Customer Application Note 116

Trace Level Determination of Eleven Antiprotozoal Agents in Eggs after Simple Matrix Clean-Up

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Key Words
Automated On-Line SPE, LC-MS/MS, Food analysis, Coccidiostats, Polyether Ionophores, SolEx HRP, Acclaim PA2 Column

Goal
Determination of eleven coccidiostats in chicken eggs at trace level as listed by the European legislation after simple matrix clean-up and automated on-line SPE.

Introduction
Coccidian protozoa are unicellular parasites of the genius *Eimeria* in the class *Sporozea*. They have a significant economic impact on high-volume commercial poultry farming by causing coccidiosis, a disease of the intestinal tract of animals. The primary symptom is bloody diarrhea which can lead to death, especially for young and immunodeficient hens. Coccidiosis is highly contagious through contact with infected feces and supported by the humid and warm conditions of intensive farming. Hence, coccidiosis is able to affect the entire breeding stock within a few days.

Coccidiostats are antiprotozoal agents preventing coccidiosis and, in most cases, are licensed for use as poultry feed additives in a prescribed concentration and during a certain time interval. According to Regulation 1831/2003/EC of the European Union[1], eleven coccidiostats are authorized for use: decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, nicarbazin, robenidine, salinomycin, and semduramicin. Most coccidiostats are naturally produced by streptomyces and belong to the group of polyether ionophores. Nicarbazin is a fifty-fifty mixture of 4,4-dinitrocarbanilide and 4,6-dimethyl-2-hydroxypyrimidine, the latter one being excreted rapidly. Consequently, residue analyses for nicarbazin are based on 4,4-dinitrocarbanilide.

According to Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of the European Union[1] and Council Directive 70/524/EEC[2], coccidiostats are allowed for laying hens during their epitaxial growth

(16 weeks from birth), unless these eggs are intended for human consumption; except for lasalocid with a maximum residue limit of 150 µg per kilogram wet weight of chicken eggs as described in Regulation 37/2010/EC.[3] Although coccidiostats have been banned by the European Union, residues are frequently found in chicken eggs. In addition to illegal use, contamination of supposedly drug-free feed can be caused by carry-over from production or accidental cross-contamination during feeding.

Maximum residue limits of coccidiostats in chicken eggs, resulting from unavoidable carry-over, are listed by Regulation 124/2009/EC.[4] With the extensive use and diversity of coccidiostats, a suitable method must be available for detecting limits of residue occuring from improper use or cross-contamination. Most of the current HPLC methods succeed in the determination of one or more coccidiostats in eggs, with only a handful of multi-residue methods known. While all methods use LC-MS/MS as the analytical tool, none of these methods allow quantification of all of the coccidiostats of concern by the European legislation in a single analytical run.[5–8]

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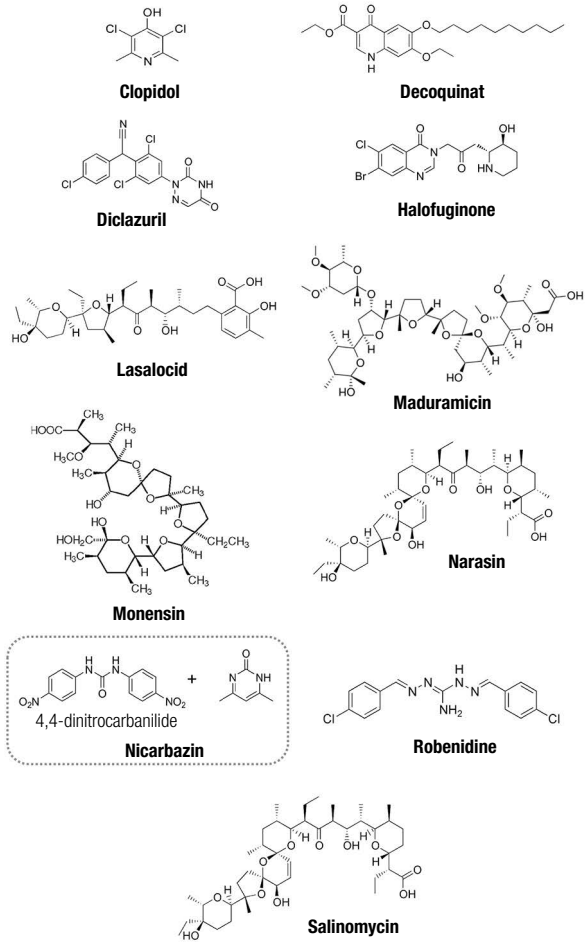

Figure 1. Chemical structures of coccidiostats.

The application shown here is a very reliable and easy-to-use on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry (on-line SPE–UHPLC–MS/MS) approach for the detection of all eleven coccidiostats in eggs. After an extraction procedure using acidified acetonitrile, the extract is injected into the on-line SPE–UHPLC system for automated clean-up and separation, followed by electrospray MS/MS quantitation (SRM in ESI^+ and ESI^-).

Studies resulted in the use of Thermo Scientific™ SolEx™ HRP SPE material for its superior trapping and desorption effects for coccidiostats. A method scouting approach showed Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2) as the most appropriate column for the analytical separation. As the polarities of the coccidiostats are very different, two methods were necessary to ensure trapping and detection of all analytes of interest. This heteropolarity, in particular the high solvent strength of certain extraction solvents, also required an additional dilution of the sample before reaching the on-line SPE column. For distinct identification, two ions of each analyte were chosen for single reaction monitoring (SRM). Validation was carried out on spiked chicken egg samples.

Experimental

Coccidiostats are extracted from the egg matrix by acidified acetonitrile. Extracts are cleaned up by on-line SPE, separated by liquid chromatography, and detected/quantified with a triplequad mass spectrometer in positive or negative mode using SRM transitions.

