Application Note: 475

High-throughput Screening Method for Multiple Classes of Antibiotics in Milk Using Automated Sample Preparation and LC-MS/MS

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Key Words

- TurboFlow Technology
- Aria TLX-2
- Food Safety
- TSQ Quantum Ultra

Introduction

Veterinary drugs are widely used to prevent the outbreak of disease in livestock and are commonly administered as feed additives or in drinking water. In addition, veterinary drugs are given to treat diseases, for drying-off purposes, or to prevent losses during transportation. Many countries, such as the United States and those in the European Union, have set maximum residue limits (MRLs) for different food products of animal origin. Japan has also set MRLs for compounds identified on the Japanese Positive List. Recently, China has defined some new national standards to monitor banned antibiotics in foods.

As a response to this, new methods are being developed for the determination of these compounds in a cost-effective way. By using the Thermo Scientific Aria TLX system powered by TurboFlow™ technology a drastic reduction in sample preparation time can be achieved while minimizing matrix interferences. LC-MS/MS is a powerful tool in food analysis, especially when combined with automated sample preparation that reduces matrix interferences. In addition, minimizing sample handling improves the performance characteristics of the method, such as recovery, repeatability, and reproducibility. However, most analytical techniques developed for quantitative analysis of antibiotic residues in food have been based on off-line methods involving solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by LC-MS[1,2]. Only recently, methods employing automated sample preparation have been reported, but usually for a specific class of compounds, rather than a multi-class method[3]. We propose a quick, high-throughput, sensitive screening method for the determination of different classes of antibiotics in milk samples.

Goal

To develop a high-throughput, sensitive and precise screening method, with minimal sample preparation, for the determination of multi-class antibiotic residues in milk samples by LC-MS/MS.

Experimental

Sample Preparation

Sample preparation involved protein precipitation, by mixing 100 µL of milk products with 900 µL of a solution of 50 mM ammonium acetate in acetonitrile (50%) and water (50%) with 7.5 mM Na_2EDTA. After centrifuging the mixtures at 10,000 rpm for 10 minutes, the supernatants were collected and injected into the Aria™ TLX LC-MS system. The sample preparation took approximately 15 minutes to complete.

TurboFlow Method Conditions:

System:	Aria TLX-2
On-line Extraction:	Thermo Scientific TurboFlow Cyclone 0.5 x 50 mm and Cyclone P 0.5 x 50 mm columns connected in tandem
Mobile Phase A:	0.10% Formic acid and 0.05% Trifluoroacetic acid in water
Mobile Phase B:	Methanol
Mobile Phase C:	Isopropanol/Acetone (50:50)
Mobile Phase D:	2.0% Acetonitrile and 0.1% ammonium hydroxide in water
Injection Volume:	50 µL

HPLC conditions:

Analytical Column:	Thermo Scientific BETASIL Phenyl/Hexyl column 3.0 x 50, 3 µm at 50 °C maintained by a Thermo Scientific HOT POCKET column heater.
Solvent A:	0.10% Formic acid and 0.01 % Trifluoroacetic acid in water
Solvent B:	Methanol

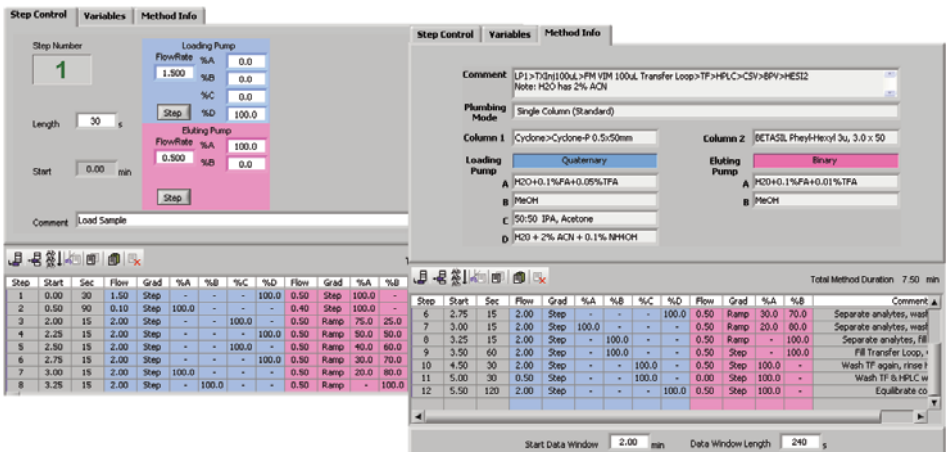

Figure 1: Aria OS provides easy-to-use software for setting up TurboFlow methods.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization probe. The MS conditions were as follows:

