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Development and optimization of confirmatory liquid chromatography—Orbitrap mass spectrometry method for the determination of 17 anticoccidials in poultry and eggs

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ABSTRACT

A new sensitive and selective multi-residue method based on liquid chromatography - Orbitrap high resolution mass spectrometry (LC-Orbitrap-HRMS) was developed and validated for the determination of 17 anticoccidials in poultry and eggs. Instrumental parameters were optimized by the means of statistical experimental designs to improve the sensitivity, precision, and repeatability of the method. Further optimization of auto-tuned MS parameters led to an increase of signal intensity by 10% to 99% for 16 out of 17 analytes. The sample preparation procedure included extraction from muscle tissue and egg samples with acetonitrile, followed by preconcentration, reconstitution, and filtration. Validation was performed according to the Commission Decision 2002/657/EC. The occurrence of anticoccidials in eggs and poultry was assessed by using the developed analytical procedure within the Latvian national monitoring program, revealing quantifiable residues for 6 analytes (marker residue of nicarbazin - 4,4′-dinitrocarbanilide (DNC), salinomycin, narasin, toltrazuril, and its two metabolites).

1. Introduction

Confirmatory analysis of veterinary drugs performed by liquid chromatography - mass spectrometry (LC—MS) requires a high level of selectivity, the criteria for which are laid down in EU Commission Regulation 2002/657/EC [1]. Anticoccidials are group B veterinary drugs that require 3 identification points (IPs) for the confirmation of their presence in samples [2], which are typically obtained by using the "gold standard" of confirmatory analysis – quadrupole tandem mass spectrometry (MS/MS). Monitoring of two product ion transitions after the precursor ion selection and fragmentation yielded a total of 4 IPs. The analysis based on high-resolution MS (HRMS) precursor ion followed by HRMS transition product yielded 4.5 IPs, thus providing a more rigorous confirmation process in the testing of real samples.

HRMS systems, which by the definition of Commission Regulation have resolution greater than 10 000, are considered by some authors to be superior compared to the conventional triple quadrupole systems (QqQ) due to higher selectivity/specificity [3–5] and the possibilities of untargeted retrospective screening [6].

However, a recent inter-laboratory study [7] has shown that for difficult matrices such as liver, muscle, and urine HRMS measurements in a full scan mode are not sufficient for the purpose of confirmatory analysis and the highest selectivity is achieved by operating the mass spectrometer in selected reaction monitoring (SRM) mode, mimicking the way QqQ systems operate. It is important to enable the application of fragmentation ion ratio criterion in this scanning mode. However, the same study noted that the ion ratio is a "chink in the armor" of Orbitrap mass detectors, leading to a great number of false negatives, especially when non-selective fragmentation modes are applied. Thus, major deviations have been observed, partly due to the narrow (±30%) tolerance windows applied. In a study comparing the QqQ and Orbitrap MS systems for the analysis of veterinary drugs, it was noted by Kaufmann et al. that selective fragmentation in an Orbitrap system showed ion ratios ranging from 30 to 85%, which were deemed acceptable [8]. Changing the Commission guidelines in favor of HRMS methods has been proposed on the basis of an inter-laboratory study, as higher selectivity was achieved with the help of the highly accurate HRMS fragment transitions.

Numerous studies have used QqQ [9–13], quadrupole linear ion trap – tandem mass spectrometers (QqLIT-MS/MS) [14], and time-

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of-flight (ToF) [15] instruments for the analysis of anticoccidials. However, to our knowledge, in only one case these compounds were determined in pig feed by an Orbitrap detector [5]. The approach used in the aforementioned study was prone to false positive results, as the selectivity was based on high-resolution full scan measurements and only a narrow mass extraction window was applied, with no confirmatory mass transitions. These limitations arose from the design of the apparatus, as it was not a quadrupole mass spectrometer. In our study, the selectivity and confirmation were achieved by the application of selective data-dependent MS^2 fragmentation of isolated full scan precursors that produced a spectrum of daughter ions. The acquisition mode was further optimized to obtain a high quality MS^2 spectrum suitable for quantification.

Another objective during our study was to optimize the Orbitrap MS system by applying the design of experiments (DoE) approach in order to enhance the analytical signal. DoE is a cost-effective method for system optimization by which the effects of multiple parameters and parameter interactions on a given response can be measured in a few experiments. The traditional approach of one factor at a time (OFAT), where only one factor is varied at a time and the others are held constant while measuring the response, relies on the intuition as well as the practical and theoretical knowledge of the experimenter. Typically, the variables and factors of MS systems are interdependent and produce combined effects [16]. A previous study by Lemonakis et al. explored the factors affecting the performance of an LTQ Orbitrap system, but the Q Exactive HRMS system may be affected by other factors due to the absence of a linear ion trap. Munoz et al. described the optimization of detection conditions for the analysis of polyfluoroalkyl and perfluoroalkyl compounds employing a laser diode thermal desorption / atmospheric pressure chemical ionization detector coupled to a Q Exactive system [17]. In our case, statistical DoE was used to separately optimize the two main parts of Q Exactive HRMS system – the HESI source (ionization conditions) and the Orbitrap mass analyzer (detection conditions).

The present work was carried out in the context of extending the scope of analytes and improving the sensitivity and selectivity of the analytical procedure for the determination of anticoccidials in poultry and eggs. The developed method was expected to be cost-efficient, relatively fast, with a high selectivity and certainty for multiclass analysis of anticoccidials regulated by the EC. The development and optimization of this LC-HRMS method in order to obtain the most suitable performance characteristics is discussed in this article.

2. Experimental

2.1. Chemicals and materials

Amprolium (APL), clopidol (CLOP), decoquinate (DEC), monensin (MON), nequinate (NEQ), toltrazuril (TOL), toltrazuril sulfone (TOLS), and toltrazuril sulfoxide (TOLX) were obtained from Sigma-Aldrich, Germany. Diclazuril (DCZ), lasalocid (LAS), and salinomycin (SAL) were purchased from Dr. Ehrenstorfer, Germany. Halofuginone (HAL) was obtained from WITEGA Laboratorien Berlin-Adlershof, Germany. The standards of maduramicin (MAD), narasin (NAR), nicarbazin (as marker compound DNC), robenidine (ROB), and semduramicin (SEM) were provided by the European Union Reference Laboratory for Residues of Veterinary Drugs (EURL). Internal standards of nigericin (NIG) (Sigma-Aldrich), robenidine-d_8 (ROB-d_8) (EURL), decoquinate-d_5 (DEC-d_5), halofuginone-$^{13}C_6$ (HAL-$^{13}C_6$), toltrazuril-d_3 (TOL-d_3), DNC-d_8 (WITEGA) were used in our study. All the compounds used in this study were of purity of 95% or higher, except for MON, NAR (86%) and SAL (80%). All compounds were dissolved in acetonitrile or DMSO and three diluted working mix solutions (two native compound solutions for each matrix and one internal standard solution) were prepared in acetonitrile at the concentrations given in Table 1. Six intermediate native compound calibration solutions for standard addition samples for each matrix were prepared by diluting 50, 100, 200, 300, 400, and 600 μL of each working standard up to 1000 μL volume in acetonitrile.

Table 1
The tolerance limits for anticoccidial drug residues in poultry muscle and eggs and the working standard concentrations.

