Determination of Multi-Residue Tetracyclines and their Metabolites in Milk by High Performance Liquid Chromatography - Tandem Mass Spectrometry

Application Note

Food

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Abstract

Tetracyclines are probably the most frequently used antibiotics in animal husbandry. In this paper, a high performance liquid chromatography tandem mass spectrometric (HPLC /MS/MS) method is developed for the simultaneous determination of 10 antibiotic residues: minocycline, 4-epioxytetracycline, 4-epitetracycline, tetracycline, 4-epichlortetracycline, demeclocycline, chlortetracycline, methacycline, doxycycline, oxytetracycline in milk and animal tissues. In the method, Agilent's novel solid phase extraction cartridge and a reversed phase Agilent ZORBAX RX C8 column (5 µm, 150 mm × 2.1 mm) are used for purification and separation. The limit of detection (LOD) is between 0.5 and 10.0 µg/kg and the limit of quantitation (LOQ) is less than 50 µg/kg. The linearity is obtained from 5 to 1000 µg/kg. Overall recoveries are between 76.4% and 101% with a relative standard deviation (RSD, n = 6) less than 8.4%. The method is rapid, sensitive, convenient and robust, and can be used to simultaneously confirm multi-residues of tetracyclines and their metabolites in milk.

Agilent Technologies

Introduction

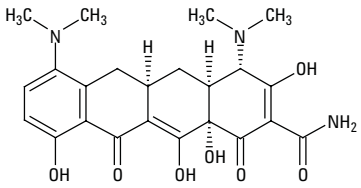
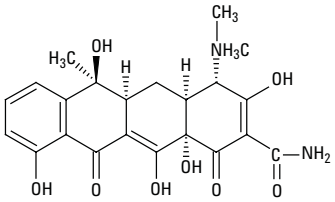
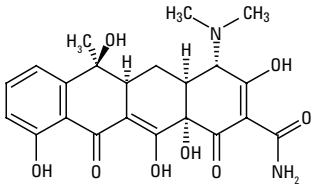
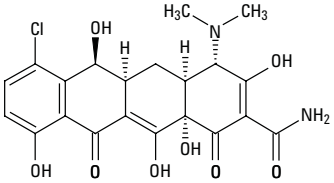
Antibiotics are used worldwide to control bacterial infection and promote healthy farm animals for milk production. Tetracyclines are broad-spectrum antibiotics, so they are widely used. However, it is undesirable to have them in the milk supply.

FDA's regulations for tetracyclines including oxytetracycline and chlortetracycline are set to provide an acceptable daily intake (ADI) and a tolerance for residues in milk. The ADI for total residues of these compounds is 25 micrograms per kilogram of body weight per day. Sixty percent (60 %) of the ADI is reserved for milk and 40 % for edible tissues. Based on the ADI, a tolerance of 300 ppb is set for the sum of residues of the tetracyclines including chlortetracycline, oxytetracycline, and tetracycline in milk. With the establishment of a tolerance of 300 ppb for the sum of residues of tetracyclines, a tolerance of 300 ppb for each of the three tetracyclines is also accepted.

In the EU, the maximum residue limit (MRL) for antibiotics is established according to (EEC) 2377/90, and for tetracyclines in milk is at 100 µg/kg (100 ppb). In China, the Government Standard (GB/T 21317-2007) also establishes the method for determination of these compounds in milk and animal tissues. This regulation took effective April 1, 2008.

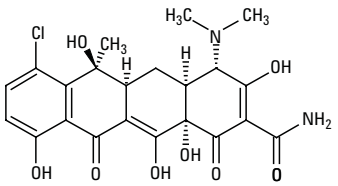
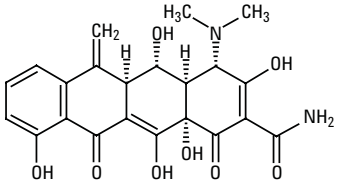
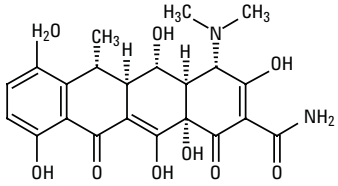
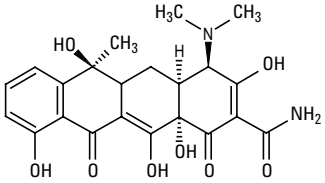
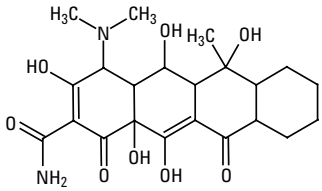
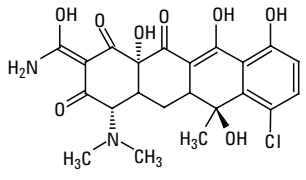
The purpose of this study is to develop a method for the Agilent 6410 LC/MS/MS to determine the presence of tetracyclines and their metabolite residues in milk. The method is rapid and easy to use. The tetracyclines and their metabolites are given in Table 1.

Table 1. The Compounds in this Study

No.	Name	CAS No.	Structure
1	Minocycline	10118-90-8	
2	Oxytetracycline	6153-64-6	
3	Tetracycline	60-54-8	
4	Demeclocycline	127-33-3	

(Continued)

Table 1. The Compounds in this Study

No.	Name	CAS No.	Structure
5	chlortetracycline	57-62-5	
6	methacycline	914-00-1	
7	doxycycline	564-25-0	
8			
8	4-epitetracycline	64-75-5	
9	4-epi oxytetracycline	35259-39-3	
10	4-epichlortetracycline	14297-93-9	

Experimental

Reagents and Chemicals

Water and methanol are HPLC grade, and they, along with formic acid were all purchased from Fluka. The standards were purchased from Sigma-Aldrich.

