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Transfer and Optimization of Existing Methods for Analysis of Antibiotics in Meat to Agilent Poroshell 120 EC-C18 Columns using MS/MS Detection

Application Note

Food

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Abstract

In this work, a generic gradient method with UV detection is used to evaluate mobile phase choices for fast method optimization of antibiotics analysis in meat, with the ultimate goal of producing a mass spectrometer compatible method. This evaluation included four buffers and two organic choices. The mobile phase combination that yielded the best separation is transferred and optimized to an Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm column. Gradient time was decreased from 45 min to 12 min. Time can be further reduced using a 3 mm × 50 mm column, at the cost of some resolution. The method is demonstrated on an Agilent 6410 triple quadrupole LC/MS System coupled with an Agilent 1200 Series Rapid Resolution LC.

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Table 1. Method Parameters for Various Column Dimensions

	4.6 × 50 mm Poroshell 120 EC-C18	3.0 × 50 mM Poroshell 120 EC-C18	2.1 × 100 mM Poroshell 120 EC-C18
Mobile Phase	A: Buffer, varies B: Organic, varies	A: 10 mm ammonium formate pH 3.8 B: Acetonitrile	A: 10 mm ammonium formate pH 3.8 B: Acetonitrile
Gradient	10-40% B	10-40% B	10-40% B
Gradient Time	12 min	12 min	12 min
Flow Rate	2 mL/min	0.85 mL/min	0.42 mL/min
Injection Volume	0.5 µL	5 µL	2.5 µL or 10 µL
Sample	0.1 mg/mL antibiotics	1 µg/mL antibiotics	1 µg/mL or 10 ng/mL antibiotics
TCC Temperature	30 °C	30 °C	30 °C
Detector	DAD: Sig = 270, 4 nm; Ref = 360, 100 nm	MS/MS: See Table 2	MS/MS: See Table 2

Introduction

Administration of antibiotics is a common practice in chicken, pork, beef and fish farming. Many domestic cattle receive various antibiotics in their feed for the prevention and control of disease caused by fungi and bacteria. Many countries regulate acceptable residue levels of these compounds in agricultural and animal products. In this work, an older method is transferred from a 5 µm, 250 mm column to a new superficially porous column to increase the speed of the analysis and change the method of detection from UV to MS/MS. An increase in throughput of 5 to 10 times is demonstrated, while minimally impacting sample preparation. Since the analysis time is shortened dramatically, time is available for optimization of mobile phase selectivity (pH, buffer types and organic modifier).

Transition methods can be developed by modifying an existing method or starting fresh. In this case, the objective was to develop a new MS-compatible separation from an existing UV separation. Consequently, a change in the mobile phase was required because 0.7 % phosphoric acid is not a desirable solvent for MS detection. A generic screening method using 0.1 % formic acid was investigated, but additional MS-compatible solvent systems were also evaluated. In this work a method is developed by first screening different mobile phase combinations using a short Agilent Poroshell 120 column using UV detection, then transferring that method to an Agilent 6410 triple quadrupole LC/MS System. A major advantage of the Agilent Poroshell 120 EC-C18 is that it uses the same 2 µm frit as the original 5 um column, negating the need for sample preparation method development.

Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm columns have similar performance to 1.8-µm totally porous Agilent ZORBAX Eclipse Plus C18 columns, but since they use 2-µm column frits similar to those found on 5-µm columns, they require no additional sample preparation. This allows for a more seamless method transfer. While some previous work demonstrates the use of Agilent Poroshell 120 columns on older Agilent 1100 systems, they are ideally used on more modern systems such as the Agilent 1200 or 1260 series UHPLC's.

Table 2. MRM Transitions for Antibiotic Compounds.

Compound name	Precursor ion	Product ion	Fragmentor voltage	Collision energy
Sulfamerazine	265	172	100	25
Sulfamerazine	265	108	100	25
Thiamphenicol	338	308	140	10
Thiamphenicol	338	118	140	50
Sulfamethazine	279	124	100	25
Sulfamethazine	279	108	100	30
Furazolidone	226	137	140	25
Furazolidone	226	122	140	25
Sulfamonomethoxine	281	126	100	25
Sulfamonomethoxine	281	108	100	25
Oxolinic acid	262	160	100	40
Oxolinic acid	262	130	100	45
Pyrimethamine	249	198	140	45
Pyrimethamine	249	128	140	60
Sulfadimethoxine	311	156	140	25
Sulfadimethoxine	311	108	140	55
Sulfaquinoxaline	301	129	100	50
Sulfaquinoxaline	301	108	100	40
Difurazone	361	222	100	15
Difurazone	361	154	100	45

Experimental

Method development is based upon the use of a generic gradient. Using a short 4.6 mm × 50 mm Poroshell 120 EC-C18, 2.7 µm column, several different mobile phases can be quickly evaluated. The generic gradient is run at 2.0 mL/min, starts at 10% and proceeds to 40% organic over 12 min. This gradient is later transferred to 2.1 mm × 100 mm and 3 mm × 50 mm columns by changing the gradient according to Equation 1. The three gradients used are listed in Table 1 with MRM transitions shown in Table 2. MS-compatible mobile phases consisting of volatile buffer components such as formic acid, ammonium formate buffer and ammonium acetate buffer are used.

An Agilent 1200 Rapid Resolution LC (RRLC) system was used for this work:

- G1312B Binary Pump SL.
- G1367C Automatic Liquid Sampler (ALS) SL.
- G1316C Agilent 1290 Infinity Thermostatted Column Compartment (TCC) SL.
- G1315C Agilent Diode Array Detector (DAD) SL using a G1315-60024 micro flow cell (3-mm path, 2-µL volume).
- G6410 Agilent Triple Quadrupole LC/MS System with Electrospray (ESI).
- ChemStation version B.04.01 was used to control the HPLC and process the data. Agilent MassHunter Version 2.0 was also used to control the Agilent 6410 Triple Quadrupole LC/MS System, the Agilent 1200 Rapid Resolution LC (RRLC), and to analyze the data.

Three Agilent Poroshell 120 EC-C18 columns were used in this work:

- 4.6 mm × 50 mm, 2.7 µm p/n 699975-902
- 3.0 mm × 50 mm, 2.7 µm p/n 699975-302
- 2.1 mm × 100 mm, 2.7 µm p/n 695775-902

The compounds of interest are shown in Figure 1, with their respective structures. Compounds were dissolved in water at 1 mg/mL. Equal aliquots were combined to produce a mixed sample. Compounds were purchased from Sigma Aldrich (Bellefonte, PA). Additionally, methanol, acetonitrile, ammonium formate, ammonium acetate, formic acid, and glacial acetic acid were purchased from Sigma Aldrich. Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Buffers used in this work were prepared by dissolving an appropriate amount of the ammonium salt to produce a 10 mM solution, adding 950 mL water and titrating the solution with either formic acid (for the ammonium formate buffers) or glacial acetic acid (for the ammonium acetate buffers). The buffer solutions were then brought to a 1 L volume.

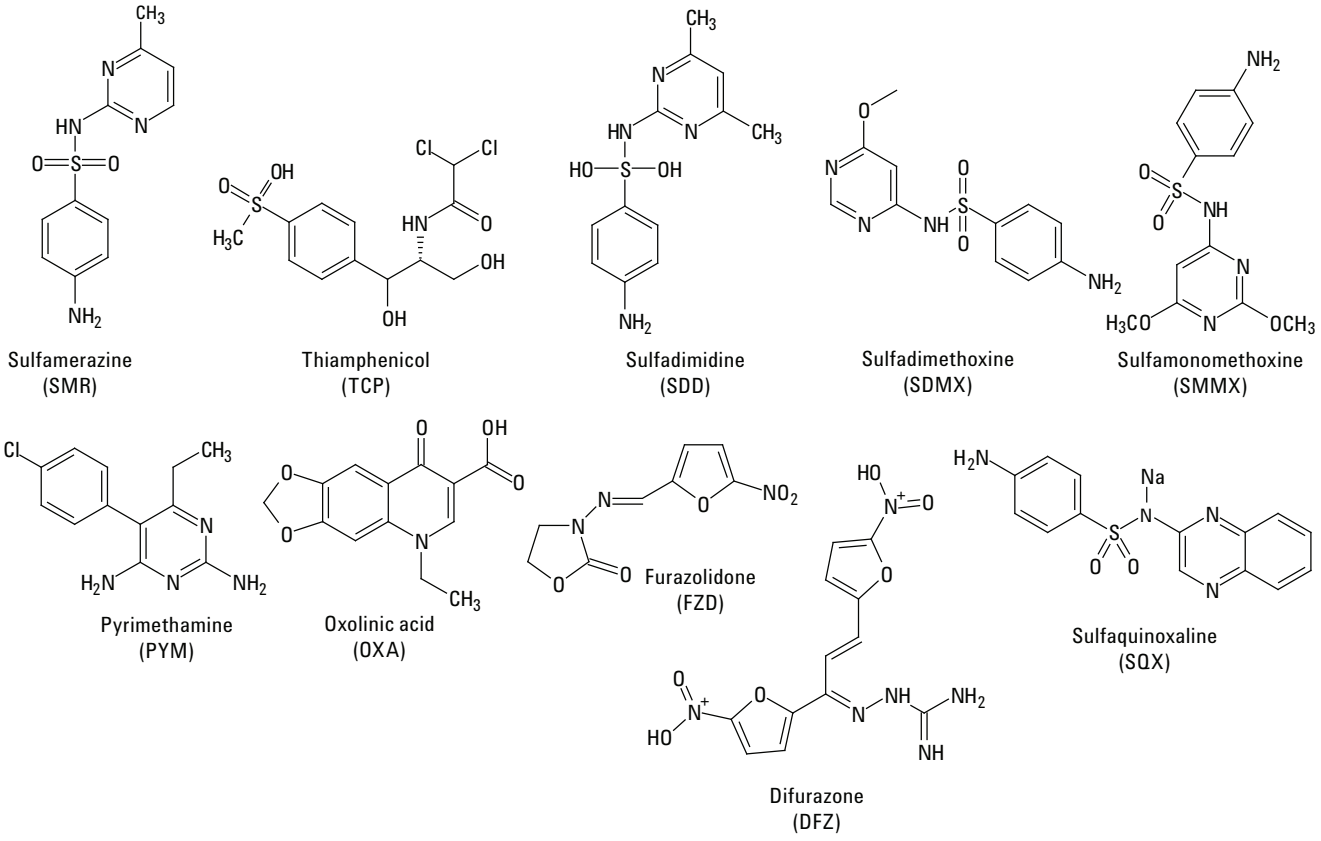


Figure 1. Compounds of interest.

Results and Discussion

The original method published in 2002 by Kumagai and Onigbinde provides an effective method for the analysis of antibiotics in meat using UV detection. As seen in Figure 2, the method separates the analytes in approximately 45 min. However the nonvolatile phosphoric acid in the mobile phase is not compatible with MS detection.

In many cases, simple scaling of a method will allow for a fast method transfer. In this case, however, a change in the mobile phase was required for LC/MS compatibility. The use of short Poroshell 120 EC-18 4.6 mm × 50 mm, 2.7 µm columns for assessing mobile phase changes has several advantages. One advantage is that they allow quick separations without sacrificing resolving power. In addition, since they are used at 2 mL/min with a generic gradient, the solvent is rapidly purged through the system. This ensures that the solvent screening experiment can be quickly performed by changing solvent bottles, with no concerns about residual solvents in the HPLC pump or the degasser. These columns can be used for LC/MS but typically smaller diameter columns such as 3.0 or 2.1 mm columns are used.

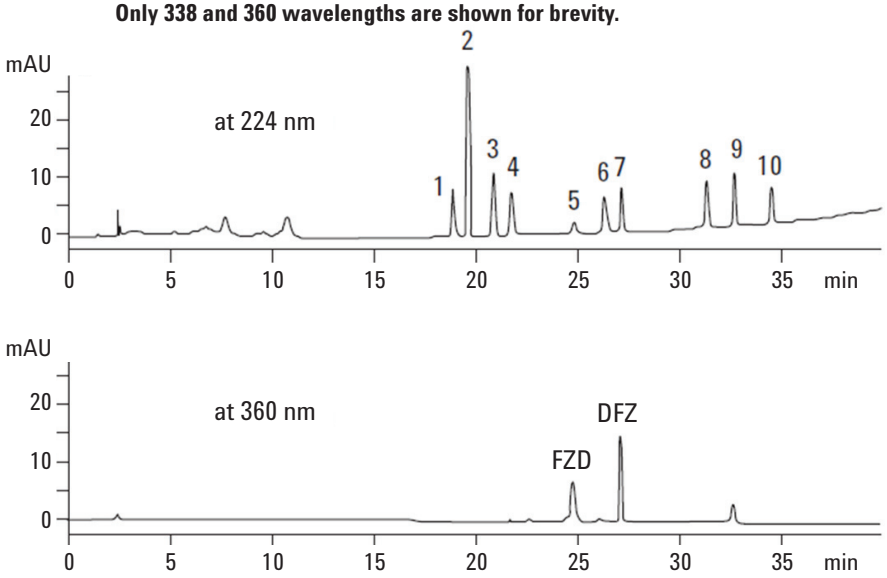
As discussed in reference 5, once a separation has been optimized according to selectivity and retention index, it is possible to further improve the chromatography by varying column length, particle size and flow rate. However the k* value must be maintained, while varying these column conditions so as not to lose selectivity.

Equation 1: $k^* = (t_g F)/(d/2)^2 L(\Delta\%B)$

Where:

t_g is the gradient time
F is the flow rate
L is the column length
d is the column internal diameter
Δ%B is the change in organic content across the gradient segment

Original Method Kumagai and Onigbinde 5988-7135 June 2002
Only 338 and 360 wavelengths are shown for brevity.



1	SMR	6	SMMX
2	PYM	7	DFZ
3	TCP	8	SDMX
4	SDD	9	SQX
5	FZD	10	OXA

Instrument:	Agilent 1100 Series HPLC
Column:	250 mm × 4 mm id, RP-18 Purospher, 5 µm, p/n 79925PU-584
Mobile phase:	A = 0.7% Phosphoric acid, B = CH_3CN
Gradient:	0.0 min 5% B; 10.0 min 5% B; 40.0 min 65% B; 45.0 min 65% B; Post Time 7.0 min 5% B
Flow rate:	1.0 mL/min
Temperature:	40 °C
Injection volume:	20 µL
Diode array detector:	A-338/10 nm, reference wavelength off B-264/8 nm, reference wavelength off C-360/8 nm, reference wavelength off

Figure 2. Original method produces excellent results on a 250 mm column with UV detection.

As illustrated in Figure 3, generic gradients using methanol or acetonitrile are used to separate the compounds of interest. The gradients using methanol generate 50% higher pressure (300 bar instead of 200 bar). While this is not critical when using a 50 mm column, this does become more important as the length of the column is increased to 100 or 150 mm.

With methanol, the last compound elutes later due to the lower solvent strength. Formic acid, while a convenient mobile phase additive, produces less optimal results than 10 mM ammonium formate buffer (pH =3), particularly for pyrimethamine. In addition to peak shape improvements, elution order changes also occur most notably with pyrimethamine.

Many selectivity improvements and changes can be produced by choice of pH or organic modifier. As noted earlier, the peak shape of many basic compounds are improved when using methanol, however Poroshell 120 EC-C18 yields excellent peak shape for all compounds in this study. By adjusting the pH even slightly, both the elution order and peak spacing can be changed. This is most evident in Figure 3, where methanol and pH act to dramatically change the elution order. For the compounds in this study the best mobile phase combination is found at pH 3.8, ammonium formate with acetonitrile.

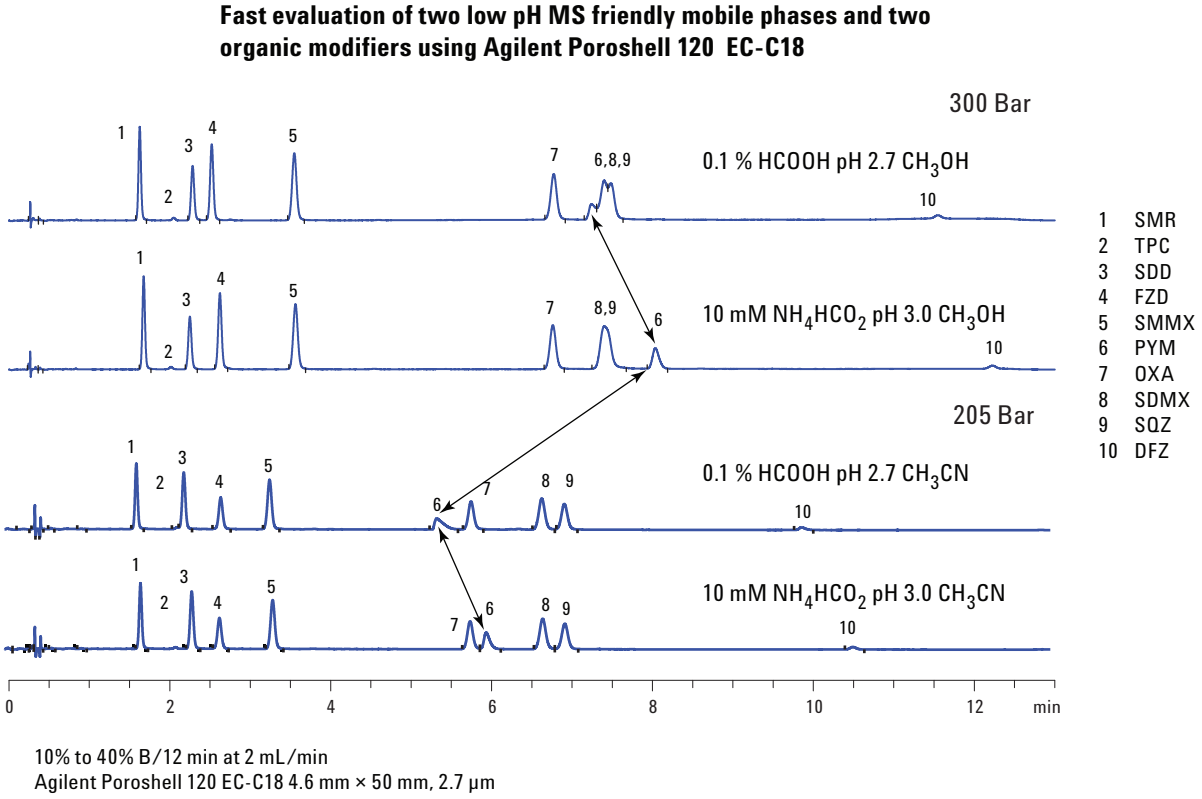


Figure 3. Comparison of chromatographic conditions: buffer, 0.1 % formic acid, CH_3OH, CH_3CN.

Acetonitrile with ammonium formate buffer yields excellent peak shape and selectivity with pH 3.8 being optimal for these analytes

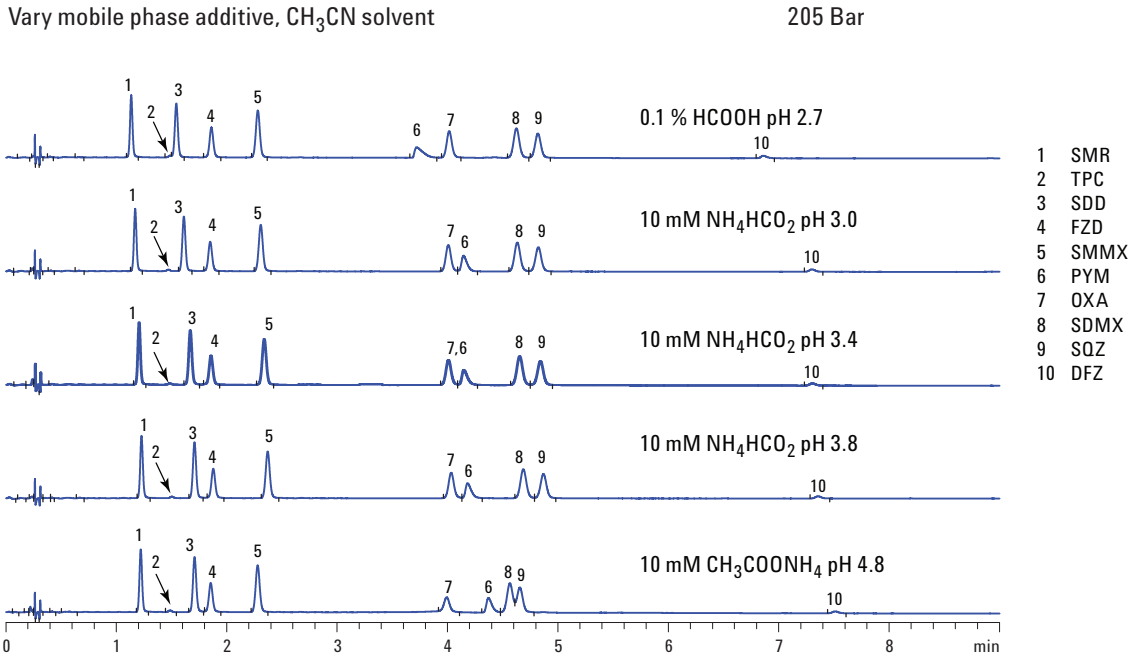
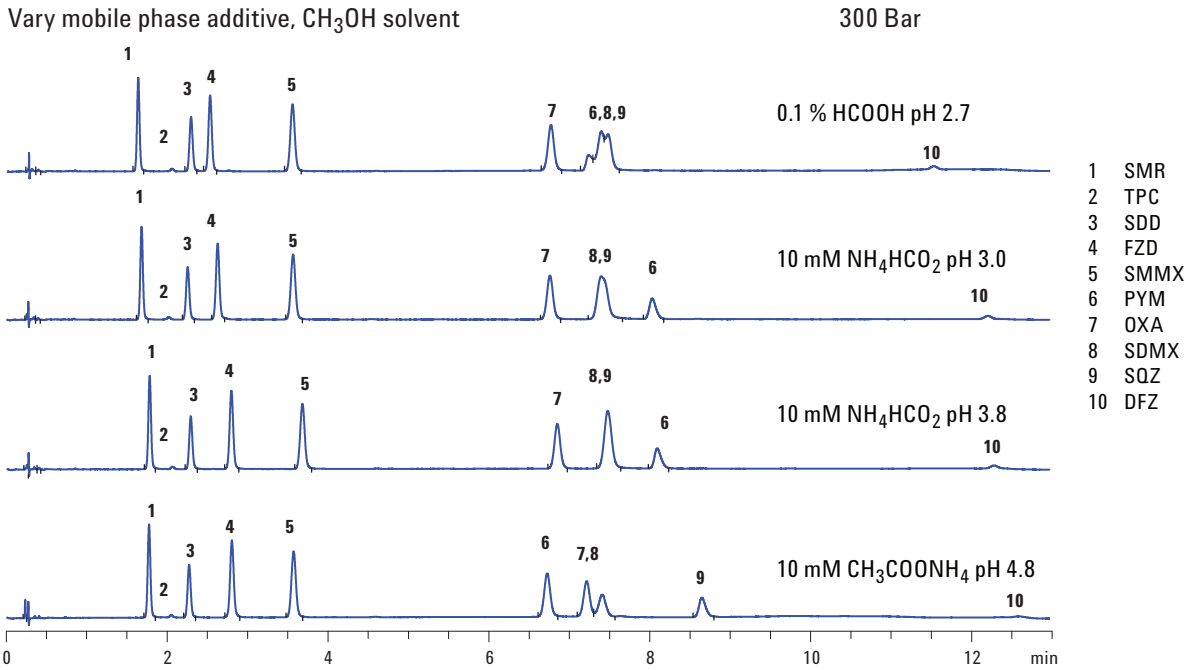


Figure 4. Comparison of buffers with CH_3CN.

Methanol with ammonium acetate buffer yields excellent peak shape with pH 4.8 being optimal for these analytes



10% to 40% B/12 min at 2 mL/min
Agilent Poroshell 120 EC-C18 4.6 mm × 50 mm, 2.7 µm

Figure 5. Comparison of buffers with CH_3OH.

Figure 6 illustrates a total ion chromatogram based on the scouting work shown in Figures 3, 4 and 5. Conditions were scaled according to Equation 1 for the 3.0 mm × 50 mm column. This easy change demonstrates that the 3 mm column can be easily used for both conventional UV and more sensitive MS. In addition, a 2.1 mm × 100 mm column is also used with the same gradient with only the flow rate changed. If the gradient had been exactly scaled, the analysis time would have been twice as long, but as illustrated, the resolution is adequate. Figure 7 shows an MRM chromatogram of the antibiotic mixture. The compounds are sufficiently separated even with a large sample volume injected on-column. Conditions are listed in Tables 1 and 2.

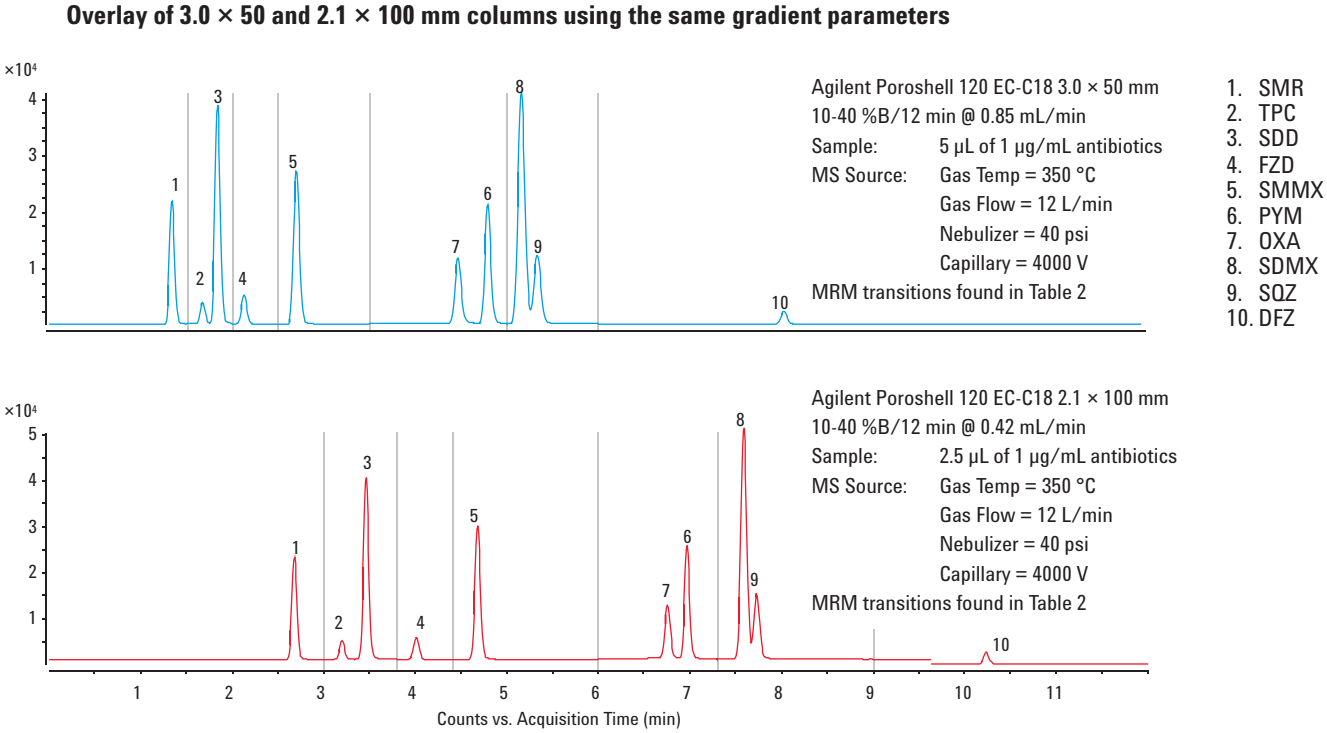
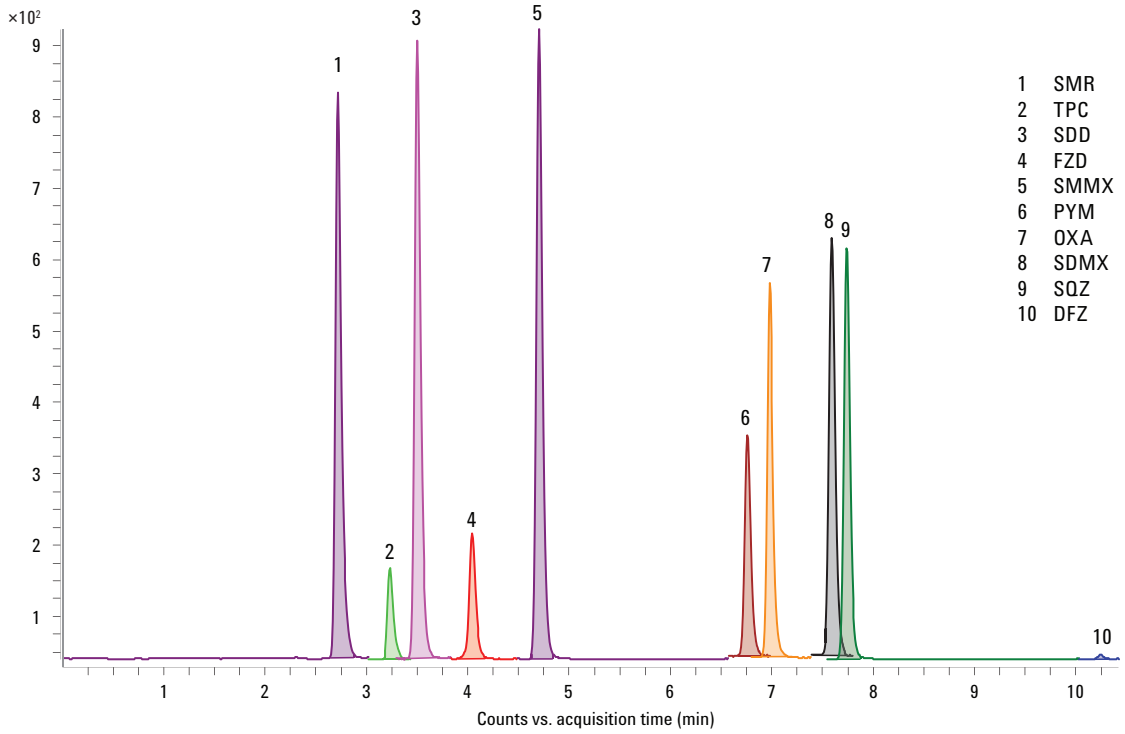


Figure 6. Total ion chromatograms of antibiotic mixture on 3 × 50 mm, and 2.1 × 100 mm Agilent Poroshell 120 EC-C18 columns.