	Tolerance limit (μg kg^{-1})		Mixed working standard concentration (μg mL^{-1})	
	Poultry muscle	Eggs	Poultry muscle	Eggs
APL	Not listed	Not listed	12.5	2.5
CLOP	Not listed	Not listed	12.5	2.5
DEC	500 [a]	20 [b]	125	5
DCZ	500 [c]	2 [d]	125	0.5
HAL	Not listed	6 [b]	2.5	1.5
LAS	60 [e]	150 [e]	15	37.5
MAD	30/2 [d,f]	12 [d]	0.5	3
MON	8 [g]	2 [h]	2	0.5
NAR	50 [i]	2 [b]	12.5	0.5
NEQ	Not listed	Not listed	2.5	2.5
DNC	50 [d]	300 [d]	12.5	75
ROB	200 [j]	25 [b]	50	6.25
SAL	5 [b]	3 [b]	1.25	0.75
SEM	Not listed	2 [b]	2.5	0.5
TOL	100 [k]	Not permitted [k]	25	2.5
TOLS	100 [k]	Not permitted [k]	25	2.5
TOLX	100 [k]	Not permitted [k]	25	2.5
DEC-d_5	ISTDs	5	5	
DNC-d_8		12.5	12.5	
HAL-$^{13}C_6$		5	5	
NIG		5	5	
ROB-d_8		5	5	
TOL-d_3		5	5	

[a] Commission Implementing Regulation No. 291/2014.
[b] Commission Regulation No. 124/2009.
[c] Commission Implementing Regulation No. 115/2013.
[d] Commission Regulation No. 610/2012.
[e] Commission Implementing Regulation No. 1277/2014.
[f] Commission Implementing Regulation No. 388/2011.
[g] Commission Implementing Regulation No. 495/2011.
[h] Commission Implementing Regulation No. 59/2013.
[i] Commission Regulation No. 885/2010.
[j] Commission Regulation No. 101/2009.
[k] Commission Regulation No. 37/2010.

Acetonitrile, methanol, formic acid (all MS grade), and DMSO (anhydrous, 99.5% purity) were purchased from Merck, Germany. Aqueous solutions were prepared in deionized water (resistivity >18 MΩ cm) obtained by using a Milli-Q purification system (Millipore, Billerica, MA, USA).

2.2. Samples

Two types of poultry – chickens raised for laying eggs and broilers, as well as two types of eggs – quail and chicken eggs were used for method validation. The samples were collected at a local chicken farm near Riga, Latvia. No information was available regarding possible previous treatment of these animals with veterinary drugs. The method specificity and the occurrence of anticoccidials were studied using another set of a variety of egg and poultry samples collected within the State monitoring program performed by the Institute of Food Safety, Animal Health and Environment – "BIOR" (Riga, Latvia).

The samples were homogenized using a Ultra-Turrax® T25 homogenizer system (IKA, USA) and frozen (−21 °C) immediately after collection, and thawed in small batches.

2.3. LC-HRMS instrumentation and conditions

Chromatographic analyses were conducted on an UltiMate 3000 (Dionex, Olten/Switzerland) HPLC system using a Kinetex C_{18} (100 mm × 2.1 mm i.d., 1.7 μm) analytical column (Phenomenex, Torrance, CA, USA). The column and autosampler temperatures were held at 35 °C and 10 °C, respectively. The mobile phase for baseline separation consisted of A – water, B – acetonitrile, and C – methanol, with all components containing 0.1% of formic acid. The following gradient elution program was used: the initial pre-run composition with equilibration for 6.0 min prior to injection – 7% B; 0–4.0 min – 7–80% B; 4.0–4.1 min – 80–95% B; 4.1–6 min – 95–100% B; 2 min hold at 100% B; 8.0–8.5 min – 100% B to 100% C; 2 min column washing at 100% C; 11.5–12.0 min – return to the initial conditions. The flow rate was 0.300 mL min^{-1}. The total run time was 18 min. The injection volume was 4 μL. Acetonitrile-water (30:70, v/v) mixture was employed as the solvent system for filling the sample loop and washing the needle.

The chromatographic system was coupled to a Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a Heated Electrospray Ionization Source II (HESI II). The qualification and quantification analyses were performed under heated electrospray ionization conditions in full scan/data-dependent MS^2 (FS-ddMS^2) mode in two separate runs for negative and positive ionization. Full mass range scans were used for quantification and ddMS^2 – for confirmatory analysis. The optimized HESI II conditions were: sheath gas, 20 arbitrary units (a.u.); auxillary gas, 6 a.u. (positive ionization, +) or 10 a.u. (negative ionization, -); spray voltage, 4.5 kV (+) or -3.5 kV (-); capillary temperature, 265 °C (+) or 293 °C (-); heater temperature, 400 °C. In the FS-ddMS^2 mode the Q Exactive HRMS instrument performed a full scan followed by a dd-MS^2 scan. The full scan mass range was set to 100-1000 *m/z* with a resolution of 35 000 (FWHM at 200 *m/z*). The optimized automatic gain control (AGC) target was assigned a value of $5.0 \cdot 10^6$ (the maximum number of ions filling C-Trap) with the maximum ion injection time (IT) of 80 ms. For ddMS^2 scans, the precursor quadrupole isolation window was set to 0.6 *m/z*. The data-dependent settings (underfill ratio, apex trigger, charge exclusion, dynamic exclusion, excluded isotopes, and peptide match) were disabled. Selected precursor ions (Table 2) were admitted for the activation of MS^2 fragmentation with collision energies specified in the inclusion list of the software. The default charge state was set to 1. The multiplex and loop count were set to 1 and 2, respectively, as appropriate for a Top 2 ddMS^2 method. Orbitrap resolution was set to 17 500 FWHM, the AGC target at $1.0 \cdot 10^5$, and IT was set at 20 ms for the ddMS^2 scan period. The instrument was calibrated using Pierce LTQ Velos ESI positive and negative ion calibration solutions.

Thermo Scientific Xcalibur™ and TraceFinder™ software suites were used for both qualitative and quantitative assessment of the obtained data. The native compounds and isotopically labeled internal standards were quantified by measuring a ratio of peak areas in FS EICs. The ddMS^2 transitions (qualifiers) were used to confirm the identity of the native analytes, but did not contribute to the quantification results.

2.4. Sample preparation

Homogeneous samples (5.00 ± 0.02 *g*) were weighed in 50 mL polypropylene (PP) tubes. Internal standard mixture (100 μL, 5 μg mL^{-1} of all compounds, 12.5 μg mL^{-1} of DNC-d_8) was added to all samples. Matrix-fortified calibration samples were prepared by adding 100 μL of the intermediate calibration solutions to blank control samples resulting in a total of six calibration levels for each matrix. The calibration concentrations were prepared at 0.25, 0.5, 1, 1.5, 2.0, and 3.0 times the ML or MRL values. The samples were vortexed and equilibrated for 20–30 min, then diluted with acetoni-

Table 2
Mass spectral properties of the investigated analytes.

