The Quantitation and Identification of Coccidiostats in Food by LC-MS/MS using the AB SCIEX 4000 Q TRAP® System

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Introduction

Coccidiostats are antiprotozoal agents that act upon parasites. In animal production, particularly in intensive animal rearing coccidiostats are used to treat infections and as such meat, chicken, egg and milk are regularly tested for these compounds. Recently maximum levels for these compounds (due to unavoidable carry-over of authorized coccidiostats to non-target feed) were set by the EU in Commission Regulations [(EC) No 124/2009][1] so methods for their detection were required. This work compares the traditional approach to sample preparation of solid phase extraction (SPE) followed by separation on a conventional 5µm particle column with that of the quicker and simpler QuEChERS[2-3] technique followed by separation with a newer 2.6 µm particle column and shows how liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) can be used to detect coccidiostats including Narasin, Diclazuril and Monensin in milk.

Experimental

1) Conventional Approach

Sample Preparation

Milk (2.0 g) in a Polypropylene Tube was mixed with acetonitrile (2 mL) and vortexed for 40 seconds. Another 2 mL of acetonitrile was added and the the tube was sealed, shaken by hand and then continually mixed using a head over head mixer for 15 minutes. The sample was then centrifuged for 15 minutes (3600 g at 4°C). The supernatant was removed and water (16 mL) and ammonia solution (1 mL, 25%) were added and this mixture was shaken. The whole extract was loaded onto an OASIS HLB SPE cartridge (3 cm^3, 60 mg) which previously had been conditioned with methanol (3 mL) and water (3 mL). The cartridge was washed with ammonia (5 mL, 1.25%) dried for 2 minutes under vacuum and eluted with methanol (5 mL). The eluent was evaporated to dryness, the sample was reconstituted in methanol/water (1 mL, 50/50), vortexed, and sonicated for 5 minutes before injection.

LC

Column: Agilent Zorbax Eclipse XDB-C8, 5 µm, 150 x 4.6 mm
Flow rate: 400 µL/min
Oven temperature: 40 ºC
Injection Volume: 40 µL
Mobile Phase A: water + 0.2% acetic acid
Mobile Phase B: methanol + 0.2% acetic acid

Table 1. LC gradient profile of conventional approach

Step	Time (min)	A (%)	B (%)
1	0.5	100	0
2	1.5	20	80
3	10	10	90
4	13	0	100
5	18	0	100
6	18.5	100	0
7	23	100	0

2) New Approach

Sample Preparation

The sample extraction was based on a QuEChERS method by Anastassiades et al. and Lehotay et al.[2-3] Milk in a polypropylene tube (50 mL) was roller mixed with acetonitrile. To this mixture anhydrous magnesium sulfate and sodium acetate were added and samples were shaken vigorously and centrifuged. Anhydrous magnesium sulfate, PSA and C18 were added to an aliquot (2 mL) of the upper layer and these samples were shaken by hand. This mixture was centrifuged and the supernatant transferred into an autosampler vial for analysis.

LC

Column: Phenomenex Kinetex C8, 2.6 µm, 100 x 4.6 mm
Flow: 600 µL/min
Oven temperature: 40 ºC
Injection Volume: 40 µL
Mobile Phase A: water + 0.2% acetic acid
Mobile Phase B: methanol + 0.2% acetic acid

Table 2. LC gradient profile of new approach with a Phenomenex Kinetex column using 2.6 µm core-shell particles for increased efficiency and improved performance

Step	Time (min)	A (%)	B (%)
1	1.0	100	0
2	2.5	20	80
3	5.0	10	90
4	7.5	0	100
5	9.2	0	100
6	9.5	100	0
7	11.5	100	0

MS/MS

The AB SCIEX 4000 Q TRAP® system was used with Turbo V™ source and Electrospray Ionization (ESI) probe. The source was heated to 600ºC with 45 psi nebulizer and heater gas.

Negative and positive polarities were used with polarity switching during, the chromatographic run, to cover all target analytes.