Optimized MRM of 10 antibiotics in less than 11 minutes on Agilent Poroshell 120 EC-C18



Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm, 2.7 µm
10% CH_3CN at t_0, ramp to 40% CH_3CN in 12 min (buffer 10 mM NH_4HCO_2 pH 3.8 adjusted with concentrated formic acid), 0.42 mL/min
Sample: 10 uL of 10 ng/mL antibiotics
using dynamic MRM mode on MS/MS

Figure 7. Dynamic MRM of antibiotic mixture on Agilent Poroshell 120.

Conclusions

This work shows that in method migration, modern colums and fast liquid chromatographs make it easier to start fresh. Using a generic gradient on short columns, 10 mobile phase combinations are quickly evaluated. Following basic scaling equations, a method can easily be transferred to a column of another dimension. By optimizing the mobile phase using a UV detector, the method is partially developed on an instrument that may be commonly used in a lab rather than the more expensive and possibly less available instrument that the method will be transferred to.

Poroshell 120 columns are good to use for LC/MS of complex samples at low pressure. Regardless of the analytical power of the triple quadrupole mass spectrometer, a better separation simplifies data analysis, which may shorten cycle time. Baseline separated compounds also allow the mass spectrometer to maximize dwell time for a given peak to yield more accurate and reproducible results. This ensures the best possible quantitation. Additionally, less chance of ion suppression is possible caused by coeluting compounds.

Several additional factors are also demonstrated. Optimal conditions for this mixture are found using the fast scouting method in acetonitrile ammonium formate buffer pH 3.8 (8 min). The analysis also works in methanol with pH 4.8, ammonium acetate (13 min). This could easily be shortened by changing the gradient to elute the last peak more quickly. For example, ramp organic more quickly at the end with a second step; however this would increase pressure further. The use of a "true buffer" such as 10 mM ammonium formate provides better peak shape for bases than a buffering solution such as 0.1 % formic acid at similar pH. The method as shown is chromatographically optimized and work is in progress to optimize detection conditions.

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Determination of Sulfonamide Residues in Chicken Muscle by Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD

Application Note

Food

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Abstract

An HPLC-Florescence detection (FLD) method has been developed and validated for the determination of nine sulfonamides in chicken muscle, after precolumn derivatization with fluorescamine. The analyzed drugs include sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole and sulfadimethoxine with sulfapyridine as an internal standard. The derivatives were separated on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm) using a gradient elution with a binary system of methanol–0.05 M acetate buffer (pH 4.5) and fluorescence detection at excitation and emission wavelengths of 406 and 496 nm, respectively. The method employs a multiresidue sample preparation procedure based on QuEChERS (quick, easy, cheap, effective, rugged and safe) which was adopted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and cleanup. The recoveries ranged from 76.8% to 95.2% with relative standard deviation from 1.5% to 4.7% at the 50, 100 and 150 ng/g fortification levels. The limits of detection and quantification ranged from 0.02 to 0.39 and 0.25 to 1.30 ng/g respectively.

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Introduction

Sulfonamides are a broad spectrum of antimicrobial drugs used mainly in veterinary practice for prophylactic, therapeutic or growth promoting purposes [1]. They are the treatment of choice for disease control of coccidiosis in poultry management [2-3]. Their use in human therapy has since become limited due to the advent of antibiotics [4]. There is a health risk associated with consumption of animal products contaminated with sulfonamide residues. The residues are usually the result of inappropriate administration or withdrawal from these drugs. The presence of sulfonamide residues can trigger adverse side effects such as allergic reactions in hypersensitive individuals and are potential carcinogens in the long term. Furthermore, prolonged exposure to sulfonamide residues may give rise to an increase in drug-resistant bacteria [5]. In order to protect consumers from risks related to the drug residues, maximum residue limits (MRL) have been established by law in many countries. In Europe (EU Regulation 1999), Canada and USA (FDA Regulation 1991) the MRL for the total sulfonamide concentration in edible tissue is 100 µg/kg while it is 20 µg/kg in Japan [6-7].

The AOAC QuEChERS method has been widely applied in the analysis of pesticides in food since it was introduced by USDA scientists [8]. In general, there are two major steps: extraction and dispersive SPE cleanup. The method uses a single-step buffered acetonitrile (1% HOAc) extraction while simultaneously salting out from the aqueous sample using anhydrous magnesium sulphate ($MgSO_4$) to induce liquid-liquid partitioning. For cleanup, a dispersive solid phase extraction (dSPE) step is employed using a combination of primary secondary amine (PSA) to remove fatty acids and other components, and anhydrous $MgSO_4$ to reduce the remaining water in the extract. Other sorbents may be added in this step, such as graphitized carbon black (GCB), to remove pigments and sterol, or C_{18} to remove more lipids and waxes.

This application note presents a method for the determination of sulfonamide drugs in chicken muscle with HPLC-FLD after a precolumn derivatization with fluorescamine, which is a fluorogenic reagent specific for primary aliphatic and aromatic amines [9–10] such as the sulfonamides in the study (Figure 1). The method includes sample preparation with SampliQ AOAC Buffered Extraction kit (p/n 5982-5755) and SampliQ AOAC Fatty Dispersive SPE 15 ml kit (p/n 5982-5158).

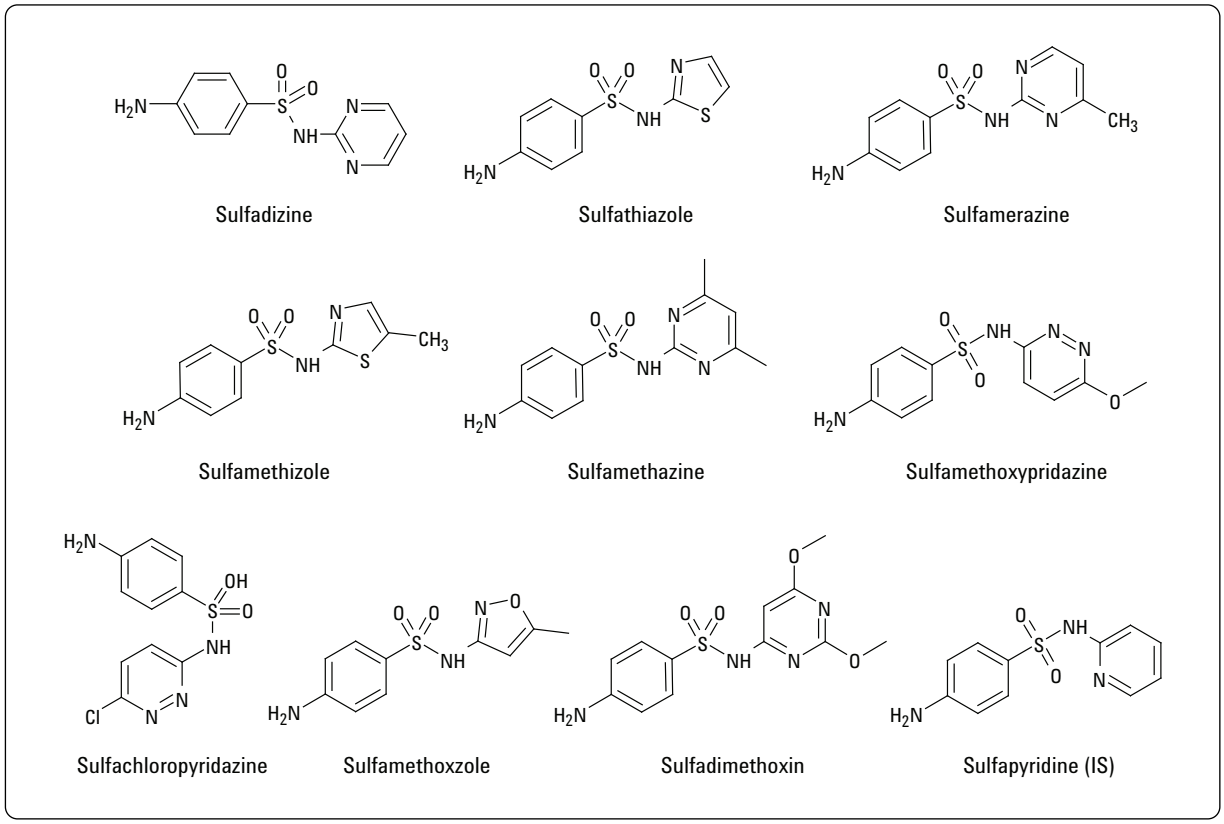

Figure 1. Chemical structures for the sulfonamide drugs used in the study.

Experimental

Reagents and Chemicals

All reagents were analytical or HPLC grade. Methanol (MeOH) was purchased from Merck KGaA (Darmstadt, Germany) while acetonitrile (ACN), acetone and glacial acetic acid (HOAc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate (NaOAc) was from Saarchem Analytical (Krugersdorp, South Africa). Fluorescamine (98%) and sulfonamide drugs including the internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used was from a MilliQ system (Milford, Mass, USA).

Solutions and Standards

A stock solution of 0.05 M sodium acetate was prepared by dissolving 4.1 g NaOAc in 1.0 L of ultrapure water and filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size). The pH was adjusted using HOAc. Fluorescamine reagent (0.02%) was prepared by dissolving 20 mg Fluram in 10 mL of acetone. The solution was stored at 4 °C. A 1% HOAc in ACN solution was prepared by diluting 10 mL HOAc to 1.0 L with ACN.

Standard and internal standard primary stock solutions (1 mg/mL) were prepared in ACN and stored at –20 °C. From the primary stock solution, 10 µg/mL standard mixtures also in ACN were prepared for the calibration curves. All working solutions were prepared daily by serial dilution in 0.05 M NaOAc (pH 3.5). All the solution vials were wrapped with aluminium foil because some of the sulfonamide drugs are light-sensitive.

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a binary pump and a fluorescence detector (FLD) set at λ_{ex} = 405 nm and λ_{em} = 495 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm, p/n 959933-902). The data was processed by HPLC 2D Chemstation software.

Extraction and cleanup were carried out with an Agilent SampliQ Buffered QuEChERS AOAC Extraction kit, p/n 5982-5755 and an Agilent SampliQ QuEChERS AOAC Dispersive SPE kit, p/n 5982-5158, (Agilent Technologies).

A Jenway 3510 pH meter (Jenway, London, UK) monitored the pH of the solutions, and a Kenwood grinder (Kenwood, Grahamstown, South Africa) homogenized the chicken sample.

Instrument conditions

HPLC conditions

Table 1. HPLC Conditions Used for Separation and Analysis

Column:	Agilent ZORBAX Eclipse Plus C18 4.6 × 75 mm, 3.5 µm	
Flow rate:	1 mL/min	
Column temperature:	25 °C	
Injection volume:	5 µL	
Mobile phase:	A = 0.05 M Sodium Acetate pH 4.5 B = MeOH	
Gradient:		
	T (min)	% B
	0	35
	35	41
	50	55
Detection:	Ex = 405 nm	Em = 495 nm

Sample preparation

The chicken muscle was purchased from a local food store, minced and deep frozen until analysis.

Extraction

Figure 2 outlines the methodology used in the QuEChERS experiments. A 2-g portion of chicken muscle homogenate was placed into a 50-mL centrifuge tube from the SampliQ QuEChERS AOAC Extraction kit. The tube was centrifuged for 20 s. Samples were then spiked with appropriate spiking solutions to yield 50, 100, and 150 ng/g sample concentrations for recoveries and reproducibility studies. A 100-µL IS spiking solution was added to all the samples except the blank. After shaking vigorously for 1 min, 8 mL Milli-Q water was added followed by further shaking for 30 s. Next, 10 mL 1% HOAc in ACN was added followed by the Agilent SampliQ QuEChERS AOAC Extraction salt packet (p/n 5982-5755). The packet contained 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous NaOAc. The sample tubes were hand shaken vigorously for 1 min then further centrifuged at 4000 rpm for 5 min.

Dispersive SPE cleanup

A 6-ml aliquot of the upper ACN layer was transferred into a SampliQ QuEChERS AOAC Dispersive SPE 15 mL tube. This SPE tube contained 400 mg of PSA, 400 mg of C18EC, and 1200 mg of anhydrous $MgSO_4$. The tubes were then centrifuged at 4000 rpm for 5 min. Next, 4 mL of the extract was transferred to a test tube and dried with N_2 gas at 35 °C. Samples (200 µL) were reconstituted into 600 µL of 0.05 M NaOAc (pH 3.5).

Derivatization

Aliquots of 400 µL working standard mixtures of sulfonamides, dissolved in 0.05 M acetate buffer (pH 3.4), were filtered through a 0.45 µm PVDF syringe filter then transferred

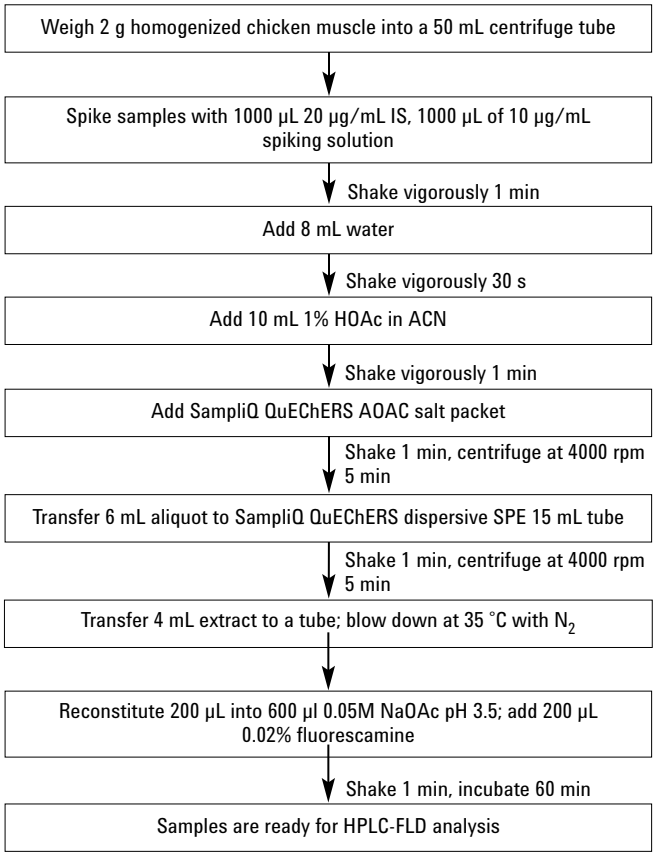

Figure 2. Flow chart for the QuEChERS AOAC sample preparation procedure.

to reaction vials. A 200 µL 0.02% w/v amount of fluorescamine solution in acetone was added. The mixtures were shaken for 1 min and the reaction left to proceed for 60 min at ambient temperature. Aliquots of 10 µL of the derivatized solutions were directly injected into the liquid chromatograph.

Results and Discussion

Derivatization of sulfonamide drugs

Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines that produce fluorophors of a high fluorescence yield [9]. This reagent and its hydrolysis products do not fluoresce, which eliminates the extensive cleanup step. Fluorescamine was therefore used in this application note to derivatize sulfonamides in the precolumn mode. The results indicated that the reaction time is the most important factor. The reaction was complete within 60 – 100 min and for reproducibility 60 min was the chosen time. The derivatised sulfonamides were detected with a single pair of wavelengths, λ_{ex} = 405 nm and λ_{em} = 495 nm.

Chromatographic results

The chromatogram of the standard mixture of these sulfonamide derivatives is shown in Figure 3. Figure 4 is the chromatogram for the blank chicken muscle extract, and Figure 5 is that of the spiked chicken muscle. All chromatograms of standards, blanks, and spiked extracts were run using the conditions outlined in Table I.

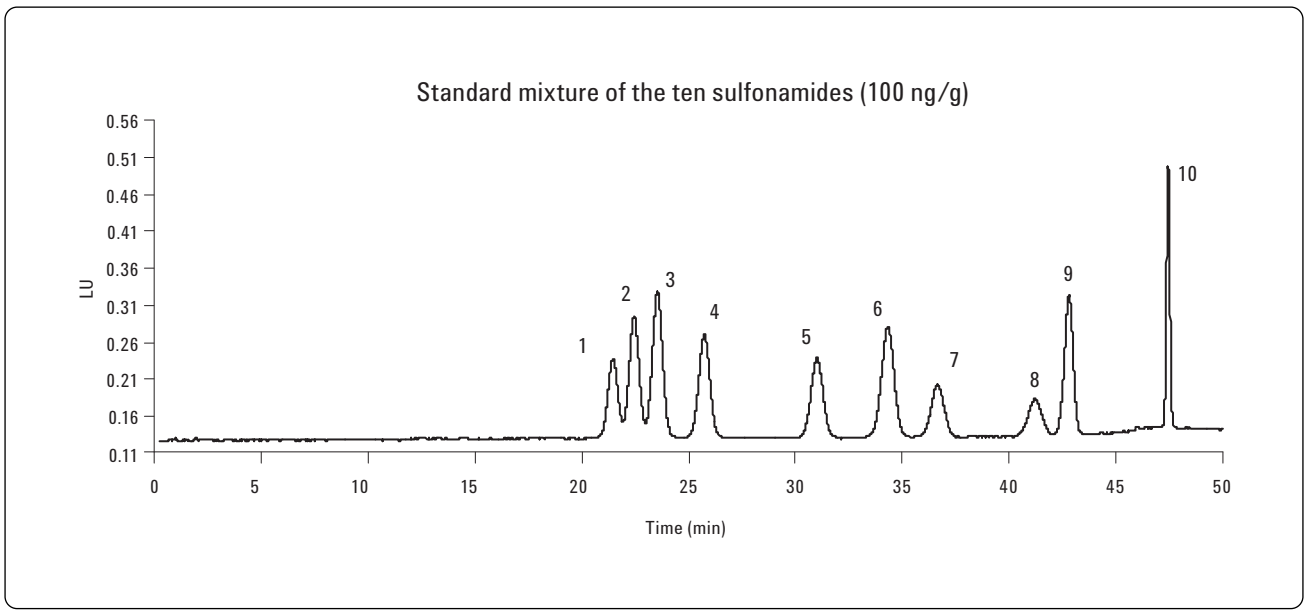

Figure 3. Chromatogram of the standard mixture of the sulfonamides (100 ng/g): 1. Sulfadiazine; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

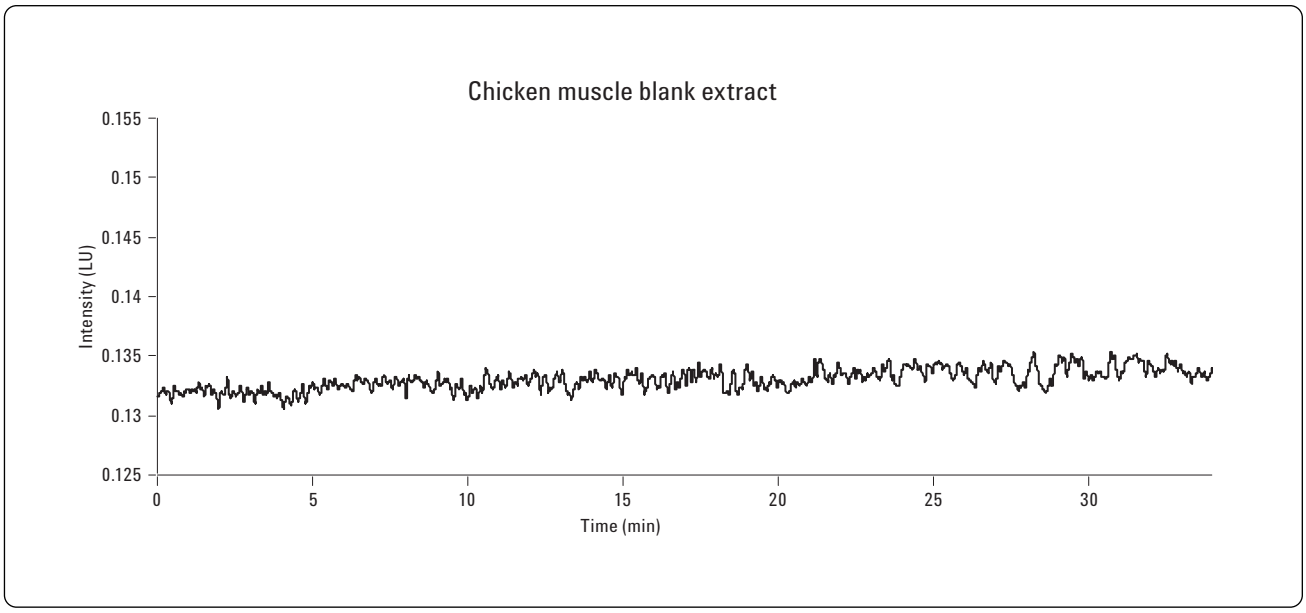

Figure 4. Chromatogram of the blank chicken muscle extract.

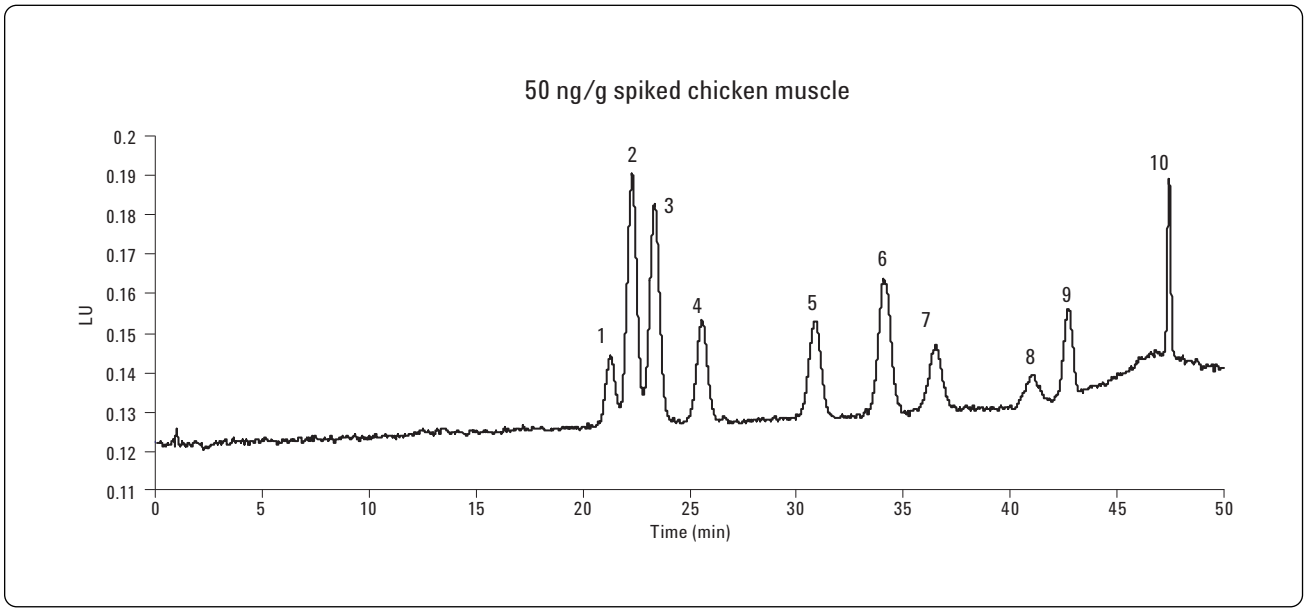

Figure 5. Chromatogram of the spiked chicken muscle extract at 50 ng/g level: 1. Sulfadiazine ; 2. Sulfathiazole; 3. Sulfapyridine (IS); 4. Sulfamerazine; 5. Sulfamethazine; 6. Sulfamethizole; 7. Sulfamethoxypyridazine; 8. Sulfachloropyridazine; 9. Sulfamethoxazole; 10. Sulfadimethoxine.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Linearity

The linear calibration curves were obtained by plotting the relative responses of analytes (peak area of analyte/peak area of IS) verses the relative concentration of analytes (concentration of analyte/concentration of IS). They were generated by spiking the sample blanks at levels of 10, 50, 100, 150, 200, 300 and 400 ng/g.

Limits of Detection and Quantification

The limits of detection and quantification were estimated from the concentration of sulfonamides required to give signal-to-noise ratios of 3 and 10 respectively. Table 2 shows the regression equation, correlation coefficients, and very acceptable limits of detection and quantification.

Recovery and Reproducibility

The recovery and reproducibility (RSD) were evaluated on spiked samples at MRL (100 µg/kg), half MRL (50 µg/kg) and one and a half times the MRL (150 µg/kg). The analysis was performed in replicates of six (n = 6) at each level. Table 3 shows the recoveries and RSD values for the nine sulfonamides.

Table 2. Linearity, LOD and LOQ for the Nine Sulfonamides

Sulfonamide	Regression equation	R^2	LOD ng/g	LOQ ng/g
Sulfadiazine	Y = 0.4154x + 0.0112	0.9995	0.26	0.87
Sulfathiozole	Y = 1.0231x – 0.0757	0.9991	0.02	0.27
Sulfamerazine	Y = 0.6735x + 0.0184	0.9993	0.14	0.46
Sulfamethazine	Y = 0.6735x + 0.0042	0.9996	0.08	0.26
Sulfamethizole	Y = 0.9751x + 0.0115	0.9995	0.30	1.00
Sulfamethoxypyridine	Y = 0.4713x – 0.0069	0.9994	0.24	0.80
Sulfachloropyridazine	Y = 0. 2769x + 0.0190	0.9992	0.33	1.10
Sulfamethoxazole	Y = 0.6996x + 0.0421	0.9991	0.39	1.30
Sulfadimethoxine	Y = 0.5008x + 0.0329	0.9991	0.08	0.25

Table 3. Recovery and Repeatability for Sulfonamides in Spiked Chicken Muscle (n = 6)

Sulfonamide	Level of spiking (ng/g)					
	50		100		150	
	%Recovery	%RSD	%Recovery	%RSD	%Recovery	%RSD
Sulfadiazine	77.8	2.6	78.1	2.1	78	1.9
Sulfathiazole	83.0	3.9	88.2	2.4	85.2	2.2
Sulfamerazine	85.7	2.9	85.5	3.1	88.4	1.6
Sulfamethazine	80.3	3.1	81.3	2.2	80.3	1.5
Sulfamethizole	95.2	4.1	89.5	2.6	87.2	2.5
Sulfamethoxypyridazine	91.4	2.3	90.7	2.1	90.5	1.7
Sulfachloropyridazine	78.1	3.0	89.4	2.6	80.7	2.2
Sulfamethoxazole	76.8	3.5	87.2	2.5	89.1	1.5
Sulfadimethoxine	90.3	4.7	92.8	2.2	89.2	2.0

Conclusions

A simple and fast mulitiresidue method based on SampliQ QuEChERS AOAC Method 2007.01 and HPLC-FLD with pre-column derivatization has been developed for the simultaneous determination of nine sulfonamide residues in spiked chicken muscle. The recoveries were good with excellent RSD and the LOQs were well below the MRL in animal food products. This method can therefore be recommended for residue control purposes.

