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Simultaneous analysis of spectinomycin, halquinol, zilpaterol, and melamine in feedingstuffs by ion-pair liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method for the simultaneous analysis of veterinary drug residues (spectinomycin, halquinol, and zilpaterol) and contaminants (melamine) in feedingstuffs by liquid chromatography–tandem mass spectrometry was developed. Method performance for all analytes was evaluated by reversed-phase liquid chromatography, reversed-phase with altered chemical equilibrium, and hydrophilic interaction (HILIC) as chromatographic modes. Validation was in accordance to Commission Decision 657/2002/CE, by considering the best chromatographic approach. Ion-pair liquid chromatography with C_{18} as stationary phase led to the lowest random uncertainties, effective analyte separation and shorter time of analysis. Low precision deviations and good recovery rates were obtained and thus method reliability and sensitivity could be consolidated. Method applicability was evaluated by the analysis of samples of feedingstuffs, such as cattle, pig, and poultry feeds, feed ingredients of both animal and vegetable origins, and mineral feeds. Some samples showed quantifiable concentrations of halquinol and zilpaterol, reinforcing the importance of this new analytical control method.

1. Introduction

The world consumption of animal protein has grown significantly in recent decades. Given this new consumption profile, many veterinary drugs have been administered to animals, aiming to increase production. The prevention of diseases, physiological and behavioral modifications, and growth promotion are among the main reasons for employing such chemicals in animal production [1].

Halquinol (chlorohydroxyquinoline) is a blend of the chemical species 5,7-dichloroquinolin-8-ol (5,7-HAL), 5-chloroquinolin-8-ol (5-HAL), and 7-chloroquinolin-8-ol (Fig. 1, structure A), And it is very effective against a wide variety of bacteria and parasites. Despite the importance of this veterinary drug, only a limited number of analytical methods are available in the current literature. Thin-layer chromatography (TLC), molecular absorption spectrometry, and high performance liquid chromatography with UV–vis detection are among the most frequently reported techniques [2–4].

Spectinomycin (SPE) is an aminoglycoside antibiotic (Fig. 1, structure B). It has been widely used in therapeutic doses to fight bacterial and parasitic infections in poultry and swine, or as growth promoters. The difficulty of analyzing this chemical species by separation techniques such as reversed phase-liquid chromatography and UV–vis detection has been reported. The polarity of these molecules and the absence of chromophore groups in their structure are among the main challenges for the development of analytical methods [5]. As a consequence, methods using hydrophilic interaction and liquid chromatography coupled to mass spectrometry have been proposed [5–7].

Zilpaterol (ZIL) is a performance enhancer that belongs to the β-agonist class (Fig. 1, structure C). It increases the production of lean meat, decreasing fat formation and thus improving feed conversion and carcass yield [8–10]. In several countries, its use has been banned from animal production, due to possible negative effects on animal behavior and welfare. The increase in body tempera-

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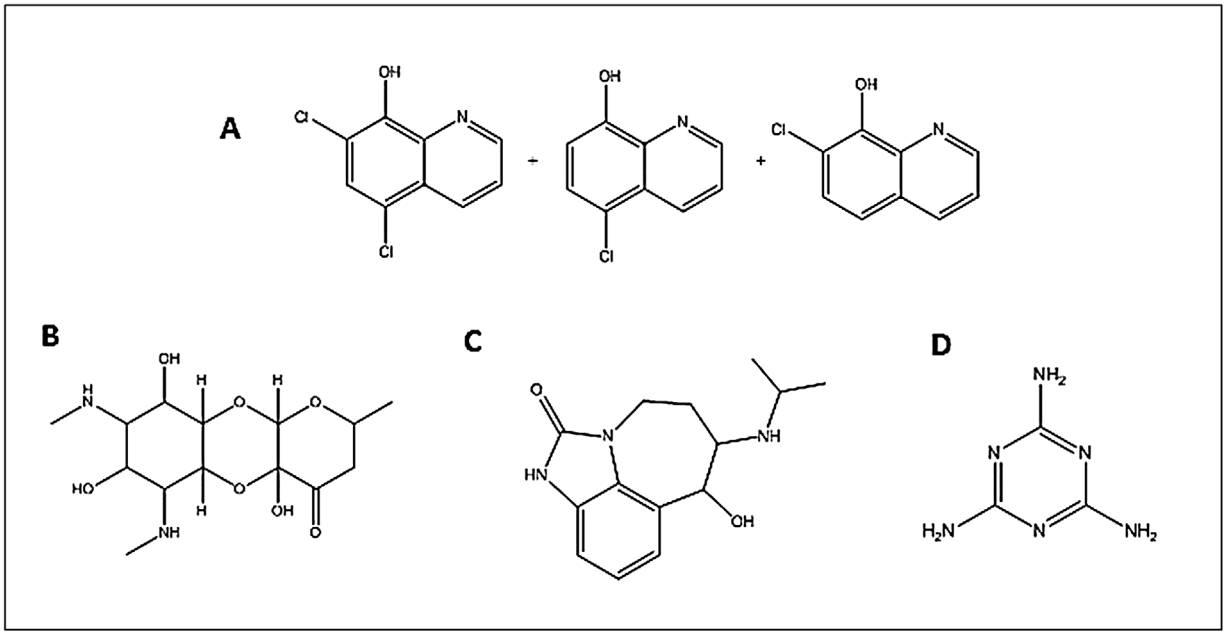

Fig. 1. Chemical structures of 5,7-dichloroquinolin-8-ol, 5-chloroquinolin-8-ol, and 7-chloroquinolin-8-ol (A), spectinomycin (B), zilpaterol (C), and melamine (D).

ture and respiration rate, in addition to possible damages to public health, have been reported [11–13]. Thus, several analytical methods for the determination of zilpaterol in tissues, body fluids, and feedingstuffs have been reported [14–16].