For best selectivity and sensitivity Multiple Reaction Monitoring (MRM) mode was used for detection. Two MRM transitions were detected per compound to allow quantitation and identification by MRM ratios (Table 4). However, since detection in MRM mode only can lead to false positive results full scan MS/MS spectra were additionally acquired to increase confidence in compound identification using mass spectral library searching. In this mode an information dependent acquisition (IDA) experiment was used to automatically trigger the MS/MS spectra acquisition when a chromatographic MRM signal exceeded a threshold of 1000 cps.

Results and Discussion

The maximum residue limits for the coccidiostats vary with analyte (Table 3). The analysis is further complicated by the fact that Diclazuril ionizes in negative polarity so to maximize sensitivity the method contains periods, so it switches from positive to negative and back to positive as shown in Figure 1.

Table 3. Maximum Residue limits (MRL) for some coccidiostats[1]

Coccidiostats	MRL in milk (µg/kg)
Diclazuril	5
Lasalocid	1
Maduramycin	2
Monensin	2
Narasin	1
Robenidine	5
Salinomycin	2

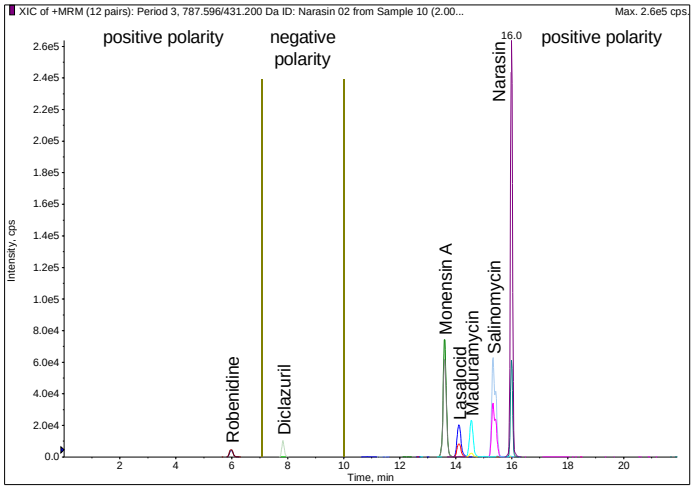
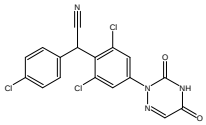
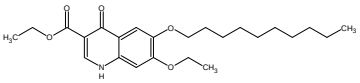
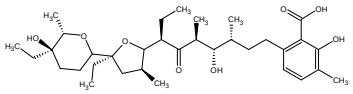
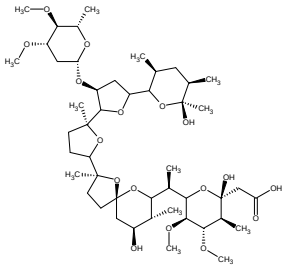
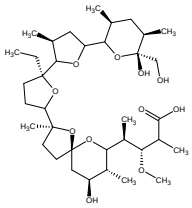
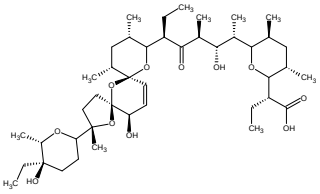
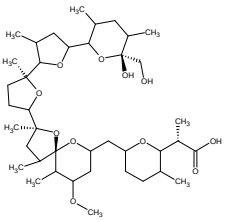
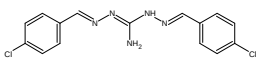
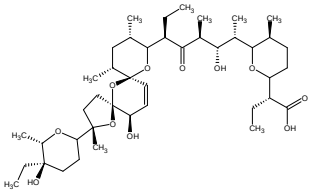


Figure 1. Example of an LC-MS/MS chromatogram from a milk matrix matched calibration standard (concentration of coccidiostats ranging from 2 to 10 µg/kg) prepared and analyzed using the conventional approach

Table 4. Targeted coccidiostats with retention times, polarity, and detected MRM transitions using the Phenomenex Kinetex C8 column