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5990-5395EN

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Extraction of Sulfa Drugs in Honey with Polymeric SPE Cation Exchange, Bond Elut Plexa PCX

Application Note

Authors

William Hudson and Rich Motyka
Agilent Technologies, Inc.

Introduction

Antibiotics and other drugs in agricultural foods is a recurrent problem that can cause serious harm or death to the allergic or sensitive consumer. In recent years, various sources of honey have been shown to be contaminated with residues of antibiotics and sulfonamides. These contaminants may occur after direct treatment of bacterial diseases of honey bees, such as American or European foulbrood and nosemosis. To address this issue, several countries have regulated use of sulfonamides and require routine testing of honey for their presence.

Honey is a complex matrix consisting of mostly carbohydrates and water. In contrast to less viscous samples, for example milk, in order to utilize SPE with a viscous matrix like honey, it is necessary to take additional steps including sonication and acidification prior to SPE. Herein, we describe a method to extract and analyze sulfa drugs in honey using cation exchange SPE and LC/MS/MS analysis.

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Materials and Methods

Table 1. SPE Reagents and Solutions

4% Phosphoric Acid	Add 40 µL of concentrated H_3PO_4 to 1 mL of DI water
Methanol	Reagent grade or better
2% Formic Acid	Add 20 µL of concentrated formic acid to 1 mL of DI water
Methanol:acetonitrile (1:1, v/v)	Add 1 mL of methanol to 1 mL of acetonitrile
5% NH_4OH Methanol:acetonitrile (1:1, v/v)	Add 50 µL of concentrated ammonium hydroxide to 1 mL of methanol:acetonitrile (1:1, v/v)
Bond Elut Plexa 10 mg 96 well plate (part number A4968010)	

Table 2. SPE Method

Sample Pre-treatment	1.0 g Honey. Add 1 mL 4% H_3PO_4 and sonicate for 20 mins. Dilute with 3 mL of 2% H_3PO_4.
Condition	1. 500 µL CH_3OH 2. 500 µL DI H_2O
Wash 1	500 µL 2% formic acid
Wash 2 (acids, neutral)	500 µL methanol:acetonitrile (1:1, v/v)
Elution (bases)	500 µL 5% NH_3 methanol:acetonitrile

All samples are evaporated to dryness and reconstituted in 100 µL of 80:20 0.1% Aq formic acid: CH_3OH.

Results and Discussion

LC Conditions

Mobile Phase: A: 5 mM Formic acid
B: Methanol

Gradient:
t = 0-0.5 min 90% A : 10% B
t = 5.0-6.0 min 50% A : 50% B
t = 6.01-7.0 min 90% A : 10% B

Column: Pursuit C18 3 µm, 50 x 2.0 mm (part number A3051050X020)

MS Conditions

Transition ions and collision energy were:

Compound	Q1	Q3	CE
Sulfathiazole	256.0	156.0	18.0V
Sulfamethazine	279.0	186.0	21.0V
Sulfaquinoxaline	301.0	156.0	19.5V
Sulfadimethoxine	311.0	92.1	37.0V

Capillary = 70 V, Dry gas temp = 350 °C, 30 psi, CID = Argon

Polarity: Negative

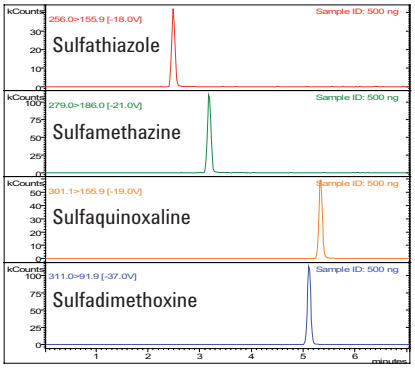

Figure 1. Chromatograms of a 50 ng/mL extract

The procedure describes a method for extracting four sulfonamide antibiotics from honey (Figure 1). The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 25 ng/g. Recoveries were calculated from a 1st order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 5.0 mg/mL with correlation coefficients all above 0.999. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). As shown in Table 3, the extractions produced reproducibly high recoveries.

Table 3. Analyte relative recoveries

Analyte	log P	pKa	% Rec (50 ng/mL)	% RSD	% Rec (500 ng/mL)	% RSD
Sulfathiazole	0.05	7.2	102	6	93	7
Sulfamethazine	0.89	7.4	103	7	87	4
Sulfaquinoxaline	1.68	5.9	107	7	100	7
Sulfadimethoxine	1.63	5.9	100	4	103	5

Conclusions

With excellent flow characteristics Bond Elut Plexa PCX is an ideal choice when working with difficult to process samples such as honey. Similar to other widely used cation exchangers, standard adjustments of pH and organic content with Bond Elut Plexa PCX yields fast and effective analyte recoveries approaching 100% with the four sulfa drugs present in honey. These data suggest Bond Elut Plexa PCX lends itself to the extraction of sulfa and similar drugs from foods and other complex matrices.

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Agilent Technologies

Analysis of tetracyclines by HPLC

Rainer Schuster

Food

Abstract
Tetracyclines are used worldwide as oral or parenteral medication in the form of additives in animal feed. In food-producing animals, these drugs exhibit a high degree of activity toward a wide range of bacteria.[1, 2]

Sample preparation
After homogenization or mincing and addition of mineral acids to dissociate tetracyclines from proteins, the samples were extracted using liquid/liquid extraction followed by degreasing and/or deproteinization, purification, and concentration.[3]

Chromatographic conditions
The HPLC method presented here for the analysis of meat is based on reversed-phase chromatography and UV-visible diode-array detection.
UV spectra were evaluated as an additional identification tool.

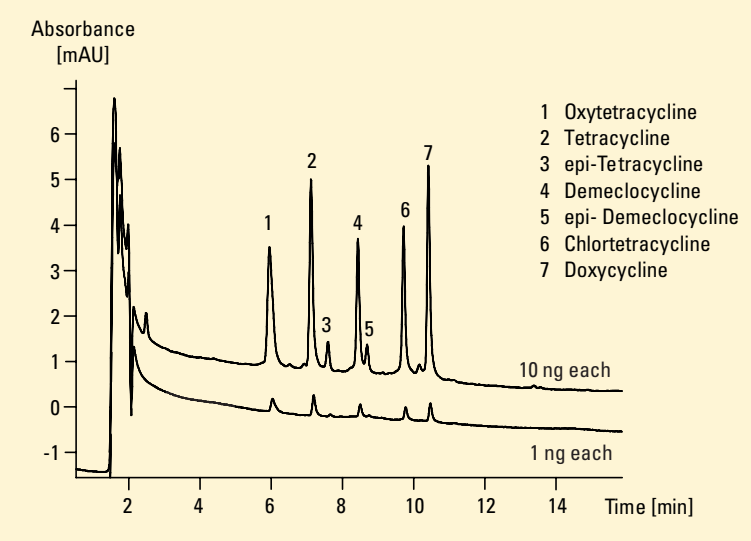

Figure 1
Analysis of tetracyclines by HPLC

Conditions

Column: 100 ˘ 4 mm Hypersil BDS, 3 µm
Mobile phase:
A = water, pH = 2.1 with sulfuric acid
B = ACN
Gradient: start with 15 % B at 10min 60% B
Flow rate: 0.5 ml/min
Column compartment: 25 ºC
Detector:
UV-DAD detection wavelength 355 nm/20 nm, reference wavelength 600/100 nm

Sample preparation

1. 1 g sample was mixed with citric acid (100 mg).
2. add 1 ml nitric acid (30 %) or 0.1 m oxalic acid
3. add 4 ml methanol 5 min in the ultrasonic bath
4. add water up to 10 ml total volume
5. centrifuge
6. inject

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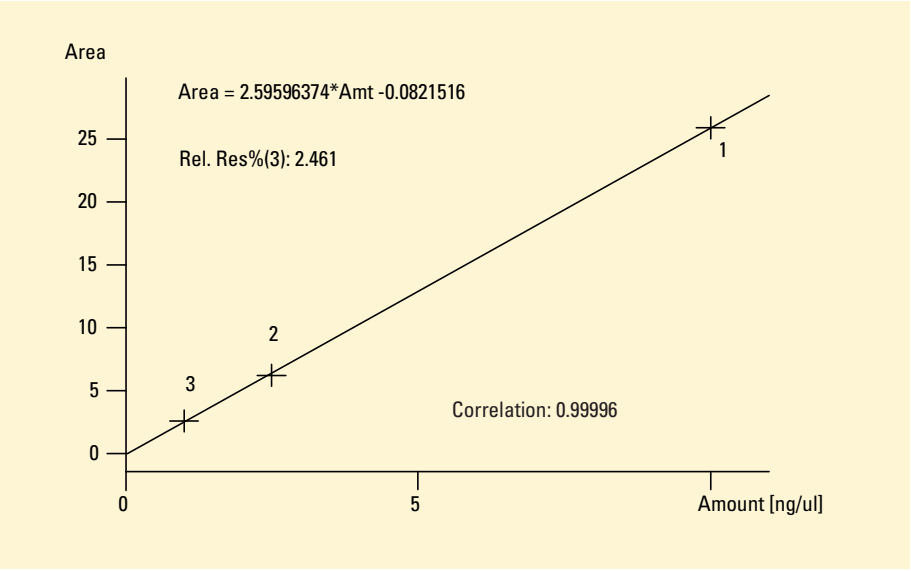

Figure 2
Linearity for oxytetracycline 1-10 ng

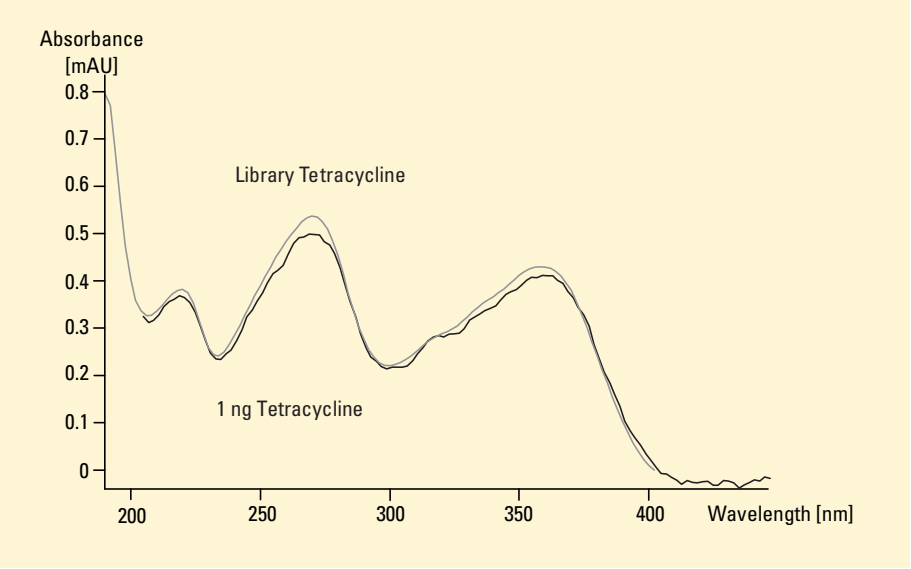

Figure 3
Analysis of tetracyclines at 100 ppb by HPLC

Rainer Schuster is application chemist at Agilent Technologies, Waldbronn, Germany.

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Equipment

Agilent 1100 Series
- vacuum degasser
- quaternary pump
- autosampler
- thermostatted column compartment
- diode array detector, Agilent ChemStation + software

HPLC method performance

Limit of detection
for UV-DAD 100 ppb
Repeatability
of RT over 10 runs <0.2 %
of areas over 10 runs <2 %

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Publication Number 5966-1619E

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Analysis of Residual Synthetic Antibacterials in Meat by HPLC

Application

Food

Hiroki Kumagai, Adebayo Onigbinde

Many domestic cattle receive various antibacterials in their feed for the prevention and control of disease caused by fungi and bacteria. Residues of antibacterials are found in food made from the meat of these animals. Since many antibiotics are toxic, many countries regulate acceptable residue levels of compounds allowable in agricultural and animal products. Many alkyl-C18 columns tail with basic compounds and have a shorter life time at low pH. Purospher® column separated basic antibacterials with good resolution, peak shape,and efficiency.

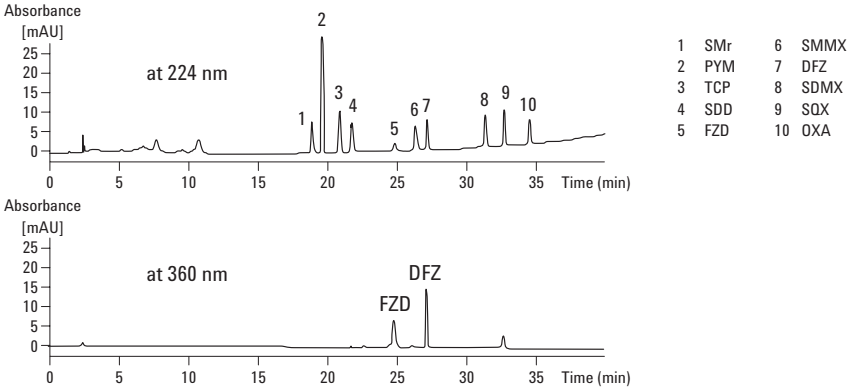

Figure 1. Chromatogram of standard solution, 2 µg/mL each analyte.

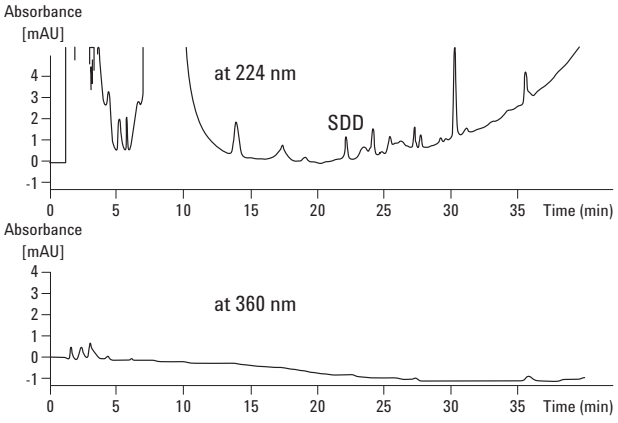

Figure 1. Chromatogram of extract of bovine muscle.

Highlights

- Separation of 10 antibacterials in meat at low pH
- Excellent and rapid resolution of antibacterials at low sample concentration
- Elution of antibacterials from the column with good peak shape and narrow peak width
- Separation of low level amounts of a wide range of pharmaceutical compounds with differing structures in a single analysis by Purospher® column

Analyzed Compounds

- Sulfamerazine (SMR)
- Sulfadimidine (SDD)
- Sulfamonomethoxine (SMMX)
- Sulfadimethoxine (SDMX
- Sulfaquinoxaline (SQX)
- Pyrimethamine (PYM)
- Thiamphenicol (TPC)
- Furazolidone (FZD)
- Difurazone (DFZ)
- Oxolinic acid (OXA)

Sample: Extracts from bovine muscle

Sample preparation: According to the official procedure of the Japanese food sanitation law.

Instrument: Agilent 1100 Series HPLC; **Column:** 250 mm × 4 mm id, RP-18 Purospher®, 5 µm, Part no. 79925PU-584; **Mobile phase:** A = 0.7 % Phosphoric acid, B = CH_3CN; **Gradient:** 0.0 min 5% B; 10.0 min 65% B; 40.0 min 65% B; 45.0 min 65% B; Post Time 7.0 min 5% B; **Flow rate:** 1.0 mL/min; **Temperature:** 40 °C; **Injection volume:** 20 µL; **Diode array detector:** A–338/10 nm, reference wavelength off; B–264/8 nm, reference wavelength off; C–360/8 nm, reference wavelength off

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Adebayo Onigbinde is an application chemist based at Agilent Technologies, Wilmington, Delaware, USA.

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5988-7135EN

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HPLC Analysis of Antibacterial Drugs with Penicillin-Like Structure

Application

Drug Development

Udo Huber, Adebayo O. Onigbinde

Penicillins can be isolated from the culture medium of certain fungi-producing natural penicillin, such as Penicillium notatumor and P. chrysogenum. Other penicillins can be synthesized semisynthetically or by precursor-indicated biosynthesis. Total synthesis would not be economical.

Penicillin inhibits the polymerization of murin, which is responsible for the stability of the bacteria's cell wall. Because many antibacterials are toxic, various countries regulate the level of antibacterial residues in agricultural, veterinary, dairy, and meat-based food products.

Figure 1 shows the HPLC separation of four common antibacterial drugs with pencillin-like structure (amoxicillin, ampicillin, penicillin G, and penicillin V) on an SB-C18 reversed phase column.

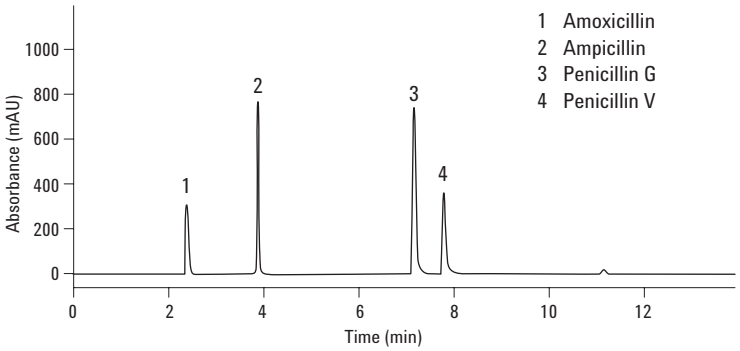

Figure 1. Separation of four penicillin analogs.

Highlights

- There is excellent resolution of penicillin analogs without ion pairing agent.
- There is rapid resolution of the penicillin analogs on the SB-C18 column at low pH and buffer concentration.
- Penicillins are eluted from the column with good and narrow peak shape.
- Extreme stability of sterically protected SB-C18 bonded phases allows for excellent separation at low pH.
- The SB-C18 column provides excellent peak shape and selectivity for antibacterial drugs.
- The HPLC method shows an easy but reliable and precise analysis of the antibacterial drugs.
- The values for limit of detection (LOD), precision of retention time (RT) and area show the good performance of the HPLC analysis.

Experimental Conditions

Equipment: Agilent 1100 Series HPLC; **UV Detector:** Variable wavelength detector, 204 nm, standard cell; **Column:** Zorbax SB-C18, 3. 5 µm, 4.6 × 75 mm (part number 866953-902); **Mobile phase:** A = 0.025 M KH_2PO_4 in water (pH = 3), B = acetonitrile; **Injection volume:** 5 µL; **Temp:** 40 °C; **Flow rate:** 1.0 mL/min; **Gradient:** at 5% B to 60% in 10 min; **Column wash:** 60% B to 5% B in 2 min; **Stop time:** 12 min; **Post time:** 5 min

Table 1. HPLC Method Performance of Antibacterial Drugs with Penicillin-Like Structure

Compound	LOD for S/N = 2 (mg/L)*	Precision of RT (RSD of 10 runs) (100 mg/L)*	Precision of area (RSD of 10 runs) (100 mg/L)*
Ampicillin	1.0	0.32	0.54
Amoxicillin	1.0	0.32	0.55
Penicillin G	1.0	0.32	0.49
Penicillin V	1.0	0.25	0.48

*Injection volume: 5 µL

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HPLC Separation of Antibacterial Drugs with Tetracycline Structure

Application

Drug Development

Udo Huber, Adebayo O. Onigbinde

Tetracyclines occur naturally in some streptomyces species. Besides being used in human and veterinary medicine, they are fed as nutritional antibiotics in pig and poultry farming. Because of their long half-life and resistance, there is a high restriction on their usage in some European countries, such as Germany. Figure 1 shows the HPLC separation of three common tetracycline analogs on a Zorbax SB-C18 reversed phase column.

This application demonstrates separation without ion pairing and the use of an alternative mobile phase to TFA in separating antibacterial drugs.

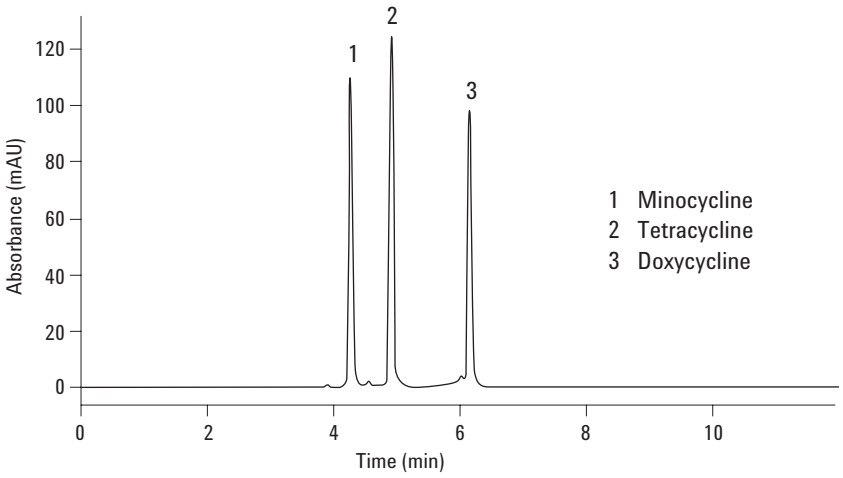

Figure 1. Separation of three antibacterial drugs with tetracycline structure.

Highlights

- The SB-C18 column provides excellent peak shape and selectivity for basic antibacterial drugs.
- The SB-C18 column shows excellent stability at low pH.
- The SB-C18 column shows excellent and rapid resolution of antibiotics at low pH and buffer concentration.
- The HPLC method shows an easy but reliable and precise analysis of the antibacterial drugs.
- The values for limit of detection (LOD), precision of retention time (RT), and area show the good performance of the HPLC analysis.

Experimental Conditions

Equipment: Agilent 1100 Series HPLC; **UV Detector:** Variable wavelength detector, 350 nm, standard cell; **Column:** Zorbax SB-C18, 3. 5 µm, 4.6 × 75 mm (part number 866953-902); **Mobile phase:** A = 0.025 M KH_2PO_4 in water (pH = 3), B = acetonitrile; **Injection volume:** 5 µL; **Temp:** 25 °C; **Flow rate:** 1.0 mL/min; **Gradient:** at 5% B to 60% B in 10 min; **Column wash:** 60% B to 5% B in 2 min

Table 1. HPLC Method Performance of Antibacterial Drugs with Tetracycline Structure

Compound	LOD for S/N = 2 (mg/L)*	Precision of RT (RSD of 10 runs) (100 mg/L)*	Precision of area (RSD of 10 runs) (100 mg/L)*
Minocycline	0.1	0.06	0.14
Tetracycline	0.1	0.05	0.13
Doxycycline	0.1	0.04	0.21

*Injection volume: 5 µL

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Agilent Technologies

Determination of the Metabolites of Nitrofuran Antibacterial Drugs in Chicken Tissue by Liquid Chromatograph-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Application

Food, Environmental

Author

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Abstract

A liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) method was developed for the simultaneous determination of the metabolites of four nitrofuran antibacterial drugs in chicken tissues: furazolidone, furaltadone, nitrofurazone, and nitrofurantoin. Sample clean-up and analyte enrichment were performed by liquid-liquid extraction with ethyl acetate followed by solvent washing, hydrolysis of the protein-bound drug metabolites, and derivatization with 2-nitrobenzaldehyde (2-NBA). ESI parameters were optimized, and the chromatographic separation of all metabolites was examined. Each metabolite produced a simple mass spectrum containing a strong signal corresponding to $[M+H]^+$. Metabolite calibration curves, in the 0.25 to 1 ng/mL range, exhibited correlation coefficients greater than 0.999. The limit of detection (LOD) for each analyte ranged from 0.02 to 0.06 ng/mL.

Introduction

The four drugs shown in Figure 1, furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, belong to the group of nitrofuran antibacterial drugs. These drugs have been widely used as feed additives to prevent bacterial enteritis by *Escherichia coli* and *Salmonella* in cattle, fish, swine, and poultry. The occurrence of furazolidone residue in edible tissue is a major human health concern. Effective June 1995, these drugs were banned from use in food animal production in the European Union (EU) because of concerns about their carcinogenicity and mutagenicity (Commission Regulation 1442/95).

Nitrofuran antibacterial drugs are characterized by their rapid metabolism, with in vivo half-lives of less than a few hours. Therefore, the detection of parent drugs in animal tissue is not practical. Studies using radioactive-labeled furazolidone have shown that protein-bound metabolites are formed in tissues [1-3]. The tissue-bound metabolites are detectable for several weeks after administration. Hence, the analysis of nitrofuran drugs is based on the detection of the tissue-bound metabolites of the parent drugs.

These tissue-bound metabolites are very small molecules which are not UV absorbing, and they elute too quickly out of a column. To induce UV absorption in the molecule and to be reasonably retained on a column, they are derivatized. It is possible to release these metabolites from the proteins under moderately acidic conditions and derivatize the metabolites with 2-nitrobenzaldehyde (2-NBA) to produce 2-NBA-derivatives for liquid chromatography (LC), UV detection, and mass spectrometry (MS) confirmation. The goal of this study is to develop a routine analytical method to simultaneously detect the target nitrofuran metabolites. Because no maximum residue limit (MRL) has

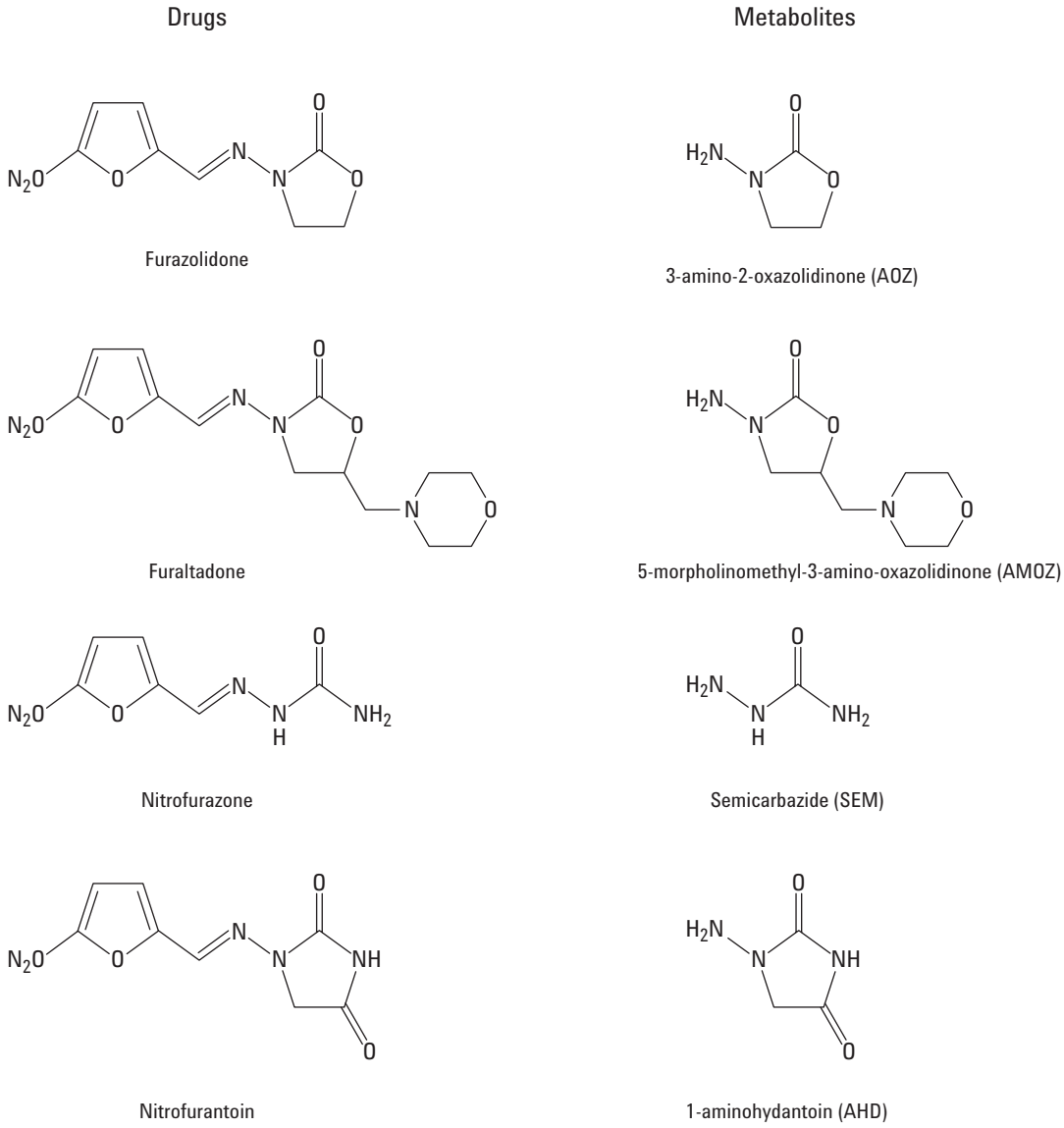

Figure 1. Structure of the nitrofuran antibacterial drugs and their metabolites .

been set by any regulatory agency, the goal of the analytical method was to estimate the lowest possible detection limit.