Melamine (MEL, 1,3,5-triazine-2,4,6-triamine) is an industrial chemical used in the production of melamine-formaldehyde resins. When combined to formaldehyde, melamine may result in a resin used by plastics and foams manufacturing industries [17]. On the other hand, due to the high amount of nitrogen present in its structure (Fig. 1, structure D), this compound can be illegally added to food and feed in order to adulterate their amount of net protein. The intake of high concentrations of melamine may induce serious renal damage in animals, since insoluble melamine cyanide crystals may be formed in the kidneys [18,19]. Besides such possible food adulterations, melamine also may be formed in the degradation of the pesticide cyromazine. Consequently, the residual maximum limit of 2.5 mg kg^{-1} has been established for food and feed [20].

The compliance assessment of the administration of veterinary drugs by feed, as well as the control over banned veterinary chemicals and the analysis of contaminants, are quite important to assure the quality of the whole food production chain. In this way, sensitive, specific, fast and highly reliable analytical methods are increasingly required as monitoring tools for feed and food inspection [21]. Methods based on liquid chromatography coupled to mass spectrometry (LC–MS) have been prominent in feed control due to their high selectivity and the ability to achieve low analytical limits, even when dealing with complex matrices [22,23]. However, high polarity chemical species may represent a challenge in the use of this technique. As a consequence, hydrophilic interaction liquid chromatography (HILIC) is becoming increasingly widespread and required for the separation of these compounds [5,24]. On the other hand, HILIC and reversed phase liquid chromatography with modified chemical equilibrium can be viable strategies to achieve this aim. Alternatively, ion-pair liquid chromatography, although well-known, is poorly exploited in reversed polar phase separation and mass spectrometry detection.

Recently, our research group developed a LC–MS method for analyzing 62 residues of veterinary drugs in feedingstuffs, covering several classes of chemicals. This method has been an effective tool to support feed inspection [23]. On the other hand, method scope extension is still required to increase the control of veterinary drugs residues. In addition, controlling the use of melamine is also important to avoid frauds and contaminations in feed production. Since there is a lack of analytical methods capable of determining halquinol residues by ion-pair liquid chromatography-tandem mass spectrometry, we have developed a reliable, selective, and sensitive tool for its measurements in feedingstuffs. To the best of our knowledge, for the first time a method capable of determining halquinol residues in feedingstuffs by LC–MS is described. Moreover, only a limited number of analytical methods for the determination of quite different molecules in feedingstuffs is available in the current literature. We found that ion-pair liquid chromatography, although well-known, is poorly explored in reversed polar phase separation and mass spectrometry detection. In this context, this work aimed to develop and validate an analytical method for the simultaneous analysis of spectinomycin, halquinol, zilpaterol, and melamine in animal feed. Its suitability for the purpose of increasing feed control was evaluated by the analysis of several real samples in our analytical routine.

2. Material and methods

2.1. Reagents, standards, and analytical blank

All solvents were in chromatographic grade. Other reagents were in analytical grade. Acetonitrile was purchased from Tedia Company, Inc. (Fairfield, USA). Methanol was supplied by Sigma-Aldrich Brasil Ltda. (Duque de Caxias, Brazil). Formic acid was supplied in LC–MS grade by J. T. Baker (Deventer, The Netherlands). Water was in ultrapure grade. Ion-pairing agent was the heptafluorobutyric acid (HFBA) supplied by Sigma-Aldrich Co. (St. Louis, USA).

Reference materials of spectinomycin and melamine were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany); halquinol was supplied by Farmabase (Jaguariúna, Brazil); zilpaterol (ZIL) was supplied by Toronto Research Chemicals Inc. (North York, Canada). Stock solutions were separately prepared at 1000 mg L^{-1}. Halquinol and spectinomycin solutions were prepared in methanol; melamine in acetonitrile:water (50:50), and zilpaterol in acetonitrile. Working solutions were prepared

by diluting each stock solution in acetonitrile:water (50:50) to 100 mg L^{-1}. All standard solutions were kept at −20 °C.

For method development and validation purposes, a blank feed sample was prepared in-house by mixing corn meal (40%), rice bran (20%), soybean meal (18%), wheat meal (18%), meat and bone meal (2%), vitamin premix (1%), phosphate (0.5%), and sodium chloride (0.5%).

2.2. Extraction method

Samples (1.00 ± 0.05 g) were weighed into 50 mL polypropylene tubes and formic acid 80% was added to each tube (5 mL). The tubes were shaken at a "wrist-action" platform for 20 min and centrifuged for 10 min at 4 °C and 3488 g-force. The tubes were kept at – 30 ± 10 °C and another centrifugation was performed for 20 min at 4 °C and 3488 g-force. Aliquots of the extracts (100 μL) were pipetted into polypropylene microtubes and diluted 10-fold with acetonitrile 80%. Centrifugation was then performed at 4 ± 2 °C and 17,300 g-force for 10 min. The supernatant (800 μL) was pipetted into glass vials and injected onto the LC–MS system.