Coccidiostats	CAS	Structure	RT (min)	Polarity	Q1 (amu)	Q3 (amu)
Diclazuril	101831-37-2		5.3	negative	405 407	334 336
Decoquinate	18507-89-6		6.4	positive	418	204 372
Lasalocid	25999-31-9		7.1	positive	613	377 595
Maduramycin	84878-61-5		7.2	positive	939	877 895
Monensin A	17090-79-8		7.0	positive	693	461 479
Narasin	55134-13-9		6.8	positive	787	279 431 531
Nigericin	28643-80-3		7.8	positive	747	703 501
Robenidine	25875-51-8		4.6	positive	334	138 111
Salinomycin	53003-10-4		6.6	positive	773	431 531 265
Decoquinate D5 (internal standard)			6.4	positive	423	377

The conventional approach using a 5 µm column, as shown in Figure 1, produced peaks with peak widths in the range of 12 to 30 seconds and a run time of 23 minutes. When this method was switched to the Kinetex core-shell particle column the peak widths were reduced to between 7 and 12 seconds and the run time could be reduced to 11.5 minutes (Figure 2).

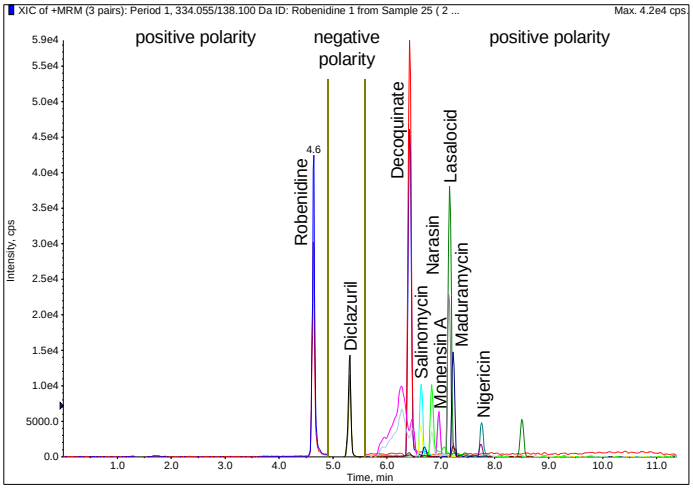


Figure 2. Example of an LC-MS/MS chromatogram from a milk matrix matched calibration standard (concentration of coccidiostats ranging from 2 to 10 µg/kg) prepared and analyzed using the new approach

To further speed up the analysis the off-line SPE was replaced by the simpler QuEChERS sample preparation technique, which is commonly used in pesticide residue analysis. The resulting simplification of the extraction produced dirtier extracts but the background interferences did not co-elute with analytes so this approach was shown to be a feasible alternative.

To assess the sensitivity of the developed method the coccidiostats were spiked into milk and extracted using the QuEChERS procedure. The results showed that this technique was capable of detecting all the coccidiostats reproducibly in milk at concentrations below 1 µg/L.

When both approaches, the conventional using SPE and the new one using QuEChERS, were compared both showed coefficients of variation (% CV) of less than 10% at or below the LOD levels needed except for Robenidine whose CV was 19% using the SPE methodology (Table 5). This showed that both methods could be applied to food samples. Both approaches produced linear responses and r values > 0.985 (see examples in Figure 3). This included the QuEChERS method which used spiked calibration standards whose concentration ranged from 0.2 to 50 µg/L with the exception of Decoquinate whose fit was quadratic over this range. The internal standard Decoquinate D5 was later used to correct the non linearity and additional internal standards could further improve these results.