Experimental

Chemicals and Solvents

Three metabolites: 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD) were purchased from Sigma Aldrich Japan (Tokyo, Japan). The purity of these compounds was greater than 99%. The 2-NBA derivatives of these metabolites were prepared by the Livestock Department in Thailand (Palm Thani, Thailand) using the procedure described by Leitner [4]. Stock solutions of these three 2-NBA derivatives were prepared in methanol at 1000 ng/mL and stored in the dark at 4 °C. The stock solution was diluted to the desired concentration just prior to its use for the optimization of ESI parameters.

Acetonitrile, ethyl acetate, formic acid, and dimethyl sulfoxide (DMSO) were supplied by Wako Chemical (Osaka, Japan). Hydrochloric acid and 2-NBA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan).

Sample Preparation

Sample preparation procedures included solvent wash and acid extraction by homogenization and derivatization with 2-NBA. Chicken muscle and liver were prepared by the Livestock Department in Thailand.

Calibration curves for the four nitrofuran metabolites (from the Livestock Department) were constructed in the range 0.25 to 1.0 ng/mL. The derivatization and sample preparation procedures used by the Livestock Department are the following:

1. The four metabolite solutions in water, 12.5, 25.0, 37.5, and 50 µL at 100 ng/mL, were transferred to separate 40 mL glass vials with screw caps.
2. A solution of 10 mL HCl (125 mM in water) and 200 µL 2-NBA (50 mM in DMSO) were added to each vial.
3. The reaction mixtures were kept in a water bath at 37 °C for 16 hours.
4. The solutions were cooled to room temperature.
5. The pH was adjusted to about 7.4 by adding 0.1 M aqueous $KHPO_4$ or 0.8 M aqueous NaOH.
6. A 5-mL measure of ethyl acetate was added to each reaction mixture, and shaken for 2 min.
7. Each ethyl acetate phase was transferred to a separate glass vial and evaporated under a stream of nitrogen.
8. Finally, each residue was reconstituted in 5 mL of 1:1 methanol:water (V/V).

The calibration curve was based on the metabolite concentration in clean solvent and derivatization using 2-NBA. Previous studies done by the Livestock Department showed that recovery of all metabolites from chicken extracts was above 80%. Therefore, the amounts of metabolite in chicken extract can be calculated by comparing the responses of 2-NBA derivatives from the samples against the calibration curve.

Instrument and Experimental Conditions

An Agilent 1100 series LC, with a solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, and a column thermostat, was used for separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. The column and MS conditions are described in Table 1.

Table 1. Instrument Parameters

LC:	Agilent 1100 series
Column:	Inertsil ODS3, 150 mm × 2.1 mm, 5 µm (GL Science, Tokyo, Japan)
Solvent A:	Acetonitrile
Solvent B:	Aqueous 0.5% formic acid
Gradient:	20/80 A/B to 70/30 A/B in 20 min
Column temp	20 °C
Sample volume	30 µL
Flow rate:	200 µL/min
MS:	Agilent 1100 MSD, SL
Ionization:	ESI (Positive)
Scan range:	100–500 *m/z* for optimization
SIM ion:	Base peak for quantitation
Drying gas:	Nitrogen, 10 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 or 140 V
V_{cap}	2000 V

Quantitative analysis was carried out using selective ion monitoring (SIM) of the base peak ions according to the program shown in Table 2. To confirm the presence of the target analytes in chicken extract, the sodium adduct ions (qualifier ions) of all target analytes were also monitored.

Table 2. SIM Program

Group	Time window min	Analyte(s)	Target ion	Qualifier ion	Dwell time msec	Fragmentor voltage, V
1	0–6	2-NBA-AMOZ	335	357	500	140
2	6–12.5	2-NBA-SEM and 2-NBA-AHD	209 and 249	231 and 271	250 and 250	120 and 140
3	12.5–14	2-NBA-AOZ	236	258	500	140

System Optimization

Positive ion mass spectra were acquired over the scan range *m/z* 100–500 using a step size of 0.1 amu and a scan rate of 2 seconds per scan for the optimization of fragmentor voltage. Ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program.

Using the analytical column and three 2-NBA derivatives (AOZ, SEM, and AHD) at 100 ng/mL, instrument performance was optimized by adjusting the four major ESI parameters: the capillary voltage, fragmentor voltage, the nebulizer gas pressure, and the drying gas flow rate. However, significant variation in the intensity of analytes was not observed when the drying gas flow rate and nebulizer gas pressure were varied from 4 L/min to 13 L/min and 20 psi to 60 psi, respectively.

Capillary and fragmentor voltages applied to the inlet and exit end of the capillary affected the ion transmission significantly. Fragmentor voltage also affected the fragmentation of sample ions. In general, higher fragmentor voltage helps the transmission of ions through the relatively high-pressure region between the exit of the capillary and the entrance of the skimmer. High fragmentor voltage can cause fragmentation to occur which provides structural information of the ion. For compounds that do not fragment easily, higher fragmentor voltage often results in better ion transmission. Optimal fragmentor voltage is compound dependent. Evaluation of the fragmentor voltages for the three 2-NBA-metabolites was done under the same chromatographic conditions as the analysis. Mass spectra of three 2-NBA-metabolites are shown in Figure 2. Each mass spectrum exhibited $[M+H]^+$ as the base peak. Adducts ions $[M+NH4]^+$ and $[M+Na]^+$ were observed at lower fragmentor voltage (120 V) and some fragment ions (*m/z*=178 and 192) were observed at higher fragmentor voltage (180 V). Interestingly, the $[M+NH_4]^+$ ion was not observed at

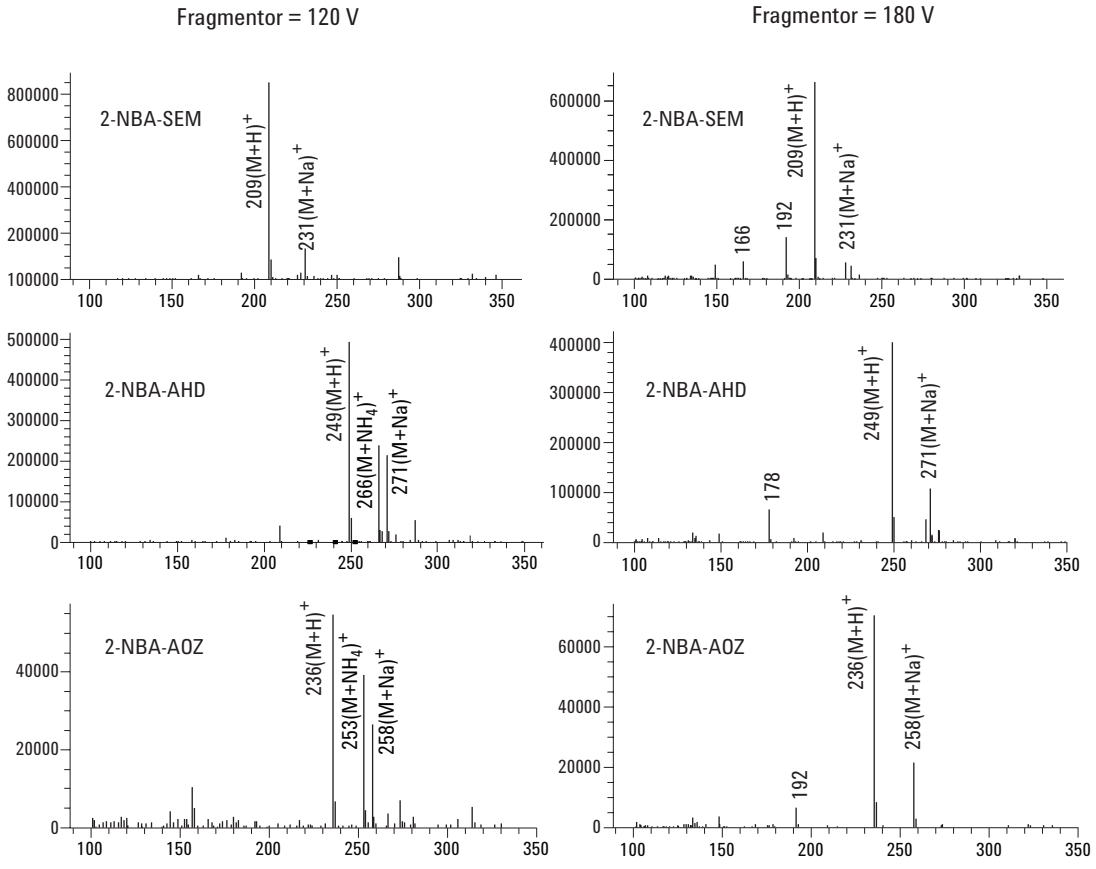

Figure 2. Mass spectra of 2-NBA-SEM, 2-NBA-AHD, and 2-NBA-AOZ from two ESI fragmentor voltages.

180 V fragmentor voltage due to its stability. As seen in Figure 3, in order to ensure the best sensitivity, the fragmentor voltage for 2-NBA-SEM was set to 120 V and that of 2-NBA-AHD and 2-NBA-AOZ was set to 140 V for the analysis. Although 2-NBA-AMOZ was not examined, fragmentor voltage of this compound was set to 140 V because of its structural similarity to 2-NBA-AOZ. For the capillary voltage varied between 1500 and 4500 V, the optimal voltage was found to be 2000 V for all three metabolites.

Linearity, Detection Limits, and Precision

In order to achieve optimal sensitivity, all quantitation experiments were carried out under SIM conditions, and the $[M+H]^+$ ions were monitored for all 2-NBA-metabolites. To evaluate the linearity of the calibration curves, various metabolite solutions ranging from 0.25 ng/mL to 1 ng/mL were derivatized and then analyzed. As shown in Table 3, the linearity was very good for all 2-NBA-metabolites with correlation coefficients (r^2) greater than 0.999.

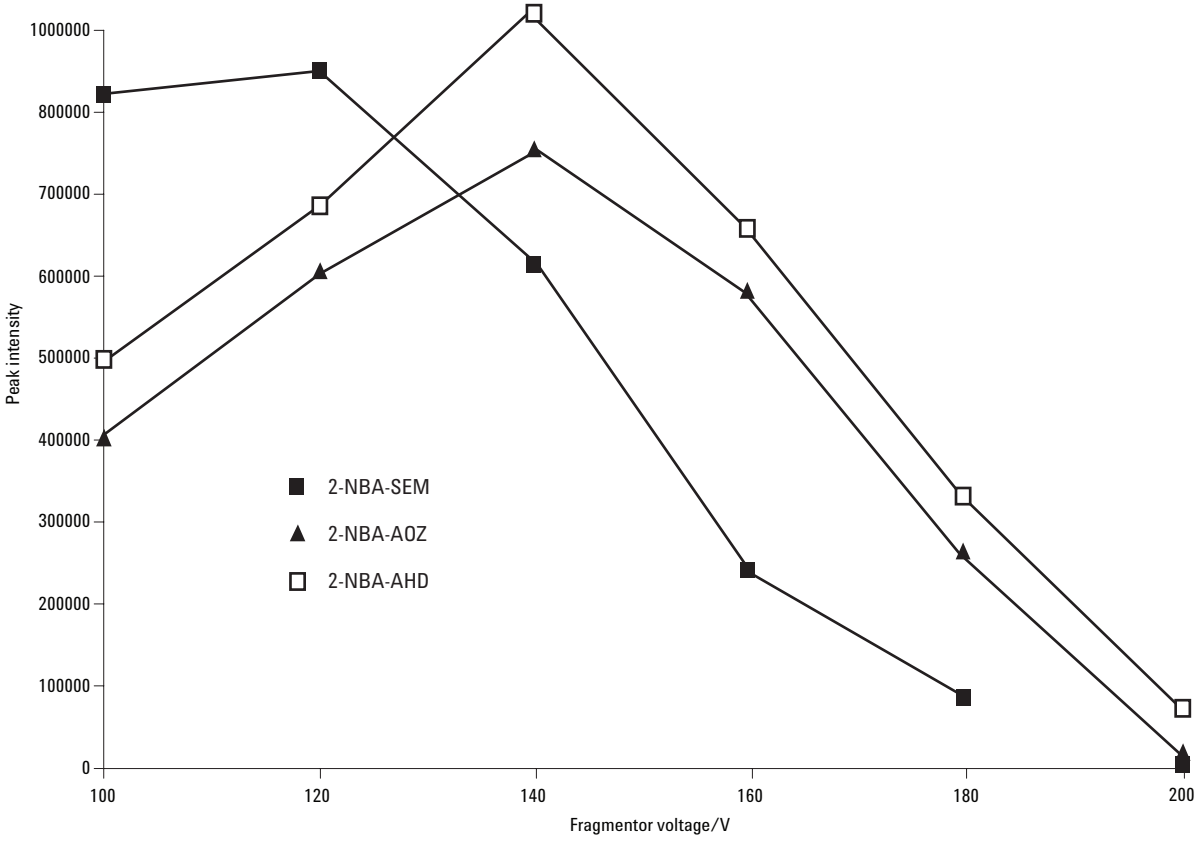

Figure 3. Effect of fragmentor voltage on peak intensity. Mobile phase, 20% acetonitrile/80% water 0.1% formic acid; Analyte concentration, 100 ng/mL.

The LOD for all 2-NBA-metabolites was estimated by extrapolating to a signal-to-noise ratio (S/N) of 3 using the signal from the standard solution at 0.25 ng/mL. These SIM chromatograms are shown in Figure 4. The LODs of the metabolites were in the range of 0.02 ng/mL to 0.06 ng/mL. These LODs were lower than those of the LC/MS/MS method developed by Leitner [4]. The intraday instrument precision (repeatability) was determined by injecting aqueous standard solutions containing all of the 2-NBA-metabolites at 0.5 ng/mL five times during a working day. The interday instrument precision (reproducibility) was evaluated by analyzing the same sample three times over 3 working days. The precision for all analytes ranged from 3.1% to 8.2%, as seen in Table 3.

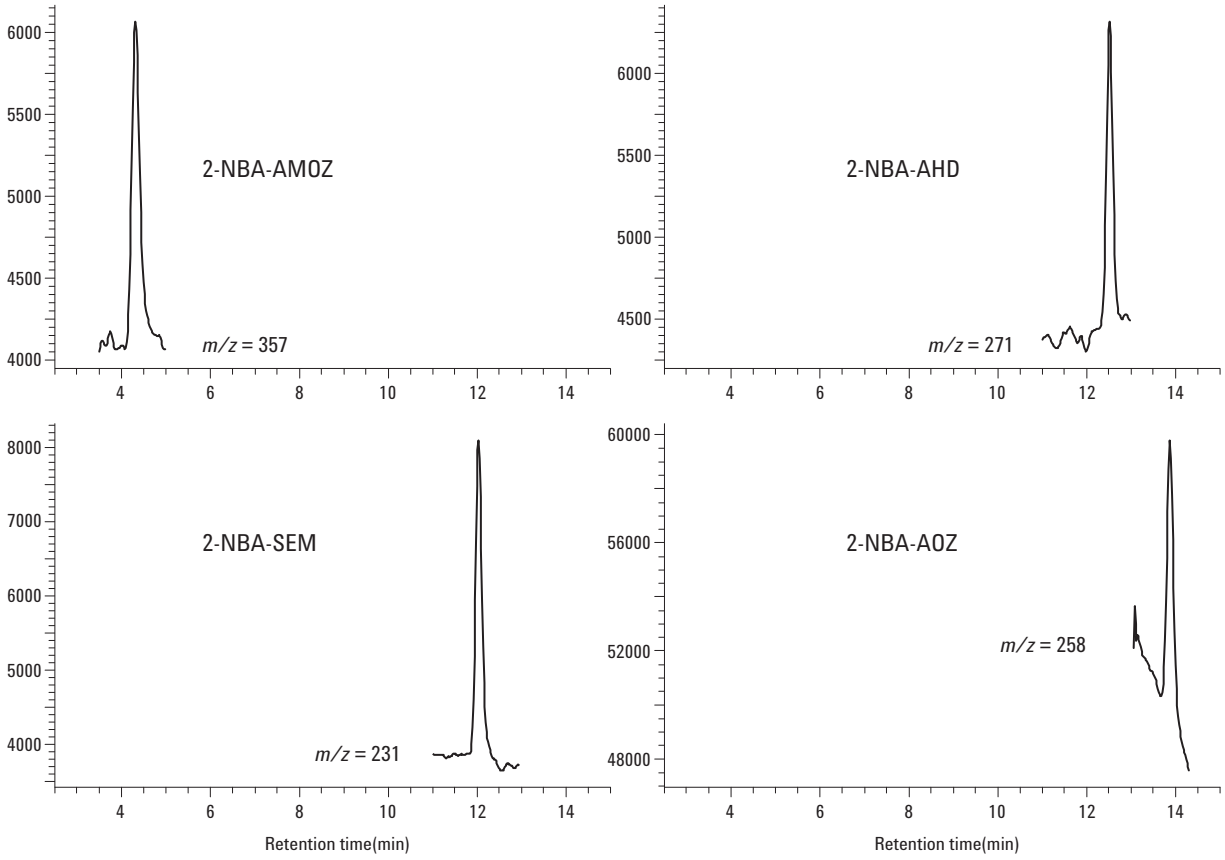

Figure 4. SIM chromatograms of aqueous 2-NBA nitrofuran metabolites solution at 0.25 ng/mL.

Table 3. Linearity, LOD, and Instrument Precision of Metabolites in Aqueous Solutions

			Instrument precision (%RSD)	
Metabolites	**r^2**	**LOD* (ng/mL)**	**Repeatability****	**Reproducibility*****
AMOZ	0.9999	0.04	5.0	7.3
SEM	0.9998	0.02	4.7	8.1
AHD	0.9989	0.06	4.9	7.9
AOZ	0.9997	0.06	3.1	8.2

*Detection limit is LOD defined as S/N = 3 for standard solution at 0.25 ng/mL

**Repeatability was calculated based on five replicates at 0.5 ng/mL within 1 day

***Reproducibility was calculated based on once per day for 3 days at 0.5 ng/mL

Evaluation of Chromatographic Separation

Several reverse-phase columns were evaluated for HPLC performance. In terms of minimizing the inherent matrix suppression effects on the ESI process, Inertsil ODS3 column provided the best separation between analytes and the majority of the matrix components with the given mobile phase. Further, the linear solvent gradient gave the best compromise between short analysis time and sufficient matrix and analytes separation. Figure 5 shows individual SIM chromatograms for the four metabolite derivatives in spiked chicken muscle at 0.2 ng/g. No interference peaks were observed for 2-NBA-AMOZ and 2-NBA-AOZ, but it was difficult to separate 2-NBA-SEM and 2-NBA-AHD from the interfering matrix peaks. However, these peaks could still be identified by comparison with the blank sample, and the analyte amounts could then be calculated.

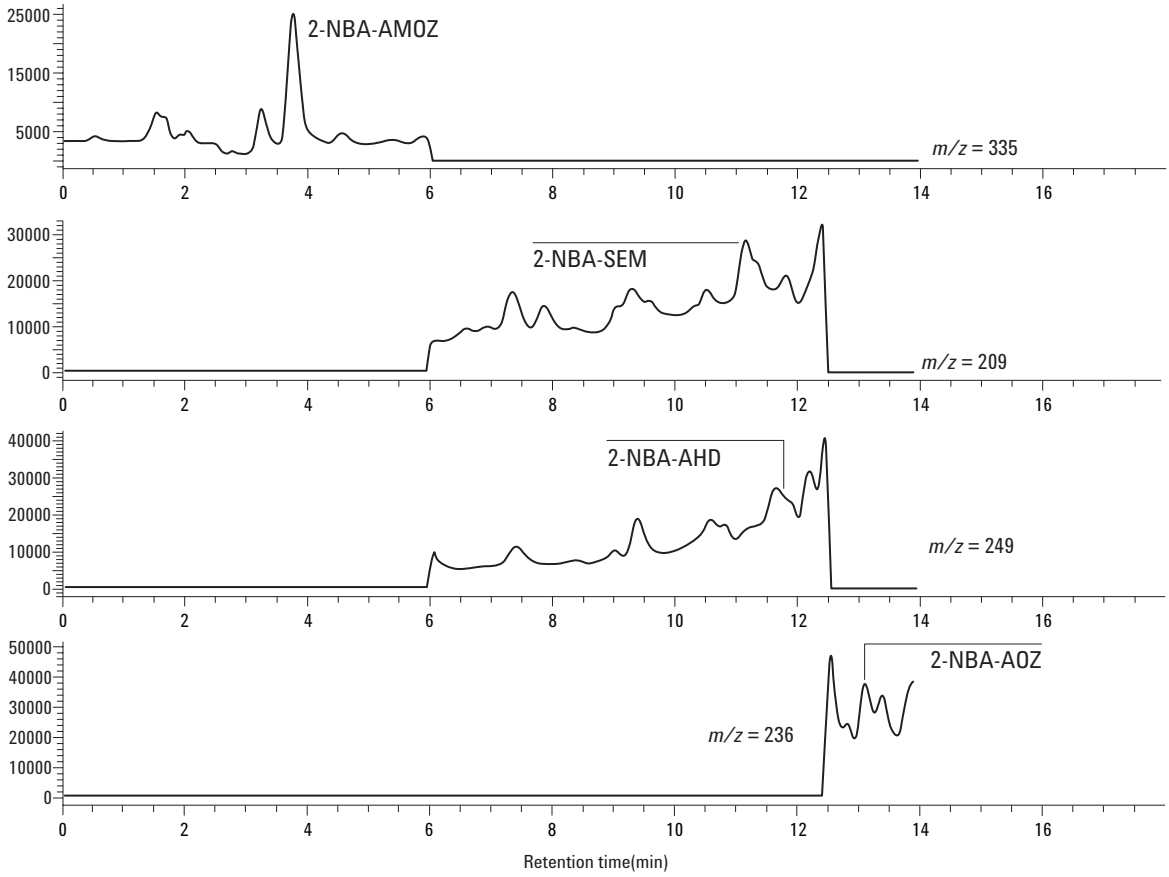

Figure 5. SIM chromatograms of a spiked chicken muscle tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Application of the Method to Chicken Liver Samples

It has been reported that AOZ concentrations in liver tissue are several times higher than in muscle tissue [1,2]. This indicates that detection of nitrofuran metabolites in liver would be possible over an even longer period of time. Since the nature of the liver matrix is considered to be different from muscle and more difficult to separate target compounds from the interfering matrix, the developed LC/MS muscle method was also tested for the applicability to liver matrix. Figure 6 shows individual SIM chromatograms of the four metabolite derivatives in spiked chicken liver tissue. AMOZ, SEM, and AOZ derivatives were identified unambiguously and quantified down to 0.2 ppb. However, the AHD derivative overlapped with the matrix component and was difficult to quantify.

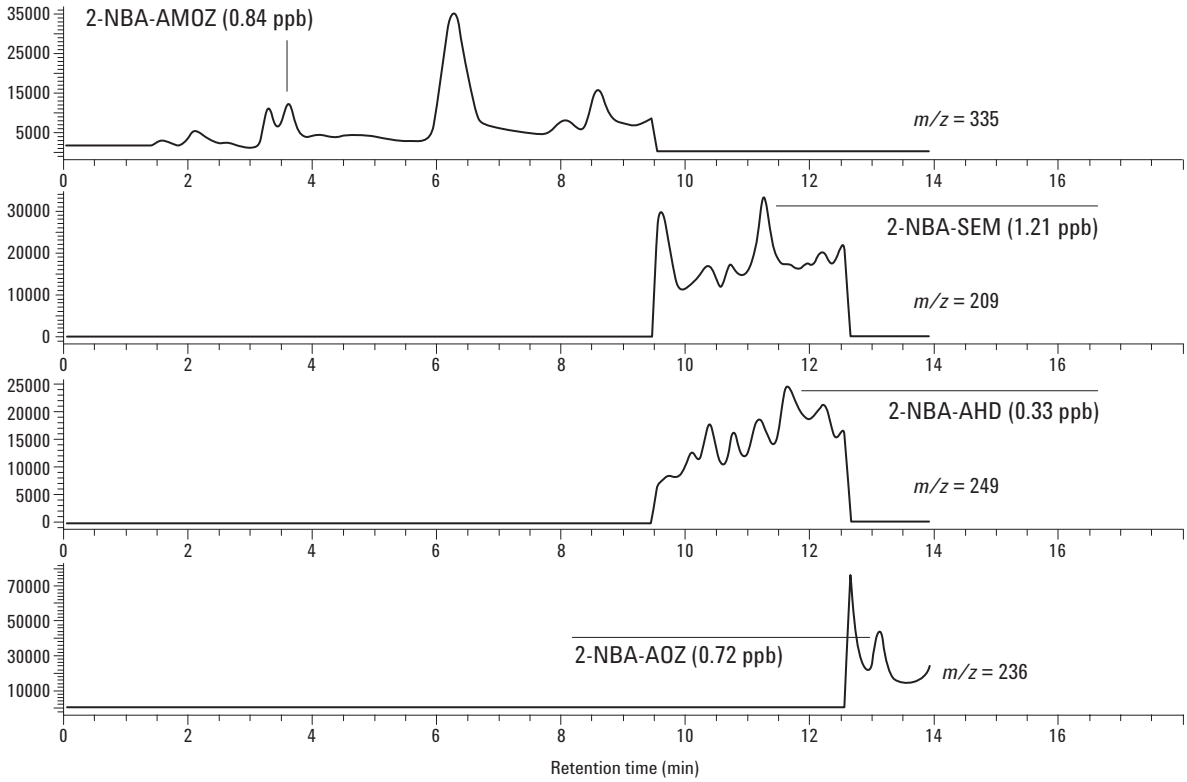

Figure 6. SIM chromatograms of a spiked chicken liver tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Conclusion

The development of a routine and sensitive LC/MS method allows for the simultaneous detection of four nitrofuran metabolite derivatives. The detection limit of each analyte ranges from 0.05 to 0.2 ng/g in chicken muscle and liver tissues.

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Acknowledgement

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Determination of Chloramphenicol in Fish Meat by Liquid Chromatograph-Atmospheric Pressure Photo Ionization-Mass Spectrometry (LC-APPI-MS)

Application

Foods, Environmental

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Abstract

A liquid chromatography-atmospheric pressure photoionization-mass spectrometry method was developed for the determination of chloramphenicol antibiotics in fish meats. For the optimization of APPI, several ion source parameters were examined. Using the optimized parameters, simple mass spectra and a strong signal corresponding to $[M-H]^-$ was observed. The samples were extracted with ethylacetate and evaporated to dryness followed by a clean-up step using liquid-liquid distribution by acetonitrile and n-hexane. Mean recoveries of chloramphenicol from young yellowtail meat and flatfish meat spiked at 0.1–2 ng/g were 89.3%–102.5% and 87.4%–94.8%, respectively. The limit of detection (signal-to-noise = 3) of the young yellowtail meat and the flatfish meat were 0.27 and 0.10 ng/g.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic, that exhibits activity against a variety of aerobic and anaerobic microorganisms. Its action works through interference with or inhibition of protein synthesis. However, weeks or months of CAP therapy can result in a well-understood and irreversible type bone marrow depression called aplasia or hypolasia. This, in turn, can lead to aplastic anemia and although uncommon, it is often fatal. Because of these health concerns, a joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives has proclaimed that CAP residues in the human food supply are unacceptable [1]. The use of CAP in food products has been banned in EU and U.S.A. However, CAP's broad-spectrum activity, ready availability, and low cost attract its use by some third world countries. Admittedly, whenever CAP is accessible, indiscriminate and illegal use potentially exists. In fact, the presence of CAP has been detected in shrimp imported from China and Vietnam that was intended for human consumption.

Liquid chromatography/mass spectrometry (LC/MS) methods are very useful in analyzing CAP in food because of the high selectivity and sensitivity of MS detection [2-7]. Atmospheric pressure ionization (API) interfaces, represented by atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are commonly used in LC/MS.