			Precursor ions (quantifiers)				Product ions (confirmatory)			
	ISTD	Charge	[M ± Adduct]	Monoisotopic exact mass (*m/z*)	Absolute mass error[b] (ppm)		NCE[a]	[M]	Ion 1 exact mass (*m/z*)	[M]	Ion 2 exact mass (*m/z*)
					Egg matrix	Poultry matrix					
APL	HAL-$^{13}C_6$	+	$[C_{14}H_{18}N_4+H]$	243.1604	3.6	4.6	10	$[C_8H_{12}N_3]$	150.1026	$[C_6H_8N]$	94.0651
CLOP	HAL-$^{13}C_6$	+	$[C_7H_7Cl_2NO+H]$	191.9977	4.4	3.9	90	$[C_5H_6Cl]$	101.0153	$[C_4H_4Cl]$	86.9996
DEC	DEC-d_5	+	$[C_{24}H_{35}NO_5+H]$	418.2588	4.7	4.1	35	$[C_{22}H_{32}NO_5]$	390.2275	$[C_{22}H_{30}NO_4]$	372.2169
DCZ	TOL-d_3	–	$[C_{17}H_9Cl_3N_4O_2-H]$	404.9718	0.2	0.2	25	$[C_{15}H_7Cl_3N_3]$	333.9711	$[C_{15}H_7Cl_2N_3]$	299.0023
HAL	HAL-$^{13}C_6$	+	$[C_{16}H_{17}{}^{80}BrClN_3O_3+H]$	416.0193	4.8	5.4	30	$[C_5H_{10}NO]$	100.0757	$[C_8H_{10}N]$	120.0808
LAS	HAL-$^{13}C_6$	+	$[C_{34}H_{54}O_8+Na]$	613.3711	5.3	3.6	35	$[C_{21}H_{38}NaO_4]$	377.2662	$[C_{34}H_{50}NaO_6]$	577.3470
MAD	HAL-$^{13}C_6$	+	$[C_{47}H_{80}O_{17}+Na]$	939.5288	4.6	5.8	20	$[C_{46}H_{78}NaO_{14}]$	877.5284	$[C_{46}H_{80}NaO_{15}]$	895.5389
MON	HAL-$^{13}C_6$	+	$[C_{36}H_{62}O_{11}+Na]$	693.4184	4.8	4.0	50	$[C_{25}H_{42}NaO_6]$	461.2874	$[C_{25}H_{44}NaO_7]$	479.2979
NAR	NIG	+	$[C_{43}H_{72}O_{11}+Na]$	787.4967	4.5	3.9	40	$[C_{23}H_{36}NaO_6]$	431.2404	$[C_{29}H_{48}NaO_7]$	531.3292
NEQ	HAL-$^{13}C_6$	+	$[C_{22}H_{23}NO_4+H]$	366.1700	6.5	4.8	30	$[C_{21}H_{22}NO_4]$	352.1543	$[C_{21}H_{20}NO_3]$	334.1438
DNC	DNC-d_8	–	$[C_{13}H_{10}N_4O_5-H]$	301.0578	0.1	0.6	10	$[C_6H_5N_2O_2]$	137.0346	$[C_3H_7O_4]$	107.0350
ROB	ROB-d_8	+	$[C_{15}H_{13}Cl_2N_5+H]$	334.0621	5.3	5.6	40	$[C_7H_5ClN]$	138.0105	$[C_7H_8ClN_2]$	155.0371
SAL	HAL-$^{13}C_6$	+	$[C_{42}H_{70}O_{11}+Na]$	773.4810	5.4	4.3	40	$[C_{23}H_{36}NaO_6]$	431.2404	$[C_{29}H_{48}NaO_7]$	531.3292
SEM	HAL-$^{13}C_6$	+	$[C_{45}H_{76}O_{16}+Na]$	895.5026	5.3	3.7	20	$[C_{44}H_{74}NaO_{13}]$	833.5022	$[C_{44}H_{76}NaO_{14}]$	851.5049
TOL	TOL-d_3	–	$[C_{18}H_{14}F_3N_3O_4S-H]$	424.0584	0.9	0.5	–	$[C_{18}H_{14}F_3N_3O_4S-H]$	425.0608	$[C_{18}H_{14}F_3N_3O_4S-H]$	426.0576
TOLS	TOL-d_3	–	$[C_{18}H_{14}F_3N_3O_5S-H]$	440.0533	0.8	0.4	–	$[C_{18}H_{14}F_3N_3O_5S-H]$	441.0557	$[C_{18}H_{14}F_3N_3O_5S-H]$	442.0526
TOLX	TOL-d_3	–	$[C_{18}H_{14}F_3N_3O_5S-H]$	456.0483	0.1	0	–	$[C_{18}H_{14}F_3N_3O_6S-H]$	457.0506	$[C_{18}H_{14}F_3N_3O_6S-H]$	458.0477
DEC-d_5		+	$[C_{24}D_5H_{30}NO_5+H]$	423.2902	4.5	4.4					
DNC-d_8		+	$[^{13}C_6C_{10}H_{17}{}^{80}BrClN_3O_3+H]$	422.0393	5.9	5.3					
HAL-$^{13}C_6$	–	–	$[C_{13}D_8H_2N_4O_5-H]$	309.1081	0	0.1	–				
NIG		+	$[C_{40}H_{68}O_{11}+Na]$	747.4654	6.1	4.8					
ROB-d_8		+	$[C_{15}H_5D_8Cl_2N_5+H]$	342.1123	6.8	5.9					
TOL-d_3		–	$[C_{18}D_3H_{11}F_3N_3O_4S-H]$	427.0773	0.9	0.8					

[a] The actual HCD energy is calculated on a basis of mass and charge of the selected precursor ion.
[b] Average mass error obtained at validation level for each matrix during 4 day validation period. (n = 8).

Table 3
The optimization steps performed and statistical methods used in the study.

	Parameter	Abbrevation	−1	0	1
1. Optimization of HESI conditions (BBD)	Sheath gas flow[a]	A	20	40	60
	Auxiliary gas flow[a]	B	6	12	18
	Spray voltage, positive (kV)	C_1	+2.5	+3.5	+4.5
	Spray voltage, negative (kV)	C_2	−1.5	−2.5	−3.5
	Capillary temperature (°C)	D	250	300	350
	Auxiliary gas temperature (°C)	E	300	350	400
2. Optimization of mass spectrometer parameters (BBD)	Resolution	F	35 000	70 000	140 000
	AGC	G	$5.0{\cdot}10^4$	$5.0{\cdot}10^5$	$5.0{\cdot}10^6$
	Injection time (ms)	H	20	100	180
		Variables			
3. The effect of injection time in the presence of matrix (OFAT)	Injection time (ms)	20	40	60	80
		100	120	140	180

[a] Arbitrary units.

trile (20 mL) and vortexed again for 10 s. The samples were agitated in a rotary shaker at high speed for 20 min and then centrifuged for 15 min at 4750 rpm while cooling at 0 °C, followed by rapid (up to 5 min) transfer of 10 mL aliquots to 15 mL PP vials. The sample extracts were found to be the most stable at this point and could be kept in a freezer (-20 °C) for at least one week. The extracts were then evaporated to dryness under nitrogen stream at 60 °C using a Turbovap LV system and then reconstituted in 500 μL solution of acetonitrile-aqueous sodium acetate (5 mM) (30:70, v/v). The samples were vortexed (1 min) and sonicated (5 min) prior to the LC-HRMS analysis. The final extracts were found to be stable for two days at −21 °C.

2.5. Procedures

A stepwise plan of all procedures performed in this work is shown in Fig. S1.

2.5.1. Stepwise optimization

In order to obtain the optimal performance of mass spectrometer, such as signal stability and sensitivity, a chemometric approach was applied by using the MiniTab 17 software (Minitab, State College, PA, USA). The optimization strategy consisted of several steps, during which particular experiments were performed.