Ion Source Polarity	Positive Ion Mode
Spray Voltage	3500 V
Vaporizer Temperature	475 °C
Sheath Gas Pressure (N_2)	50 units
Auxiliary Gas Pressure (N_2)	25 units
Ion Sweep Gas Pressure	2 units
Capillary Temperature	250 °C
Collision Gas (Ar)	1.5 mTorr
Q1/Q3 Peak Resolution	0.7 u (unit mass resolution)
Scan Time	0.100 s
Scan Width	0.020 m/z
Data Acquisition Mode	SRM

The optimization of Selective Reaction Monitoring (SRM) parameters was performed by direct infusion of standards using positive electrospray ionization (ESI). Collision induced dissociation (CID) mass spectra were recorded for each analyte and the optimum collision energies obtained for the selected ion transitions. Table 1 summarizes these parameters.

Table 1: Selected ion transitions *(m/z)* and collision energy (CE) for studied compounds.

Analyte	Precursor Ion *(m/z)*	Product Ion *(m/z)*	CE (V)
1. Albendazole	266.1	234.0	15
		191.0	31
2. Sulphamethazine	279.1	124.2	14
		108.0	16
3. Phenylbutazone	309.2	211.3	16
		188.3	15
4. Difloxacin	400.1	356.1	20
		299.1	27
5. Spiramycin	422.0	174.0	35
		350.5	12
6. Tetracycline	445.5	410.0	17
		427.0	6
7. Oxytetracycline	461.2	426.0	19
		201.0	36
8. Salinomycine Na	773.4	265.4	50
		432.0	44

Results and Discussion

Liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry is currently the method of choice for the quantitative determination of antibiotics in food matrices. The advantages of this technique include high specificity, sensitivity, and throughput. Representative SRM chromatograms of a neat standard, whole milk, and fat-free milk sample containing 100 ppb of the veterinary drugs are shown in Figure 2.