Atmospheric pressure photoionization (APPI) is a new ionization technique for LC/MS [8, 9]. The APPI source is based on a high-fluence gas discharge lamp that generates vacuum-ultraviolet (VUV) photons of 10 and 10.6 eV energy. The energy of this discharge lamp is normally greater

than a first ionization potential (IP) of an analyte because many organic compounds have IPs in the range of 7–10 eV. On the other hand, the IPs of the most common LC solvents, which are used as a mobile phase, have higher values (water, IP = 12.6 eV; methanol, IP = 10.8 eV; acetonitrile, IP = 12.2 eV). This provides ionization of many analytes with lower IPs without interference from the mobile phase. To our knowledge, APPI has not yet been applied to residual analysis in food.

This application note describes how parameters affect the ionization efficiency of APPI for the analysis of CAP. In addition, the suitability of LC/MS and liquid-liquid extraction using the APPI technique is evaluated for the determination of CAP in fish meat.

Experimental

Chemicals and Solvents

CAP was purchased from Sigma-Aldrich Japan (Tokyo, Japan). The purity of this compound was greater than 99%. Stock solutions at 1 mg/mL were prepared in methanol, stored in the dark at 4 °C, and diluted to the desired concentrations prior to use. Ammonium acetate, pesticide-grade ethyl acetate, anhydrous sodium sulfate, acetonitrile, HPLC-grade methanol and n-hexane were obtained from Wako Chemical (Osaka, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan). A nylon-type 0.22 µm centrifuge filter was obtained from Toyo Soda (Tokyo, Japan).

Sample Preparation

The samples analyzed (young yellowtail and flatfish) were obtained from a local market. To a centrifuge tube, 5 g fish meat and 5 g anhydrous sodium sulfate were weighed and 10 mL ethyl acetate was added. The mixture was homogenized for 20 s with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Staufen, Germany). After centrifugation for 5 min at 6000 rpm, the supernatant was removed and transferred to a round flask. The extraction step was repeated twice, each with 10 mL ethyl acetate. The combined ethyl acetate extract was then evaporated in a rotary evaporator at 40 °C under vacuum. One mL acetonitrile and 1 mL n-hexane was added to the residue, transferred into a graduated glass stopper reagent bottle, and shaken. The n-hexane phase was discarded. The step was repeated with another 1 mL of n-hexane. Finally, the acetonitrile phase was evaporated to dryness under a stream of dry nitrogen using a heating block at 50 °C, redissolved in 5 mL of a 10% acetonitrile in 10 mM ammonium acetate water solution, and filtered through a 0.22 µm nylon centrifuge filter. The samples were spiked with 0.1–100 ng/mL of CAP after the homogenation step to generate a calibration by LC/APPI-MS selected ion monitoring (SIM).

LC/MS

An Agilent 1100 series LC, consisting of a vacuum solvent degassing unit, a binary high-pressure gradient pump, a standard automatic sample injector, and a column thermostat, was used for the separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. See Table 1. The separation was performed on a 150 × 3 mm id column packed with 5 µm Zorbax Eclipse XDB C18 (Agilent Technologies, Palo Alto, USA). A 15-min linear solvent gradient was used for elution with the mobile phase. Quantitative analysis was carried out using SIM of *m/z* 321 with a dwell time of 500 msec.

The following six parameters were optimized using

Table 1. Instrument Parameters

LC:	**1100 series LC**
Column:	Zorbax Eclipse XDB C18 (150 mm × 3 mm, 5 µm)
Solvent A:	Water with 10 mM ammonium acetate
Solvent B:	Methanol
Dopant:	Acetone at 0.05 mL/min
Gradient:	90/10 A/B 15 min to 70/30 A/B
Column temp:	40 °C
Sample volume:	20 µL
Flow rate:	0.5 mL/min
MS:	**1100 MSD, SL**
Ionization:	APPI (Negative)
Scan range:	*m/z* 100–400 for optimization
SIM ion:	*m/z* 321; $(M\text{-}H)^-$
Drying gas:	Nitrogen, 7 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 V
Capillary:	3500 V
Vaporizer temp:	350 °C

the analytical column with CAP at 100 ng/mL: the voltages for in-source-fragmentation (the fragmentor voltage), the capillary voltage (V_{cap}), the drying gas flow rate, the nebulizer pressure, the mobile phase composition, and the mobile phase flow rate. The ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program. Negative ion mass spectra were acquired over the scan range *m/z* 100–400 using a step size of 0.1 amu and a scan rate of 2 s per scan for the optimization of fragmentor voltage.

Results and Discussion

Optimization of the APPI Parameters

To optimize the APPI conditions, parameters that influence the ionization efficiency were investigated. The drying gas flow, the nebulizer gas pressure, the vaporizer temperature, the capillary voltage, and the mobile phase composition were evaluated under the chromatographic conditions mentioned in the Experimental section by SIM mode using the *m/z* 321 ion as the target ion. It was found that modification of drying gas flow rate and nebulizer gas pressure did not drastically improve the sensitivity of CAP. In addition, the fragmentor voltage was included in optimization because of its compound dependence and its significant effect on the mass spectral response.

Effect of Capillary Voltage

The capillary voltage is applied to the inlet of the capillary and influences the transmission efficiency of the ions through the capillary sampling orifice. To establish the optimum capillary voltage, this parameter was varied from 1000 to 4000 V. As shown in Figure 1, 1500 V was found optimum. A tremendous effect of this parameter on the intensity of CAP was observed in the case where acetone was not used as the dopant. On the other hand, when acetone was introduced into the APPI source as the dopant, the maximum intensity of the ion was found at 3500 V. The intensity found at 3500 V with the dopant was higher than the maximum intensity without the dopant. Based on the above results, the capillary voltage was set at 3500 V with acetone.

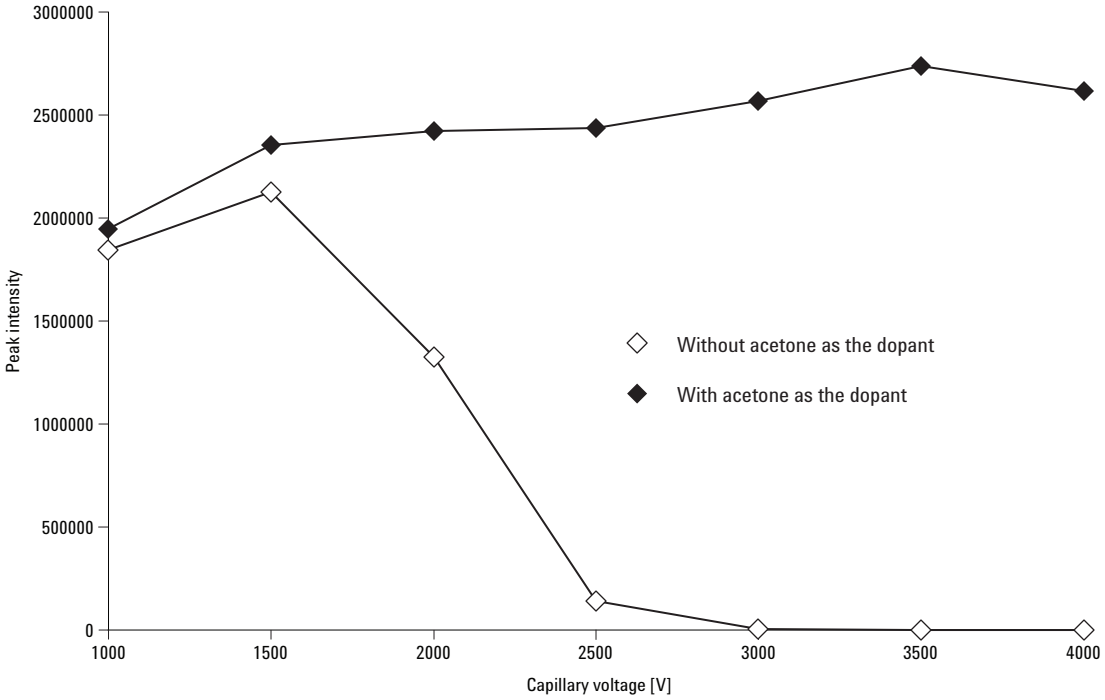

Figure 1. The effect of the capillary voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

Effect of Vaporizer Temperature

In APPI, the vaporizer temperature plays a key role for the complete evaporation of CAP because ionization occurs in the vapor state like APCI. Thus, in the case of using linear gradient elution, this temperature must be kept sufficiently high so that the change of mobile phase composition does not influence the ion intensity of CAP. Under high temperature, however, the risk of thermal degradation occurs. In this study, the vaporizer temperature was modified between 250 and 450 °C to optimize the intensity and the *S/N* ratio. The highest temperature for a maximum intensity and *S/N* ratio of CAP was observed at 350 °C. The intensity of CAP decreased as the vaporizer temperature was increased over 400 °C. In addition, intense fragmentation was observed in the mass spectrum at 400 °C. Therefore, the decrease in intensity above 400 °C seems to be a result of the thermal degradation. Based on the above results, the vaporizer temperature was set at 350 °C.

Optimization of Fragmentor Voltage

The fragmentor voltage is applied to the exit of the capillary and affects the transmission and fragmentation of sample ions between the exit of the capillary and the skimmer at relatively high pressure (3 torr). In general, the higher the fragmentor voltage (which helps the transfer of ions), the more fragmentation will occur. To establish the optimum fragmentor voltage for the analysis of CAP, the intensity of this compound versus the fragmentor voltage was studied in the range from 80 to 200 V. As shown in Figure 2, the optimum fragmentor voltage was found at 120 V, whereas at higher values a significant intensity reduction was observed. Further, the best *S/N* ratio was also observed at 120 V. The mass spectra of CAP at optimal and higher fragmentor voltages are shown in Figure 3. The deprotonated molecule $(M\text{-}H)^-$ was the predominant ion at 120 V, and this included isotopic ions (*m/z* 321, Cl^{35} Cl^{35}; *m/z* 323, Cl^{35} Cl^{37}; *m/z* 325, Cl^{37} Cl^{37}) because CAP includes two

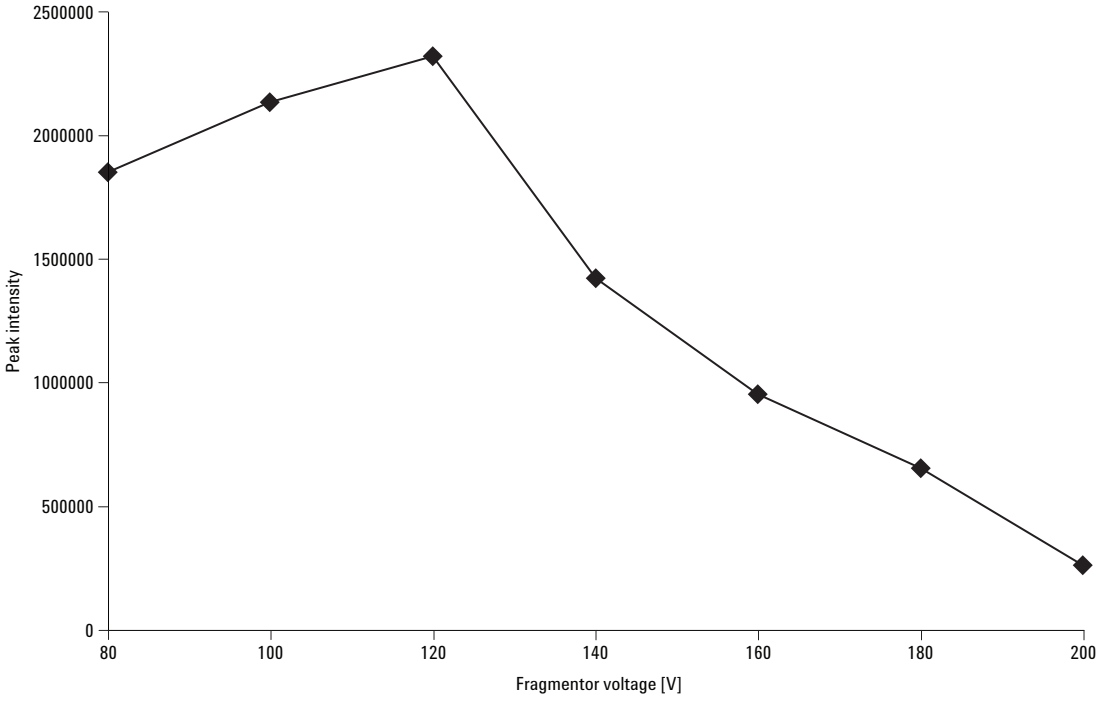

Figure. 2. The effect of the fragmentor voltage on the peak intensity of CAP concentration : 1 ng/mL. For the other conditions, see Experimental section.

chlorines. A higher fragmentor voltage (180 V) generated structurally relevant fragment ions. The *m/z* 152 fragment ion gives the greatest intensity and might be produced by the cleavage of the carbon-carbon bond on the alkyl branch as shown in Figure 3. Other fragment ions are observed at *m/z* 121 and 257. The *m/z* 121 may be the nitrophenyl fragment. The *m/z* 257 fragment might be explained by a charge migration hydrogen shift with a concerted loss of HCl and CO. These observed fragment ions in the APPI source corresponded with the fragment ions in an ESI source and an APCI source. Based on the above results, the fragmentor voltage was set to 120 V.

Optimization of the Chromatographic Conditions

The separation of CAP from sample matrix peaks was optimized using acetonitrile, methanol, water, and ammonium acetate. The combination of methanol and ammonium acetate was found optimum for the separation of CAP. When methanol was replaced with acetonitrile, a significant signal intensity and *S/N* decrease was observed. This result indicates that methanol may be a source of electrons for the hydrogen abstraction from CAP. Therefore, methanol and 10 mM ammonium acetate was used as the mobile phase in this study. The flow rate was set at 0.5 mL/min considering the size of the used column.

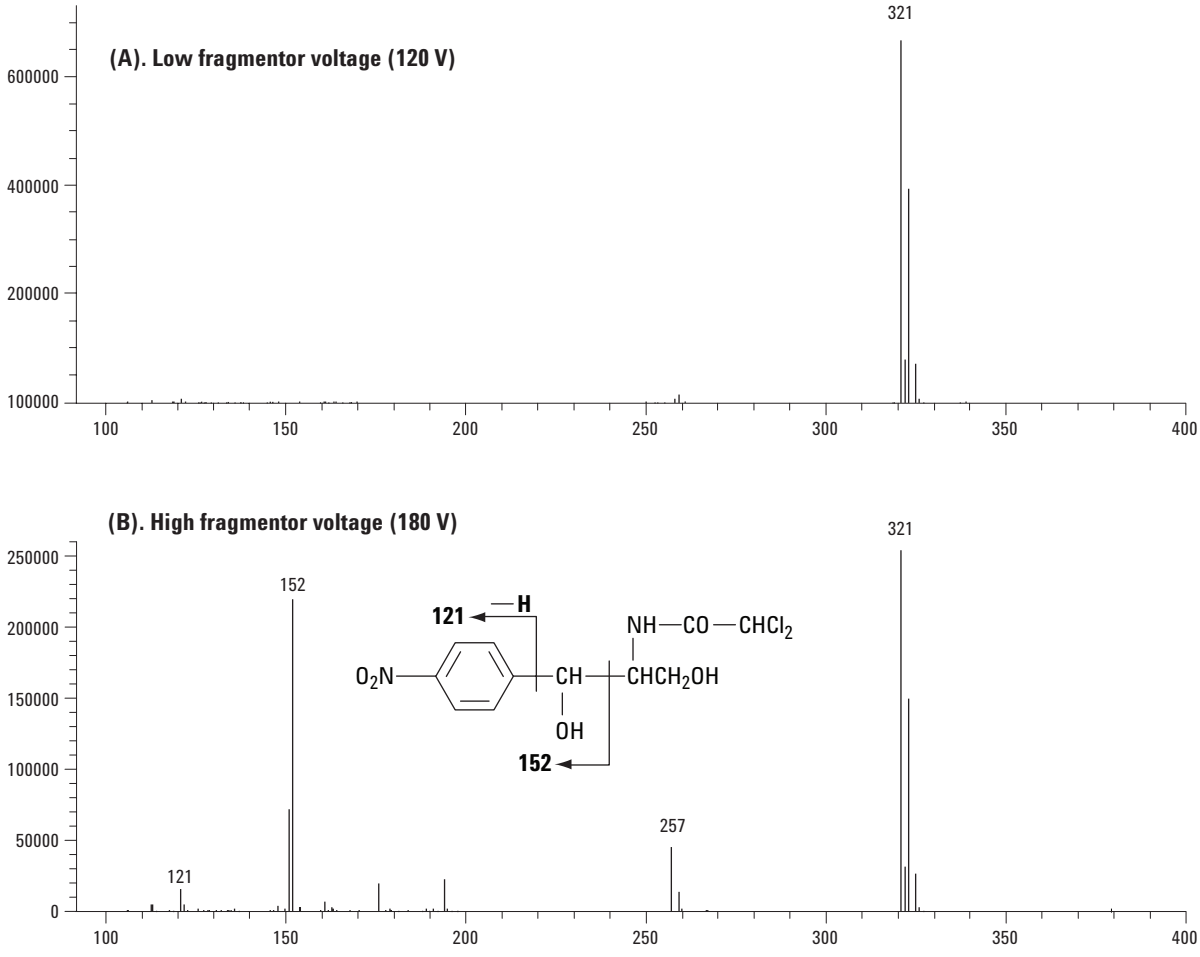

Figure 3. The mass spectra of CAP at two different fragmentor voltages.

Linearity, Detection Limit and Precision of LC/APPI-MS System

The analytical performance characteristics of the optimized LC/APPI-MS were first determined on standard solutions of CAP in pure solvent. See Figure 4. In order to achieve optimum sensitivity, all experiments were carried out under SIM mode using the mass corresponding to the $[M\text{-}H]^-$ ions for CAP. To test the linearity of the calibration curves, various concentrations of CAP ranging from 0.1 to 100 ng/mL were analyzed. The calibration curves of APPI showed good linearity with correlation coefficients (r^2) = 0.9998. The repeatability of APPI for a standard solution was calculated on the basis of five replicates at 0.5 ng/mL in the same day. The limit of detection (LOD) was calculated by using a *S/N* ratio of 3 at 0.1 ng/mL. The SIM chromatogram of CAP with APPI is shown in Figure 4 (the *S/N* ratio of this chromatogram was 4.2); LOD and RSD were 0.07 ng/mL and 2.1%.

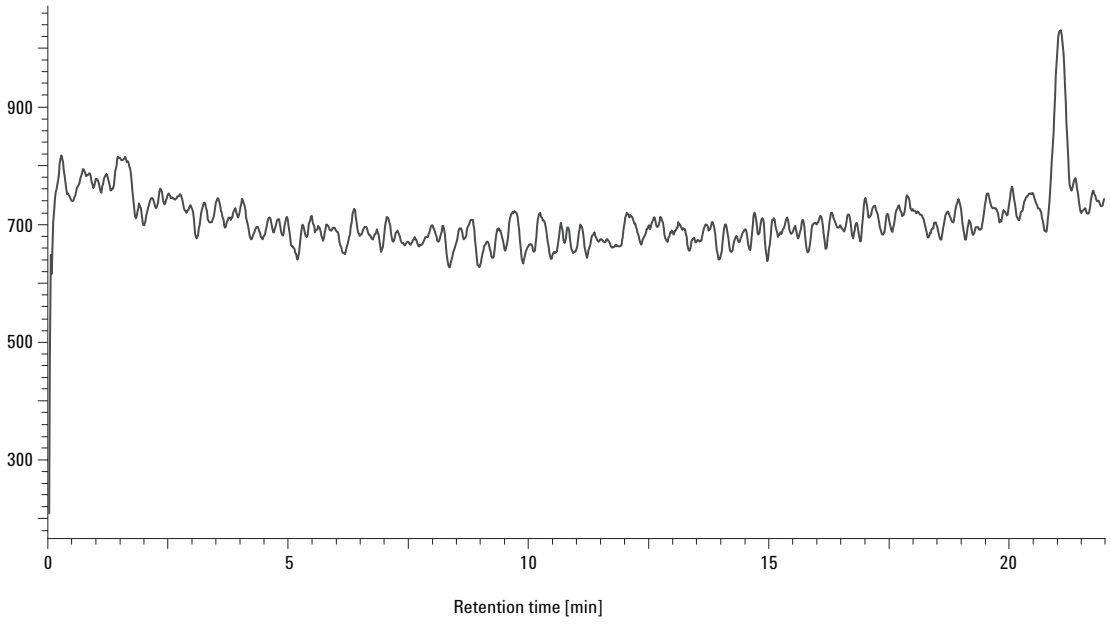

Figure 4. SIM chromatogram of CAP in pure solvent at 0.1 ng/mL with APPI.

APPI Method Evaluation

To evaluate recoveries, the proposed method was applied to the analysis of spiked CAP-free samples of young yellowtail and flatfish meat. Eighteen samples of two different fish were each spiked with CAP and each sample was spiked at three levels. The spiking levels ranged from 0.1 to 2 ng/g. Typical chromatograms from the fish meat extracts spiked at 1 ng/g and 0.1 ng/g are shown in Figure 5.

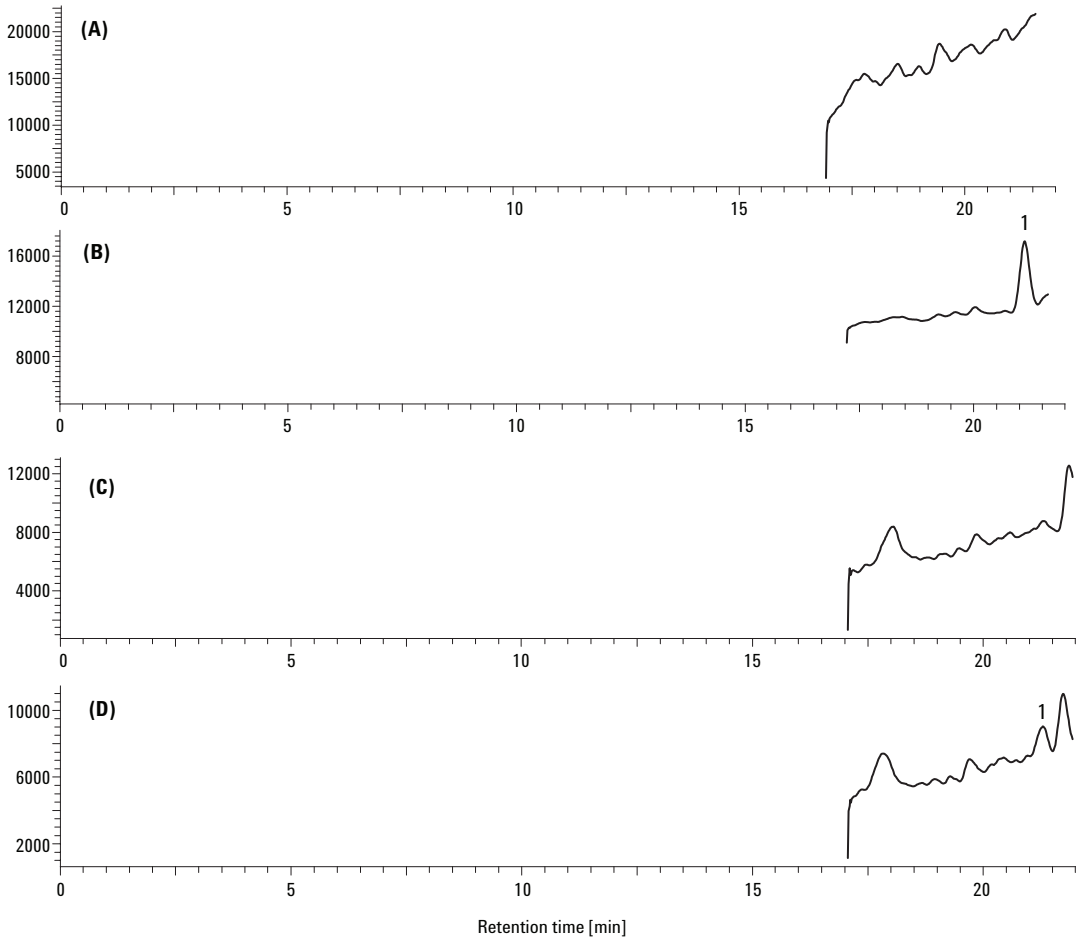

Figure 5. SIM chromatograms of A) Young yellowtail meat, B) Spiked young yellowtail meat at 1 ng/g CAP, C) a flatfish meat, and D) a spiked flatfish meat at 0.1 ng/g CAP.

Data from 18 spiked samples led to recoveries and RSD are summarized in Table 2.

Table 2 Recovery of CAP for Spiked Fish Meat

Spiking levels (ng/g)	Recovery [±RSD (%)]* Young yellowtail	Flatfish
0.1	89.3 ±5.1	87.4 ±6.1
0.5	102.5 ±4.9	94.8 ±6.7
2.0	96.1 ±4.3	91.8 ±4.9

*Three spiked samples at the same amount were analyzed.

Mean recoveries ranged from 87.4% to 102.5% with RSD of 4.3% to 6.7%. The LODs of CAP in fish meats were determined by the signal corresponding to three times the background noise on SIM chromatogram of spiked sample at 1 ng/g and 0.1 ng/g and shown in Table 3. The intraday precision (repeatability) was estimated by injecting the same spiked fish meat extract at 0.1 ng/g five times during a working day. The interday precision (reproducibility) was evaluated by analyzing the same sample over 5 working days. The repeatability and reproducibility for CAP in fish meats were 4.8%, 9.4% and 2.1%, 7.3%, respectively. These results indicate that this LC/APPI-MS method is suitable for the analysis of residues of CAP in fish meats.

Table 3. LODs, Repeatability, and Reproducibility of CAP in Standard Solution Using APPI

Fish meats	LODs* (ng/g)	Repeatability** (RSD, %)	Reproducibility*** (RSD, %)
Young yellowtail	0.27	4.8	9.4
Flatfish	0.10	2.1	7.3

*Detection limit is LOD defined as $S/N = 3$ at 0.1 ng/mL.

**Repeatability was calculated on the basis of five replicates at 0.5 ng/mL within 1 day.

***Reproducibility was calculated by analyzing one fish meat spiked at 0.1 ng mL^{-1} per day during 5 days.

Conclusion

APPI is an ideal ionization technique because of high sensitivity and high selectivity for the determination of CAP in fish meats. An important advantage of using APPI for CAP content of fish meats is that sample matrix did not significantly affect ion intensity of CAP. The data presented here demonstrate that this method is convenient for routine analysis of CAP residues in fish meats at trace levels, as excellent recoveries and precision for different samples were obtained.

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Detection, Confirmation, and Quantification of Chloramphenicol in Honey and Shrimp at Regulatory Levels Using Quadrupole and Ion Trap LC/MS

Application

Foods, Environmental

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Abstract

Methodology capable of meeting regulatory requirements has been developed for the determination of chloramphenicol in honey and shrimp. Samples of the two foodstuffs are extracted with Isolute HN-M cartridges and analyzed with both the Agilent 1100 LC/MSD Trap (SL) and the Agilent 1100 LC/MSD (SL) quadrupole with negative mode electrospray ionization. Using deuterated internal standard and one simple sample extraction procedure, both instruments provide a limit of detection at or below 0.1 ppb in both shrimp and honey. Detection limits are lower using the ion trap for shrimp because of less matrix interference. The Agilent 1100 LC/MSD gives quantitative results and the Agilent 1100 LC/MSD Trap gives full spectrum confirmation.

Introduction

Chloramphenicol is a broad range antibiotic that has found its way into foodstuffs such as honey and shrimp. Because it has displayed significant toxicological effects on humans, it has been banned from foods in the European community and the United States at levels greater than 0.1 ppb. Analytical methods used to determine this limit must achieve both the required sensitivity and maintain sufficient selectively. LC/MS has been demonstrated by the US Food and Drug Administration for these analysis [1-3]. In addition, the Commission of European Communities has issued guidelines stipulating that for mass spectral detection, a molecular ion (or quasimolecular ion) and at least two fragment ions are needed for positive confirmation [4]. For quantitative analysis the Agilent 1100 LC/MSD provides excellent results and can give some confirmation information. The Agilent 1100 LC/MSD Trap gives excellent full spectrum confirmation at the regulated concentration.