2.3. Instrumentation

Liquid chromatography was performed in the 1290 Infinity system, manufactured by Agilent Technologies, Deutschland GmbH (Waldbronn, Germany), equipped with binary pump and column heater. It was coupled to the 5500 QTrap hybrid triple quadrupole-linear ion trap mass spectrometer, manufactured by Sciex (Framingham, USA), equipped with ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) sources.

Mass spectrometry was optimized by individual infusions set to 10 μL min^{-1} flow and concentrations among 5 and 50 μg L^{-1}. The multiple reaction monitoring mode (MRM) was used for analyte identification in positive modes (ESI^+), as well for the selection of two stable transitions (for confirmation and quantitation). The optimization of the quadrupoles and the ionization source was performed to achieve the greater signal intensity. With this purpose, source temperature (TEM), declustering potential (DP), collision energy (CE), entrance potential in Q1 (EP), exit collision cell potential (CXP) and the nitrogen source pressure (drying, nebulization, and curtain gas) were optimized.

2.4. Liquid chromatography approaches

Different chromatographic approaches were conducted to optimize instrumental parameters. For this purpose, a mixed solution with each analyte at 100 μg L^{-1} was diluted in mobile phase and blank sample extract (20%). Several stationary phases and chromatographic techniques were assessed, aiming at the best instrumental performance. Univariate experiments were conducted to achieve the best condition of each chromatographic mode. With this aim, variables such as column size, injection volume, column temperature, flow, mobile phases, among others, were set.

Hydrophilic interaction liquid chromatography (HILIC) was performed with the Hypersil Gold HILIC column (150 × 3.0 mm, 5 μm), manufactured by Thermo Fisher Inc. (Rockford, USA). Water and acetonitrile were used as mobile phases A and B, respectively, with ammonium formate (5 mmol L^{-1}) and formic acid (0.1%) in each phase. The elution gradient was set to 5% A (1–4 min), 50% A (4–6 min), and 90% A (6–10 min), plus 5 min to system's auto-equilibrium. The injection volume was set to 5 μL in 350 μL min^{-1} flow. The column was kept at 40 °C.

Reversed phase-liquid chromatography was conducted by modifying the chemical equilibrium with HFBA as pairing agent. The Symmetry C_{18} (2,1 × 50 mm, 3.5 μm) was used as stationary phase (Waters Corporation, Milford, USA) with the Security Guard C_{18} guard column (4.0 × 3.0 mm), supplied by Phenomenex (Torrance, EUA). Water (A) and acetonitrile (B) were used as mobile phases, both containing HFBA (0.12%). The column oven was kept at 40 °C. Gradient elution was set to 95% A (0–2 min), 75% A (2–3 min), 10% A (3–5 min), 75% A (5–6 min), 95% A (6–8 min), plus 4 min for system's auto-equilibrium. The injection volume was 5 μL in 300 μL min^{-1} flow.

Primary chemical equilibrium-reversed phase-liquid chromatography with polar groups, such as cyanopropyl and phenyl-hexyl, bound to silica, was also assessed. The Zorbax 300SB-CN (4.6 × 50 mm, 3.5 μm) and the Poroshell 120 phenyl-hexyl (3.0 × 50 mm, 4 μm) columns from Agilent Technologies (Santa Clara, USA) were used. The column oven was kept at 40 °C. Water and acetonitrile were used as eluents A and B, respectively, both containing formic acid (0.1%). The elution gradient was set to 95% A (0–2 min), 70% A (2–3 min), 10% A (3–5 min), 70% A (5–6 min), 95% A (6–8 min), plus 2 min for system's auto-equilibrium. The injection volume was 5 μL in 300 μL min^{-1} flow.

Finally, all liquid chromatography approaches were compared in order to obtain the shortest running time, higher analyte retention and separation, as well as good reproducibility. The random standard uncertainty was calculated by the analyte areas (in cps) mean standard deviation obtained by successive injections (n = 20) without measurement variables; this value was divided by 4.47 (68% probability Student t-distribution). The random standard uncertainty was expanded to 95% by multiplying the value by 2. Outliers were previously discarded by the Grubbs test (95%) and point scatter plot, to verify trends.

2.5. Method validation

After the best chromatographic condition was set, method validation was conducted in accordance to Commission Decision 2002/657/CE [25]. Validation was performed in terms of selectivity, linearity, precision, accuracy, extract stability, minimum required performance level (MRPL), decision limit (CC_α), and detection capability (CC_β). Additionally, matrix effect was assessed in accordance to Hoff et al. [26].

Method selectivity was assessed by the analyzing at least 20 spiked blank commercial samples of different kinds of feedstuffs, in order to check possible interferences around the analytes retention times. The relative intensity among ions was set to 20% (minimum). Furthermore, the occurrence of contamination among different runs was checked in order to set the need for system's auto-equilibrium.

Linearity was checked by preparing triplicate analytical curves with six concentration levels (including zero), under conditions of in-house reproducibility. Analytical curves were prepared by unweighted linear relation among peak areas (y-axis) versus concentrations (x-axis). The criterion of acceptance was that the mean regression coefficient (R^2) should be greater than 0.98.

Matrix effect was calculated in accordance to Hoff et al. [26]. For this calculation, analytical curves were prepared in pure solvent and in matrix (by spiking blank sample before and after the extraction procedure). All analytical curves were assessed with the same criteria considered for linearity.