Instrument Settings

Table 2. LC/MS/MS Conditions

HPLC	
Column	ZORBAX RX-C8, 2.1 mm × 150 mm, 5 µm (p/n 883700-906)
Flow rate	0.3 mL/min
Mobile phase	A: Water/ 0.1 % Formic Acid B: Methanol
Gradient	0–10 min, B from 5% to 30% 10–12 min, B from 30% to 40% 12.5–18 min, B 65% 18.5–25 min, B 95% 25.5 min, B 5.0%
Total run	28 min
Post time	5 min
Temp	30 °C
injection	5 µL
MS Source settings	
Source	ESI
Ion polarity	Positive
Drying Gas temp.	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	4000V

MRM Setting

Name	Frag.	Precursor ion	Product ion	CE	Rt. (min)
Minocycline	120	458	352	35	
			441	20	8.58
4-Epitetracycline	120	445	410	20	
			427	10	8.60
4-Epioxytetracycline	120	461	426	20	
			444	15	9.47
Tetracycline	120	445	410	20	
			427	15	9.90
Oxytetracycline	120	461	426	20	
			443	10	9.95
Demethylclocycline	120	465	430	25	
			448	15	11.25
4-Epichlortetracycline	120	479	444	22	
			462	15	11.59
Chlortetracycline	120	479	444	22	
			462	15	12.95
Methacycline	120	443	381	25	
			426	15	13.98
Doxycycline	120	445	154	30	
			428	15	14.08

Sample Preparation

Extraction:

1. Weigh a 5 g-milk sample (accurate to 0.01 g) into a 50-mL colorimetric tube, and dissolve with 0.1 mol/L Na_2EDTA-McIlvaine buffer solution and bring volume to 50 mL.
2. Vortex for 1 min and ultrasonicate the extract in an ice water bath for 10 min.
3. Transfer the sample to a 50-mL polypropylene centrifuge tube and cool to 0 °C ~ 4 °C.
4. Centrifuge the sample at a speed of 5000 rpm for 10 min (below 15 °C).
5. Filter with fast filter paper.

Purification:

1. Accurately draw 10 mL of the extract (equivalent to 1 g sample) and put it through the SampliQ OPT cartridge (p/n 5982-3036) at a speed of 1 drop/s.
2. After it elutes completely, clean the cartridge with 3 mL water adjusted to pH 4.5 with trifluoroacetic acid and then discard the entire effluent.
3. Under a negative pressure below 2.0 kPa, drain the cartridge for 5 min.
4. Elute with 10 mL of 10 mmol oxalic acid in methanol.
5. Collect the eluent and dry with nitrogen below 40 °C.
6. Dissolve the residue with 1.0 mL of the initial mobile phase.
7. Filter with a 0.45-µm filter membrane and inject.

Results and Discussion

Optimization and Separation

Fragmentor and Collision Energy (CE) optimization

It is well known that the LC/MS/MS QQQ is the best tool to identify, confirm and quantify target analytes in food matrices. In order to get the best response, only two parameters need to be optimized for each compound on this instrument, the fragmentor and the collision energy. The correct fragmentor voltage allows the highest transmission of the precursor ion into the mass analyzer. The correct collision energy provides the highest intensity of quantitation of the qualifier product ion.

One method of optimization is to inject the sample multiple times at the different fragmentor voltages set within segments of a single run. This is shown in Figure 1 for minocycline. For this compound, there is a small increase in detection as the voltage is increased. Collision energy is optimized in the same way and the results for tetracycline is shown in Figure 2.

Recently, Agilent introduced the "Optimizer" program that automatically determines the optimum fragmentor voltage and collision energy and stores the results in the Optimizer Database. Using this program and flow injection with or without a column, the user enters the compounds to be optimized and their molecular formulas. The nominal mass of the compound is automatically calculated from the formula. The user then specifies the adducts expected for positive and negative modes, the low mass cutoff, any ions to be excluded, and the method to be used (mobile phase conditions etc.). Once started the program will inject the sample, determine the precursor ion, and optimize the fragmentor voltage by stepping through the increments that the user selected for one injection. The program then selects the voltage producing the highest intensity for the precursor ion. For tetracycline, this is shown in Figure 3.

The program then performs a product ion scan on a second injection of the sample, and chooses the four most prevalent product ions. It reinjects the sample again and performs MRMs of each ion collected with collision energies in increments covering the range of voltage selected by the user. The collision energy that generates the maximum signal for each product ion is then automatically determined and can be stored in the database. The data from this collision energy optimization for tetracycline is shown in Figure 4 along with the ion breakdown curve shown in Figure 5. Compounds with product ions can be imported directly into the users' acquisition method.