Materials and Methods

Equipment

- Thermo Scientific™ CL10 Centrifuge (or equivalent model providing 30,000 g)
- Thermo Scientific™ Savant™ SPD2010 SpeedVac™ Concentrator
- Thermo Scientific™ Titer Plate Shaker
- Thermo Scientific™ Dionex™ UltiMate™ 3000 ×2 Dual Standard System, including:
  - SRD-3600 Integrated Solvent and Degasser Rack
  - DGP-3600BM Dual Ternary Low Pressure Gradient Micro Pump
  - SRD-3200 Integrated Solvent and Degasser Rack
  - HPG-3200SD Binary High Pressure Gradient Pump
  - WPS-3000SL Well plate Autosampler
  - TCC-3000SD Thermostatted Column Compartment
- Thermo Scientific™ Dionex™ Valve Pod for Two-Position 10-Port HP Valve (alt.: Pod for Two-Position 6-Port HP Valve)
- Thermo Scientific™ Dionex™ Valve Actuator Kit HP
- SolEX HRP, 12–14 µm, 2.1 × 20 mm SPE-Cartridge (P/N 074400)
- Acclaim PolarAdvantage II (PA2) 3 µm, 2.1 × 150 mm Analytical Column (P/N 063187)
- Thermo Scientific™ Dionex™ DCMS^{Link}™ software
- Thermo Scientific™ Dionex™ Viper™ On-Line SPE Kit, RS
- Viper SST capillary, 550 × 0.13 mm (connection line from right DPG to T-piece)
- 2× Viper SST capillary, 65 × 0.13 mm (not required if a pod for two-position 6-port HP valve is used)
- T-piece, i.d. 500 µm
- Viper SST capillary (0.10 mm I.D.) or Thermo Scientific™ Dionex™ nanoViper™ Fingertight fitting (0.075 mm i.d.) of appropriate length as MS connection line
- Thermo Scientific™ TurboFlow Cyclone-P, 50 × 0.5 mm cartridge (P/N CH-953289)
- Triple Quadrupole Tandem Mass Spectrometer

Viper Fingertight Fitting System Connections

All flexible stainless steel capillary tubing used in this application is precut and has Viper fingertight fittings allowing for virtually zero-dead-volume connections. All Viper fingertight fitting capillaries needed for making the on-line SPE connections are provided in a Viper on-line SPE kit, apart from the parts required to extend this conventional configuration to an on-line dilution setup

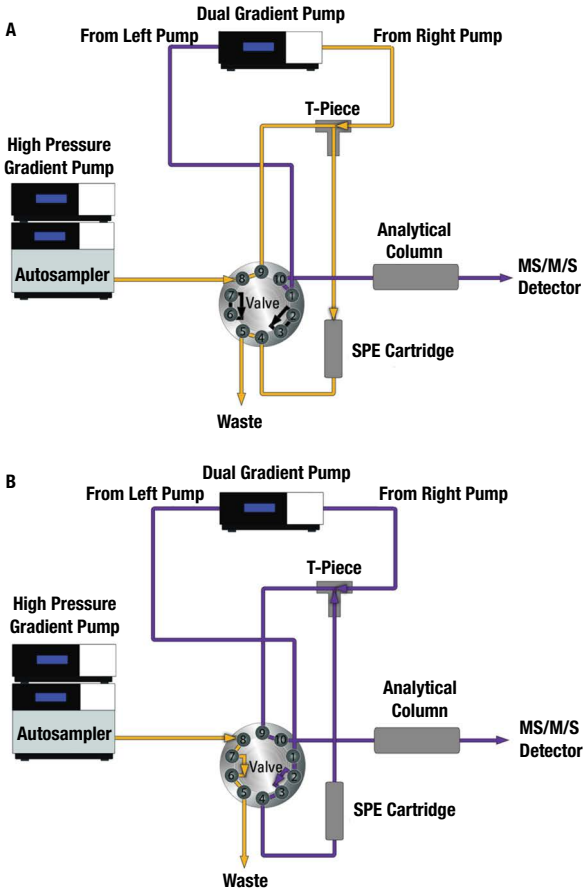

Figure 2. Flow schemes of the advanced on-line SPE configuration for loading/washing (A) and transfer/analysis (B).

using a third pump: The t-piece, the Viper fingertight fitting capillaries to loop through valve ports 2 and 3 as well as ports 6 and 7 of the two-position 10-port valve (Figure 2), and the Viper fingertight fitting capillary needed to connect the right pump of the DGP with the T-piece are not included in a Viper on-line SPE kit and therefore must be ordered separately. Alternatively, a two-position 6-port valve, which eliminates the need for additional shortcut fluidics, could be used.

Reagents

- 2-Propanol (2-PrOH), LiChrosolv® (Merck® KGaA, P/N 1.01040)
- Acetic Acid (AA), pro analysis (Merck KGaA, P/N 1.00063)
- Acetonitrile (MeCN), ULC/MS (Biosolve b.v., P/N 01204102)
- Acetone (DMK), LiChrosolv (Merck KGaA, P/N 1.00020)
- Dimethylformamide (DMF), pro analysis (Fluka/Sigma-Aldrich®, P/N 40243)
- Dimethyl Sulfoxide (DMSO), Uvasol® (Merck K GaA, P/N 1.02950)
- Ethanol (EtOH), LiChrosolv (Merck KGaA, P/N 1.11727)
- Formic Acid (FA), pro analysis (Fluka/Sigma-Aldrich, P/N 56302)
- Methanol (MeOH), ULC/MS (Biosolve b.v., P/N 13684102)
- Sodium Chloride (NaCl), pro analysis (Merck KGaA, P/N 1.06404)
- Triethylamine (TEA), purissimum (Fluka/Sigma-Aldrich, P/N 90340)
- Water (H_2O), demineralized by Kantonales Laboratorium Bern

Standards

- Clopidol (Sigma-Aldrich, P/N 33988)
- Decoquinate (Sigma-Aldrich, P/N 33823)
- Decoquinate-d_5 (Sigma-Aldrich, P/N 32552)
- Diclazuril (Sigma-Aldrich, P/N 34057)
- Halofuginone Lactate (Dr. Ehrenstorfer GmbH, P/N XA14059300AL)
- Lasalocid A Sodium Salt (Fluka/Sigma-Aldrich, P/N 33339)
- Maduramicin (Sigma-Aldrich, P/N 34069)
- Monensin Sodium Salt (Sigma-Aldrich, P/N 46468)
- Narasin (Sigma-Aldrich, P/N N1271)
- Nicarbazin (4,4-Dinitrocarbanilide) (Sigma-Aldrich, P/N 32409)
- Nicarbazin-d_8 (4,4-Dinitrocarbanilide-d_8), (Witega Laboratorien Berlin-Adlershof GmbH, P/N OP001)
- Robenidine Hydrochloride (Sigma-Aldrich, P/N 33979)
- Salinomycin (Sigma-Aldrich, P/N S4526)

Preparation of Standards

Stock Solutions

In consideration of their purity, stock solutions of each standard substance are prepared with a target concentration of 1 mg/mL by using the solvents as noted in Table 1.