Precision (in terms of repeatability and in-house reproducibility) and accuracy (under recovery) were checked by applying measurement variables, such as analysts (n = 2) and day of analysis (n = 3). Blank samples (n = 18) were spiked in three concentration levels (125, 250, and 375 μg kg^{-1}), depending on the measurand range in analytical routine. The acceptance criteria were that coefficient of variation (CV) should be lower than 20% for the results dispersion and the recovery rate should be among 80 and 110%

MRPL was evaluated by successive injections of blank sample spiked at the lower detected concentration and CV < 30%, in order to assess method sensitivity. The limit of decision (CC_α) was set by multiplying the mean standard deviation found for spiking by the factor 1.64. The detection capability (CC_β) was set by multiplying the mean standard deviation found for spiking by the factor 1.64 plus the CC_α value.

Analytes stability was checked by storing triplicate extracts at $4 \pm 2\,^{\circ}C$ for 1, 2, 6, 10, and 15 days. Firstly, test variability was assessed. The mean deviation (n = 21) was considered as centerline of the standard deviation control chart. The upper and lower limits were defined by multiplying the mean standard deviation by 2.57 and 0, respectively, depending on the number of repetitions needed to the calculation of each standard deviation of the chart (n = 3). Analysis of variance (ANOVA single factor, 95% significance level) was performed to check if any significant difference among the concentrations could be detected during the experiment.

2.6. Method applicability

After validation procedures, method applicability was assessed by routine analysis. In total, 21 samples of feedingstuffs, collected by the official inspection service of the Brazilian Ministry of Agriculture, Livestock and Food Supply, were analyzed. The samples scope covered cattle feeds (n = 4), poultry feeds (n = 3), pig feeds (n = 2), mineral feeds (n = 5), vegetable feed ingredients (rice, wheat, and sorghum, n = 4), and animal by-products (meat and bone meal, feathers meal, and poultry offal meal, n = 3). Samples were also spiked for the evaluation of recovery rates.

3. Results and discussion

3.1. Method development

Feedingstuffs are complex matrices that usually contain carbohydrates, proteins, and lipids in high concentrations. Thus, an extraction procedure shall minimize matrix interference and concomitant agents. One of the main challenges faced in the development of a multi-residue method, is to perform a single extraction procedure for all analytes, without compromising performance parameters [23]. The aqueous extraction with 80% formic acid yielded proper recoveries for all analytes. This was an already expected finding, since other well-succeeded aqueous extraction protocols have been reported [27,28]. Low temperature partitioning-solid liquid extraction contributed to improve our results, providing satisfactory clean-up, and resulting in clearer extracts compared to the turbid extracts initially obtained. Thus, the use of lead acetate, hexane, and other toxic reagents was not necessary. Good sensitivity was provided by the quadrupoles optimization for the transitions of the analytes (Table 1). Considering that halquinol is a blend of three chemical species, the two species were present in higher concentration and thus monitored in this study. Melamine, zilpaterol, and spectinomycin transitions yielded similar data to the literature [27,29,30].

According to the Commission Decision 2002/657/CE [25], a precursor ion and two second generation ions are required to meet the compound identification criteria set for liquid chromatography-tandem mass spectrometry methods. In our method, the fragments of more intense signals were used for quantitation and the others for compound identification. Relative intensities were all above 20%, ensuring adequate detection. The ESI source optimization increased sensitivity by employing the worst performing analyte (spectinomycin). In addition, the analytes fragmentation at the ionization source was not evidenced. Finally, the source was optimized in positive mode and the parameters were set to 5000 V (capillary voltage), 600 °C (source temperature), 55 psi (drying and nebulization), and 20 psi (curtain gas pressure).

Table 1
Parameters of multiple reaction monitoring using electrospray ionization mass spectrometry in positive mode.

Analytes	*m/z*	DP (V)	CE (V)	EP (V)	CXP (V)
Melamine					
Precursor ion	127.0	–	–	–	–
Quantitation transition	85.0	60	25	10	14
Confirmation transition	67.9	60	37	10	28
Zilpaterol					
Precursor ion	262.1	–	–	–	–
Quantitation transition	244.1	51	17	10	12
Confirmation transition	185.1	51	33	10	12
Spectinomycin					
Precursor ion	351.1	–	–	–	–
Quantitation transition	333.1	70	25	10	16
Confirmation transition	207.1	70	31	10	14
5-chloroquinolin-8-ol					
Precursor ion	180.1	–	–	–	–
Quantitation transition	145.0	41	33	10	6
Confirmation transition	116.9	41	39	10	14
5,7-dichloroquinolin-8-ol					
Precursor ion	214.0	–	–	–	–
Quantitation transition	150.0	60	39	10	10
Confirmation transition	179.0	60	35	10	10

Liquid chromatography in different modes proved to be, for the most part, viable methods for the determination of the analytes. However, not all approaches led to required retention of the molecules, since a by-pass valve was used during the first minutes of each run. This is an essential step in the LC–MS feed analysis, since it discards part of the extracted compounds that should not be retained, reducing dirt and oxidation of the ionization source in the mass spectrometer. Thus, shorter time was required for system auto-equilibration and cleaning.