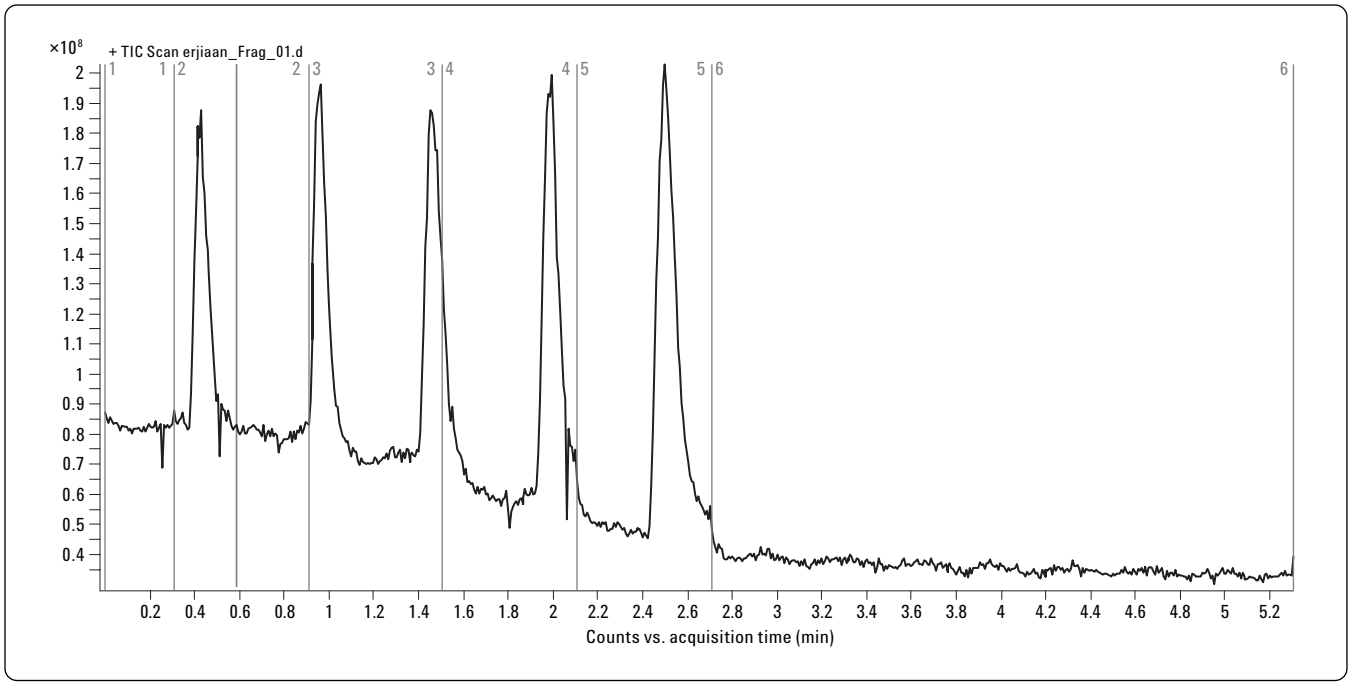


Figure 1. Optimization of fragmentor voltage for minocycline from 60-160 by steps of 20 V.

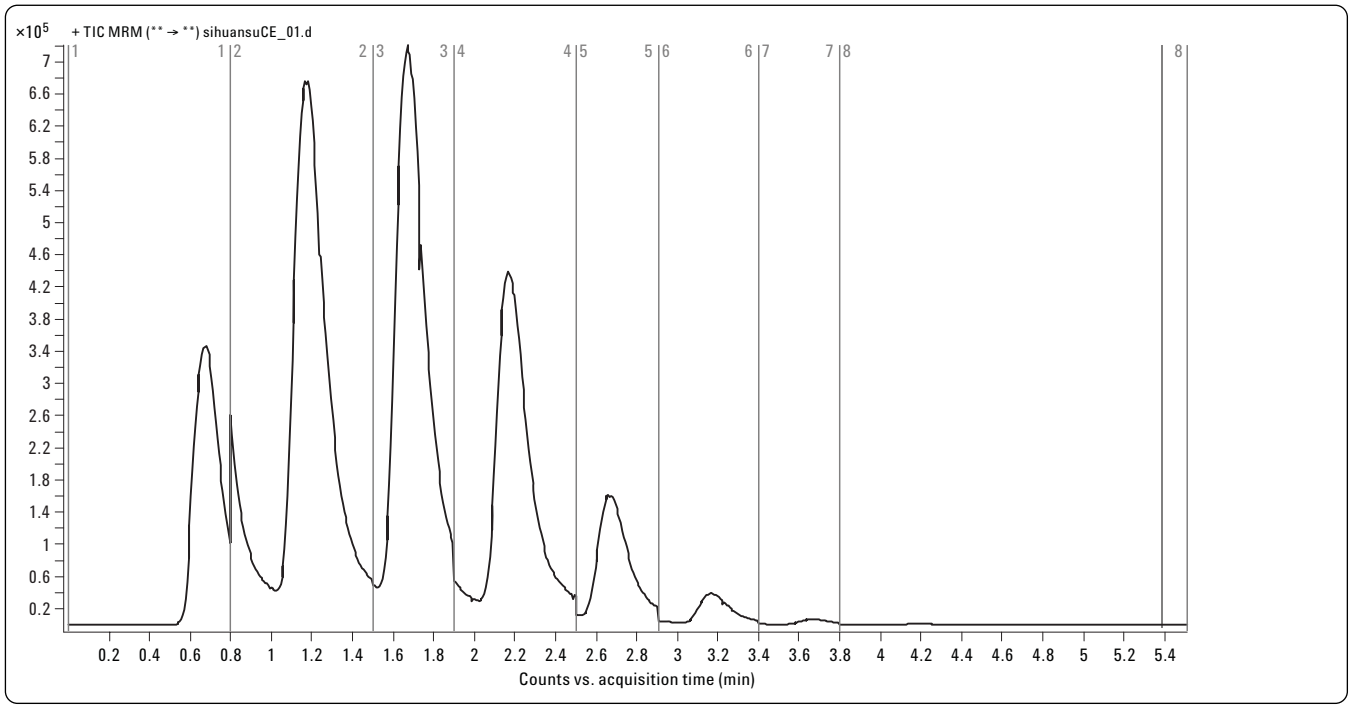


Figure 2. Manual collision energy optimization of tetracycline.