Table 1. Stock solutions.

Standard Substance	Solvent
Nicarbazin (DNC)	Dimethyl Sulfoxide
Nicarbazin-d_8 (DNC)	Dimethyl Sulfoxide
Clopidol	Dimethylformamide
Decoquinate	Methanol + 1% Triethylamine
Decoquinate-d_5	Methanol + 1% Triethylamine
Diclazuril	Dimethylformamide
Halofuginone	Methanol
Lasalocid	Methanol
Maduramicin	Methanol
Monensin	Methanol
Narasin	Acetonitrile
Robenidine	Ethanol
Salinomycin	Acetonitrile

Standard Solutions

Prepare two types of solutions, an Internal Standard Solution (ISTD) and a Calibration Standard Solution (CSTD). The ISTD solution contains all deuterium-labeled reference standards; the CSTD solution consists of a mixture of all non-labeled target analytes and serves as a stock solution to prepare all further standards. Achieve the target concentration of 10 ng/µL for each standard solution by adding the appropriate volume of each stock solution into a 10 mL volumetric flask and fill with methanol. These standard solutions are used to spike the egg samples as well as to prepare the various standard types for method quality control and system suitability tests, as described in "Sample Types" and "Sample Preparation".

Sample Types

Quantitation of the egg samples is achieved via calibration standards. In addition to the calibration standards, several types of standards are run within a sequence to ensure and monitor the performance of the separation and MS detection. The following sample types are run (see Figure 3 for clarity):

Calibrate Standard

- Blank egg matrix, spiked with several defined amounts of target analytes (CSTD) and ISTDs before preparation
- ISTDs and target analytes added prior to sample preparation
- The Calibrate Standard is used for quantitation of the coccidiostats in eggs

Control Standard

- Blank egg matrix, spiked with a defined amount of both target analytes (CSTD) and ISTD (30 µg/kg) before preparation
- ISTDs and target analytes added prior to sample preparation
- The Control Standard is used for method control and recovery rate determination

Matrix Standard

- Blank egg matrix, spiked with a defined amount of both target analytes (CSTD) and ISTD (30 µg/kg) after preparation
- ISTDs and target analytes added after sample preparation
- The Matrix Standard is used for method control, recovery rate, and determination of matrix effects on MS signal

Sample

- Chicken egg sample
- ISTDs added prior to sample preparation

External Standard

- Pure ISTDs and target analytes (CSTD) in a matrix-free solution
- The External Standard is used for method control and MS signal stability/detection performance monitoring

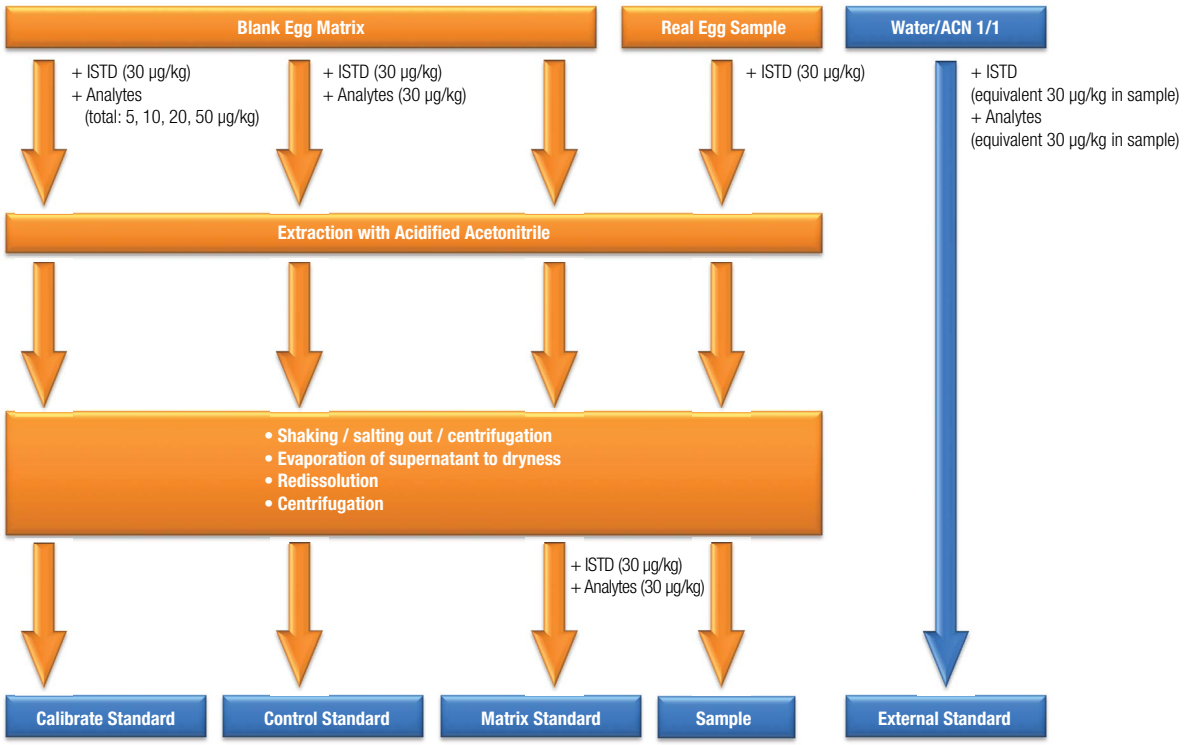

Figure 3. Sample type and preparation overview.

Sample Preparation

External Standard

The External Standard sample is prepared by adding an appropriate amount of both ISTD and CSTD solution to 50% acetonitrile in water to give a final concentration of 0.14 ng/µL (equivalent 30 µg/kg in eggs) for each analyte.