The separation by hydrophilic interaction yielded good results, despite the low retention of halquinol (1.12 min). This mode employed a traditional polar phase and a blend of acetonitrile, buffer solution, and water as mobile phase. It has been shown to be an alternative to the separation of analytes that were not retained in the reversed phase without alteration of the primary chemical equilibrium mode, such as melamine, zilpaterol, and spectinomycin. This explains the increasing use of this chromatographic mode for the separation of very polar chemical species, especially drug residues and contaminants [7]. However, this approach presented some disadvantages in relation to the ion-pair reversed phase mode. The equilibrium time required for reproducibility was higher (5 min), thus resulting in longer time of analysis. In addition, this mode was sensitive to pH variations, requiring mobile phase buffering. In high throughput control laboratories, shorter chromatographic runs imply lower expenses. In addition, the stationary phases in HILIC mode are generally more costly in relation to the stationary phases employed in the reversed phase mode, such as C_{18}, which uses to be widely available in laboratories.

On the other hand, ion-pair liquid chromatography was successfully employed for the separation of all analytes. In addition, unlike the HILIC mode, this chromatographic mode provided the effective separation of 5,7-HAL and 5-HAL, increasing method selectivity. Ion-pair liquid chromatography is a high performance version of ion exchange chromatography in which the mobile phase pH is adjusted to promote ionization of the sample components. HFBA is a volatile species, with opposite charge to the analytes. By adding HFBA, a neutral ion-pair was formed which, upon contact with the stationary phase (C_{18}), was adsorbed due to interactions with the surfactant's aliphatic tail, thus allowing their separation. The

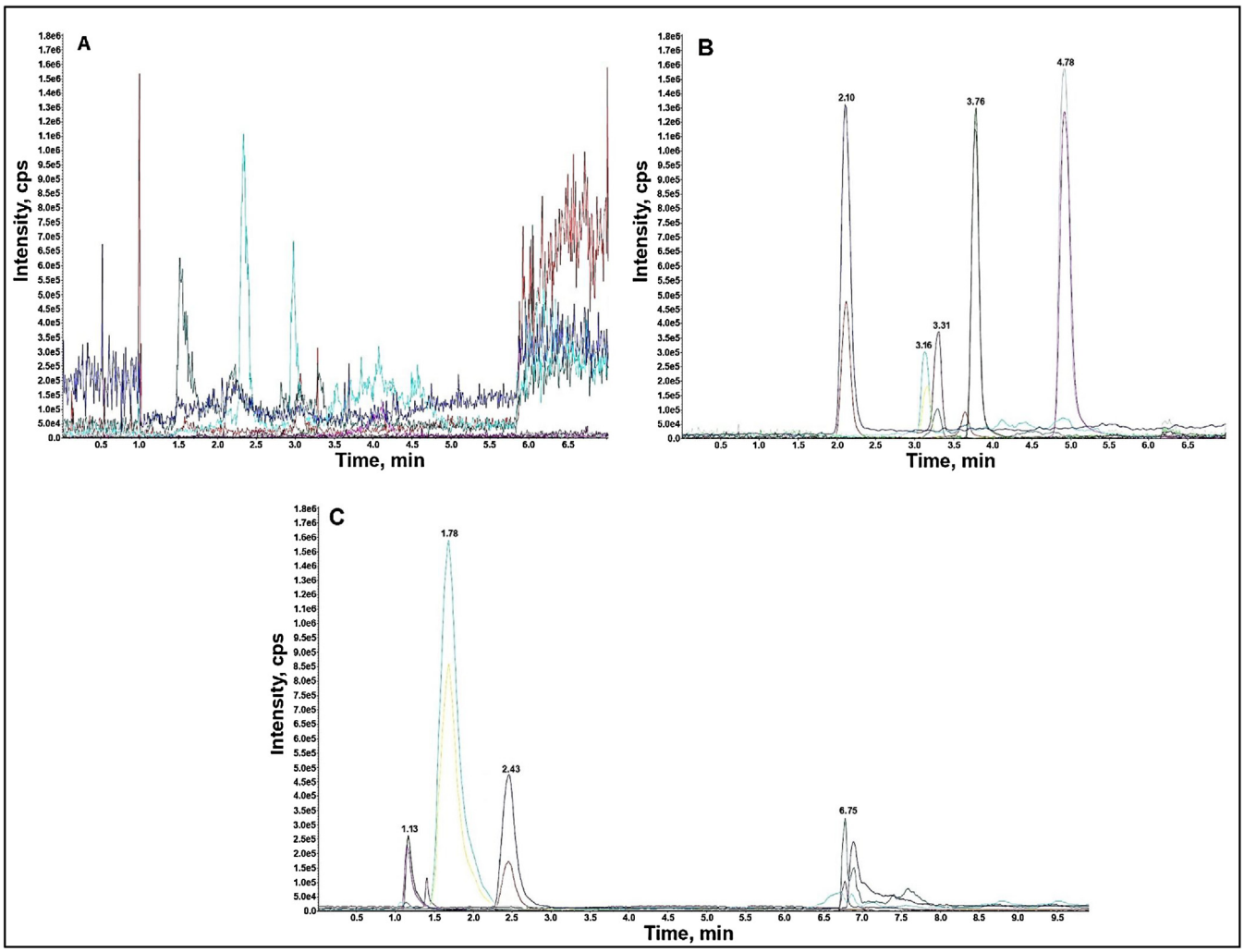

Fig. 2. Part A - blank sample ion-pair liquid chromatogram. Part B - spiked blank sample ion-pair liquid chromatogram of spectinomycin (2.10 min), melamine (3.16 min), 5-chloroquinolin-8-ol (3.31 min), 5,7-dichloroquinolin-8-ol (3.76 min), and zilpaterol (4.78 min). Part C - spiked blank sample HILIC chromatogram of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol (1.13 min), zilpaterol (1.78 min), melamine (2.43 min), and spectinomycin (6.75 min).

elution occurred in descending order of polarity (SPE > MEL > 5-HAL > 5,7-HAL > ZIL), due to the nonpolar stationary phase and to the elution gradient, which started with aqueous composition. As it can be seen in Fig. 2, higher retention and effective separation of the analytes occurred in relation to the HILIC mode.