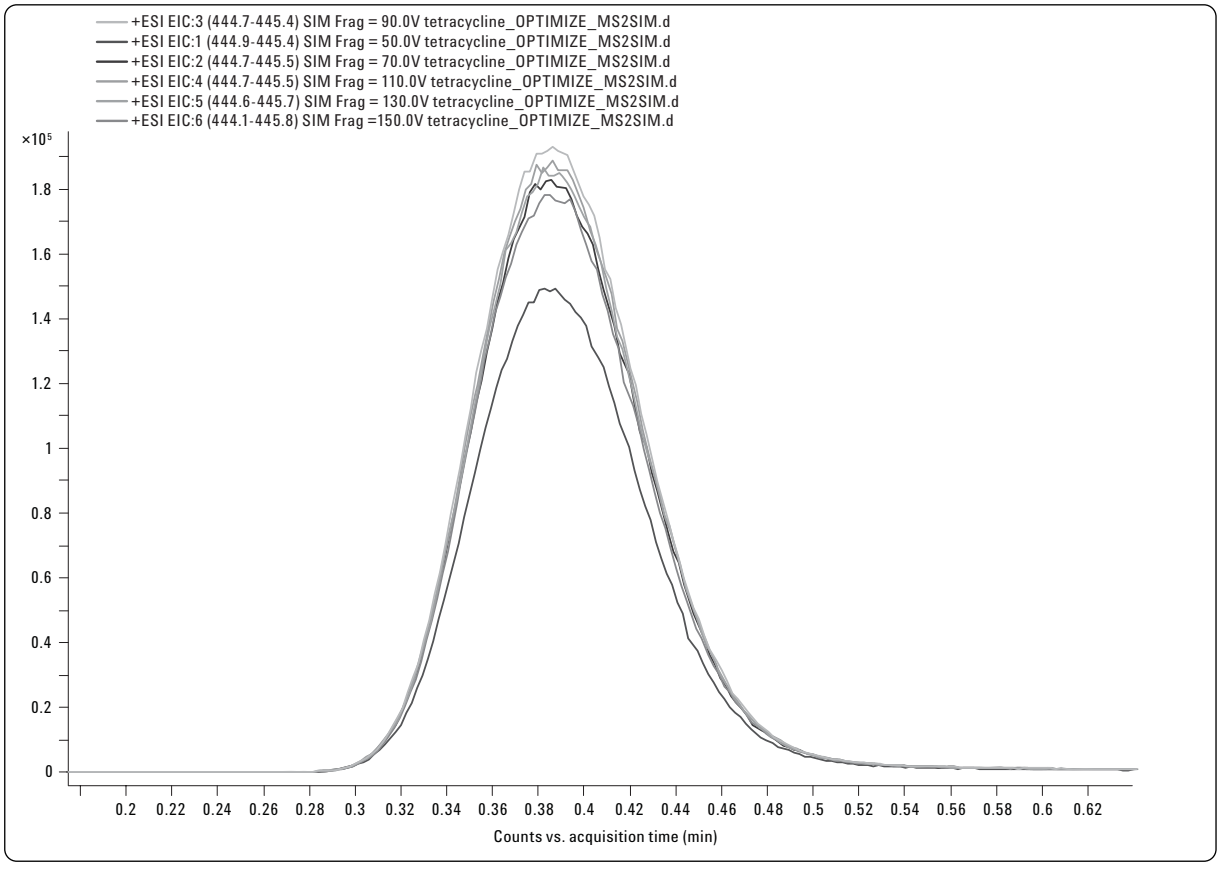


Figure 3. Single injection automatic determination of fragmentor voltage for tetracycline using the Optimizer program.

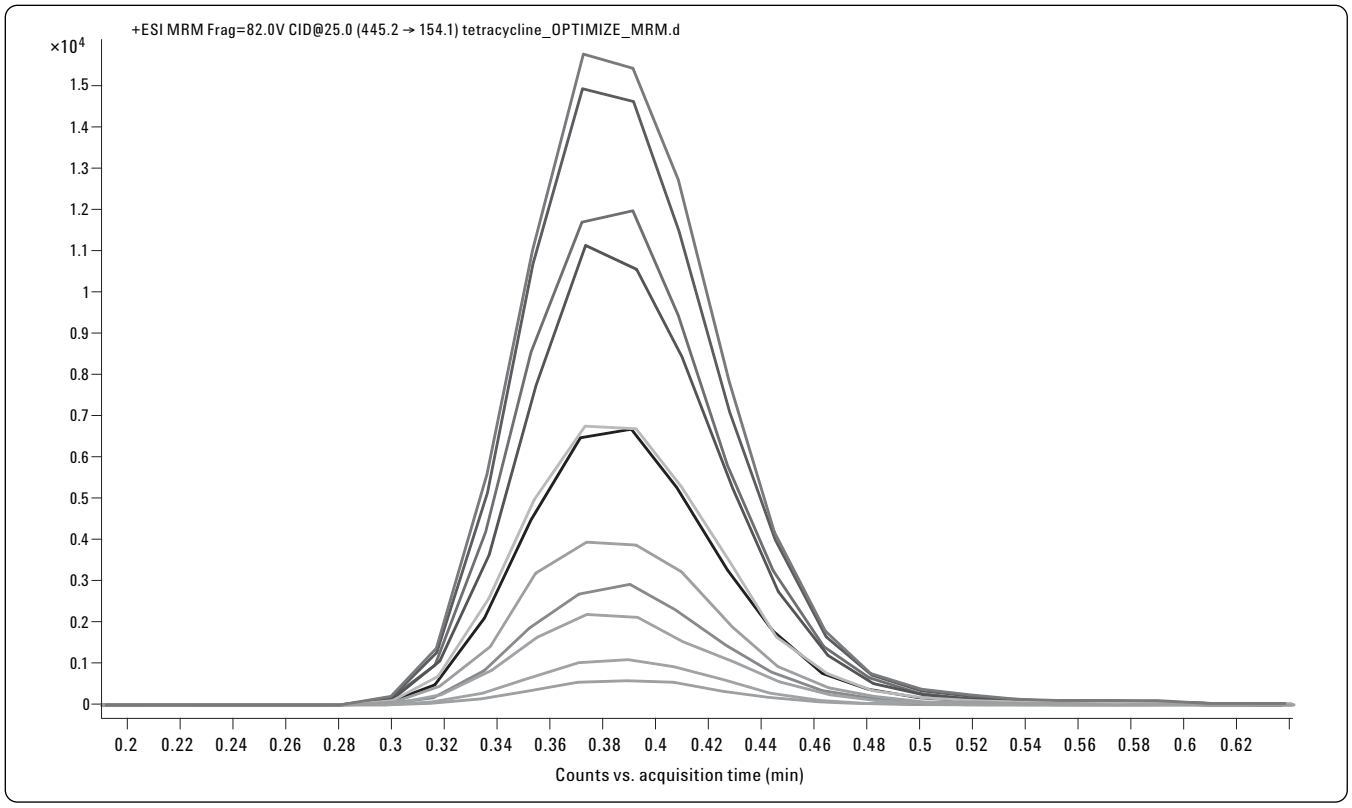


Figure 4. Single injection automated collision energy determination using Optimizer program for tetracycline.