Experimental

Reagents and Materials

ISOLUTE HM-N cartridges from IST (Hengoed, UK, Part-nr. 800-1300-FM)

Ethyl acetate from Vel (Merck Eurolab, Leuven, Belgium)

Methanol HPLC-grade from Merck (LiChrosolv, Darmstadt, Germany)

Deuterated (d5) CAP internal standard from Cambridge Isotope Laboratories (CIL, Andover, MA, USA)

Syringe filters (0.2 µm, PTFE) from Alltech Associates Inc. (Lokeren, Belgium)

Sample Preparation

For honey, 5 g of sample is diluted to 20 mL with water and 5 µL of 1 ng/µL internal standard (IS) is added. The solution is loaded on the cartridge and allowed to stand for 5 minutes. Elution is performed with 50 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL water/methanol (9/1, v/v) and put in an ultrasonic bath for 1 minute. The solution is filtered, using a syringe filter, before injection. No additional clean-up of the sample solution is performed.

For shrimp, a portion of at least 10 g of frozen shrimp is defrosted and mixed in a blender. To 10 g of the mixed shrimp, 30 mL of water and 10 µL of 1 ng/µL IS is added. This portion is centrifuged for 10 minutes (2000 rpm). A 20-mL portion of the supernatant is loaded on the cartridge and allowed to stand for 5 minutes. Elution is performed with 50 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL water/methanol (9/1, v/v) and put in an ultrasonic bath for 1 minute. The solution is filtered before injection.

LC/MS Conditions

The LC/MS systems were the Agilent 1100 LC/MSD quadrupole mass spectrometer and the Agilent 1100 LC/MSD Trap. Both were equipped with Agilent 1100 binary pumps and 1100 well plate autosamplers. See Table 1.

Table 1. LC/MS Conditions

HPLC		
Column	Eclipse XDB C18, 4.6 mm × 150 mm, 5 µm (p/n 993967.902)	
Flow-rate	0.9 mL/min	
Mobile phase	10 mM ammonium acetate in water (solvent A) Methanol/acetonitrile 1/9 (solvent B) both from Merck (LiChrosolv, Darmstadt, Germany)	
Gradient	0–1 min	30% B
	1–8 min	30%–70% B
	8–8.5 min	70%–100% B
	8.5–12 min	100% B
	Post time	4 min at 30% B
Injection	100 µL with needle wash (methanol)	
Injection solvent	Water/methanol (9/1 v/v) for both standards and samples	
Column temperature	30 °C	
MSD source settings		
Source	ESI	
Ion polarity	Negative	
Drying gas temperature	340 °C	
Drying gas flow-rate	11 L/min	
Nebulizer pressure	50 psig	
V_{cap}	3500 V	
Quadrupole MSD		
MSD acquisition on	Between 3 and 7.5 min	
Fragmentor	160 V	
SIM settings	*m/z* 257, 321, 323 (CAP) *m/z* 262, 326, 328 (CAP-d5)	

Table 1. LC/MS Conditions (continued)

Trap MSD	
MSD acquisition on	Between 3 and 7.5 min
Target mass (SPS)	323 *m/z*
Trap parameters	
Max. accumulation time	300 ms
ICC target	30,000
Scan range	160–340
Averaging	2
Fragmentation parameters (MS/MS)	
Smart Frag	On, 30%–200% (default)
Isolation mass	*m/z* 325.0
Isolation width	10.0 *m/z*
Fragmentation amplitude	1.0 V
Fragmentation cutoff	*m/z* 88

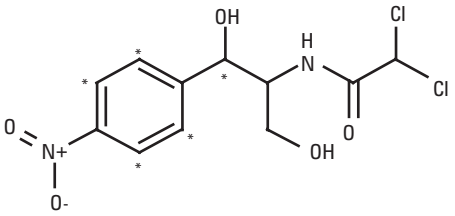
Results and Discussion

Spectral Quality and Sensitivity of Standards

For analysis with the quadrupole LC/MSD, selected ion monitoring (SIM) was used to obtain the required sensitivity. Table 2 shows the structure, fragment ions and identity of CAP and CAP-d5. Figure 1 shows the analysis of a standard mixture containing 2.5 pg/µL CAP and 5 pg/µL CAP-d5. By applying a fragmentor voltage of 160 V, fragment ions at *m/z* 257 and 262 are detected for confirmation purposes. Lowering the fragmentor voltage to optimize for the *m/z* 321 and *m/z* 326 and monitoring those ion alone would obtain greater sensitivity. However, the confirmation of the fragment ions would be lost. For screening analysis without confirmation this would be acceptable and provide a much lower limit of detection (LOD).

Table 2 Structure and Fragment Ions and Identity of CAP and CAP-d5 (* Indicates Deuterated Positions for the CAP-d5 IS)

Chloramphenicol structure



	m/z	Identity
CAP	257	$[M-H-HCOCl]^-$
	249	$[M-H-2HCl]^-$
	194	$[M-H-NH2CoCl2H]^-$
	176	$[M-H-NH2CoCl2H-H2O]^-$
CAP-d5	262	$[M-H-HCOCl]^-$
	254	$[M-H2HCl]^-$
	199	$[M-H-NH_2COCl_2H]^-$
	180	$[M-H-NH_2COCl_2H-HDO]^-$

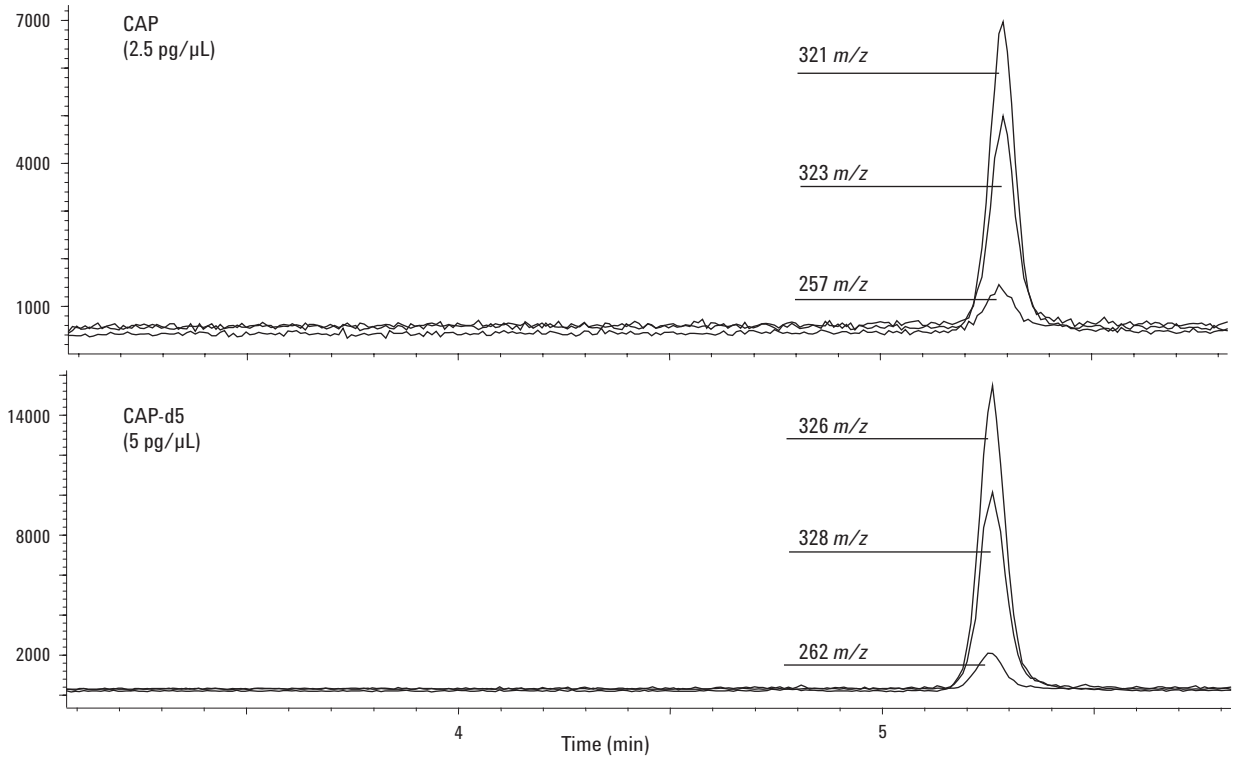


Figure 1. Analysis of a standard solution containing 2.5 ppb of CAP and 5 ppb of CAP-d5 (IS) on the quadrupole MSD. The extracted ion chromatogram for the corresponding ions are shown.

Using the LC/MSD Trap in MS/MS mode both the needed sensitivity (through reduction in chemical noise) and selectivity (for confirmation) is obtained. The compound shows a clear and reproducible fragmentation pattern. An example of the analysis of the standard mixture together with the corresponding MS/MS spectra is shown in Figure 2. Optimizing the fragmentation energy [turning off Smart Frag] and fragmentation cutoff in the ion trap will increase sensitivity even further than shown here. Using an isolation width of 10 *m/z* allows inclusion of the chlorine isotopes in the resulting full scan mass spectra of the analyte and the Cl^{35} isotope of the internal standard. Contact Agilent for more details on these and other ion trap settings.

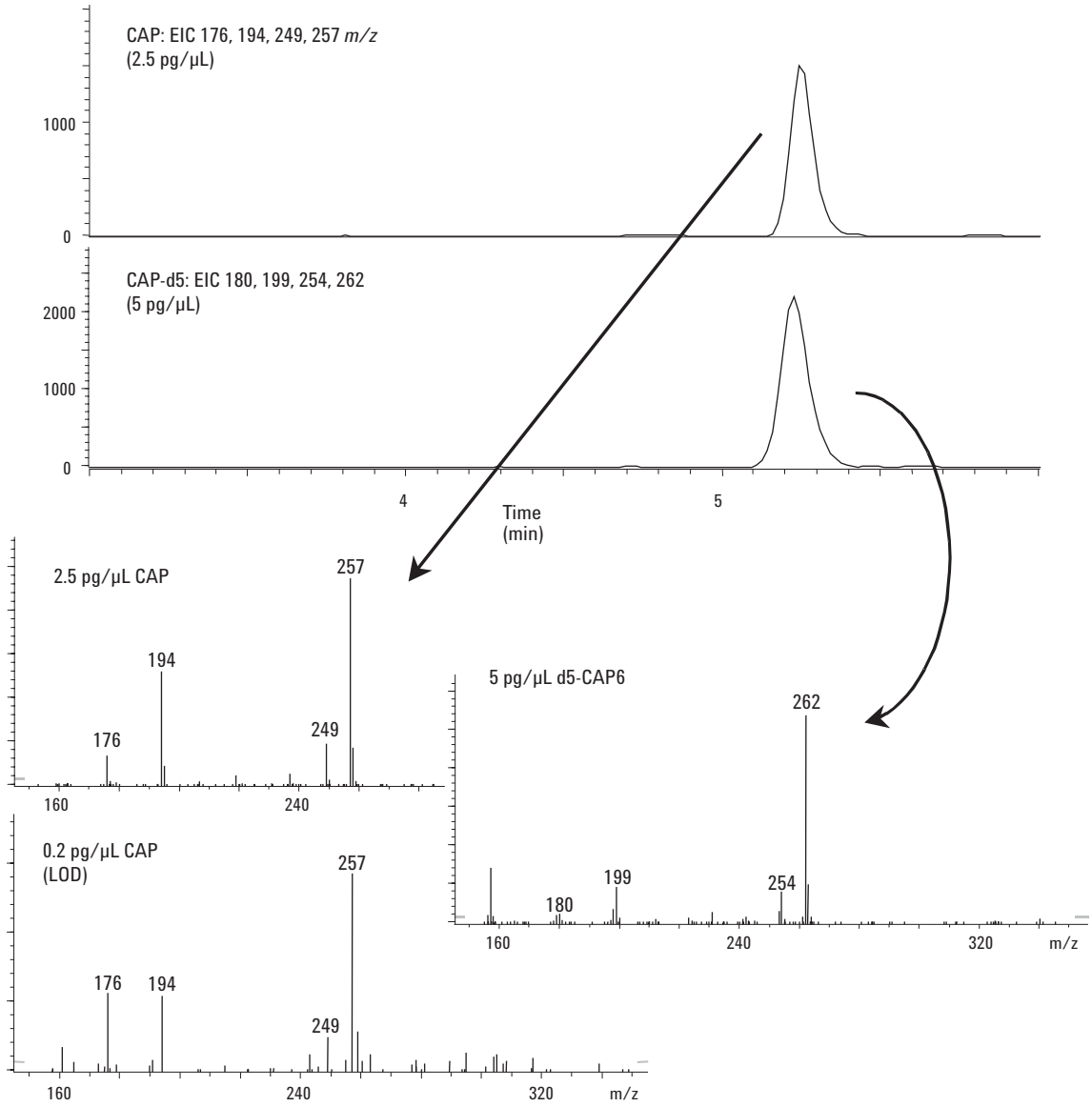


Figure 2. Analysis of a standard solution containing 2.5 pg/µL CAP and 5 pg/µL CAP-d5 (IS) on the LC/MSD Trap together with the corresponding MS/MS spectra and the MS/MS spectrum resulting from an analysis of a standard solution containing 0.2 pg/µL CAP.

Method Performance

Standard solutions of CAP containing 5 pg/µL of CAP-d5 were injected six consecutive times to test repeatability of injection on the mass selective detector (MSD) quadrupole instrument. This was done at two concentration levels. Each time, the response of CAP relative to CAP-d5 was recorded. For a solution containing 0.5 pg/µL CAP the relative standard deviations (RSDs) on the relative response were 5.05%. This 0.5-pg/µL level would correspond to a sample containing approximately 0.1 ppb CAP with the five-fold concentration step. When a solution containing 5 pg/µL CAP was analyzed, RSDs on the relative response were 1.28% for the quadrupole.

A calibration line was constructed by injecting standard solutions of CAP with a concentration of 0 to 25 pg/µL with 5 pg/µL of the IS added to each solution. One injection was performed per concentration. The quadrupole showed a linear response for CAP in this concentration range. Calibration curves and correlation coefficients are shown in Figure 3. The LOD with this method was determined to be ca. 0.2 pg/µL in a standard solution for both mass spectrometers. With the 100-µL injection used, this corresponds with 20 pg on-column.

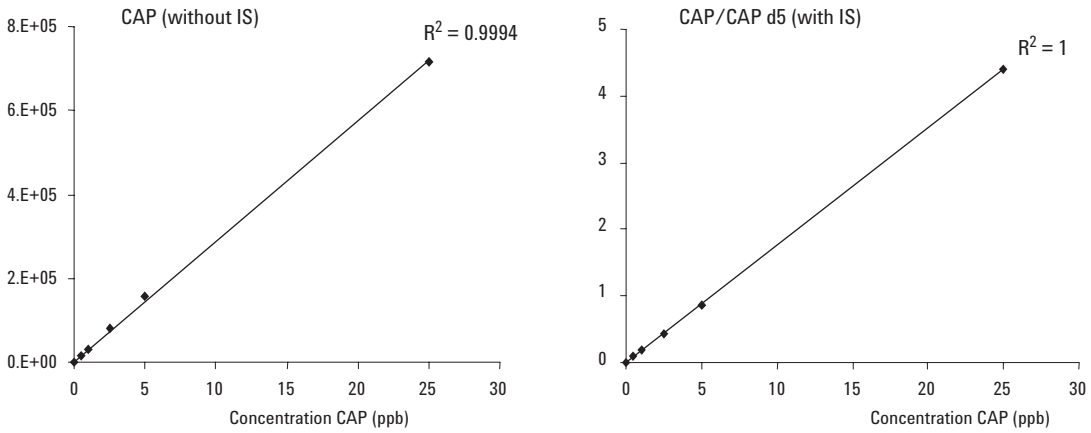


Figure 3. Calibration graphs for standard solutions of CAP on the quadrupole with and without CAP-d5 (IS).

Extraction Recovery and Repeatability of Extraction

The extraction procedure was evaluated on repeatability and linearity with the quadrupole instrument. Blank honey was spiked with 1 ppb CAP and 1 ppb CAP-d5. The extraction procedure was carried out six times and the recovery was calculated. The recovery for CAP varied from 85.31% to 94.94% and the mean recovery was 90.60%. The RSD on the recovery was 4.34% for CAP and 3.39% when the IS was taken into account. An analysis of blank honey spiked only with the IS is shown in Figure 4 run on both instruments. With the quadrupole, LC/MSD matrix interferences are present but chromatographically separated from the CAP signal. The ion trap results show that no matrix interference is present in the isolation window from *m/z* 318 to 328. The data suggest that other endogenous compounds in honey produce fragments at the same *m/z* as CAP. This supports an even lower detection limit for this matrix if a screening analysis were conducted with a lower fragmentor voltage monitoring only the *m/z* 321.

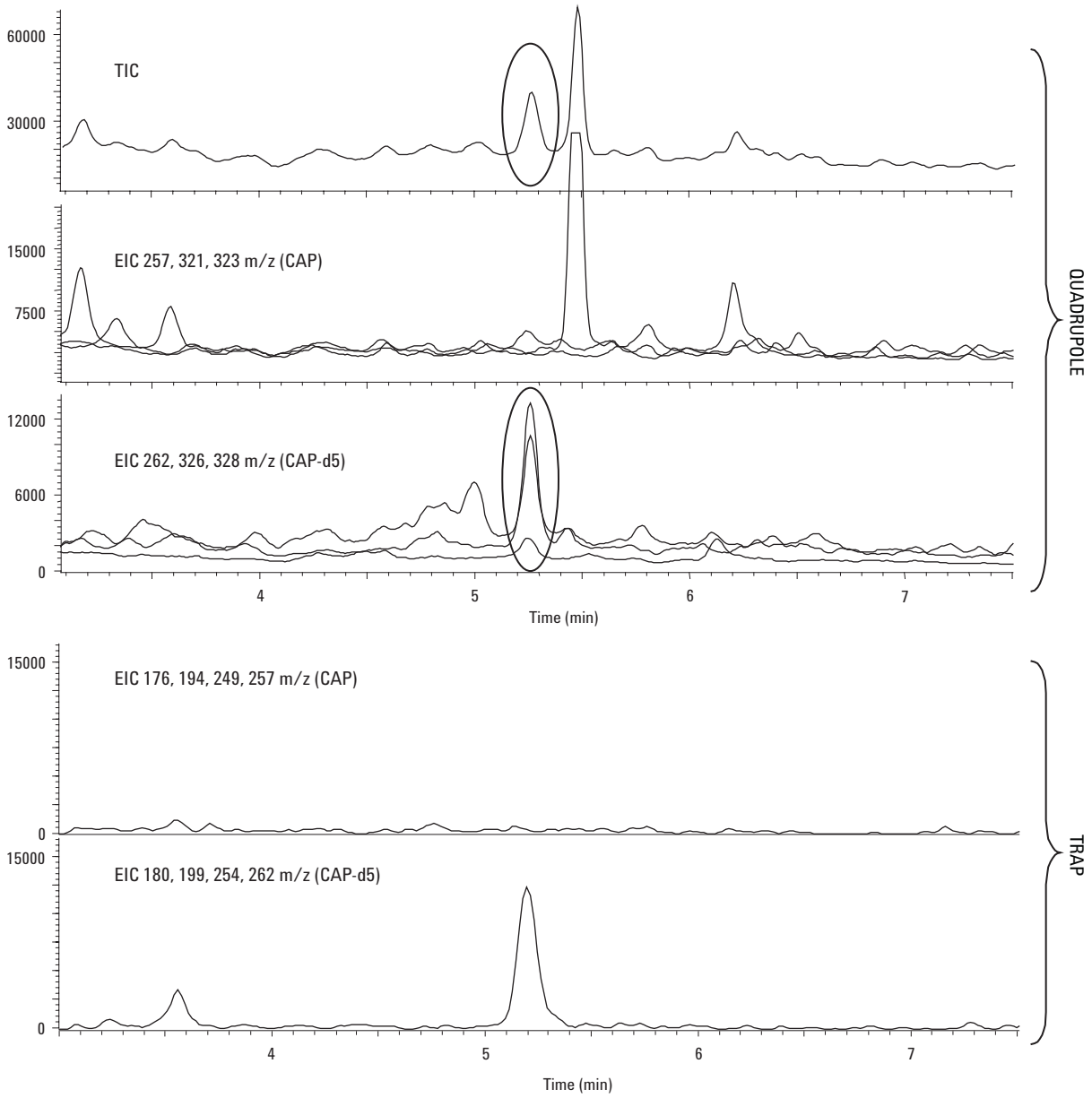


Figure 4. Analysis of a blank honey sample containing 1 ppb CAP-d5.

A calibration curve was constructed with blank honey samples spiked with 0, 0.1, 0.2, 0.5, 1.0, and 2.0 ppb CAP. The samples also contained 1 ppb of the IS. The correlation coefficients were 0.9997 and 0.9998 without and with correction with the IS, respectively. The slope for the calibration curve constructed with these extracts for CAP with correction with the IS was 0.1822. This is in good agreement with the slope obtained with the standard solutions, which is 0.1758 (see Figure 3).

Spectra on the trap were similar for standard solutions and real samples. An example of an MS/MS spectrum of an extract of a honey sample spiked with 0.5 ppb CAP and 1 ppb CAP-d5 is shown in Figure 5. Since the analyte and the IS coelute, a mixed spectrum is obtained. This could be avoided by using a smaller isolation width and the multiple reaction monitoring (MRM) function of the ion trap. Note that the chlorine isotope for $Cl^{35}Cl^{37}$ is not observed for the deuterated internal standard because its precursor ion is at the edge of the isolation width and thus not trapped.

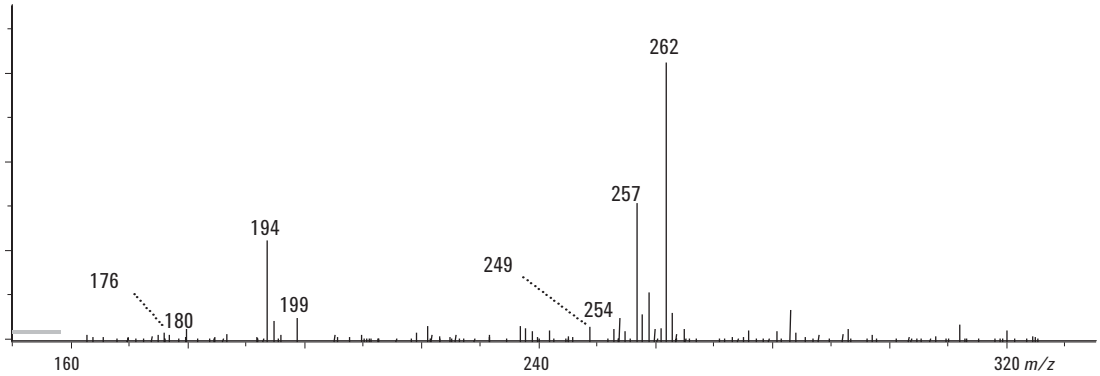


Figure 5. Ion trap MS/MS spectrum from analysis of a honey sample spiked with 0.5 ppb CAP and 1 ppb CAP-d5.

Analysis of Honey

The extraction procedure and LC/MS methods were applied to the analysis of honey samples that were known to contain CAP. Sample results obtained with the quadrupole and trap MSD were compared (Figure 6).

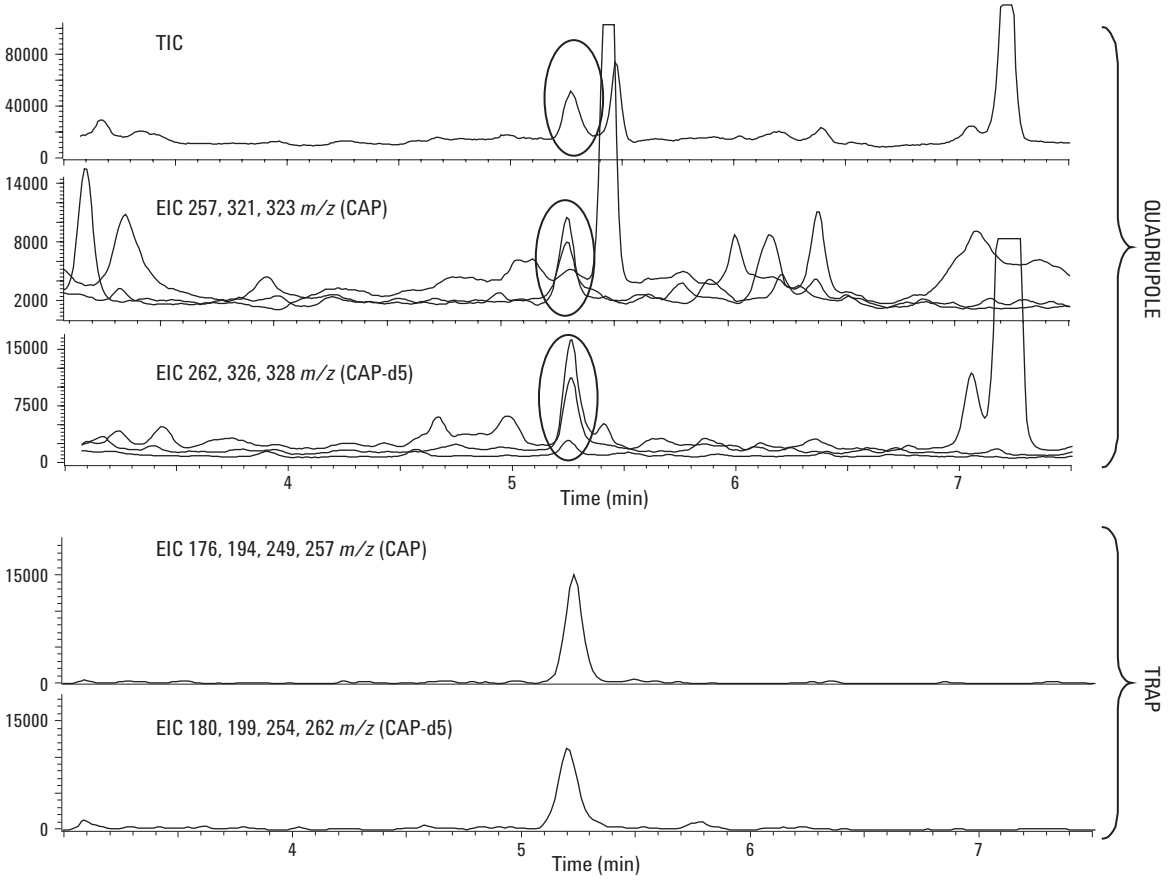


Figure 6. Analysis of a honey sample containing 0.5 ppb CAP and 1 ppb CAP-d5.

The LOD for the honey samples varies between detectors. For the quadrupole, it is found to be 0.5 pg/µL in the analytical solution. This corresponds with 50 pg on-column. Taking into account the sample preparation with a five-fold concentration, samples containing 0.1 ppb CAP can be detected. It is obvious that the sample matrix interferes with the sensitivity (Figures 4 and 6). Due to the increased selectivity using MS/MS in the trap, the LOD with this MS is similar for honey samples as for the standard solutions and is ca. 0.2 pg/µL in the analytical solution. This is equivalent to 0.04 ppb CAP in the sample because of the five-fold concentration step.

Analysis of Shrimp

The same sample preparation method was applied to the analysis of shrimp. The total volume of shrimp and water added was about 40 mL. Taking 20 mL of the 10 g shrimp aliquot for the Isolute sample preparation and reconstituting the dried extract in 1 mL produced a five-fold concentration as with the honey. This sample preparation shows less matrix interference with the analysis compared to honey samples. An example of an analysis of shrimp is shown in Figure 7. Due to the reduced matrix effect, the LOD with the quadrupole is lowered to nearly the same level as for the trap (0.05 ppb in the sample with the five-fold concentration). A concentration of 0.35 ppb was recovered in the shrimp sample by both the quadrupole and the trap MSD. Extraction recovery was approximately 85%.