Ion-pair chromatography is more complex than reversed phase chromatography with primary chemical equilibrium. In order to establish the equilibrium between the surfactant and the stationary phase, some precautions are required, especially with regard to variations in temperature and pH. Although it is not discussed in the literature how HFBA promotes reversed phase separation, its hydrophobic part seems to interact with the stationary phase, whereas the carboxylate ion interacts with the analytes. Thus, the elution was set in gradient mode, based on other methods with ion-pairing agents [5,31]. However, to our knowledge, this is the first time that these analytes are determined in the same chromatographic run by this chromatographic mode. In this context, the best chromatographic condition was achieved with water as mobile phase A and acetonitrile as mobile phase B, both containing 0.12% of HFBA.

Chromatograms obtained by reversed phase liquid chromatography without alteration of the primary chemical equilibrium employing cyanopropyl and phenyl-hexyl groups are shown in Fig. 3. The cyanopropyl phase promoted desirable retention of the analytes, including those which were not retained in C_{18}. Differently, the phenyl-hexyl phase did not retain melamine, zilpaterol, and spectinomycin, which came out in the extra column (dead) volume. However, neither of the phases presented adequate repeatability or reproducibility.

However, as it can be seen in Fig. 4, ion-pair liquid chromatography and HILIC modes were the only ones that presented satisfactory results for all the analytes. Although the other approaches presented higher sensitivity for melamine and zilpaterol, reproducibility and sensitivity were lower for other analytes, turning those approaches into impracticable alternatives for method validation. The highest random uncertainty was determined with the phenyl-hexyl phase, generating unfavorable analysis conditions. Ion-pair liquid chromatography employing C_{18} as stationary phase conferred the lowest random uncertainties, effective separation of the analytes and short time of analysis compared to the HILIC mode. Thus, this was the adopted approach adopted for method validation. To the best of our knowledge, this is the first time that these analytes are determined by the same chromatographic run and by this chromatographic mode. Compared to other veterinary drugs residues analytical methods, our method is simple to perform, dispensing the need for purification steps such as the use of SPE cartridges which are often necessary for the determination of β-agonists [27].

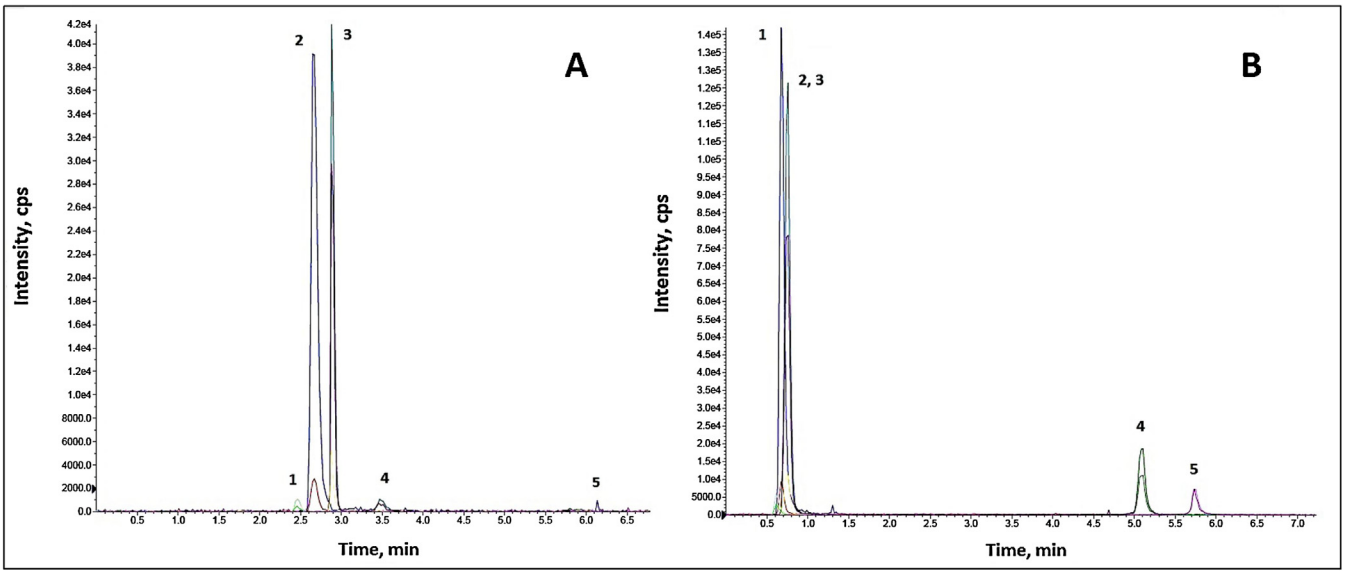

Fig. 3. Part A - chromatogram yielded by cyanopropyl as stationary phase, in which peaks correspond to spectinomycin, 2.30 min (1); melamine, 2.60 min (2); zilpaterol, 2.90 min (3); 5-chloroquinolin-8-ol, 3.50 min (4), and 5,7-dichloroquinolin-8-ol, 6.25 min (5). Part B – chromatogram yielded by phenyl-hexyl as stationary phase, in which peaks correspond to spectinomycin, 0.8 min (1); melamine, 0.8 min (2); zilpaterol, 0.80 min (3); 5-chloroquinolin-8-ol, 5.10 min (4); and 5,7-dichloroquinolin-8-ol, 5.70 min (5).