For all egg samples, the white and yolk of six eggs from each batch are homogenized. The workup process differs between the individual sample types:

Calibrate Standards

Add 10 g of homogenized blank egg which contain no coccidiostats by design to a 50 mL PP-tube four times, then add 20 mL acetonitrile, 1 mL of 1 M acetic acid, and 30 µL ISTD solution. The samples are spiked with 5, 10, 20, and 50 µg/kg of analyte.

Control Standard

Add 10 g of homogenized blank egg which contain no coccidiostats by design to a 50 mL PP-tube, then add 20 mL acetonitrile, 1 mL of 1 M acetic acid, 30 µL of ISTD solution, and 30 µL of CSTD solution.

Matrix Standard

Add 10 g of homogenized blank egg which contain no coccidiostats by design to a 50 mL PP-tube, then add 20 mL acetonitrile and 1 mL of 1 M acetic acid.

Sample

For the chicken egg samples, 10 g of homogenized egg are added to a 50 mL PP-tube, then 20 mL acetonitrile, 1 mL of 1 M acetic acid, and 30 µL ISTD solution are added.

- All egg-based samples are shaken extensively for 30 s by vortex and then for 30 min on a plate shaker at the highest speed setting
- Add 5 g of sodium chloride to each sample and shake extensively by hand
- Centrifuge the samples for 20 min at 2550 g and 4 °C.
- Transfer 2 mL of the supernatant into a SpeedVac tube
- Evaporate the vessels to dryness in the SpeedVac concentrator for 60 min at 55 °C and maximum vacuum
- Re-dissolve each dried residue with 200 µL of 50% acetonitrile in water and transfer into a micro-tube
- Centrifuge the micro-tubes for 10 min at 30,000 g and 4 °C
- Transfer the supernatant into an HPLC micro-vial

The coccidiostats are quantitated using the Calibrate Standards by linear regression in a four-point calibration. All Calibrate Standards should be injected in triplicate. External Standard and Control Standard should be injected at least three times distributed equally over the sequence, e.g. at the beginning, in the middle, and at the end of the sequence, to monitor retention time precision and MS sensitivity.

LC/MS Analysis

Table 2. Method 1 for clopidol, robenidine, halofuginone, nicarbazin (DNC), and diclazuril.

Method 1			
Column	Acclaim Polar Advantage II (PA2), 3 µm, 2.1 × 150 mm		
SPE Cartridge	SolEx HRP, 12–14 µm, 2.1 × 20 mm		
Injection Volume	10 µL		
Temperature (TCC)	25 °C		
Temperature (WPS)	20 °C		
2-Position 10-Port HP Valve			
Time		**Position**	
0.0		10–1	
3.0		1–2	
6.0		10–1	
High Pressure Gradient Pump (HPG) used as loading pump			
Mobile Phase A	H_2O with 0.1% FA		
Mobile Phase B	30% MeCN/30% DMK/40% 2-PrOH		
Time	**Flow (mL/min)**	**%A**	**%B**
0.0	0.1	100	0
0.4	0.1	100	0
0.41	0.75	100	0
3.0	0.75	100	0
3.1	0.75	0	100
3.2	0.05	0	100
6.0	0.05	0	100
6.1	0.75	0	100
12.0	0.75	0	100
12.1	0.75	100	0
15.0	0.75	100	0
15.1	0.1	100	0
23.0	0.1	100	0
Left pump of Dual Gradient Pump (DGP) used as analytical pump			
Mobile Phase A	H_2O with 0.1% FA		
Mobile Phase B	MeOH		
Time	**Flow (µL/min)**	**%A**	**%B**
0.0	300	85	15
3.0	300	85	15
3.001	300	70	30
3.002	150	70	30
6.0	150	70	30
6.001	150	85	15
6.002	300	85	15
12.0	300	30	70
12.2	300	0	100
18.0	300	0	100
18.1	300	85	15
23.0	300	85	15
Right pump of Dual Gradient Pump (DGP) used as dilution pump			
Mobile Phase A	H_2O		
Time	**Flow (µL/min)**	**%A**	
0.0	650	100	
0.3	650	100	
0.4	10	100	
3.0	10	100	
3.001	150	100	
6.0	150	100	
6.001	10	100	
23.0	10	100	

Table 3. Method 2 for decoquinate, lasalocid, maduramicin, monensin, narasin, and salinomycin.

Method 2			
Column	Acclaim Polar Advantage II (PA2), 3 µm, 2.1 × 150 mm		
SPE Cartridge	SolEx HRP, 12–14 µm, 2.1 × 20 mm		
Injection Volume	10 µL		
Temperature (TCC)	40 °C		
Temperature (WPS)	20 °C		
2-Position 10-Port HP Valve			
Time		**Position**	
0.0		10–1	
3.0		1–2	
6.0		10–1	
Mobile Phase A	H_2O with 0.1% FA		
Mobile Phase B	30% MeCN/30% DMK/40% 2-PrOH		
Time	**Flow (mL/min)**	**%A**	**%B**
0.0	0.1	100	0
0.5	0.1	100	0
0.51	0.75	100	0
3.0	0.75	100	0
3.1	0.75	0	100
3.2	0.05	0	100
6.0	0.05	0	100
6.1	0.75	0	100
12.0	0.75	0	100
12.1	0.75	100	0
15.0	0.75	100	0
15.1	0.05	100	0
20.7	0.05	100	0
20.8	0.1	100	0
Left pump of Dual Gradient Pump (DGP) used as analytical pump			
Mobile Phase A	H_2O with 0.1% FA		
Mobile Phase B	MeOH		
Time	**Flow (µL/min)**	**%A**	**%B**
0.0	300	55	45
3.0	300	55	45
3.001	300	10	90
3.002	150	10	90
6.0	150	10	90
6.001	150	55	45
6.002	300	55	45
10.0	300	0	100
15.0	300	0	100
15.1	300	55	45
20.8	300	55	45
Right pump of Dual Gradient Pump (DGP) used as dilution pump			
Mobile Phase A	H_2O		
Time	**Flow (µL/min)**	**%A**	
0.0	650	100	
0.5	650	100	
0.51	10	100	
3.0	10	100	
3.001	150	100	
6.0	150	100	
6.001	10	100	
20.8	10	100	

Table 4. Conditions of the MS/MS detector for both methods.