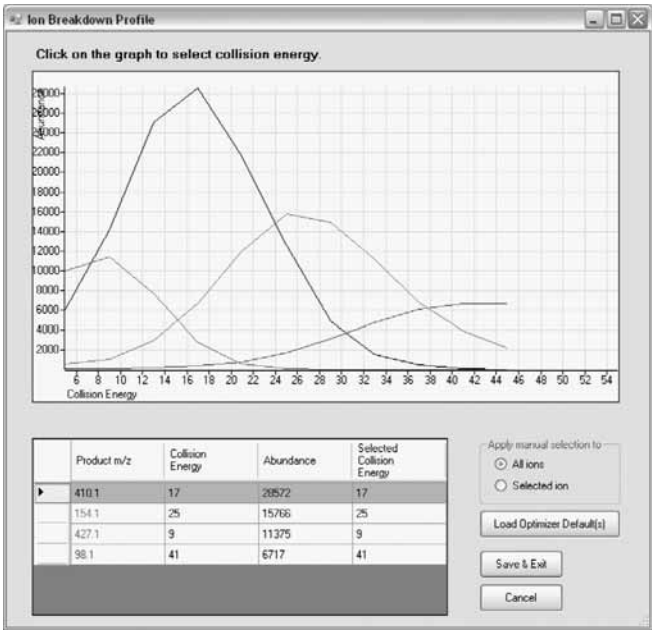


Figure 5. Ion breakdown profile for tetracycline as determined by the Optimizer program.

Separation

Sample preparation and separation of tetracycline, chlortetracyline and oxytetracycline is important. The challenge in separating these kinds of compounds is that they easily degrade under conditions of weak acid, strong acid, strong base, and heat converting the diasteriomer to its diaxial epimer.

The typical process is shown below with tetracycline:

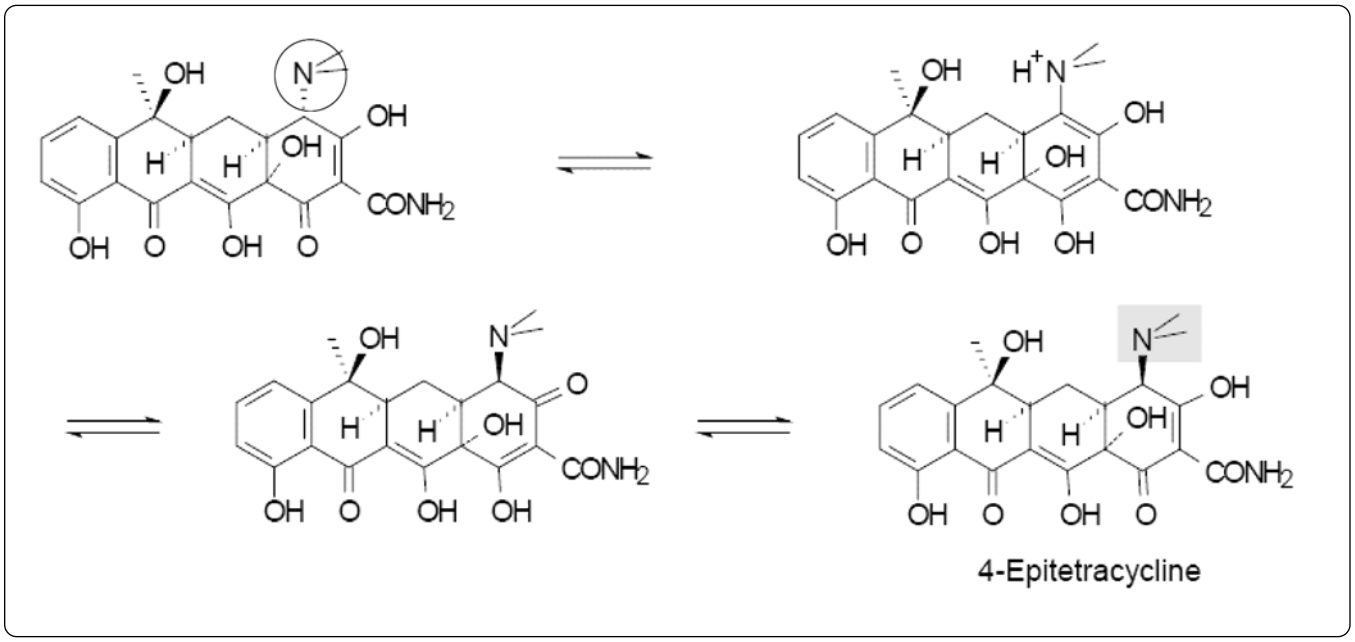


Figure 6. The degradation of tetracycline to 4-epitetracycline.

Tetracyclines and their degradants are diasteriomers with the same formula and the same fragment ions are formed in MS/MS. Therefore, they have the same precursor ions, qualitative ions, and quantitation ions. In order to identify and confirm them in the Rapid Resolution liquid chromatograph (RRLC), separation is important for this analysis. Using the Agilent ZORBAX Rx-C8, 2.1 mm × 150 mm, 5-µm particle size column and a simple gradient, the three epimer pairs of these compounds are well separated. This is shown with the retention times given in Table 2. Figure 7 shows the graphic representation of the separation of tetracycline and its epimer.