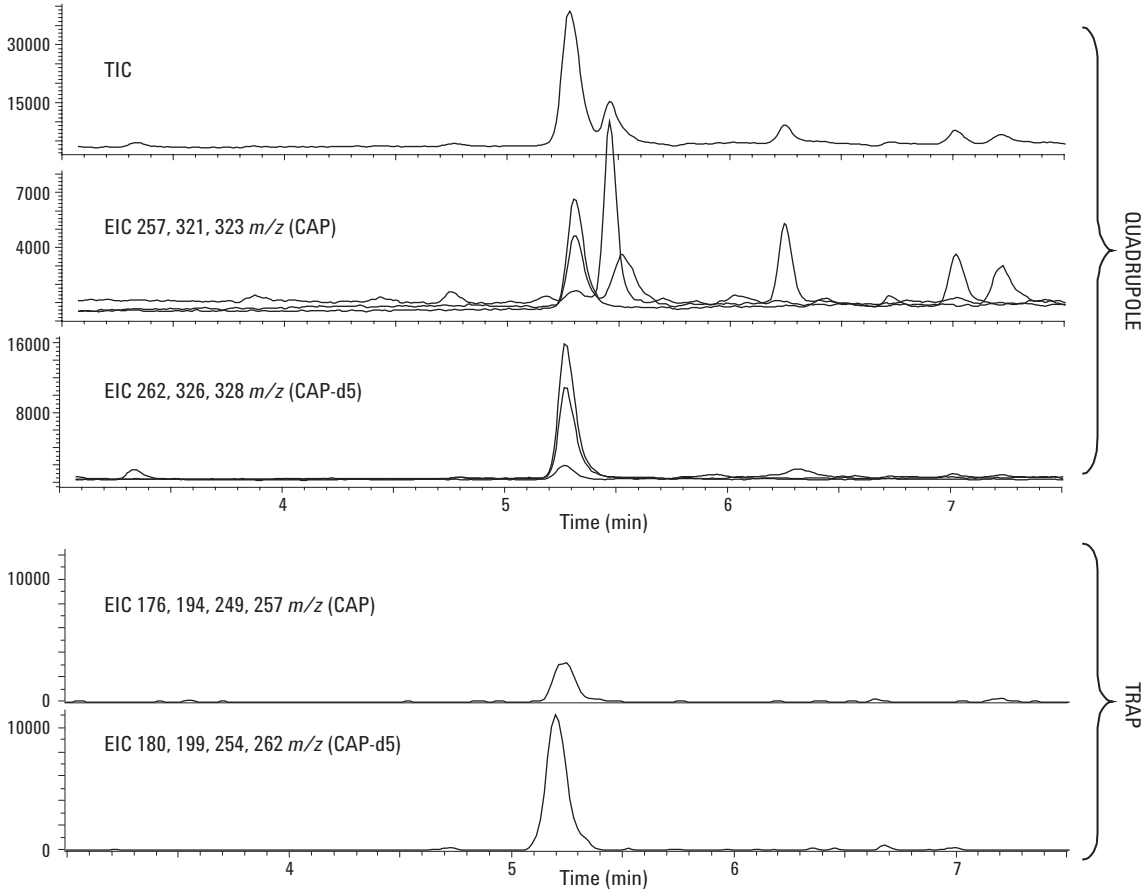


Figure 7. Analysis of a shrimp containing 0.35 ppb CAP and 1 ppb CAP-d5.

Conclusion

Honey and shrimp samples were successfully analyzed for CAP with both the quadrupole and trap MSD. A simple liquid-liquid extraction procedure using ISOLUTE HM-N cartridges was found to perform excellently in view of recovery and repeatability. The LC method used a standard 4.6-mm id column and produced the required sensitivity on both instruments. The LC/MSD quadrupole instrument produced excellent linearity and demonstrated its quantitative ability. The LC/MSD Trap showed the needed sensitivity with excellent full scan capability below the regulated limit in both sample matrices. The use of a broad isolation window for full scan spectra using the ion trap produced more transition ions than required for confirmation.

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Agilent Technologies

A Validated Atmospheric Pressure Chemical Ionization Method for Analyzing Sulfonamides in Pork Muscle

Application

Food

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Abstract

This application note presents a simple method for the analysis of sulfonamide antibiotics in pork muscle. Samples were extracted with acidified methanol, centrifuged, and a portion of the extract was diluted with water. This dilution was analyzed directly by HPLC mass spectrometry using chemical ionization, with all compounds eluting in less than 5 minutes. Using an internal standard, recoveries for seven sulfonamides ranged from 84%–118% at a spiking level of 50 ppb (ng/g). The statistically derived detection limit was 10–25 ppb. A comparison was made to the cleaned extracts using solid phase extraction, as well as a comparison of mass selective detector settings for both screening (maximum sensitivity) and confirmation (greater fragmentation). The enhanced sensitivity of the Agilent quadrupole mass selective detector allows this dilution cleanup technique to be used in labs where high throughput is required.

Introduction

Meat, edible organs, animal feed, and animal waste may contain antibiotics, growth hormones, and other chemicals that can enter the food supply. These compounds are added to maintain animal health, to increase animal growth rate, and to reduce stress. Human exposure can result from eating contaminated meat, or contacting runoff and leaching from manure and compost. Health specialists warn that there may be reduced options for effectively treating disease with antibiotics, such as penicillin and sulfa drugs, since antibiotic-resistant strains of bacteria may develop from the low-level exposure.

Sulfonamides are broad-spectrum antimicrobials used in both humans and animals. The maximum residue limit (MRL) in Canada for sulfonamides in meat is 100 ppb (ng/g), and 10 ppb in milk, while the MRL in the European Union is 100 ppb for both of these matrices. The Canadian Food Inspection Agency method for sulfonamides in meat tissue calls for extraction in ethyl acetate, partitioning with glycine buffer, followed by a pH-adjusted back extraction into methylene chloride [1]. Extracts are evaporated, reconstituted, then separated by thin layer chromatography (TLC), derivatized, and quantitated by densitometry. Alberta Agriculture has improved the quantitative and qualitative aspects by using liquid chromatography/mass spectrometry (LC/MS) with atmospheric pressure chemical ionization (APCI) for the final analysis [2]. There are a number of

extraction steps in the Alberta method, and a faster method would greatly benefit laboratories monitoring the food supply for residues.

The goal of this method was to reliably quantitate the sulfa drugs at one-half of the regulatory limit or lower, with minimal sample preparation, and a maximum injection cycle time of 10 minutes. Maximum sensitivity is generally obtained by forming as many parent ions $[M+H]^+$ as possible and minimizing fragmentation. Due to the operational complexity of triple quadrupole instruments, it is also desirable to confirm positive findings on a single quadrupole. This could be achieved by using collision induced dissociation (CID) to enhance fragment ions characteristic of the compounds.

Experimental

Chemicals and Materials

All sulfonamide standards were purchased from Sigma Aldrich Canada, with a minimum purity of 99%. Stock solutions were prepared at 2 mg/mL in acetone, with the exceptions of sulfadiazine and the sodium salt of sulfaquinoxaline. Three mL of 0.2N NaOH was added in order to completely dissolve these compounds. Standard solutions at different concentrations were prepared for spiking and quantitation by diluting with de-ionized water.

Internal standard (IS): sulfachloropyridazine (SCPD) at 2 mg/mL in de-ionized water.

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid (min. 98%), was purchased from EM Science.

Acidified methanol was prepared by adding about 100 µL of 98% formic acid to 100-mL methanol.

Ultra-Turrax T8 homogenizer with 8-mm diameter dispersing element, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

Oasis HLB (3 cc, 60 mg) solid phase extraction (SPE) cartridges were purchased from Waters.

Sample Preparation

1. For pork muscle, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. The samples were homogenized for 3 minutes with 10 mL acidified methanol using the Ultra-Turrax homogenizer.
3. The samples were then centrifuged for 10 minutes, and the supernatant decanted into a clean test tube.
4. The samples were then re-extracted with a further 10 mL acidified methanol, and centrifuged again.
5. The supernatants were combined, and 1 mL IS (2 mg) was added to the combined extract.
6. The extract was diluted with de-ionized water 1 in 4 (250 µL extract + 750 µL water), filtered through a 0.2 µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the combined extracts, there is no need to measure the final volume of the extract. The IS calculations performed by the ChemStation measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Sample extracts were also taken through SPE cleanup cartridges in order to compare with the dilution-only extracts. The 60-mg Oasis HLB cartridges are prewashed by eluting 1.5 mL acidified methanol, followed by 1.5 mL de-ionized water. The 1 mL extract was diluted to 10 mL with de-ionized water, eluted through the cartridges, and the eluant was discarded. The sulfa drugs were then eluted with 1.5 mL acidified methanol. This eluant was evaporated to near dryness under nitrogen. Samples were reconstituted in 1 mL of 25% methanol in water, filtered, and analyzed by LC/MS.

A further comparison was done by evaporating 1 mL methanol extract to near dryness, and reconstituting it in 1 mL of 25% methanol in water without the SPE cleanup. This gave the sample extract the proper solvent composition for HPLC analysis, but without the dilution step to negatively affect the detection limits (DL) of the compounds.

LC/MS Conditions

The LC/MS system was made up of Agilent Technologies 1100 Series solvent degasser, binary pump, autosampler, column oven, diode array detector, and quadrupole mass selective detector (MSD) (Table 1).

Compound Identification and Confirmation

In general, the goal of a monitoring method for target analytes is to separate the compounds from potential interferences and maximize sensitivity on the instrument. Using mass spectrometry (MS), maximum sensitivity is achieved by the production of a single ion, for example, the protonated parent ion $[M+H]^+$ in LC electrospray ionization (ESI) or APCI in target ion mode. However, once a positive is detected, a confirmation must be made as to whether the suspect peak is actually the target analyte, or simply a co-eluting compound that produces the same ion. There are a number of ways to perform the confirmation: re-extract the sample with a different solvent system; further clean up the sample to a higher final concentration, to allow detection of additional confirmation ions or analysis in scan mode; derivatize and analyze by gas chromatography/mass spectrometry (GC/MS); or re-analyze the extract on a triple quadrupole LC/MS/MS. All of these techniques are useful, but the drawback is the additional time and expense involved, especially with LC/MS/MS.

Table 1. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (p/n 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_3 = 90% B $t_{6.5}$ = 90% B Post time = 1.5 min
Flow rate	1.0 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	APCI (positive ion mode)
Ion dwell time	8 Ions at 63 ms each
Fragmentor	70 V
Drying gas	6.0 L/min
Nebulizer pressure	60 psi
Drying gas temperature	350 °C
Vaporizer temperature	400 °C
Capillary voltage	3000 V
Corona current	4 µA

The Agilent 1100 MSD has the capability of acquiring up to four separate MS signals during the same run, where each signal can be made up of a number of selected ions (SIM) or a full scan spectrum. For example, Signal 1, with a low fragmentor voltage to maximize parent ion response, can include each of the $[M+H]^+$ ions in the target list, while Signal 2, at higher fragmentor voltages can acquire the confirmatory fragment ions. For analytes expected at higher concentrations, Signal 1 could acquire in SIM mode for quantitation, while Signal 2 could be set for scan mode for identification. Figure 1 demonstrates the former example, with the Fragmentor set to 70 V for Signal 1 (MSD1), and 200 V for Signal 2 (MSD2).

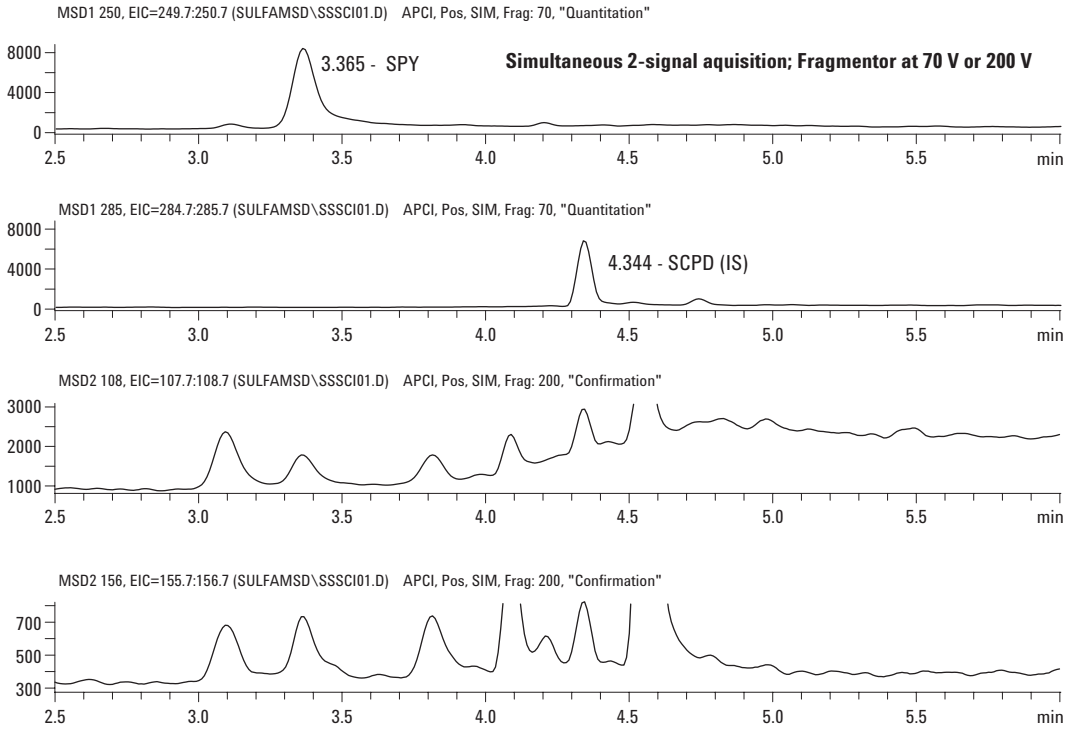


Figure 1. Dual MSD acquisition signals (Masses 108 and 156 are class-specific fragments for sulfonamides).

Table 2 shows the mass spectra for the sulfonamides using various fragmentor voltages. Masses 108 and 156 are class-specific fragments for sulfonamides ($H_2N^+=[C_6H_4]=O$ and $H_2N^+=[C_6H_4]=SO_2$, respectively), and, as such, are very useful diagnostic ions, when acquired along with the protonated molecular ion.

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages

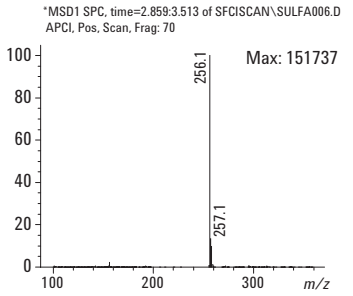
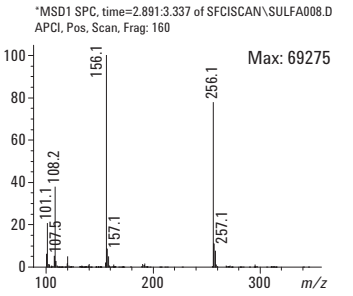
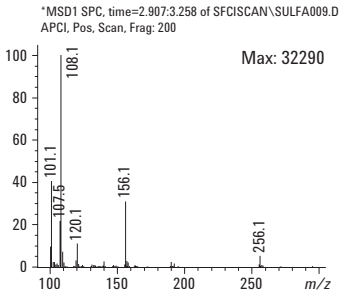
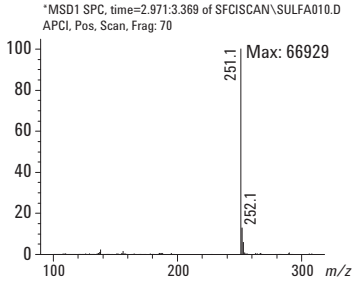
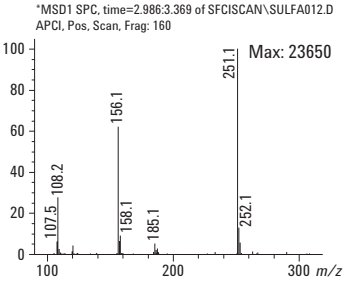
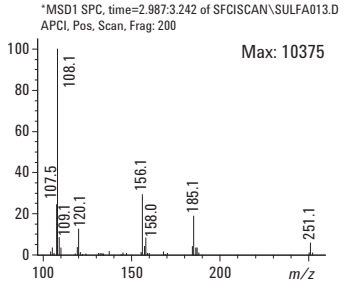
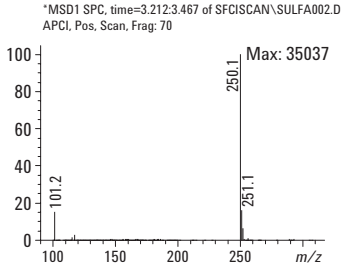
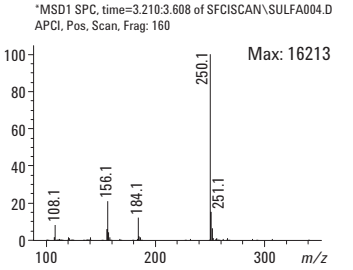
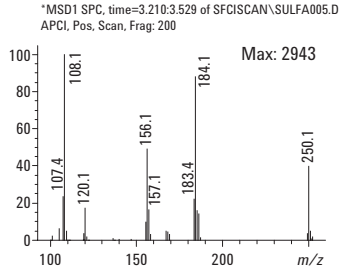
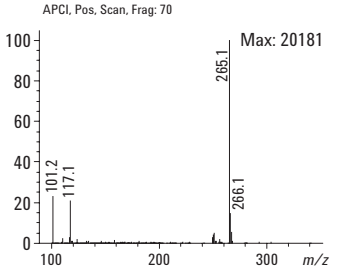
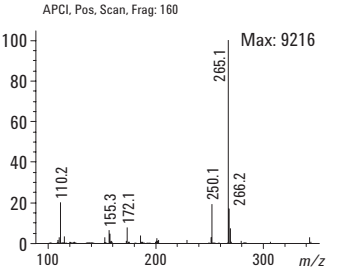
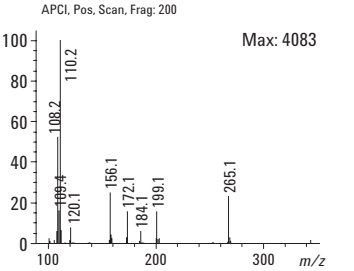
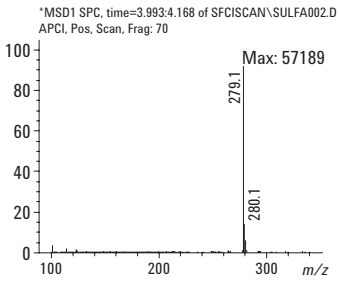
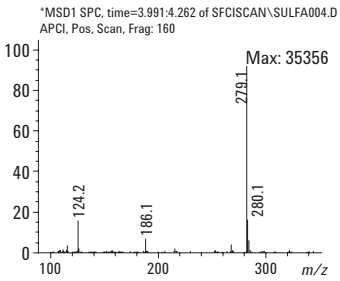
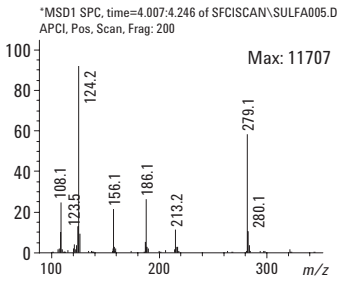
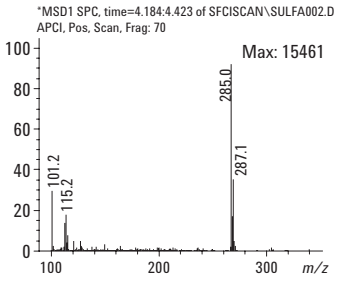
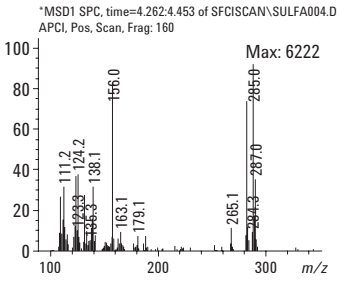
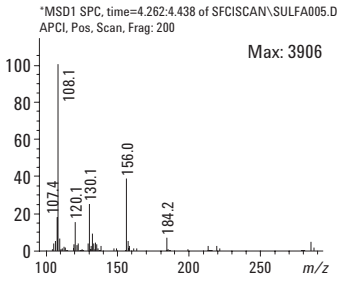
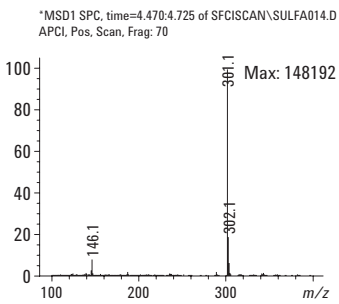
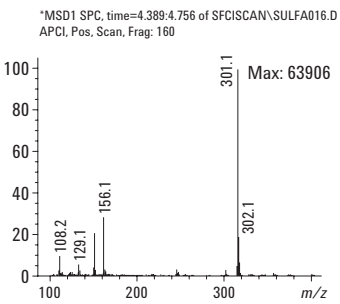
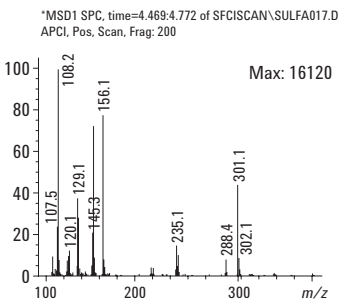
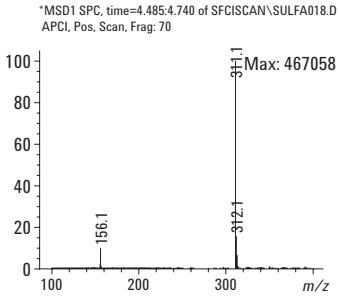
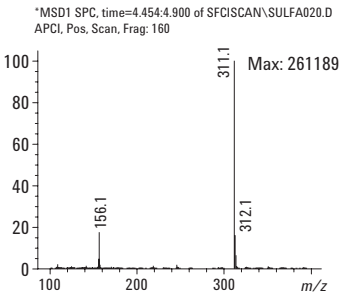
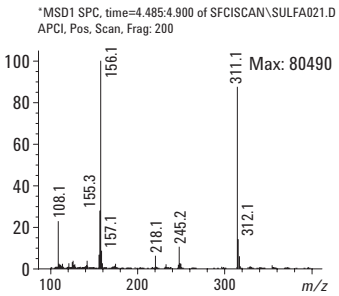
Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfathiazole (STZ) $C_9H_9N_3S_2O_2$ MW = 255 RT = 3.05 min			
Sulfadiazine (SDZ) $C_{10}H_{10}N_4SO_2$ MW = 250 RT = 3.09 min			
Sulfapyridine (SPY) $C_{11}H_{11}N_3SO_2$ MW = 249 RT = 3.33 min			
Sulfamerazine (SMR) $C_{11}H_{12}N_4SO_2$ MW = 264 RT = 3.78 min			

Table 2. APCI Spectra of Sulfonamides, Using Various Fragmentor Voltages (Continued)

Compound	Fragmentor 70 V	Fragmentor 160 V	Fragmentor 200 V
Sulfamethazine (SMZ) $C_{12}H_{14}N_4SO_2$ MW = 278 RT = 4.06 min			
Sulfachloropyridazine (SCPD); istd $C_{10}H_9N_4SO_2Cl$ MW = 284 RT = 4.31 min			
Sulfaquinoxaline (SQ) $C_{14}H_{12}N_4SO_2$ MW = 300 RT = 4.51 min			
Sulfadimethoxine (SDMX) $C_{14}H_{12}N_4SO_2$ MW = 310 RT = 4.54 min			

Chromatography

While complete separation of target compounds is not always necessary when using mass spectral detection, it is, however, essential when common ions are present. For example, the protonated molecular ion of SPY is 250 mass units. Due to the naturally-occurring C^{13} isotope, ions 251 coexist with the parent ions 250. Separating SPY from SDZ (m/z = 251) was, therefore, important when trying to optimize the chromatographic conditions, and was achieved as shown in Figure 2. While this results in a slightly longer chromatographic run than would otherwise be necessary, there is more consistent integration of the peaks during data analysis; the chromatogram is easier to interpret; and the amount of SDZ is not underestimated due to co-elution of SPY in the standard mix.

A recently published application shows four sulfonamides were analyzed with an injection cycle time of 1.1 minutes, using a 2-position 10-port valve, two analytical columns in parallel, and a second binary pump [3]. Since most labs do not have such high sample volume requirements, the method described in this application note was developed using more conventional techniques, without the additional hardware costs. Conditions were set up to provide good chromatographic separation in a relatively short time of 6 minutes (total cycle time was 10 minutes).

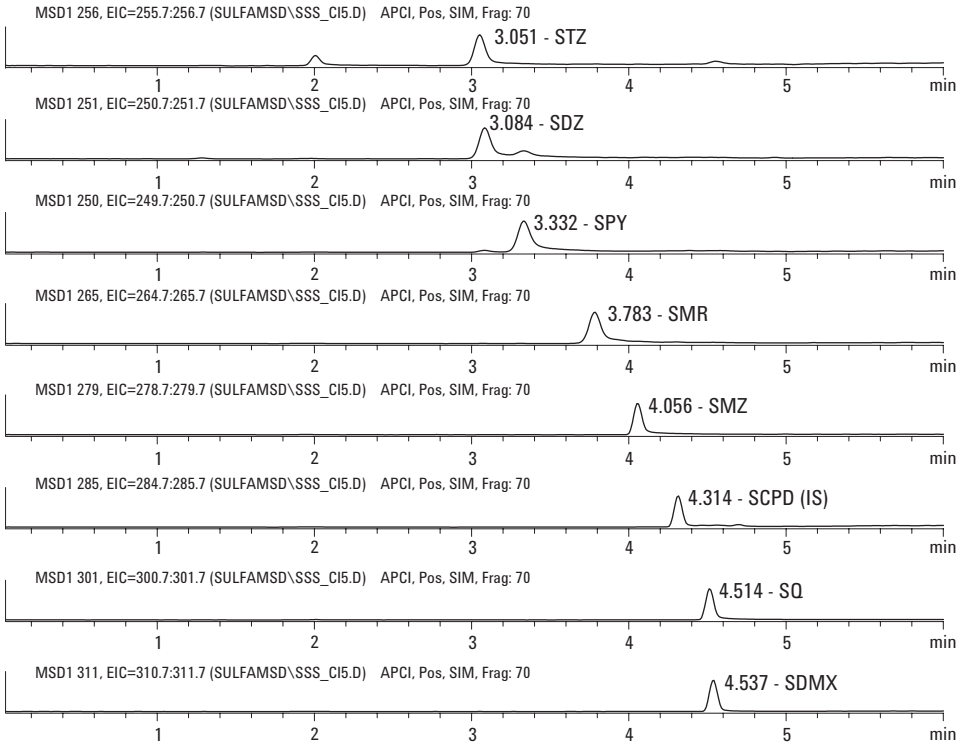


Figure 2. Sulfonamide standard mix, 500 pg each (SIM).

Sample Cleanup

The total ion chromatograms (TIC) in Figure 3 show that there is considerable matrix background from the samples. A simple solvent exchange was performed, where 1 mL of extract was evaporated under nitrogen, and reconstituted in 25% methanol in water. One of the problems with solvent exchange only is the amount of matrix material that is injected onto the HPLC column. Peak shape can be negatively affected by overloading, and eventually the performance of the column will deteriorate. All of this matrix material is also introduced into the MSD. Frequent cleaning and maintenance may be required for the MSD, further reducing productivity.

In order to develop a high-throughput method, keep the number of required steps to a minimum. The Agilent liquid chromatography/mass selective detector (LC/MSD) has enough sensitivity to allow simple dilution of the extracts with water to act as a cleanup technique. This eliminates the need for costly SPE cartridges and analyst time to further prepare the samples. Minimal sample handling can also improve recoveries, since losses are possible at each step.

The third chromatogram in Figure 3 shows how the use of SPE cleanup techniques can remove the majority of co-extracted materials, allowing for a more concentrated final extract and ultimately lower DLs. This also results in a simpler chromatogram for integration and interpretation.

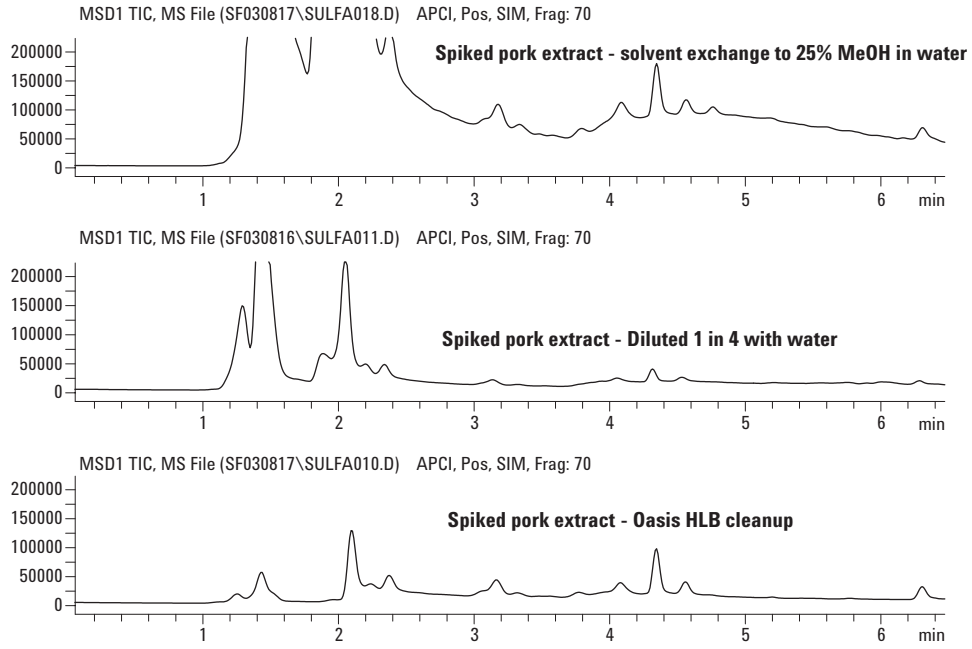


Figure 3. TIC comparisons of various cleanup techniques.