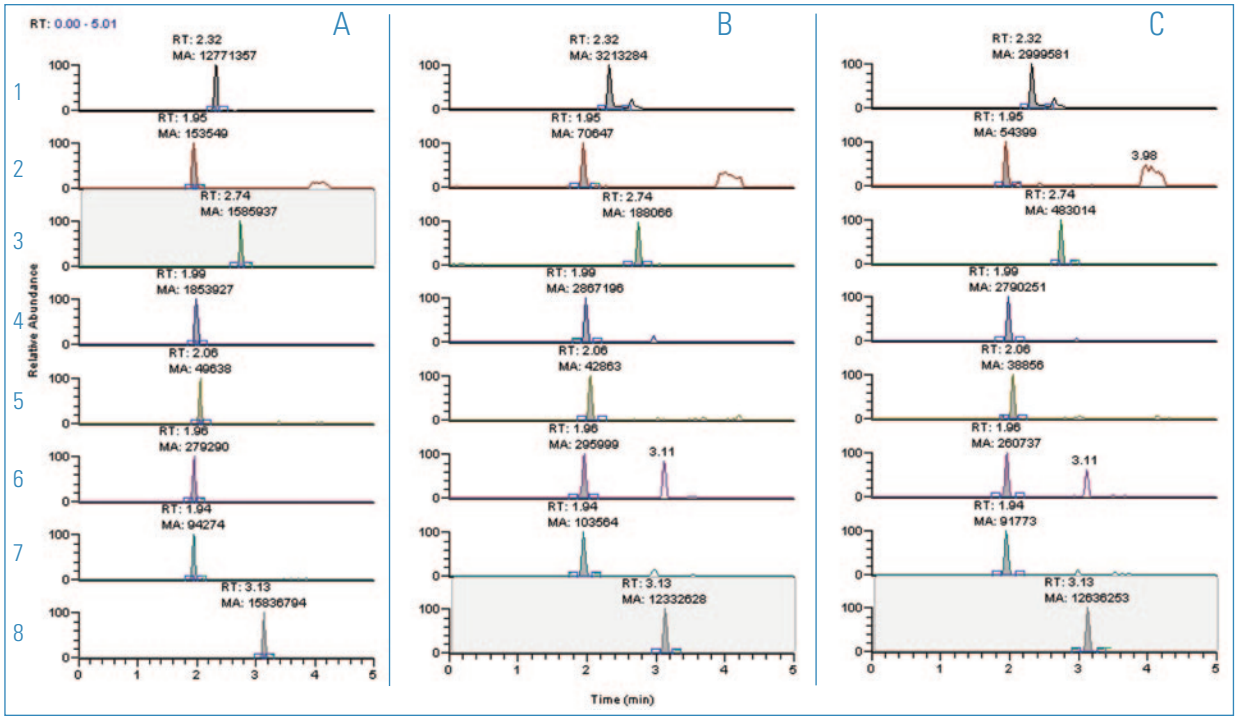

Figure 2: Representative SRM chromatograms of a neat standard (A) and milk samples (B-whole fat milk; C-low fat milk) containing antibiotics at 100 ppb level. 1-albendazole; 2-sulphamethazine; 3-phenylbutazone; 4-difloxacin; 5-spiramycin; 6-tetracycline; 7-oxytetracycline; 8-salinomycine Na

Table 2 presents linearity and precision data for the range of concentration studied in three types of commercially available milk samples. The analysis of a blank sample showed no major interferences present (Figure 3). The method proved to be linear in the studied range as well as reproducible (n=3) and precise. However, the amount of fat present in the sample seemed to influence the precision of the method for difloxacin and sulphamethazine at the highest level of the fortification (n<3).

Table 2: Linearity (r^2), precision (RSD %) for the different fortification levels when studying various fat content milk samples (Brand A)

Milk Samples – Brand A		Non-Fat				Low-Fat (2%)				Whole Fat			
Fortification Levels		50	100	250	500	50	100	250	500	50	100	250	500
Albendazole	r^2	0.9984				0.9967				0.9928			
	(RSD %)	1.6	1.7	1.7	2.7	6.3	3.2	3.6	4.2	2.6	6.2	1.2	2.9
Sulphamethazine	r^2	0.9964				0.9908				0.9970			
	(RSD %)	2.4	7.2	4.9	2.4	6.6	14.5	5.2	5.6	8.9	1.2	5.1	n/a*
Phenylbutazone	r^2	0.9947				0.9922				0.9963			
	(RSD %)	2.9	3.3	0.8	2.6	8.1	4.1	4.9	3.1	0.6	0.9	0.7	0.3
Difloxacin	r^2	0.9958				0.9907				0.9968			
	(RSD %)	12.2	4.3	6.0	2.4	10.8	4.6	2.7	5.5	2.6	6.1	5.1	n/a*
Spiramycin	r^2	0.9920				0.9740				0.9951			
	(RSD %)	11.1	11.8	8.4	4.1	10.9	4.0	10.0	9.4	13.3	6.5	5.2	0.2
Tetracycline	r^2	0.9923				0.9948				0.9903			
	(RSD %)	6.2	6.4	5.4	3.7	7.3	4.8	5.9	4.5	4.1	9.5	6.5	5.5
Oxytetracycline	r^2	0.9947				0.9922				0.9663			
	(RSD %)	2.9	3.3	0.8	2.6	8.1	4.1	4.9	3.1	0.6	0.9	0.7	0.3
Salinomycine Na	r^2	0.9993				0.9966				0.9984			
	(RSD %)	1.2	0.7	1.2	1.5	1.5	0.8	3.1	0.4	2.8	3.1	1.3	1.2

*n/a: n<3

A study evaluating the matrix effect was performed because it is well known that molecules originating from the sample matrix that co-elute with the compounds of interest can interfere with the ionization, causing either suppression or enhancement of the signal. The response areas of the neat standards were compared with the spiked milk samples for the 100 ppb level, for two different brands. Table 3 shows the relative response (%) as well as carry-over values and limits of detection (LOD). Carry-over was determined by injecting the higher calibration level standard (500 ppb) in triplicate, followed by a blank, and was found to be minimal.