The compound-specific collision energies were optimized by adding the individual standards at 1 $\mu g\,mL^{-1}$ concentration in reconstitution solvent with a syringe at a flow rate of 5 $\mu L\,min^{-1}$. Using the Q Exactive tune program, the precursor ions of the corresponding anticoccidial drug residues were selected by setting the quadrupole in the t-MS/MS mode and the intensities of product ion signals were assessed by increasing the Normalized Collision Energy (NCE). The value of NCE was optimal when complete fragmentation of precursor ions occurred. The monoisotopic masses of the monitored ions and the optimal NCE values are shown in Table 2. The LC conditions were defined at this point by selecting the appropriate mobile and stationary phases to achieve the necessary resolution and reasonable chromatographic peak shape.

The HESI II ionization source parameters and Orbitrap MS detector conditions were optimized using Box-Behnken response surface designs (BBD). The campaign of experiments for optimization of ion source parameters involved 5 different factors at 3 levels, for a total of 46 runs. The Orbitrap parameters (AGC, IT, and resolution) were optimized at three levels for a total of 15 runs. A total of 5 replicate runs were planned for the ion source and 3 replicate runs for the optimization of Orbitrap parameters. The experiments were conducted in both positive and negative ionization modes and in randomized order.

The ion source and Orbitrap analyzer conditions were optimized using analytical standards at 20 $\mu g\,kg^{-1}$ concentration. The injection time was studied by one factor at a time (OFAT) optimization using matrix-matched standards at 20 $\mu g\,kg^{-1}$ concentration for both matrices. The experimental factors were tested by both BBD and OFAT approaches. The optimization experiments are compiled in Table 3.

2.5.2. Validation of the method

To evaluate the performance and suitability of the developed LC-HRMS method for the determination of anticoccidial veterinary drugs, it was validated according to the predefined criteria summarized in Table 4. A matrix-comprehensive in-house validation concept was applied using the InterVAL software according to 2002/657/EC [1] for a quantitative confirmatory method.

The validation levels for poultry and eggs were chosen according to the ML and MRL values of the target anticoccidial drugs in the relevant European Commission regulations and were set at 0.5, 1, 1.5, and 2 times the ML and MRL values. Substances without defined MRL were validated at 5, 10, 15, and 20 $\mu g\,kg^{-1}$ concentrations. The whole validation study for each matrix type consisted of 8 replicate standard addition experiments, which were performed by two operators over two days. Fresh matrix calibration series and three matrix blank samples fortified with the internal standard were additionally prepared on both days in order to prove the specificity and the lack of susceptibility to matrix interference during the validation. Four validation levels per experiment, two calibration and blank series resulted in a total of 50 samples per each type of eggs (chicken and quail eggs) and 50 samples per each poultry (chickens raised for laying eggs and broilers) matrix. Additional selectivity evaluation experiments were performed post-validation using different samples of eggs ($n = 20$) and meat ($n = 5$) to further ensure the robustness of the method.

The stability of anticoccidials in spiked (20 $\mu g\,kg^{-1}$) sample extracts was evaluated by using reconstituted extracts. The extracts were stored at -20 °C and monitored for a week, with aliquots analyzed after 1, 2, 4, and 7 days. A fresh reference sample was prepared on each day of analysis to evaluate and compare the recoveries. Five sample injections were performed and a narrow recovery threshold of -20 to +10% was applied, taking into account a previous study involving such evaluation [18].

3. Results and discussion

3.1. Sample extraction

During the initial stage of method development, a convenient sample preparation procedure for routine samples was optimized. Since poultry and eggs contain large amounts of problematic matrix components such as fats and proteins, some basic clean-up is mandatory. Considering the previous studies [13,14,19–23], ace-

Table 4
Validation criteria.

Validation parameter	Requirement/Criteria		
Selectivity	No interfering peaks at the defined analyte RT and a confirmatory fragmentation scan.		
Decision limit (CCα) and detection capability (CCβ)		Set validation levels from lowest ML/MRL (μg kg^{-1}) Egg matrix	Poultry meat matrix
	APL	10	50
	CLOP	10	50
	DEC	20	500
	DCZ	2	500
	HAL	6	10
	LAS	150	60
	MAD	2	2
	MON	2	8
	NAR	2	50
	NEQ	10	10
	DNC	300	50
	ROB	25	200
	SAL	3	5
	SEM	2	10
	TOL	10	100
	TOLS	10	100
	TOLX	10	100
	$CC_\alpha = ML/MRL + 1.64 \cdot (RSD_{wR})$ (1) $CC_\beta = CC_\alpha + 1.64 \cdot (RSD_{wR})$ (2) ,where *ML/MRL*- maximum limit/maximum residue limit (μg kg^{-1}); RSD_{wR} - standard deviation of the within-laboratory reproducibility at validation level.		
Recovery	−30 to +10% at concentration range 1–10 (μg kg^{-1}); −20 to +20% at concentrations higher than 10 (μg kg^{-1}).		
Linearity	$R^2 > 0.90$, where R^2 - coefficient of determination of calibration graph.		
Reproducibility / repeatability	< 45% at concentration range 1–10 (μg kg^{-1}); < 32% at concentration range 10–100 (μg kg^{-1}); < 23% at concentration range 100–1000 (μg kg^{-1}); < 16% at concentrations higher than 1000 (μg kg^{-1}).		
Uncertainty	$U = f(RSD_{S_{wR}}, U_{Corrected\ Recovery})$ (3) , where RSD_{wR} – standard deviation of the within-laboratory reproducibility at validation level; $U_{CorrectedRecovery}$ – uncertainty of recovery correction.		

tonitrile was chosen as the extraction solvent and partitioning clean-up at moderately low temperature (0 °C) was applied.

3.2. Liquid chromatography

The selection of UHPLC method and the choice of specific conditions were influenced by previous studies [5,12,23]. First, we tested a gradient system of 0.5% formic acid in water and methanol systems [5], which led to a good overall performance, except for polyether ionophore anticoccidials that showed poor results, including notable tailing. A modified method developed by Pereira et al. [12] was found to be applicable in our case. Excellent peak shapes for all compounds were observed using a Hypersil Gold (50 mm × 2.1 mm, 1.9 μm particle size) column, but co-elution of some peaks occurred. A longer Kinetex C18 column (100 mm × 2.1 mm, 1.7 μm particle size) was chosen as a compromise. The selected gradient program produced excellent peak shapes and chromatographic separation, while also effectively removing the matrix components and thus reducing the noise level, decreasing the risks of carry-over effect and minimizing column deterioration. During the method development, validation and sample analysis, the retention time of analytes did not deviate by more than ± 0.1 min.

A solution of acetonitrile-aqueous sodium acetate (5 mM) (30:70, v/v) was used as the injection solvent. Polyether ionophore anticoccidials are known to favor sodium adducts [23,24] and a significant increase in signal was observed. Another reason in favor of using this solution was the increased stability of analytes under mildly basic (pH = 7–8) conditions [23]. The injection volume of 4 μL was found to be satisfactory for balancing the sensitivity and selectivity of the method.

3.3. MS conditions

TOL, TOLS, and TOLX did not produce suitable fragment ions while using the electrospray ionization interface. Previous studies have shown that the confirmatory fragmentation criteria for these three compounds can be met by MS systems operating in atmospheric pressure chemical ionization mode [25]. In our work, however, the confirmation of species derived from TOL was possible by using full scan spectra and confirming the presence of monoisotopic target ions with the expected isotope pattern and applying a narrow (10 ppm) window of mass tolerance to all of the aforementioned species.