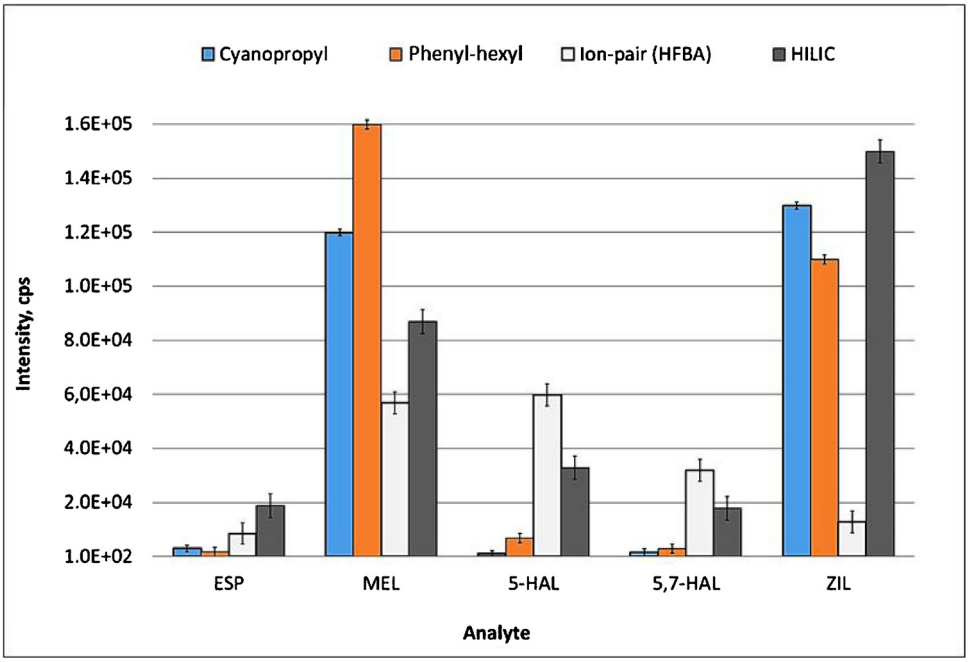

Fig. 4. Random uncertainties and intensities according to each stationary phase and liquid chromatography mode assessed.

3.2. Method validation

In practice, the fit-for-purpose of analytical methods is usually assessed during validation studies. The results of the validation procedures were considered satisfactory and all the tested requirements were in accordance with the protocol adopted. Thus, method selectivity was achieved, since no interfering transitions from endogenous and exogenous matrix compounds were evidenced during analysis (Fig. 2, part A). Only at the first level of the analytical curve, in the absence of analytical standard and in the presence of blank sample, interfering peaks were detected, close to the spectinomycin retention time. However, since a matrix-matched concentration gradient was provided, the analyte signal also increased, while the interfering transitions remain constant (Fig. 5). This could also be observed when the mass gradient of the blank sample was increased, which generated inverse data.

Linearity was achieved for all analytes in the concentration ranges among 0 and 500 $\mu g\ kg^{-1}$. The mean values of the regression coefficients were all satisfactory and above 0.98 (Table 2). On the other hand, in the presence of matrix, the analytes generated a suppression of the signal in the ESI source, notably for melamine, that yielded the greater signal loss (60%). The matrix effect was negligible for SPE (10%) in relation to other kinds of error (Table 2). In this way, calibration curves in the matrix were used to analyze all the analytes.