Analyte	Retention Time [min]	Precursor Ion (Q1) Mass [*m/z*]	Fragment Ion (Q3) Mass [*m/z*]	Ionization Mode
Clopidol	10.1	192.0 194.0	157.0 103.0	Positive
Robenidine	14.0	334.2 336.2	155.0 180.0	Positive
Halofuginone	12.1	416.1 416.1	138.1 398.0	Positive
Decoquinate	11.7	418.3 418.3	372.2 232.0	Positive
Decoquinate-d_5	11.7	423.3	377.3	Positive
Lasalocid	13.0	613.4 613.4	377.4 577.5	Positive
Monensin	11.7	693.5 693.5	675.6 461.5	Positive
Salinomycin	11.9	773.5 773.5	431.5 265.4	Positive
Narasin	12.1	787.5 787.5	531.6 431.6	Positive
Maduramicin	11.8	934.6 934.6	647.8 629.7	Positive
Nicarbazin (DNC)	15.4	301.0 301.0	136.9 106.9	Negative
Nicarbazin-d_8 (DNC-d_8)	15.4	309.0	140.9	Negative
Diclazuril	14.6	405.1 407.0	334.2 336.2	Negative

Results and Discussion

Advanced On-Line SPE Configuration

Method Development

The advanced on-line SPE configuration with three different LC pumps offered the flexible use of chromatographic sample enrichment and cleanup for a broad polarity range of both analytes and sample solvents. It allowed the individual reduction of solvent strength by water addition prior to and after the SPE material (Figure 2A). This dilution had no negative impact on the chromatographic results. In the case of polar analytes dissolved in a strong solvent (50% water/acetonitrile), retention was significantly improved when water was used to dilute the organic sample solvent plug of high elution strength. As the on-line SPE cartridge was used for analyte enrichment and matrix depletion, the dilution will not compromise the chromatographic separation on the analytical column. In case of non-polar analytes which require high amounts of organic modifier for a complete transfer from the SPE phase to the analytical column, the configuration allowed dilution of the transfer liquid (Figure 2B). The refocusing effect of the analytical column head still provided outstanding chromatography.

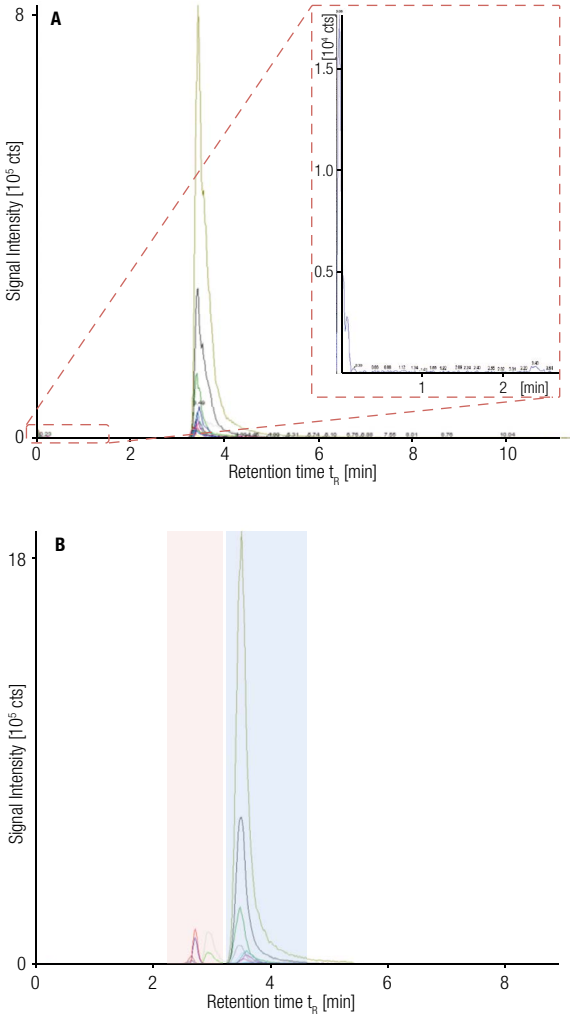

Figure 4. Foreflush on-line SPE trapping and elution of coccidiostats on Cyclone-P (A) and SolEx HRP (B) cartridges.

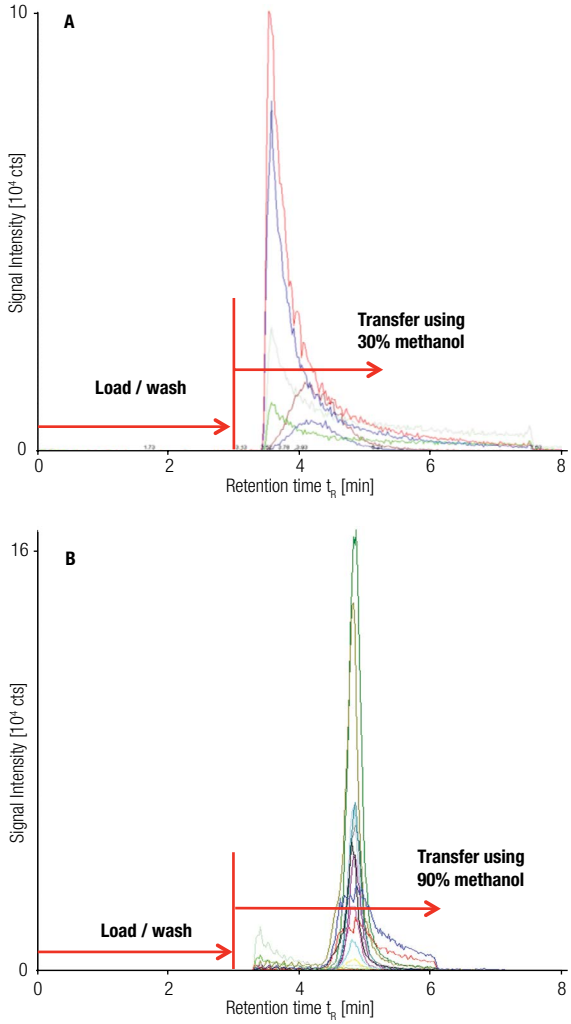

Figure 5. Backflush on-line SPE trapping and elution of polar (A) and non-polar coccidiostats (B) on SolEx HRP cartridges (no analytical column connected).