3.4. Optimization of MS parameters

The responses were assessed as absolute peak areas in arbitrary units. A series of diagnostic tests (e.g., residual normal probability test, residuals vs. run order, histograms) did not indicate the presence of any significant outliers which would indicate experimental errors for any of the statistical DoE. BBD and OFAT optimization parameters are shown in Table 3. The analysis of variance (ANOVA) results for all of the factor interactions along with the final coefficients for BBD equations in terms of actual and coded response factors regarding all of the analytes both for the ion generation

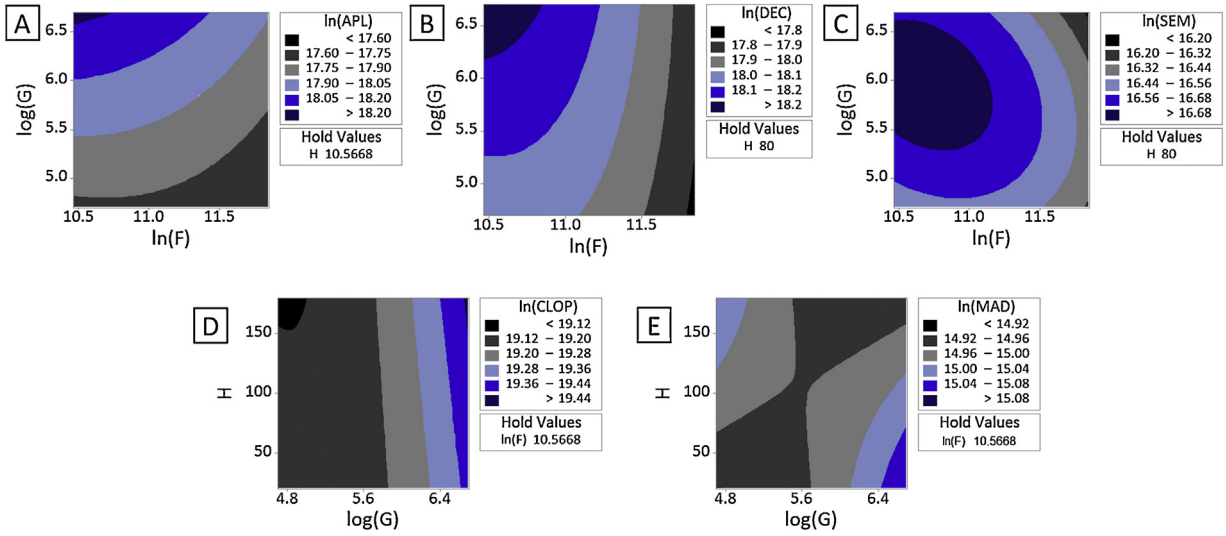

Fig. 1. Contour plots illustrating the most significant interactions of parameters and their effects on the Orbitrap detector response, where dark blue area represents the highest response. Plots: (A) – APL signal response plotted versus resolution (ln(F)) and AGC (log(G)) value, (B) – DEC vs ln(F), log(G), (C) – SEM vs ln(F), log(G), (D) – CLOP vs log(G), injection time (H), (E) – MAD vs log(g), H. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

region and the optimization of analyzer are tabulated in the Supplementary Workbook.

3.4.1. Source conditions

The HESI source factors exerting significant effects on the MS response were identified through simultaneous batch screening and optimization experiments using a BBD model. Five main source parameters – the sheath (A) and auxiliary (B) gas flow rates, spray voltage (C1 for positive and C2 for negative ionization), capillary (D) and auxiliary gas heater temperatures (E) were considered as independent variables. A wide range of values were chosen and the original Orbitrap software auto-tune settings were selected as the center point of BBD. The BBD models showed a good fit for most of the compounds analyzed, with $R^2 > 0.9$ in most of the cases. Significant lack-of-fit was observed for MAD ($p < 0.001$), MON ($p < 0.001$), and NAR ($p < 0.05$), indicating that the model did not fit the data within the observed variations among replicate samples. The fit values were insignificant for other compounds, showing a good fit with the experimental model. The terms were found to be significant at 95% confidence interval with the p values of the F-test being lower than 0.05 (statistically significant) or 0.001 (statistically highly significant).

The experimental study showed differences in the most significant parameters depending on the ionization modes used. For all of the compounds analyzed in positive ionization mode, ANOVA allowed to identify three parameters (A, B, and C1) and the corresponding linear terms that most affected the response with a high statistical significance ($p < 0.001$). Four out of 11 compounds were also significantly affected by parameters D and E. As for the squared interactions, 9 compounds out of 11 showed significant ($p < 0.05$) interaction for A^2, 5 compounds out of 11 for B^2, 4 compounds out of 11 for ${C_1}^2$ and D^2, and E^2 was found to be significant for HAL. Response surface curvature was found for some 2-way interactions and ANOVA confirmed the significance of BC (auxiliary gas flow × spray voltage) interactions for 7 compounds, as well as AB, AD, AE, and BD interactions for 2 compounds. The AC, BE, and DE interactions were significant for only one compound in each case.

The significance of the linear term A in the case of negative ionization was observed with 5 compounds out of 6, and 4 compounds out of 6 for the term C. The linear terms of B, D and E had a significant impact on individual compounds. The squared interaction of ${C_2}^2$ was evident for 3 compounds out of 6, while A^2 and D^2 affected 2 compounds. Statistically significant ($p < 0.05$) and visible (surface curvature) 2-way interactions were AB and AE for 3 compounds out of 6; BC for 2 compounds out of 6; AC and DE for 1 compound each.

The optimal source parameter values were established separately for positive and negative ionization by using the integrated response optimizer function. The target compound responses were optimized simultaneously with the goal to maximize the intensities and to achieve the highest composite desirability. The overall value of composite desirability combines the individual desirability of compounds and reflects the relative importance of the responses. The higher the desirability, the closer it is to 1 (measured on a scale from 0 to 1). Composite desirability of 0.9922 was achieved in positive ionization mode and 0.9135 in negative ionization mode for 11 and 6 target compounds, respectively. The maximum simultaneous responses for all compounds in positive ionization mode were obtained with the following parameters: sheath gas flow rate 20 a.u., auxiliary gas flow rate 6 a.u., capillary voltage + 4.5 kV, capillary temperature 265 °C, auxiliary gas heater temperature 400 °C. The finalized parameter values in negative ionization mode were: sheath gas flow rate 20 a.u., auxiliary gas flow rate 10 a.u., capillary voltage −3.5 kV, capillary temperature 293 °C, auxiliary gas heater temperature 400 °C. The desirability contour plots for anticoccidials and their residues analyzed in positive and negative ionization modes are shown in the Supplementary Fig. S2a and b, respectively. Another way to interpret the data is shown in Tables S1 and S2, which contain center point levels of source variables for methods used in the optimization and the resulting relative responses and RSD values for HESI ionization source conditions according to the BBD design.

3.4.2. Orbitrap detector conditions

The AGC values were expressed as decimal logarithms as they differed from each other by a factor of 10 (Log(G), further G) and the resolution values were normalized using natural logarithm so the difference between the levels was constant (Ln(F), further F). Center-reducing the data yielded [−1; 0; +1] values that were expected to fit in the BBD levels. The rest of the variables for the MS system, which were found in the previous steps, were set at the values optimized for these experiments.