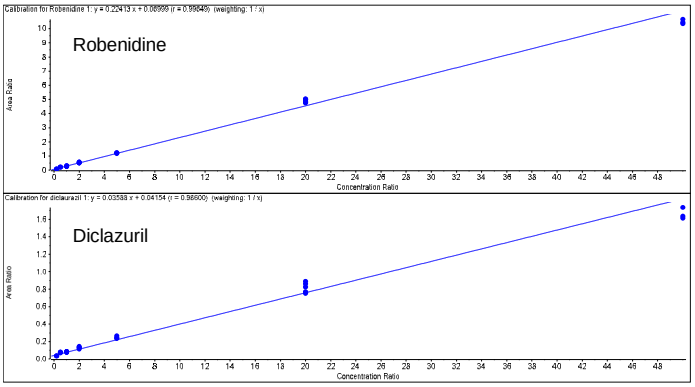


Figure 3. Calibration line for Robenidine (top) and Diclazuril (bottom) 0.2 to 50 µg/L in milk using the new approach with QuEChERS extraction and fast chromatography

Table 5. Reproducibility from the repeat analysis of a low spiked matrix matched standard

Coccidiostats	Concentration of spiked SPE extract (µg/L)	% CV (4 replicates) using the conventional approach	Concentration of QuEChERS extract (µg/L)	% CV (4 replicates) using the new approach
Diclazuril	1.25	2.6	1	7.7
Lasalocid	0.25	6.3	0.5	5.1
Maduramycin	0.5	0.7	0.5	3.5
Monensin A	0.5	2.9	0.5	3.8
Narasin	0.25	4.7	0.5	4.7
Robenidine	1.25	18.8	1	7.9
Salinomycin	0.5	3.6	0.5	7.9

There are known cases, especially in food analysis, when MRM ratios can be misleading and produce false positive results therefore additional information for identification is beneficial.

So in addition to collecting MRM data there is the possibility of automatically acquiring full scan MS/MS spectra when an MRM signal exceeds a defined threshold. These full scan MS/MS spectra [Enhanced Product Ion (EPI) spectra] are highly characteristic and sensitive using this unique scan function of a Q TRAP® system. Figure 4 shows two examples of how MRM triggered EPI spectra further aids identification of coccidiostats in food samples.

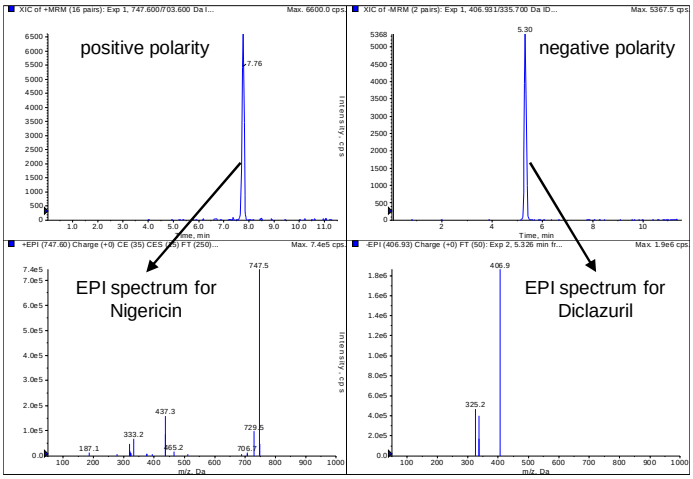


Figure 4. Example of an LC-MS/MS chromatogram from a 2 µg/L matrix matched calibration standard run in positive polarity with an EPI spectrum of Nigericin (left) and an LC-MS/MS chromatogram from the same sample run in negative polarity where a spectrum of Diclazuril has been automatically acquired

Summary

The LC-MS/MS approaches discussed in this work have been shown to be suitable for the detection of coccidiostats in food at the required sanctioned levels.

When the sample preparation was simplified using a QuEChERS procedure and a core-shell particle column was used the additional sensitivity of this assay enabled the detection of these residues below the MRL required but at over twice the speed of the conventional method which enables a reduction in cost of the analysis.

References

1 Commission Regulation (EC) No 124/2009 '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'

2 M. Anastassiades et al.: 'Fast and easy multi-residue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce' J. AOAC Int. 86 (2003) 412-431

3 S. J. Lehotay et al.: 'Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection' J. AOAC Int. 88 (2005) 595-614

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