Due to the heterogeneous polarities of the coccidiostats, on-line SPE method development is quite challenging. Thus, two solid phase materials were compared: a TurboFlow Cyclone-P, 50 × 0.5 mm cartridge and a SolEx HRP, 20 × 2.1 mm cartridge. To investigate enrichment and transfer behavior of the analytes, both columns were connected to the UHPLC system in the same manner as analytical separation columns and loaded with 10 µL of standard solutions at 0.75 mL/min in 0.1% aqueous formic acid. Then the solvent strength was raised to 100% methanol, the SPE column eluted in foreflush direction, and the effluent monitored by tandem-mass spectrometry. Although the majority of analytes were trapped without problems, the Cyclone-P cartridge exhibited immediate breakthrough of some highly polar analytes (Figure 4A). In contrast, the SolEx HRP cartridge showed a much more balanced retention for polar and non-polar compounds, eluting in two distinct fraction windows, even when a pure organic solvent was used for elution (Figure 4B).

The same can be seen in a true on-line SPE backflush configuration. After loading and washing with 100% acidified water, the analyte transfer succeeded in two ways: polar analytes (clopidol, diclazuril halofuginone, nicarbazin (DNC), and robenidine) could be transferred to the analytical column with a mobile phase composition of 30% methanol (Figure 5A), while non-polar analytes (decoquinate, lasalocid, maduramicin, monensin, narasin, and salinomycin) needed 90% methanol to elute from the enrichment phase, but still eluted moderately late (Figure 5B). The polarity mismatch between the high amount of organic solvent needed to transfer non-polar analytes and the analytical column lead to retention loss; this can easily be corrected by diluting the transfer solvent with aqueous mobile phase by T-piece infusion behind the SPE material (Figure 2), which, due to sample refocusing effect on the analytical column head, does not compromise the subsequent LC separation.

As a result, two different injections that seamlessly ran on the same on-line SPE-UHPLC-MS/MS system were required to quantify all coccidiostats of interest. All samples were dissolved in 50% water/acetonitrile and loaded onto the SPE cartridge for 3 min; diluting this sample solvent plug with 0.1% aqueous formic acid ensured retention on the SPE material despite the considerably high solvent strength of the sample plug. Method 1 trapped the polar coccidiostats and transferred them from the SPE cartridge via the left pump of the DGP-3600 dual gradient pump using a mobile phase with 30% methanol (Figure 2). Water was added to this mobile phase after the SPE cartridge via the T-piece, resulting in a final content of 15% methanol in the mobile phase before it entered the analytical column. Then, a solvent strength gradient from 15% up to 70% methanol was used to separate the analytes within 6 min. Method 2 trapped non-polar coccidiostats and transferred them from the SPE cartridge using 90% methanol. Again, water was added to this mobile phase via the T-piece in front of the analytical column, resulting in a total of 45% methanol in the mobile phase for the separation. The mobile phase gradient ran from 45% to 100% methanol within 4 min. As both methods used the same mobile and stationary phases for analytical separation, they can easily run consecutively.

In analytical laboratories, solid phase extraction is an established method used for the preparation of samples. The main objective of on-line SPE is to decrease sample preparation time, which results in decreased labor costs and increased throughput. Sample preparation for coccidiostats in eggs by manual off-line SPE typically needs a working effort of about six hours for 10 samples, while on-line SPE only requires three hours for the same number of samples. However, the advantage of this on-line SPE approach, next to throughput increase, is better repeatability and minimized exposure to hazardous chemicals, as well as a significant improvement in data quality. Figure 6 compares the SRM traces of a monensin analysis from eggs using traditional off-line (A) and on-line SPE (B). On-line SPE leads to a more than four-fold decrease in noise amplitude and thus, a significant increase in sensitivity.

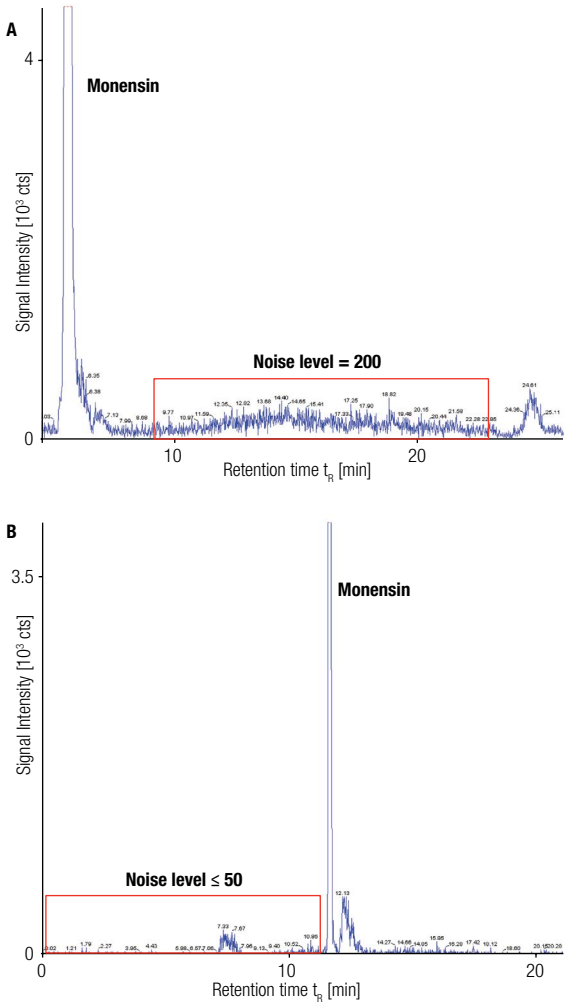

Figure 6. SRM trace (693.5 → 675.6) of a UHPLC-MS/MS analysis of 5 ppb monensin standard in egg after sample preparation by off-line (A) and on-line SPE (B).

The UHPLC separation was achieved using the Acclaim PolarAdvantage II column, which showed baseline resolution for all analytes of interest (Figure 7).