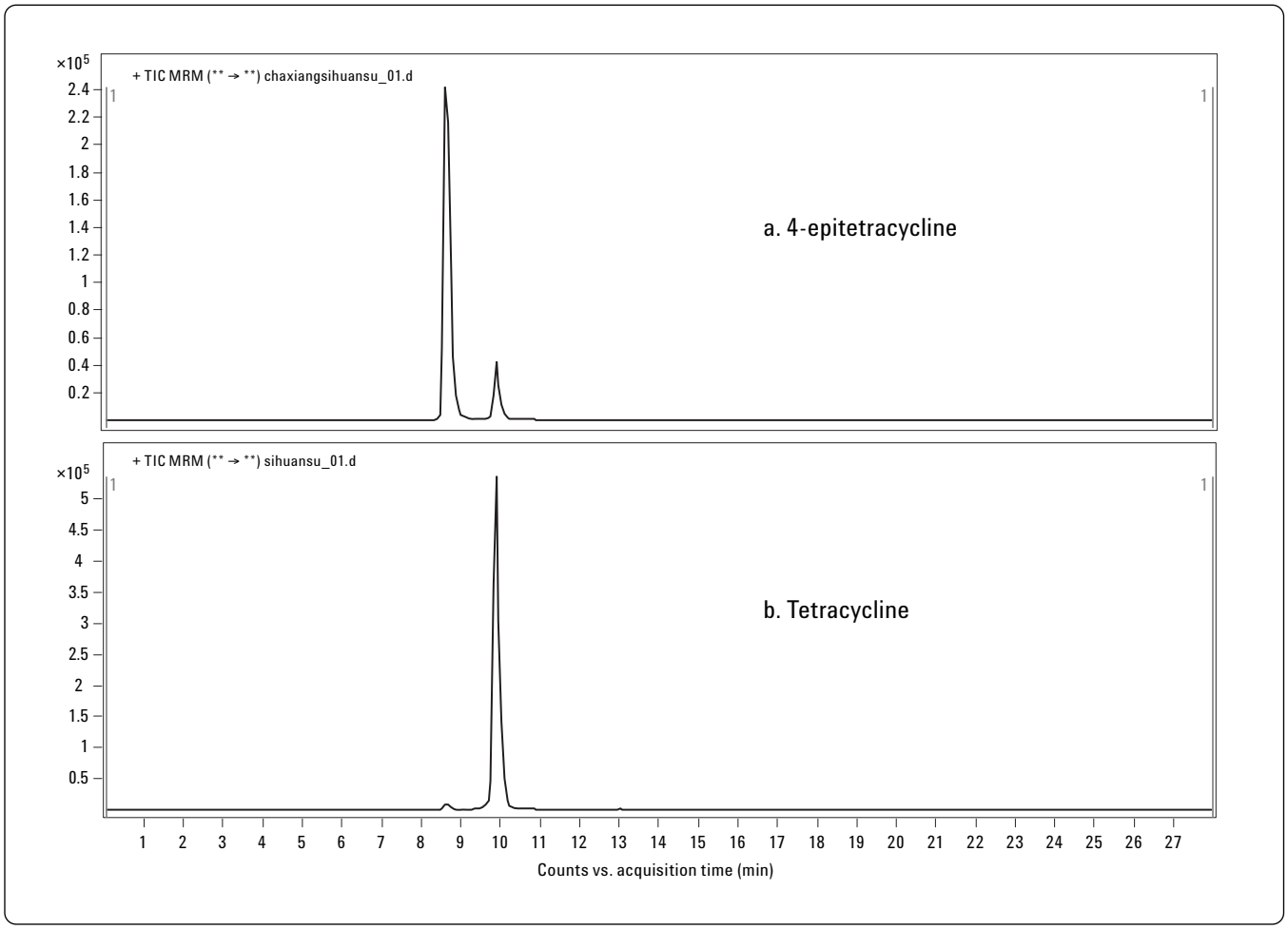


Figure 7. The separation of tetracycline and its degradation product 4-epitetracycline.

Linearity, LOD and LOQ

Linearity, LOD and LOQ were evaluated in both solvent and a milk matrix. The results are given in Table 3 and show that linearity is similar for both solvent and milk matrix and generally provide greater than 0.99 coefficient of variance. The tetracyclines do not ionize well with electrospray but the limits of detection (LOD) for each are still in the low pictograms on-column. The limit of quantitation is typically set at a signal to noise (S/N) of 10:1 but we report twice that in the solvent. The graphic representation of the calibration curve for minocycline is shown in Figure 8.

Table 3. Quantitative Performance of Tetracyclines in Solvent and Milk Matrix

	Standards in solvent*			Standards in Milk matrix*	
Name	**R^2**	**LOQ** (S/N=20) pg on column	**LOD** (S/N=3) pg on column	**R^2**	**LOD** (S/N=3) pg on column
Minocycline	0.999	41.5	6.2	0.990	16.3
4-epitetracycline	0.991	10.8	1.6	0.994	8.7
4-epioxytetracycline	0.996	14.7	2.2	0.996	12.8
Tetracycline	0.998	9.4	1.4	0.994	10.2
Oxytetracycline	0.996	10.7	1.6	0.991	8.6
Demethylclocycline	0.999	22.8	3.4	0.993	8.1
4-epichlortetracycline	0.986	38.2	5.7	0.987	11.9
Chlortetracycline	0.986	8.1	1.2	0.994	7.6
Methacycline	0.999	20.8	3.1	0.994	12.3
Doxycycline	0.999	32.2	4.8	0.995	11.2

Note: *The calibration curve range is from 1 ppb-1 ppm with injection volume of 5 uL

