Agriculture of the People's Republic of China, Beijing, 2003.
[6] Council Directive 96/22/EC, Off. J. Eur. Commun. (1996) L125.
[7] Commission Regulation (EU) No. 37/2010, Off. J. Eur. Union (2010) L15.
[8] M.D. Engelmann, D. Hinz, B.W. Wenclawiak, Anal. Bioanal. Chem. 375 (2003) 460.
[9] J.P. Wang, X.W. Li, W. Zhang, J.Z. Shen, Chromatographia 64 (2006) 613.
[10] F. Ramos, A. Matos, A. Oliveira, M.I. Noronha da Silveira, Chromatographia 50 (1999) 118.
[11] L. Wang, Y.Q. Li, Y.K. Zhou, Y. Yang, Chromatographia 71 (2010) 737.
[12] L. Amendola, C. Colamonici, F. Rossi, F. Botrè, J. Chromatogr. B 773 (2002) 7.
[13] F. Ramos, C. Santos, A. Silva, M.I. Noronha da Silveira, J. Chromatogr. B 716 (1998) 366.
[14] W. Liu, L. Zhang, Z. Wei, S. Chen, G. Chen, J. Chromatogr. A 1216 (2009) 5340.
[15] G. Van Vyncht, S. Preece, P. Gaspar, G. Maghuin-Rogister, E. DePauw, J. Chromatogr. A 750 (1996) 43.
[16] L.X. Whaites, E.J. Murby, J. Chromatogr. B 728 (1999) 67.
[17] J. Blanca, P. Muñoz, M. Morgado, N. Méndez, A. Aranda, T. Reuvers, H. Hooghuis, Anal. Chim. Acta 529 (2005) 199.
[18] L.D. Williams, M.I. Churchwell, D.R. Doerge, J. Chromatogr. B 813 (2004) 35.
[19] J. Cai, J. Henion, J. Chromatogr. B 691 (1997) 357.
[20] 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.
[21] Z. Lin, S. Zhang, M. Zhao, C. Yang, D. Chen, X. Zhang, Rapid Commun. Mass Spectrom. 22 (2008) 1882.
[22] G. Salquèbre, M. Bresson, M. Villain, V. Cirimele, P. Kintz, J. Anal. Toxicol. 31 (2007) 114.
[23] M. Thevis, T. Schebalkin, A. Thomas, W. Schänzer, Chromatographia 62 (2005) 435.
[24] 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.
[25] J. Wang, Q. Zhang, Z. Wang, X. Li, J. Chin. Mass Spectrom. Soc. 30 (2009) 153.
[26] D. Ortelli, E. Cognard, P. Jan, P. Edder, J. Chromatogr. B 877 (2009) 2363.
[27] N. Unusan, Int. J. Food Sci. Technol. 43 (2008) 617.
[28] B. Stoffel, H.H.D. Meyer, J. Anim. Sci. 71 (1993) 1875.
[29] F. Caloni, M. Montana, C. Pasqualucci, G. Brambilla, G. Pompa, Vet. Res. Commun. 19 (1995) 285.
[30] European Commission Decision 2002/657/EC, Off. J. Eur. Commun. (2002) L221.
[31] X.L. Cui, B. Shao, R. Zhao, J.Y. Yang, Y. Hu, X.M. Tu, Rapid Commun. Mass Spectrom. 20 (2006) 2355.
[32] M. McDonald, K. Granelli, P. Sjöberg, Anal. Chim. Acta 588 (2007) 20.
[33] Directive 96/23/EC Office for Official Publications of the European Communities (1996) 4.
[34] Commission Recommendation 1999/333/EC of 03/03/1999, Off. J. Eur. Commun. (May 1999) L128.