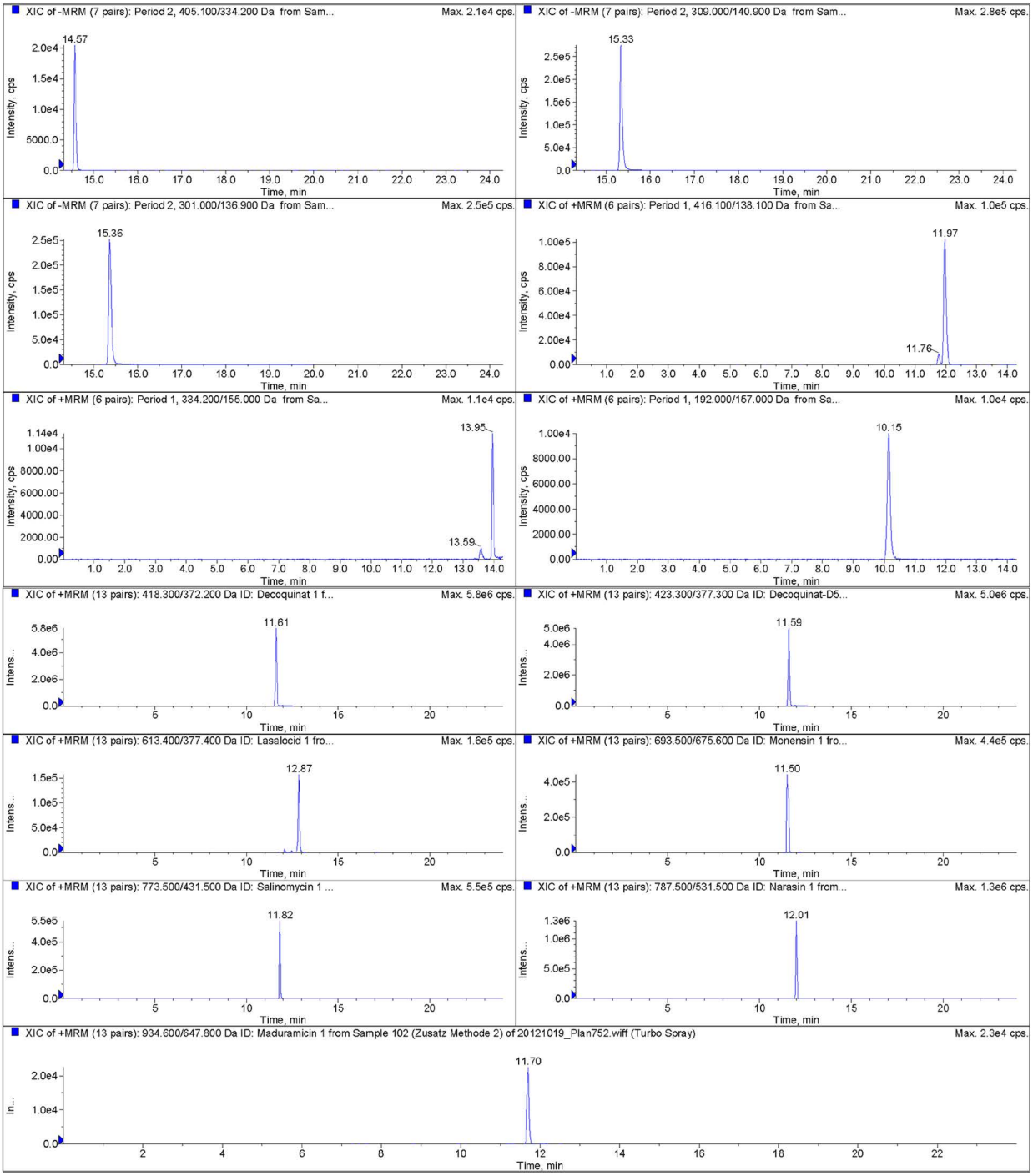

Figure 7. SRM traces of coccidiostats.

Quantitative Analysis - Screening of Egg Samples

Linearity and Sensitivity

Each Blank Value and External Standard dissolved in solvent was first injected in duplicate using method 1. For method verification, the External Standard, the Control Standard, and the Matrix Standard were injected at least three times and dispersed to different stages of the sequence. After all samples were analyzed by method 1, the system was equilibrated to the conditions given by method 2. After starting with at least two blank runs for equilibration purposes, each Blank Value and External Standard was injected in duplicate using method 2. Again, the External Standard, the Control Standard, and the Matrix Standard needed to be injected at least three times each and dispersed to different stages of the sequence. Typically, quality control of eggs starts with a screening approach, checking the samples for critical amounts of coccidiostats close to the regulatory limits. Calibration of the screening sequences was done by external calibration with calibration standards spiked to blank eggs which had been worked up following the sample preparation procedure. Any suspicious samples were then explicitly quantified based on peak areas by standard addition of the Calibration Standard solutions. All calibration curves showed excellent linearity with correlation coefficients r between 0.9879 (robenidin) and 0.9993 (DNC), see Figure 8.

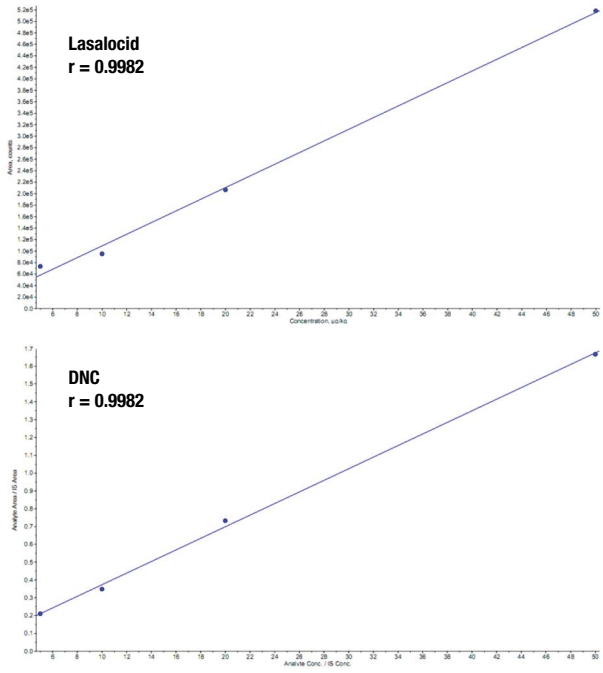

Figure 8. Calibration plots of lasalocid and DNC.