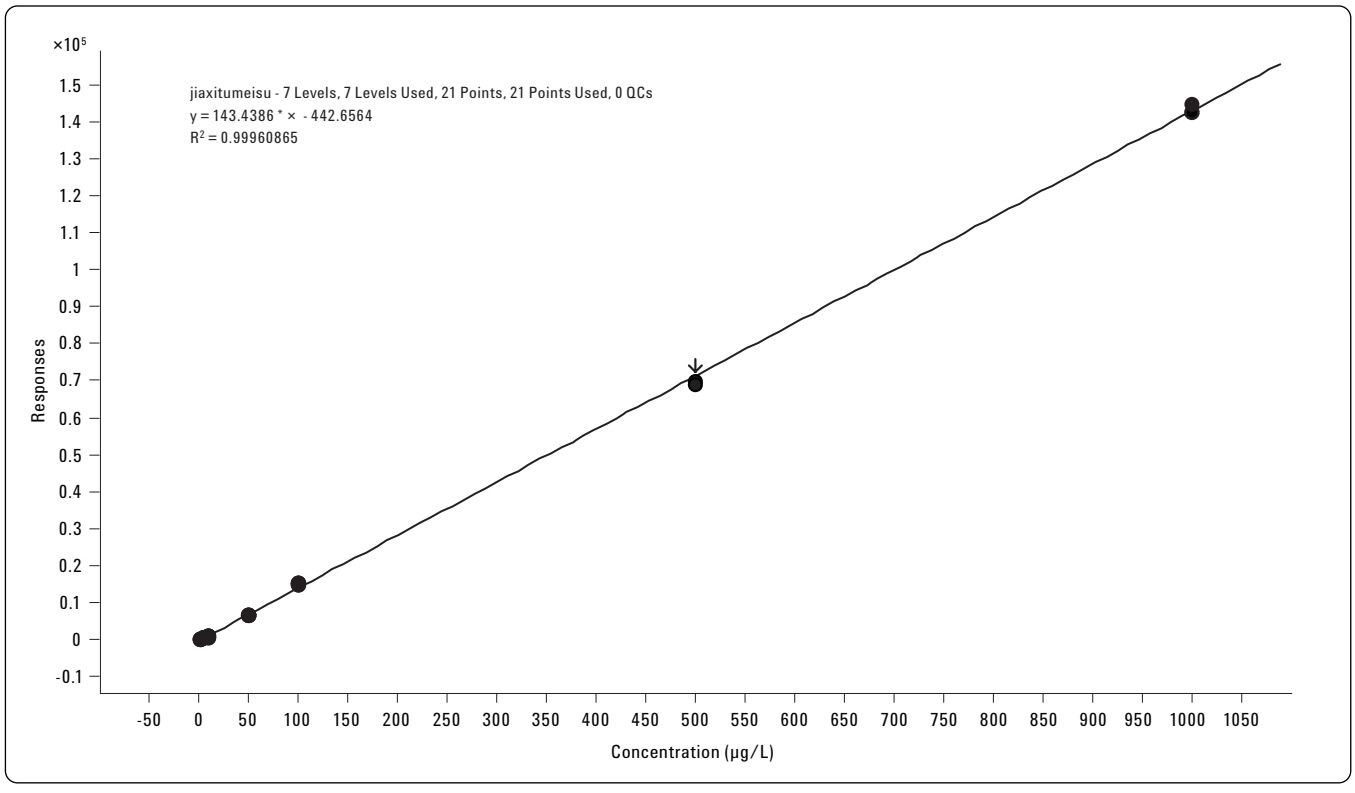


Figure 8. Tetracycline calibration curve from 1 ppb to 1000 ppb.

Recovery and Repeatability

The recovery and repeatability of the method was evaluated and the results shown in Table 4. All recoveries were greater than 80 % which is generally accepted as sufficient. In addition the precision, as shown in the table, is 5 % or better for the lower concentration and less than 2 % for the higher concentration. Ion ratios for confirmation are a very important performance criterion and these results show excellent repeatability. A graphic representation of the ion ratios for methacycline is shown in Figure 9. The ratios combined with matching retention time provide the necessary information for confirmation.

Table 4. Recovery and Repeatability in Milk Matrix

Name	Recovery in milk (Conc. 50 ppb n=6)	RSD % (Signal response n=6)	RSD % (Ion ratio n=6)	Recovery in milk (Conc. 100 ppb n=6)	RSD % (Signal response n=6)	RSD % (Ion ratio n=6)
Minocycline	96.5	4.9	2.1	101.4	1.6	1.0
4-epitetracycline	89.2	3.8	1.5	96.3	1.6	0.9
4-epioxytetracycline	84.4	5.4	1.3	88.2	0.9	0.6
Tetracycline	86.1	2.5	1.2	90.7	1.1	1.2
Oxytetracycline	77.6	3.8	1.6	82.5	1.2	0.9
Demethylclocycline	79.2	2.0	3.1	84.7	0.9	0.6
4-epichlortetracycline	76.4	5.5	5.4	84.3	1.1	0.5
Chlortetracycline	94.3	4.5	1.5	100.9	1.8	1.1
Methacycline	86.3	1.0	1.9	91.2	1.2	0.8
Doxycycline	78.7	3.6	6.7	82.4	1.0	0.8