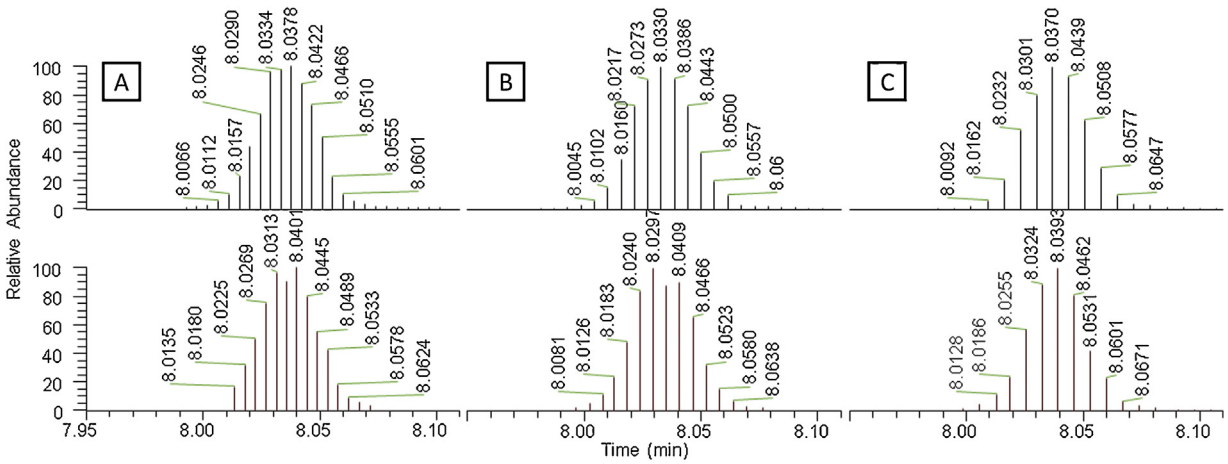

Fig. 2. The effect of ddMS^2 method loop count (N) on the number of scans per peak in full scan (first row) and data-dschemeependent MS^2 (second row). Loop count: (A) – 1, (B) – 2, (C) – 3.

The BBD models did not fit as well for the factors associated with the mass analyzer as for those of the ionization source, with generally lower R^2 values observed. Significant ($p < 0.05$) lack-of-fit was observed for APL, CLOP, MON, while the lack-of-fit was very significant ($p < 0.001$) for DEC and NEQ. Otherwise, the statistical model performed quite well. The terms were found to be significant at 95% confidence interval, with the p values of the F-test being lower than 0.05 (statistically significant) or 0.001 (statistically highly significant).

Yet again, various parameters affected the response, depending on the ionization modes used. The BBD models revealed that the most significant linear term affecting the response in positive ionization mode was resolution ($p < 0.001$ for 7 compounds out of 11; $p < 0.05$ for 3 compounds; no significance of resolution setting for 1 compound). Among the squared terms, F^2 was found to be significant ($p < 0.001$ for 7 compounds; $p < 0.01$ for 1 compound; $p < 0.05$ for 3 compounds) and the 2-way term of FG showed the p-values of < 0.01 for APL and DEC and < 0.05 for SEM.

The terms G and G^2 were found significant for 4 compounds with *p*-values <0.01. The 2-way interaction of GH was significant for CLOP and MAD, with the *p*-values of <0.05 and <0.01, as shown by the contour plots of interactions in Fig. 1D and E, accordingly. Longer injection times were favored in the case of CLOP, but not in the case of MAD, as it can be seen both in the Fig. 1E contour plot and Fig. S3a desirability plot.

For compounds analyzed in the negative ionization mode, the change of terms had barely any effect on the total response according to ANOVA. ROB was the only compound affected by the linear terms of F and G, the squared terms of F^2 and G^2, and the 2-way term FG ($p < 0.001$). As a matter of fact, the collection time of ions or the injection time (H) in both the positive and negative ion modes had no significant influence on the signal intensity. C-trap typically reached the AGC target value in fewer milliseconds than set, before the time interval allotted for ion gathering. This observation conforms with the findings by Lemonakis et al. [26] who showed that suppression effects can occur in an Orbitrap MS analyzer in the presence of a difficult matrix with barely any clean-up.

The optimal values were established separately for the positive and negative ionization modes, as two separate runs were required for the analysis. The target compound responses were optimized simultaneously with the goal to maximize the signal intensities and to achieve the highest composite desirability. The composite desirability was achieved with a desirability coefficient of 0.8740 in the positive ionization mode and 0.8386 in the negative ionization mode for 11 and 6 target compounds, respectively. The optimization results were quite similar for both ionization modes – the response was the highest when the resolution was kept low (35 000 FWHM), the AGC was set to represent the maximum loading capacity of the C-trap (target of $5{\cdot}10^6$ ions), and IT was kept low (20 ms for positive and 26 ms for negative ionization). The effect of resolution on the Orbitrap sensitivity was an interesting finding that was backed up by statistically significant experiments. A plausible explanation could be the loss of ion energy during the travel time and distance, which is more pronounced at high resolution. This observation could be particularly relevant when trying to achieve the required MRLs for specific compounds at low concentrations. We did not find a correlation between the mass to charge ratios of particular compounds and the observed effect and additional studies may be needed to further explore this phenomenon. If the aim is to look for a specific analyte in a pool of isobaric compounds, the selectivity still can be improved by using fragmentation mode transitions to compensate for the relatively low resolution.

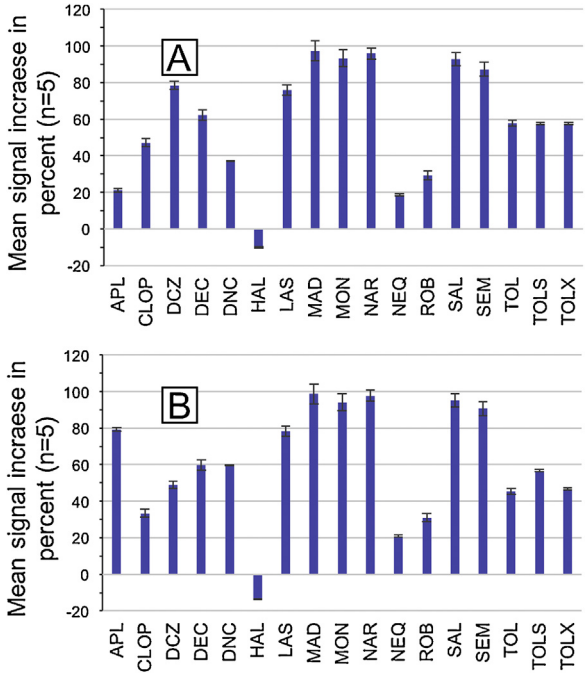

Fig. 3. Mean signal increase after the performed optimization procedures; (A) – spiked (20 μg kg^{-1}) egg sample, (B) – spiked (20 μg kg^{-1}) poultry meat sample.