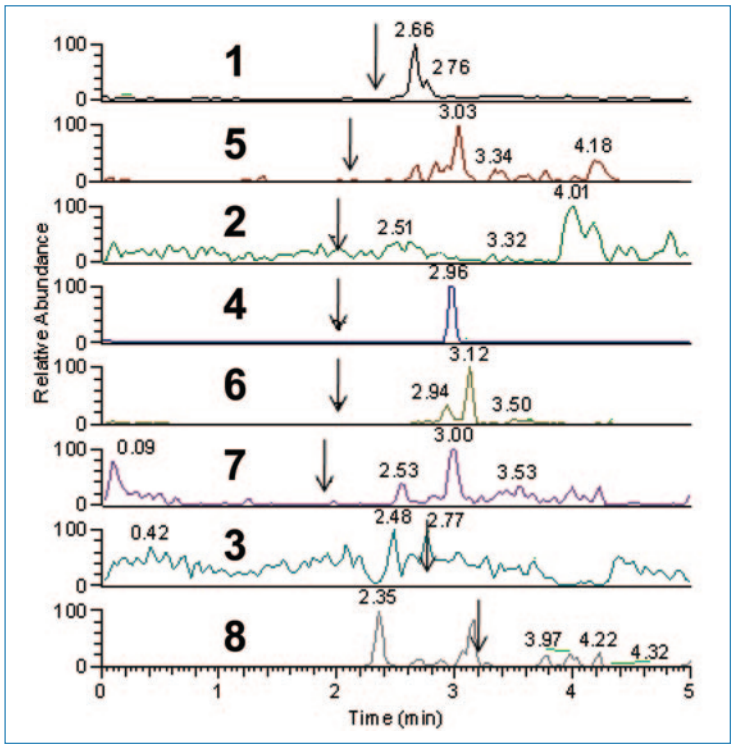

Figure 3: Representative SRM chromatogram of a blank whole milk sample. The arrows indicate the expected retention time for each of the analytes. 1-albendazole; 2-sulphamethazine; 3-phenylbutazone; 4-difloxacin; 5-spiramycin; 6-tetracycline; 7-oxytetracycline; 8-salinomycine Na

Table 3: Relative Response (%) found when running the method for two milk brands commercially available in the US market. The different milk samples were spiked with 100 ppb of stock solution and the peak areas compared with neat standards. Limits of detection of the method were calculated by linear regression analysis of the matrix matched calibration curve. Carry Over was minimal.

Analyte	Relative Response[1] (%) Brand A	Brand B	LOD (ppb)	Carry-over[2] (%)
Albendazole	- 82	- 85	0.4	1.2
Sulphamethazine	- 57	- 59	1.6	0
Phenylbutazone	- 69	- 25	1.9	0
Difloxacin	70	40	1.7	0.6
Spiramycin	- 28	- 37	5.2	0
Tetracycline	8	8	2.4	0
Oxytetracycline	31	- 5	3.0	0
Salinomycine Na	- 19	- 31	0.7	0.2

[1]*Relative Response (%) = (Area milk/Area Standard -1) x 100*
[2]*Carry-over (%) = (Area blank/Area standard) x 100*

Albendazole showed the strongest suppression because the signal was less than 20% than that of a neat standard while difloxacin showed signal enhancement indicating that the matrix is probably not completely removed. On the other hand, with two exceptions, the matrix effects seem to be similar for both brands of milk. While some matrix effects remain, the study showed that accurate quantitative data can be obtained because the method is linear in the concentration range of 50 to 500 µg/L as well as reproducible and precise (RSD <15%). Limits of detection ranged from 0.4 to 5.2 µg/L, which is well under most MRL values for veterinary drugs in milk. The use of an internal standard would compensate for the matrix effects.

The method was tested by screening a batch of real milk samples. The proposed method proved to be able to detect all the compounds presumably present in the sample.

Table 4: Screening of real milk samples

Sample	Preliminary results	Aria TLX coupled to TSQ Quantum Ultra™
01	Negative	Negative
02	Negative	Negative
03	Negative	Negative
04	Negative	Negative
05	Negative	Negative
06	Negative	Negative
07	Negative	Negative
08	Negative	Negative
09	Negative	Negative
10	Negative	Oxytetracycline 5ppb
11	Oxytetracycline 200 ppb	Oxytetracycline 1 ppm Tetracycline 5 ppb
12	Sulphamethazine 200 ppb	Sulphamethazine 200 ppb

Conclusion

This application note presents a new online LC-MS method for the simultaneous screening of different classes of antibiotics in milk. This method proved to be quick, sensitive, and reproducible. It can be successfully applied for the quantitative determination of several classes of antibiotics in milk samples. Accurate quantitative measurement of these compounds subjected to residual matrix interferences could be accomplished by using a suitable internal standard.

The automated TurboFlow LC-MS/MS method significantly improves the laboratory throughput by significantly minimizing the necessary sample preparation while still allowing limits of detection of low ppb levels.

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