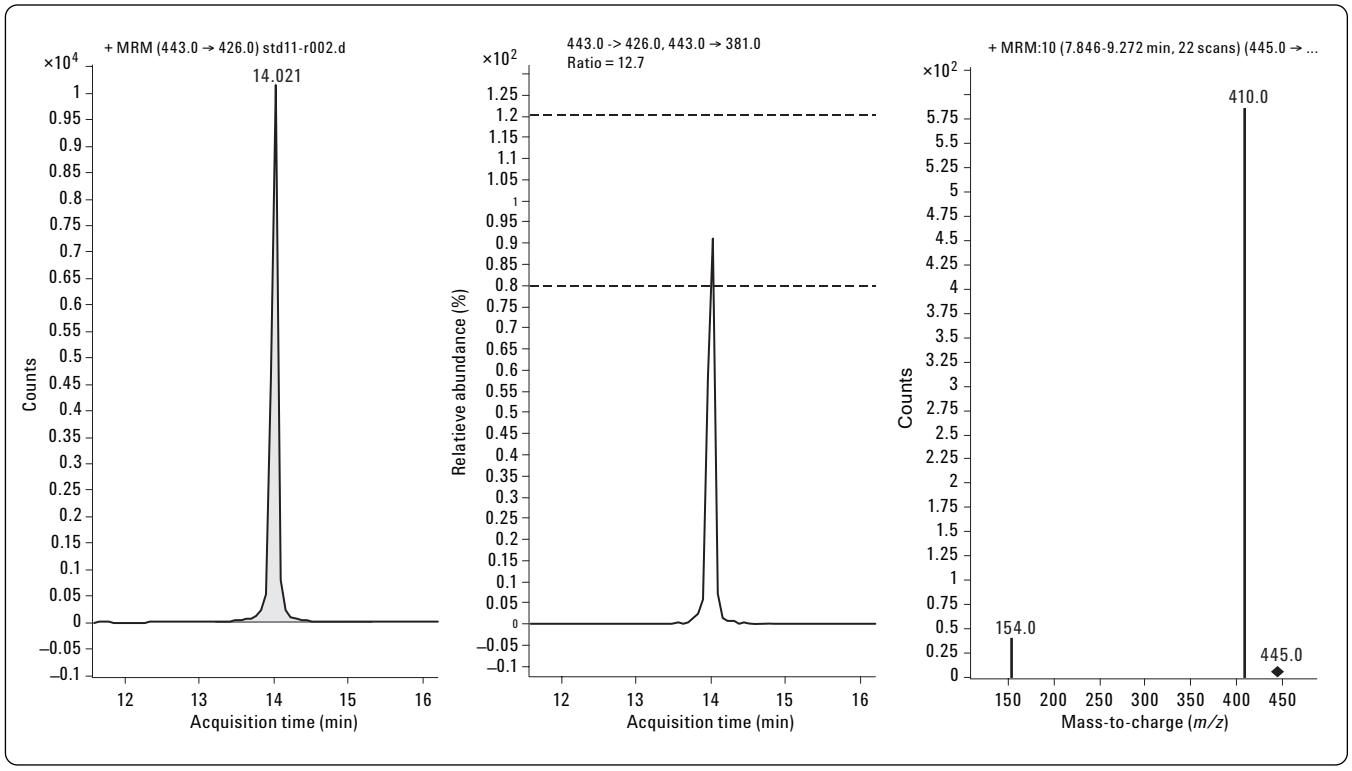


Figure 9. Shows the ion ratios for qualifier ion and the quantitation ion of methacycline.

Study of Ion Suppression

In general, tandem MS can remove chemical noise to get a "clean" spectrum even in dirty and complex food matrices. However, the matrix may contain components that suppress the ionization of the analyte. Figure 10 shows the comparison of the response of methacycline and tetracycline in solvent and milk. The difference in the slope of each curve demonstrates the suppression effect of the milk matrix.

Because of the strong suppression observed, using the external standard method (ESTD) for calibration, matrix matched standards should be prepared in antibiotic-free milk, or milk known to not contain the analytes. In this way, the calibration curve is generated with the same matrix effects as the samples.

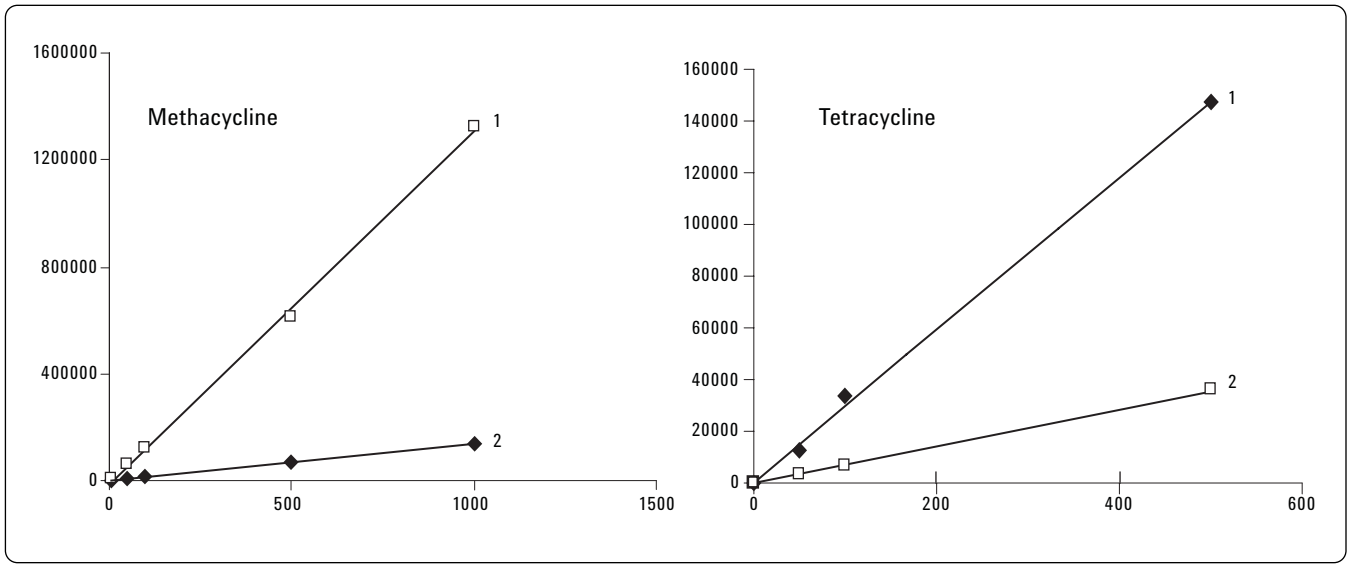


Figure 10. Ion suppression of two of the tetracyclines in milk; 1) response in solvent, 2) response in milk.

Conclusions

The results of this work show that the Agilent 6410 triple quadrupole LC/MS System is a robust, sensitive, and repeatable instrument for the study of tetracyclines residues in a milk matrix. In China, the government standard requirement (GB/T 21317-2007) sets the detection limit at 50 ppb with a 100 µL injection. This method easily meets these requirements. Additionally, these types of antibiotics readily degrade under the conditions of weak acid, base etc. The preparation method used here avoids this reaction, allowing the LC method to separate these isomers for reliable confirmation and quantitation. Finally, ion suppression is considered for the LC/MS/MS method when comparing different compounds in the same matrix to their response in solvent. Using the ESTD method, the preparation of a matrix-matched calibration curve is necessary to obtain accurate results, even though the recoveries measured for the sample preparation are better than 80%.

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