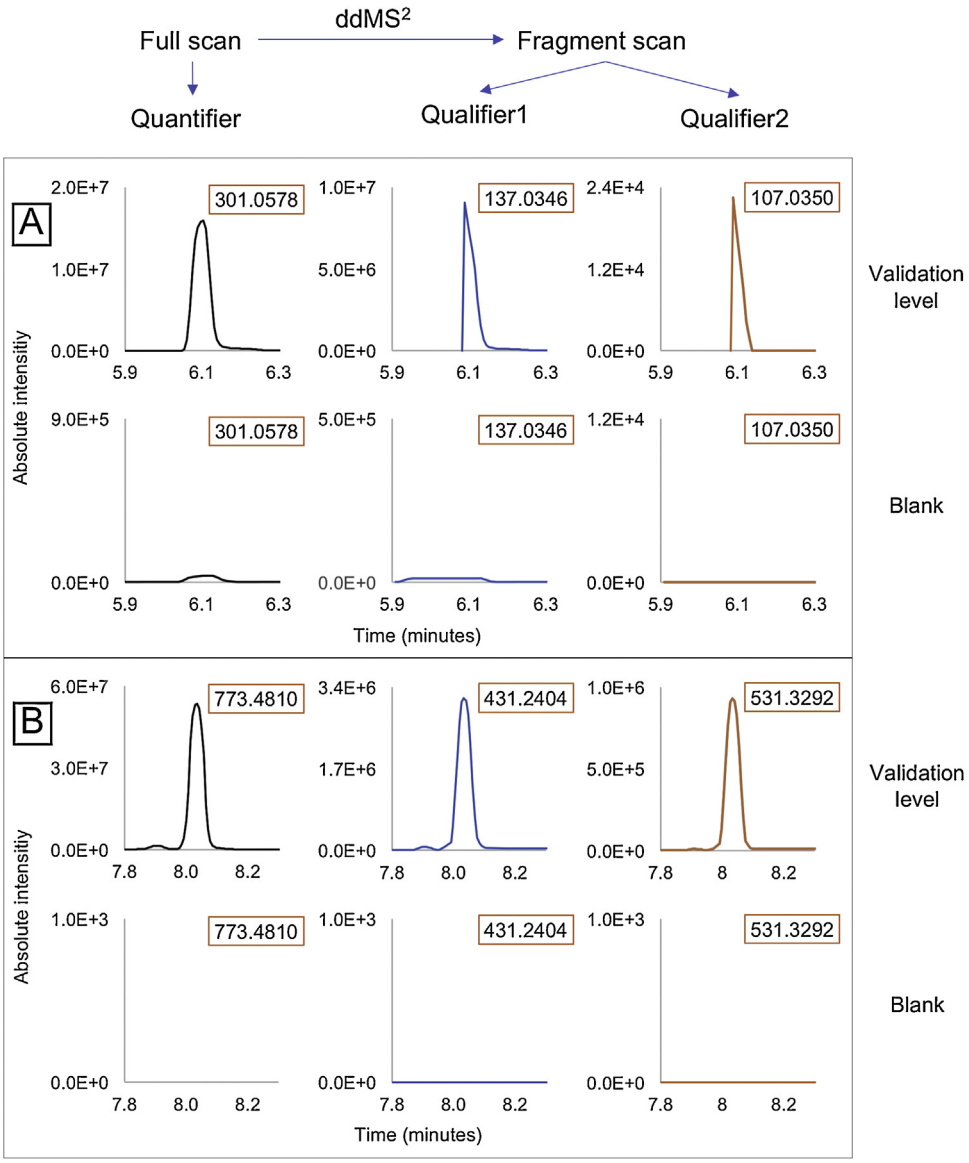

Fig. 4. Scheme of FS-ddMS^2 data acquisition. Extracted ion chromatograms for quantifier and qualifier ions for DNC (A) and SAL (B) at the validation and blank concentration levels in egg matrix are displayed.

The desirability contour plots for Orbitrap detector optimization are shown in the Supplementary Fig. S3a and b for anticoccidials and their residues analyzed in the positive and negative ionization modes, respectively. Table S3 contains the methods and levels with the corresponding relative responses and RSD values used for the optimization of Orbitrap detector conditions using the BBD design.

3.4.3. *Optimization of the ion injection time in the presence of matrix*

The injection time was further optimized by the means of OFAT experiments through a stepped increase of injection time over the range from 20 ms to 180 ms, same as previously done in BBD experiments, but this time including the food matrix. Poultry and egg samples spiked at 20 $\mu g\,kg^{-1}$ level were analyzed in five replicates for each injection time. The values were normalized against the responses obtained for injection time of 20 ms (Figs. S4 and S5). The observations for egg and poultry matrices were the same. In positive ion mode, a small suppression of signal was observed ranging from 0.5 to 3%, with the RSD values not significantly influenced by the change of injection time. On the other hand, a small but notable increase of signal by 0.5% occurred in negative ion mode for all of the analyzed compounds, while the RSD also tended to vary less. In the presence of egg matrix, the compound most affected by the injection time was MAD, due to the particularly strong signal of co-eluted unidentified matrix components observed in TIC. Short injection times (20–40 ms) produced jagged peaks and occasionally even loss of signal, so we preferred 80 ms injection time both for the positive and negative ionization modes.

3.4.4. *Optimization of data-dependent acquisition*

Data-dependent scans were introduced as a source of fragmentation criteria and used to further reduce the chance of false positive results. The ddMS^2 events occur when there is a precursor ion detected in the full scan measurement. The following scan cycles include the fragmentation scan events of the detected precursors. A single data-dependent scan provides full scan spectra of an isolated and fragmented precursor ion, which contains confirmatory fragment ions. Rajski et al. described the overall FS-ddMS^2 workflow superiority over other confirmation modes, with greater repeatability at low concentration levels and equal selectivity than that of

Table 5
The validation results for fortified egg and poultry meat samples.

	Egg matrix							Poultry matrix						
	MRL/ML level (μg kg^{-1})	[a]RSD S_r (%)	[b]RSD S_{wR} (%)	Recovery (%)	Total uncertainty [c] at VL (%)	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	MRL/ML level (μg kg^{-1})	[a]RSD S_r (%)	[b]RSD S_{wR} (%)	Recovery (%)	Total uncertainty [c] at VL (%)	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
APL	10	8.4	10.3	96.8	10.5	12	15.8	50	6.9	8.6	101.9	8.7	58.5	73.6
CLOP	10	20.3	20.9	97.7	21.3	13.8	>20.0	50	5.4	10.8	99.2	11.3	62.8	90.2
DEC	20	14.5	15.3	96.3	15.6	26.2	37.6	500	5.5	7.7	105.2	7.8	577	721
DCZ	2	11.8	12.8	105.8	13	2.54	3.54	500	8	9.4	100.8	9.5	589	749
HAL	6	5.5	7.5	97.2	7.5	6.79	8.21	10	5.5	7.5	99.2	7.5	11.4	13.9
LAS	150	10.4	11.6	97.8	11.8	183	255	60	11.8	13.4	102	13.6	77.9	109
MAD	2	15.6	16.4	99.2	16.8	2.64	3.78	2	19.4	20.1	91.6	20.4	2.64	3.74
MON	2	2	5.4	100.5	5.4	2.2	2.58	8	12.6	13.6	97.3	13.8	9.95	14.2
NAR	2	13.5	15	99.9	15.3	2.55	3.52	50	6	8.4	102.6	8.5	58.4	75.1
NEQ	10	6.8	8.6	99.5	8.7	11.6	14.4	10	6	7.8	105.7	7.9	11.5	14.1
DNC	300	3.6	6.1	101.2	6.2	336	401	50	3.6	6.2	104.4	6.3	56	67.2
ROB	25	8.9	10.2	102.9	10.4	30.1	39.3	200	7.3	9.7	99.9	9.9	238	310
SAL	3	12.7	13.6	101.2	13.9	3.77	5.13	5	9.4	11	99.1	11.2	5.97	7.79
SEM	2	19.7	20.7	94.1	21.1	2.71	3.96	10	14.3	15.1	97.2	15.4	12.9	17.9
TOL	10	1.4	5.2	101	5.2	12.5	12.9	100	1.2	5.1	100.2	5.2	110	129
TOLS	10	11.7	12.8	102.9	13	12.5	16.9	100	7.9	9.4	101.5	9.5	118	150
TOLX	10	10	11.2	99.6	11.4	12.2	16	100	7.4	8.9	102.5	9	117	147