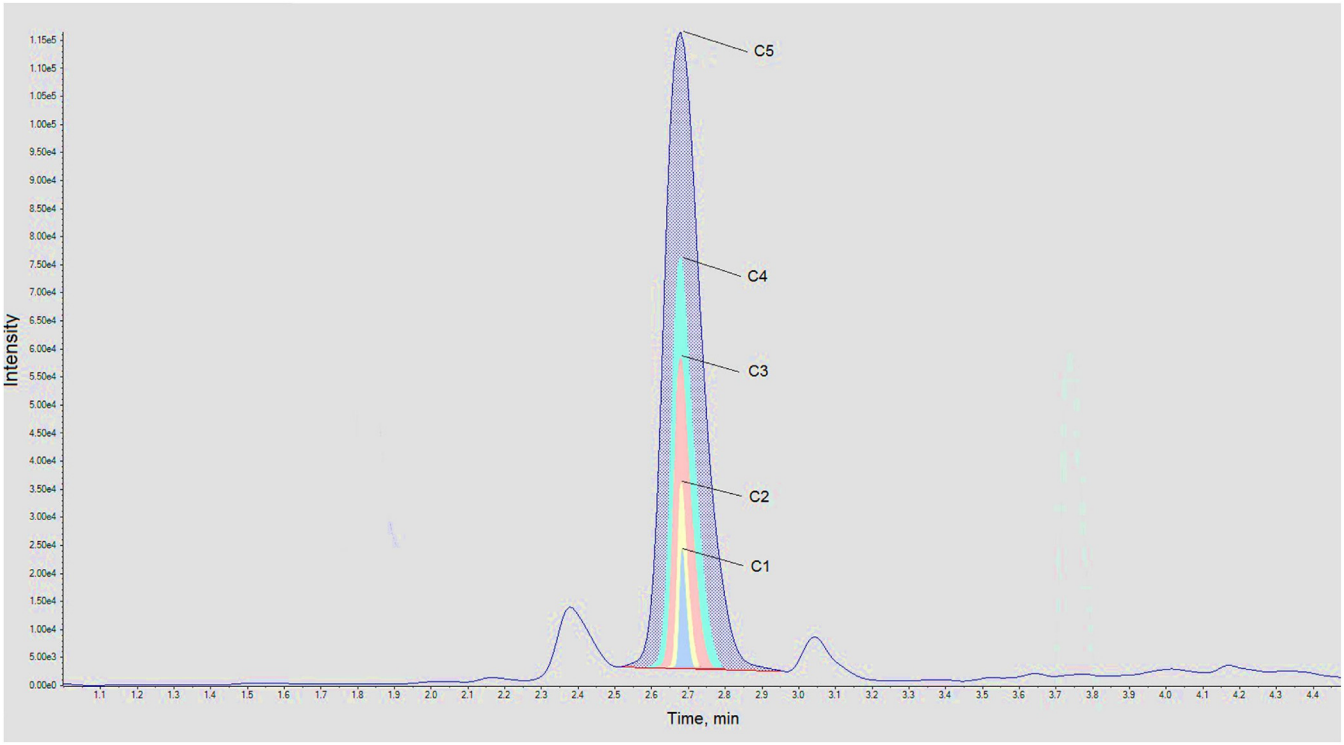

Fig. 5. Results of selectivity assessment for spectinomycin. C1, C2, C3, C4, and C5 refer to five concentration levels (μg kg^{-1}) of analytical curve.

Table 2
Results of the validation assessment.

Analytes	Recovery (%)/ Repeatability CV (%)			Intermediate precision CV (%)			Regression coefficient (R^2)	Matrix effect (%)	CC_α (μg kg^{-1})	CC_β (μg kg^{-1})	MRPL (μg kg^{-1})
	FL 1	FL 2	FL 3	FL 1	FL 2	FL 3					
Spectinomycin	81/16	80/8.9	92/10	20	10	11	0.98	−10	304	358	70
5-chloroquinolin-8-ol	85/14	80/6.7	87/9.1	14	6.8	13	0.98	−30	304	358	50
5,7-dichloroquinolin-8-ol	91/14	83/8.4	90/9.5	14	10	11	0.99	−40	304	359	50
Melamine	92/11	87/7.9	91/7.5	11	8.7	8.7	0.99	−60	292	334	20
Zilpaterol	87/13	91/7.2	88/8.0	14	7.8	10	0.98	−30	298	347	20

CV = coefficient of variation; MRPL = minimum required performance level; FL = fortification levels: 125 μg kg^{-1} (FL 1), 250 μg kg^{-1} (FL 2), and 375 μg kg^{-1} (FL 3).

Recovery rates were satisfactory, with values ranging from 80 to 110% (Table 2) and dispersions (CV < 20%). The CC_α values were between 298 and 304 μg kg^{-1}, yielding errors of less than 20% in all evaluated levels propagation (Table 2). The decision limit is the limit by which it can be concluded that a sample is non-compliant with α-error probability, which is the probability of a sample being compliant, despite presenting a non-compliant result. The detection capability is the lowest content of the analyte that can be detected or quantitated in a sample, with β-error probability, which is the probability of the analyzed sample being non-compliant, despite presenting a compliant result. These errors comprise system variability caused by random effects due to analysts and equipment when considering specific concentration ranges. An analytical result shall be considered non-compliant if the obtained value is greater than the CC_α set for the method. Thus, the CC_α values should be very close to regulatory limits. As there are no current regulatory limits set for the analytes of this study, errors were determined by considering the actual range of work required by routine analysis. On the other hand, low MRPLs were obtained, demonstrating that this method has adequate sensitivity for the studied matrices (Table 2).

Finally, the extract stability study showed that the analytes were stable at −4 °C within 15 days, since no significant difference between the concentrations could be evidenced ($p < 0.05$). The experimental deviations were lower than those established under in-house reproducibility conditions (<20%).

3.3. Method applicability

Method applicability showed its fit-for-purpose, allowing the quantitation of zilpaterol and halquinol in four of 20 samples. Concentrations between 0.6 and 19 mg kg^{-1} were obtained, with the highest values for halquinol. The other analytes could not be quantitated. However, all recoveries were between 80 and 110%, demonstrating proper applicability for routine analysis. On the other hand, mineral feed samples did not yield adequate recoveries by considering analytical curves prepared in feed matrix, since matrix effect was very pronounced (greater than 50%). In this sense, this type of matrix should be analyzed by standard addition, or blank samples with similar characteristics to samples should be used.

4. Conclusion

Ion-pair liquid chromatography was the more adequate mode compared to others, such as HILIC. Thus, the developed method provided analytical reliability after validation procedures. The method was used to analyze routine samples, and its adequate applicability

was confirmed. The presence of halquinol and zilpaterol analytes in the analyzed samples reinforces the need for controlling these drugs in feedstuffs, aiming at the protection of animals and consumers. This multipurpose method can be performed in a short time of analysis, increasing sample throughput in feed control laboratories. It may serve as a screening method, due to its low detection levels, as well as for the control feeds intended for both therapeutic and grow promotion aims. Furthermore, this method may help on indicating the violation of good manufacturing guidelines in the production of feedingstuffs.

Conflict of interest

None.

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