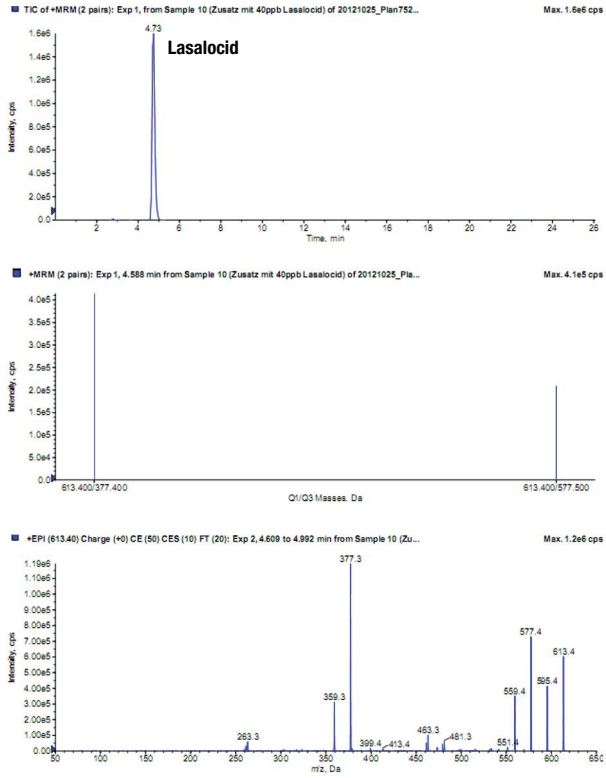

Figure 9. The enhanced product ion of lasalocid.

Identification of precursor and product ions in the positive and negative electrospray mode and preliminary optimization of instrument settings were performed by continuous infusion of the individual coccidiostats at 0.1 µg/mL dissolved in 50% methanol in water containing 0.1% formic acid. Quantitation was done with the most intense product ion. Identification of each coccidiostat was confirmed with its ion ratio and enhanced product ion (Figure 9). An injection volume of 10 µL was selected for sensitivity reasons. As an analytical method used for official control in compliance with European legislation, a single laboratory validation of the method was carried out and passed according to Commission Decision 2002/657/EC.[9] Limits of detection (LOD) ranged from 0.02–0.5 µg/kg, the limits of quantitation (LOQ) from 0.06–1.5 µg/kg, thus being 5–10 times lower than the allowed maximum residue levels of coccidiostats in eggs, with very good recovery rates for all relevant analytes (Table 5).

	LOD [µg/kg]	LOQ [µg/kg]	MRL [µg/kg]	Recovery (at 30 µg/kg) [%]	Measurement uncertainty [%]
Clopidol	0.08	0.25	2.0*	100	10
Robenidin	0.5	1.5	25.0	92.0	13
Halofuginone	0.1	0.3	6.0	90.4	10
Decoquinate	0.05	0.15	20.0	98.2	13
Lasalocid	0.4	1.2	5.0 (150)	101	13
Monensin	0.06	0.2	2.0	93.0	18
Salinomycin	0.07	0.22	3.0	104	28
Narasin	0.02	0.06	2.0	107	37
Maduramicin	0.1	0.3	2.0	118	30
Nicarbazin (DNC)	0.15	0.45	50.0	96.1	11
Diclazuril	0.13	0.4	2.0	98.7	16

*MRL of clopidol with respect to [10]

Conclusion

This simple and reliable on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry (On-Line SPE–HPLC–MS/MS) approach allows the detection of eleven coccidiostats at trace levels in eggs. The great advantage of on-line SPE over off-line SPE is a doubling of throughput–two results in the same time needed for one result with classical off-line SPE-HPLC. A common screening against external calibration in a spiked matrix allows for the identification of potentially critical samples, which then are quantified more accurately by standard addition calibration to account for matrix effects. This approach was single laboratory validated and meets all criteria of European legislation.[9] Very good values for the sensitivity, precision, and trueness of both methods were confirmed. This approach can therefore be used for official control screening for the presence and also quantitation of these eleven coccidiostats at target and trace levels.

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References

1. OJEU. Regulation (EC) No 1831/2003 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on additives for use in animal nutrition. [Online] September 22, 2003, 268, 29–30. http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/EC-1831-2003.pdf (accessed May 15, 2013).
2. OJEU. Directive 70/524/EEC, [Online] **2003,** *268,* 29–43. http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/EC-1831-2003.pdf (accessed May 15, 2013).
3. OJEU. Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their maximum residue limits in foodstuffs of animal origin. [Online] January 20, 2010, 15, 1-76. http://ec.europa.eu/health/files/mrl/mrl_20101212_consol.pdf (accessed May 15, 2013).
4. OJEU. COMMISSION REGULATION (EC) No 124/2009 of 10 February 2009 setting maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed. [Online] February 11, 2009, 7–11. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:040:0007:0011:en:PDF (accessed May 15, 2013).
5. Mortier, L.; Daeseleire, E.; Delahaut, P. Simultaneous detection of five coccidiostats in eggs by liquid chromatography–tandem mass spectrometry, *Anal. Chim. Acta* **2003,** *483,* 27–37.
6. Vincent, U.; Chedin, M.;Yasar, S.; von Holst, C. Determination of ionophore coccidiostats in feedingstuffs by liquid chromatography–tandem mass spectrometry Part I. Application to targeted feed, *J. Pharm. and Biomed. Anal.,* **2008,** *47,* 750–757.
7. Dubois, M.; Pierret, G.; Delahaut, P. Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography–electrospray tandem mass spectrometry, *J. Chromatog., B: Anal. Technol. Biomed. Life Sci.* **2004,** *813,* 181–189.
8. Delahaut, P.; Pierre, G.; Ralet, N.; Dubois, M.; Gillard, N. Multi-residue method for detecting coccidiostats at carry-over level in feed by HPLC-MS/MS, *Food Add. Contam.,* **2010,** *27*(6), 801–809.
9. OJEU. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. [Online] August 17, 2002, 657, 8-36. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF (accessed May 15, 2013).
10. OJEU. Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin [Online] August 18, 1990, 224, 1-8. http://ec.europa.eu/health/files/eudralex/vol-5/reg_1990_2377/reg_1990_2377_en.pdf (accessed May 15, 2013).
11. "Kokzidiostatika in Geflügelfleisch und Eiern; LC-MS/MS" SLMB, 1571.2, 2008.

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