[a] RSD S_r - repeatability.
[b] RSD S_{wR} - within-laboratory reproducibility.
[c] Total uncertainty takes into account the within-laboratory reproducibility standard deviation and additionally the uncertainty of recovery correction.

targeted single reaction monitoring (SRM). It was also superior to other non-targeted Orbitrap methods (all ion fragmentation (AIF) and variable data-independent analysis (vDIA)) [27]. Similar work has been done by Kumar et al. [4].

The ddMS^2 procedure was optimized by taking account the number of co-eluting analytes, which correspond to the parameter *N* in Top *N* approach. Since the maximum number of co-eluting analytes was 2 and the other analytes were baseline separated, the loop count was set to 2, thus making the method a Top 2 approach. The number of scans acquired per very narrow 3.2 s peak of SAL using a Top 2 approach is illustrated in Fig. 2B. The total scan cycle time was 340 ms, allowing for one full scan and two fragmentation scans per cycle. The peak was covered by 9 full scans for quantitative purposes and 9 fragmentation scans for qualitative purposes. When the loop count was set to 1, the Q Exactive instrument prioritized the ddMS^2 scan for the precursor ion of the highest intensity (Fig. S6). In the case of signal overlap between different compounds, this feature increased the chance of missed confirmatory scans. Thus, even though more scans were acquired, there was a slight chance of a false negative result. It should be noted that the ddMS^2 data can also be used for quantitation purposes in the case when full-scan interference is present. Fig. 4. illustrates the scheme of FS-ddMS^2 data acquisition and gives an example for the achieved selectivity of DNC and SAL, in positive and negative ionization modes, accordingly.

The ddMS^2 workflow also enabled the specificity needed for confirmation using the ion ratios. The non-targeted fragment acquisition modes such as AIF and vDIA were susceptible to altered ion ratios in the presence of isobaric fragmentation ions, while the selectivity of ddMS^2 was improved tremendously due to the narrow (0.6 *m/z*) isolation window of quadrupole. The known fragmentation patterns of precursor ions enabled the use of intensity ratios of two fragment ions determined in preliminary experiments as confirmation criteria.

The isolation of fragment ions was performed by the means of inclusion list of specified target compound masses, retention times, and compound-specific collision energies. The data-dependent scan was set to trigger when the accurate mass of precursor ion was detected within the bounds of 10 ppm. The fragment ion ratio measurements during the method development, validation and sample analysis were within ±20% from the original ratios observed in the standard solutions, thus the HCD-induced ddMS^2 fragmentation was deemed to be a suitable confirmatory tool. Table S4 contains the calculated ion ratios within the ddMS^2 scanning mode, observed in the standard sample analysis.

3.4.5. Results of the optimization

Numerical optimization steps led to an impressive overall signal improvement for most of the compounds, even in the presence of either poultry meat or egg matrices. Repeated analysis of spiked (20 μg kg^{-1}) sample extracts showed 10–99% increase of signal intensity for 16 out of the 17 anticoccidials, as illustrated in Fig. 3. A minor (10%) decrease of signal intensity was observed for HAL, but it was more than compensated by the significant gains of sensitivity obtained for other analytes.

3.5. Analytical method validation

The optimized methodology was validated to confirm its applicability for routine analysis of samples and to assess the reliability of results. The parameters evaluated were repeatability, within-laboratory reproducibility, recovery, selectivity, CCα, and CCβ. The results of this validation fulfilled the defined validation criteria and are summarized in Table 5. The recovery was corrected using isotopically labeled internal standard addition and matrix calibration to account for possible matrix effects. After the additional selectivity evaluation experiments, no significant interference was observed at the expected retention time of analytes after the analysis of negative samples (n = 20 for eggs, n = 5 for poultry meat). The final sample extracts were found to be stable for 2 days, after which the recoveries for some of the analytes dropped below 80% when compared to freshly prepared sample extracts. The results are in agreement with those of Spisso et al. [23]

Overall, the method covered a wide and unique range of analytes, with only a couple of previously published methods providing such scope for the analysis of multiclass anticoccidials in poultry and eggs.

3.6. Method applicability

The present method has been applied to eighty-one egg samples and three poultry muscle samples over a 5-month time, as a part of the 2017 Latvian national monitoring program. The results are displayed with uncertainty. One egg sample was found to be non-compliant, with 102 ± 7 μg kg^{-1} concentration of TOLS, along with TOL and TOLX at the levels of 10.2 ± 0.5 and 4.0 ± 1.1 μg kg^{-1}, respectively. The same sample contained DNC at the level of 10.1 ± 4.1 μg kg^{-1}. Among the rest of the samples, five contained the target compounds at levels lower than the MRL. DNC was found in 3 samples at the levels of 1.4 ± 0.6, 49 ± 6, and 133 ± 11.9 μg kg^{-1}. Another two samples contained TOL and its metabolites. The first sample contained 7.0 ± 1.2 μg kg^{-1} of TOLS, 1.9 ± 0.4 μg kg^{-1} of TOL, with trace levels of TOLX detected. The second sample contained 2.9 ± 1.4 μg kg^{-1} of TOLS and trace levels of TOL and TOLX. One sample contained 2.4 ± 0.4 μg kg^{-1} of SAL and 1.8 ± 0.3 μg kg^{-1} of NAR.

4. Conclusions

We have developed and successfully implemented a new HRMS method for the quantification of anticoccidials in poultry meat and eggs. The method has been optimized and validated according to the EU Commission Regulation 2002/657/EC.

A step by step optimization procedure has been employed in our work and represents a systematic approach for the optimization of Orbitrap MS parameters. We have highlighted the significance and influence of a wide range of parameters on the MS response to selected anticoccidials. Our design of experiments allowed to find the most suitable values of parameters and to enhance the signal intensity by 10%–99% for 16 out of the 17 analyzed compounds.

High selectivity was achieved by the application of FS-ddMS^2 workflow with an inclusion list, acquiring simultaneous full scan and MS^2 spectra. FS detection was chosen over targeted quantification (t-SIM and PRM), to allow for further broadening of the scope of veterinary drugs analyzed and to enable the merging of distinct sample preparation methods. The data-dependent mode was optimized to achieve an average of 9 confirmatory MS^2 scans across narrow (less than 4 s) target compound peaks. Hence, the spectra obtained in ddMS^2 mode could be used not only for confirmation, but also for quantification.

The main advantage of the proposed method is the relatively quick sample preparation procedure with indisputable confirmatory abilities. Thus, it represents a valid method for the analysis of multiclass veterinary anticoccidial drug residues, suitable for screening and quality control programs and occurrence studies.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.10.056.

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