However, where the goal of a method is to screen large numbers of samples to find potential violations of MRLs, a simple dilution technique may be preferred. Dilution could offer enough cleanup for good chromatographic separation, while remaining concentrated enough to meet DL requirements. The second chromatogram in Figure 3 shows a much improved baseline. Figures 4 through 6 show the same analyses with all the target ions in SIM mode.

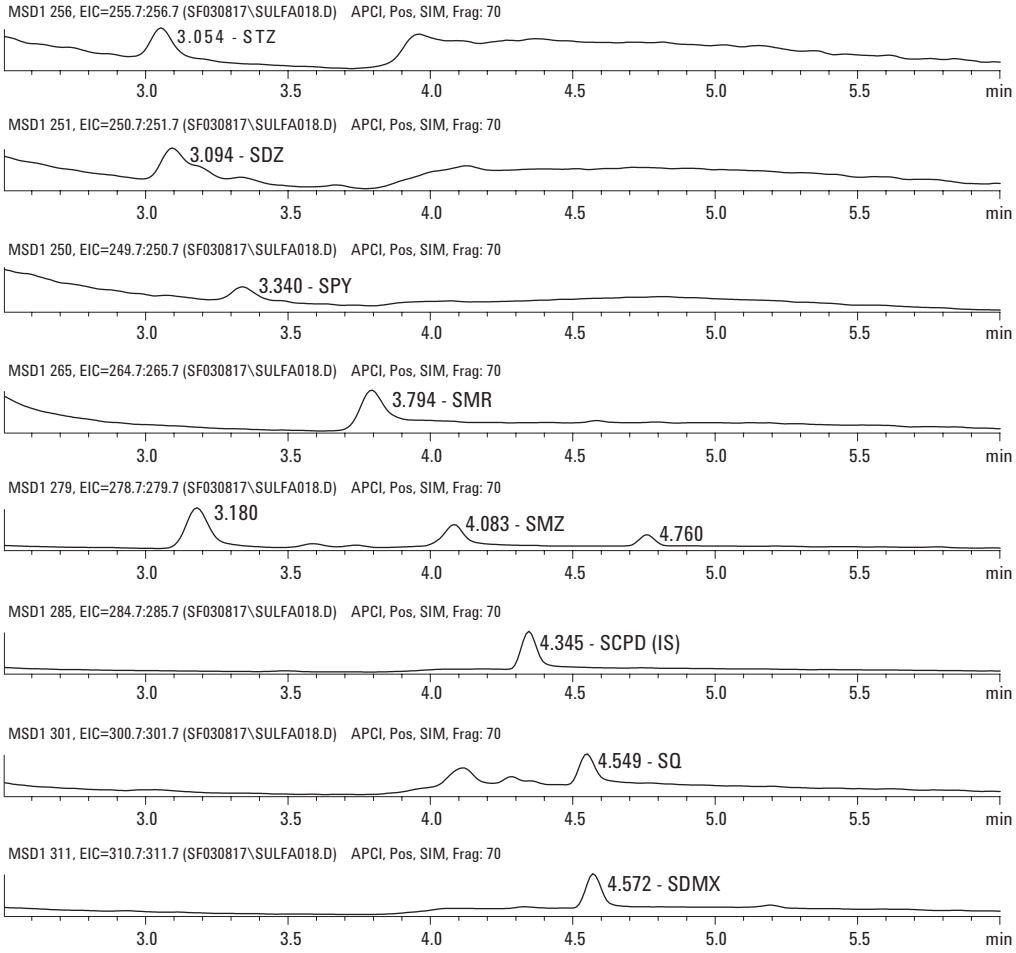


Figure 4. Solvent exchange only (SIM)

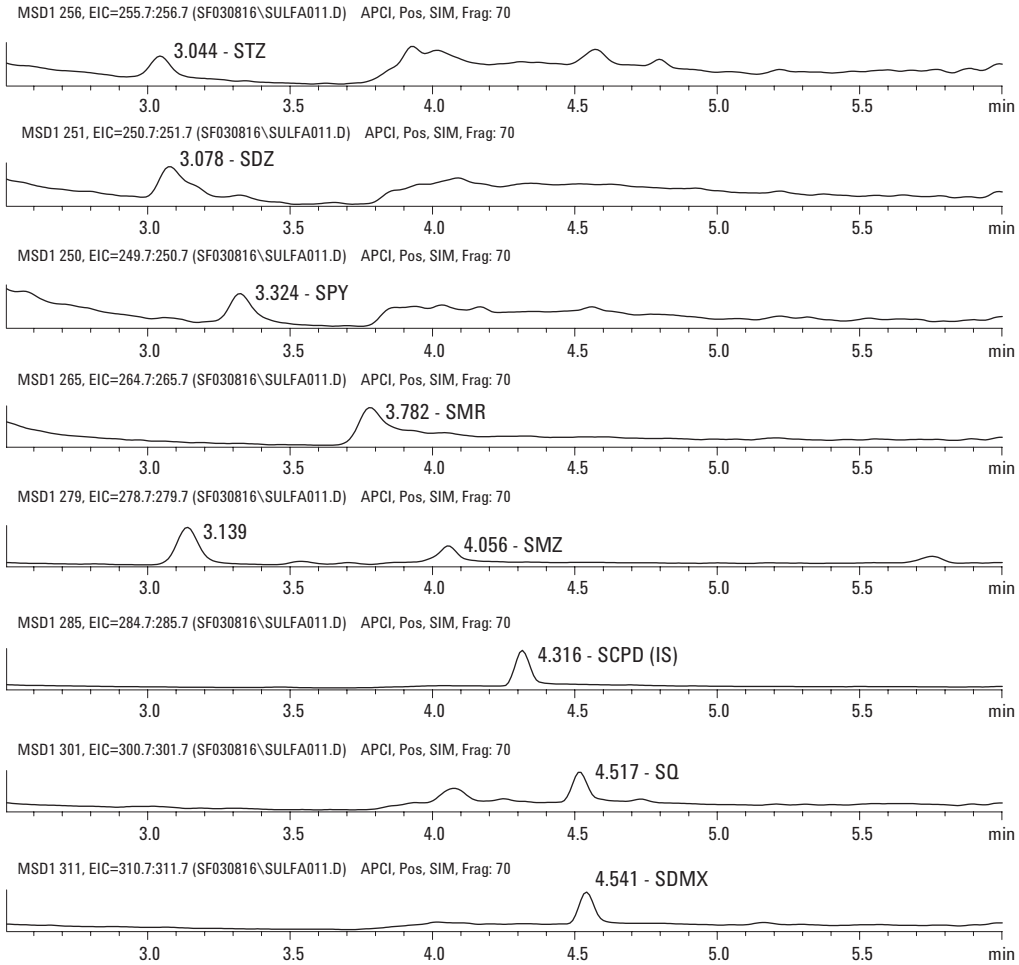


Figure 5. Diluted 1 in 4 with water.

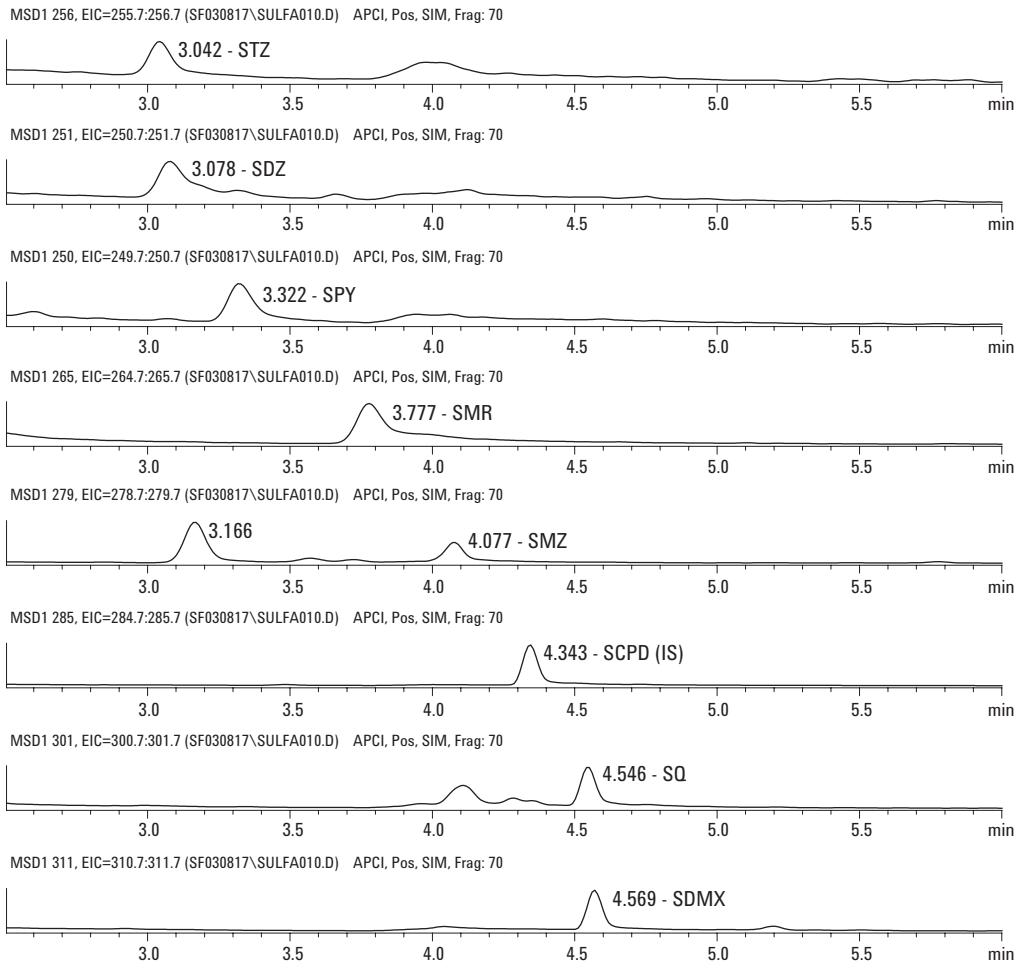


Figure 6. After HLB cleanup (SIM).

Results and Discussion

The recoveries obtained for seven samples spiked at a level of 50 ppb (150 ng of each sulfonamide in 3 g sample) appear in the following tables. The spiking solutions were added before homogenization and allowed to stand for at least 30 minutes before extraction. SMR (sulfamerazine) was added separately at 300 ng per sample before homogenization, and could be used as a surrogate. Results in Table 3 were obtained by simply diluting the extracts 4-fold with water (recovery 84%–118%), while results in Table 4 are from extracts taken through SPE cleanup (recovery 79%–104%).

In both cases, a five-point IS calibration with SCPD was used, with 20 to 200 pg of each target compound injected, plus 2,000 pg SCPD. The five standards were injected both before and after the set of seven spikes, and the curves were created by using the average responses of the two sets of standards. Peak height was used to measure response, as there was less variability compared to peak area, due to the noticeable tailing of these compounds. The linearity results (R^2) are tabulated in Tables 3 and 4.

Table 3. Recoveries of Sulfonamides by Diluting Extracts 1 in 4 with Water

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	167	172	164	317	151	2,000	148	130
Pork spike 2	168	197	68	343	164	2,000	169	137
Pork spike 3	160	183	158	315	157	2,000	133	121
Pork spike 4	158	189	167	336	156	2,000	138	129
Pork spike 5	151	169	154	295	169	2,000	133	129
Pork spike 6	147	161	144	322	143	2,000	120	112
Pork spike 7	144	72	141	272	151	2,000	124	125
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	156	178	157	314	156	2,000	138	126
SD (Precision)	9	13	11	24	9	–	17	8
MDL (SD × t-stat) ng	29	40	34	77	28	–	53	26
LOQ (SD × 10) ng	94	126	108	245	88	–	167	82
RSD (SD × 100/Mean)	6	7	7	8	6	–	12	7
Accuracy (%)	104	118	104	105	104	100	92	84
Linearity (R^2)	0.9997	0.9996	0.9997	0.9972	0.9996	1.0000	0.9984	0.9992
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 4. Recoveries of Sulfonamides Using Oasis HLB Cleanup Cartridges

	Amount recovered (ng)							
Description	**STZ**	**SDZ**	**SPY**	**SMR**	**SMZ**	**SCPD(IS)**	**SQ**	**SDMX**
Pork spike 1	161	157	132	273	149	2,000	139	126
Pork spike 2	154	156	132	293	157	2,000	153	131
Pork spike 3	149	158	124	267	155	2,000	132	113
Pork spike 4	145	152	122	279	144	2,000	119	111
Pork spike 5	151	162	127	294	149	2,000	127	121
Pork spike 6	136	147	127	274	136	2,000	116	108
Pork spike 7	148	161	128	275	155	2,000	124	116
Amount spiked (ng)	150	150	150	300	150	2,000	150	150
Mean	149	156	127	279	149	2,000	130	118
SD (Precision)	8	5	4	10	7	–	13	8
MDL (SD × t-stat) ng	24	17	11	33	23	–	40	26
LOQ (SD × 10) ng	76	53	36	104	73	–	128	82
RSD (SD × 100/Mean)	5	3	3	4	5	–	10	7
Accuracy (%)	99	104	85	93	100	100	87	79
Linearity (R^2)	0.9994	0.9994	0.9997	0.9979	0.9998	1.0000	0.9989	0.9989
t-stat (N=7)	3.14	3.14	3.14	3.14	3.14	3.14	3.14	3.14

Table 5 summarizes the comparison of recoveries when diluted with water versus using Oasis HLB cartridge cleanup. Generally there is a greater difference in recoveries for the early eluting compounds, as one might expect. Since the samples are loaded onto the cartridge with a mostly aqueous phase (10% methanol in water), the water-soluble matrix components would tend to pass through the cartridge to waste. Because these early eluting compounds were removed prior to injection on the HPLC column, the chromatograms are cleaner with more reproducible chromatography, as shown by the smaller standard deviations in recoveries. The results from the HLB cleanup exhibited smaller standard deviations and lower minimum detection levels (MDLs).

Conclusion

A fast and sensitive single quadrupole LC/APCI/MS method was developed and validated for detection of sulfonamide residues in pork. The DL ranged from 10 to 25 ng/g of tissue when analyzed by simple dilution of the extracts, and 4 to 13 ng/g when SPE cleanup is used. Instrumental conditions allow injection cycle-time of 10 minutes using typical columns and conditions for most labs.

References

1. TLC-Densitometric Procedure for Sulfonamide Residues in Animal Tissue, SUL-SP08, Canadian Food Inspection Agency, Saskatoon, Saskatchewan, Canada; 2001/04.
2. Sulfonamides in Tissue by LC/MS, Alberta Agriculture, Edmonton, Alberta, Canada, Standard Operating Procedure TX-0278-01.
3. Mark Stahl, “High-throughput analysis with the Agilent 1100 Series high-throughput LC/MS system”, Agilent Technologies, publication 5988-9638EN. www.agilent.com/chem

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Table 5. Comparison of Recoveries Obtained by Dilution vs Oasis HLB Cleanup

Description	STZ	SDZ	SPY	SMR	SMZ	SCPD(IS)	SQ	SDMX
Accuracy % (1 in 4 dilution)	104	118	104	105	104	100	92	84
SD (Precision)	9.4	12.6	10.8	24.5	8.8	–	16.7	8.2
MDL (ng)	29	40	34	77	28	–	53	26
Accuracy % (HLB cleanup)	99	104	85	93	100	100	87	79
SD (Precision)	7.6	5.3	3.6	10.4	7.3	–	12.8	8.2
MDL (ng)	24	17	11	33	23	–	40	26

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The Analysis of Fluoroquinolones in Beef Kidney Using HPLC Electrospray Mass Spectrometry

Application

Food

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Abstract

A fast and simple screening method was validated for the analysis of three fluoroquinolone antibiotics in beef kidney. Samples were extracted with acidified methanol, centrifuged, diluted with water, and filtered. The diluted extract was analyzed directly by HPLC mass spectrometry using electrospray ionization in positive ion mode. Using an internal standard, mean recoveries were 73%–96% at spiking levels of 33 µg/kg (ppb), with statistically derived detection limits of 8–19 µg/kg. This is below the European Union maximum residue limit of 200 µg/kg for enrofloxacin and ciprofloxacin in bovine kidney. The method is evaluated relative to the requirements of the European Commission Decision 2002/657/EC for use as a confirmatory method.

Introduction

Fluoroquinolones are synthetic antibacterial compounds derived from nalidixic acid, and are useful to treat animal infections that are resistant to other antibacterial agents. They have a broad spectrum of activity, acting against both gram-positive and gram-negative bacteria. The maximum residue limit (MRL) for enrofloxacin (as the sum of enrofloxacin and ciprofloxacin) was entered into Annex 1 of Council Regulation (EEC) No. 2377/90 for kidney at 200 µg/kg in bovine and ovine species, and 300 µg/kg for porcine, poultry, and rabbits. For all other food producing species, the MRL is 200 µg/kg in kidney [1].

There are a number of methods describing the analysis of fluoroquinolones in various tissues, with HPLC coupled with fluorescence and mass spectrometric detection being very popular. Most methods involve extraction into acidic or basic organic solvents, followed by some type of cleanup, most notably solid phase extraction (SPE). The Canadian Food Inspection Agency extracts animal tissue with acidic ethanol, followed by strong cation exchange SPE cleanup, and HPLC fluorescence analysis [2]. Chen and Schneider [3] described a screening method for enrofloxacin in chicken, where extracts were detected by fluorescence without cleanup, following extraction and centrifugation.

European Community Commission Decision 2002/657/EC allows the use of HPLC coupled with fluorescence detection [4] for substances in Group B of Annex I to Directive 96/23/EC. Quinolones and other veterinary drugs fall into Group B, where three identification points are required for confirmation by Selected Ion Monitoring (SIM) using mass spectrometry (MS). With low resolution HPLC/MS, one point can be earned for each ion detected, provided that the ion ratios meet relative intensity criteria. Additional requirements of Directive 2002/657/EC, based on spiking levels of 33 µg/kg carried out in this study, are as follows:

- The internal standard (IS) shall be added to the test portion at the beginning of the extraction procedure.
- In order to allow the use of data corrected for mean recovery, the range of recoveries allowed are –20% to +10%.
- The reproducibility of coefficient variation (CV) (%) is expected to be about one-half to two-thirds of the 100 µg/kg CV, which is 23%, at a concentration of half the permitted limit.
- For liquid chromatography/mass spectrometry (LC/MS) procedures, the minimum acceptable retention time (RT) for the analyte under examination is twice the RT corresponding to the void volume of the column.
- The ratio of the chromatographic RT of the analyte to that of the IS, that is, the relative RT of the analyte, shall correspond to that of the calibration solution at a tolerance of 2.5% for LC.
- The molecular ion shall preferably be one of the selected diagnostic ions.
- The maximum permitted tolerances for relative ion intensities shall meet the criteria in the Annex, (in this case, either ±25% or 30%), as reproduced in Table 6.

Experimental

Chemicals and Materials

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid, min. 98%, was purchased from EM Science.

Acidified methanol solution: 30% methanol in pH 3 deionized water (100 µL of formic acid per 100 mL of water).

Acidified methanol was prepared by adding 100 µL of 98% formic acid to 100 mL of methanol.

Acidified deionized water was prepared by adding 100 µL of 98% formic acid to 100 mL deionized water.

Ultra-Turrax T25 homogenizer, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

All fluoroquinolones, including the IS, were provided as a gift from the Canadian Food Inspection Agency, Calgary, Alberta, Canada, as stock solutions of 100 ng/µL (ppm) in 1% acetic acid in methanol. Solutions were stored at 4 °C. Standard solutions at different concentrations were prepared for spiking by dilution with acidified methanol solution. The analytes ciprofloxacin, enrofloxacin, and sarafloxacin were chosen as targets since these compounds are included in the Canadian Food Inspection Agency's proficiency check samples. The spiking standard for these compounds (1 ng/µL) was prepared by diluting 100 µL of each the stock solutions to a 10-mL volumetric flask, and made to volume with acidified deionized water. A separate IS solution at 1 ng/µL was prepared the same way, except that it only contained norfloxacin and danofloxacin.

Sample Preparation

1. For beef kidney, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. For spiked samples, 100 µL of the 1-ng/µL (100 ng) spiking solution was added, resulting in fortification levels of 33 µg/kg. Samples were allowed to stand for 1 hour before subsequent extraction.
3. For the sample blank, 100 µL of acidified methanol solution was added.
4. For all spiked samples, 100 µL of the 1-ng/µL (100 ng) IS solution was added just prior to extraction. Norfloxacin was included in this solution at the same level, to be used as an alternate IS, if required due to potential interferences for danofloxacin.
5. The samples were homogenized for 2 min with 15 mL of acidified methanol using the Ultra-Turrax homogenizer.
6. The samples were then centrifuged for 10 min, and the supernatant decanted into a clean test tube.

7. The extract was diluted with acidified deionized water 1 in 4 (250 µL of extract + 750 µL of water), filtered through a 0.2-µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the initial sample before extraction, there is no need to measure the final volume of the extracts, nor the aliquot to be diluted. The IS calculations, performed by the ChemStation, measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Standard Preparation

A 5-point calibration curve was used for the determination of each of the three target compounds, and a 1-point curve was used for norfloxacin, the alternate IS. Table 1 gives the volumes of the IS and target solutions added (1 ng/µL each) to each of five test tubes. The standards were prepared by adding 250 µL of the blank extract and 750 µL of acidified deionized water to the tubes containing the analytes, after which the solutions were filtered through 0.2-µm PVDF filters.

The final solution of each standard contained 5 ng of IS per mL of diluted extract, or 5 pg/µL. With 50 µL injected, this results in 250 pg injected. The amount of target analyte in each of the five solutions varies to produce the calibration curves, as shown in Table 1.

The correlation coefficient (R^2) for the target analytes ranged from 0.9987 to 0.9992, as shown in Table 4.

Preparation of the standards in this fashion will compensate for any ion suppression or enhancement that may occur, due to the presence of co-eluting material at the MS source, which may not otherwise occur if pure solvents alone are used.

Table 1. Preparation of Analytical Standards (50-µL Injections into LC/MSD)

Standard	IS Volume added (µL)	Target volume added (µL)	IS Amount injected (pg)	Target amount injected (pg)
1	5	1	250	50
2	5	2	250	100
3	5	5	250	250
4	5	10	250	500
5	5	20	250	1,000

LC/MS Conditions

The HPLC system was made up of an Agilent Technologies 1100 series solvent degasser, binary pump, autosampler, column oven, diode array detector (DAD), and quadrupole mass selective detector (MSD) (Table 2).

Table 2. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (P/N 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_8 = 90% B t_{15} = 90% B Post time = 2.0 min
Flow rate	0.4 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	Electrospray Ionization (ESI) (positive ion mode)
Ion dwell time	14 ions at 40 ms each
Fragmentation	Varies by ion, see Table 3
Drying gas flow	12 L/min
Nebulizer pressure	30 psi
Drying gas temperature	350 °C
Capillary voltage	4000 V

Table 3. Fragmentor Voltages for Acquired Ions in SIM (single acquisition group)

Compound	Ion	Fragmentor (V)
Norfloxacin (IS)	320	120
	302	200
	276	200
Ciprofloxacin	332	120
	314	200
	288	200
Danofloxacin (IS)	358	120
	340	220
Enrofloxacin	360	120
	342	220
	316	220
Sarafloxacin	386	120
	368	220
	342	220

All ions were included in a single acquisition group, which started at injection (time = 0). An alternative approach would be to set the group start time to a value around half a minute before the elution of the first compound, as this will keep the eluant stream diverted to waste as long as possible. This will reduce the amount of co-extracted material being introduced into the source, reducing contamination.

Another alternative is to add an additional time-programmed acquisition group to the method, and only include the ions for compounds eluting within the group times. This will take on more significance as the overall number of compounds in a method increases, and with three ions per compound required for identity confirmation.

Fragmentor voltages were chosen that maximized the response for each selected ion. For each fluoroquinolone, a value of 120 V produced only the protonated parent ion, while higher voltages were required to induce fragmentation to confirmatory ions. The ions monitored corresponded to the neutral losses of water and carbon dioxide in each case.

Note that although mass 342 is acquired for both enrofloxacin and sarafloxacin, it is only added to the MSD acquisition table once.

Chromatography

All compounds eluted between 5 and 9 minutes, however the total run time was set to 15 minutes with 90% organic solvent to allow co-extractives to elute from the column. Otherwise, their eventual elution could interfere with subsequent injections. This is more of a potential problem when methods with abbreviated cleanups, such as dilution-only, are used. The following figures compare the blank beef kidney sample to a sample fortified at 33 µg/kg. In each case, the selected ions are the protonated forms of the parent ion, as well as the protonated ions resulting from the loss of H_2O (M-18) and CO_2 (M-44).

The qualifier ion for danofloxacin, the compound used as the IS for this study, is mass 340. The matrix causes an interference at mass 340. The interference is shown as a small peak in the beef kidney blank as shown in Figure 1. Since a diagnostic qualifier ion is not required for the IS calculations, it had no impact on the results. It does, however, indicate that there is elution of co-extractive material in the samples, and that without further cleanup, ion suppression may result from its presence. All standards were prepared in blank beef kidney extract in order to compensate for these potential effects.

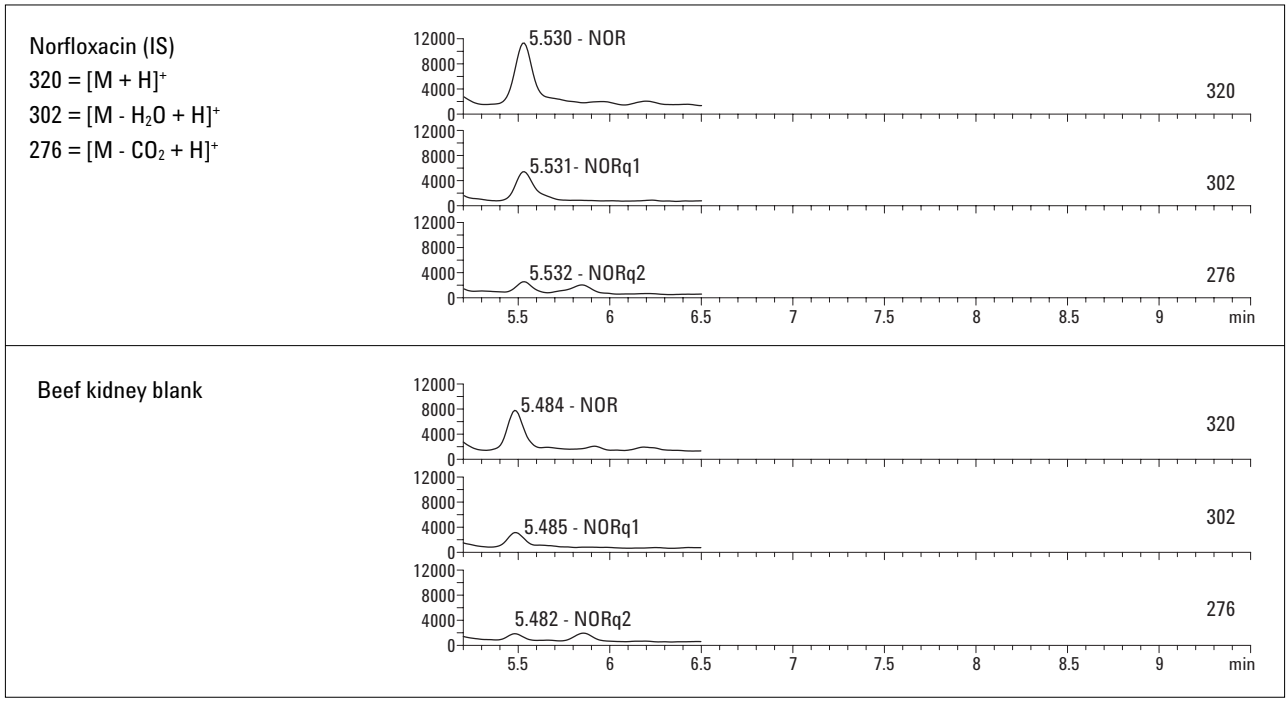
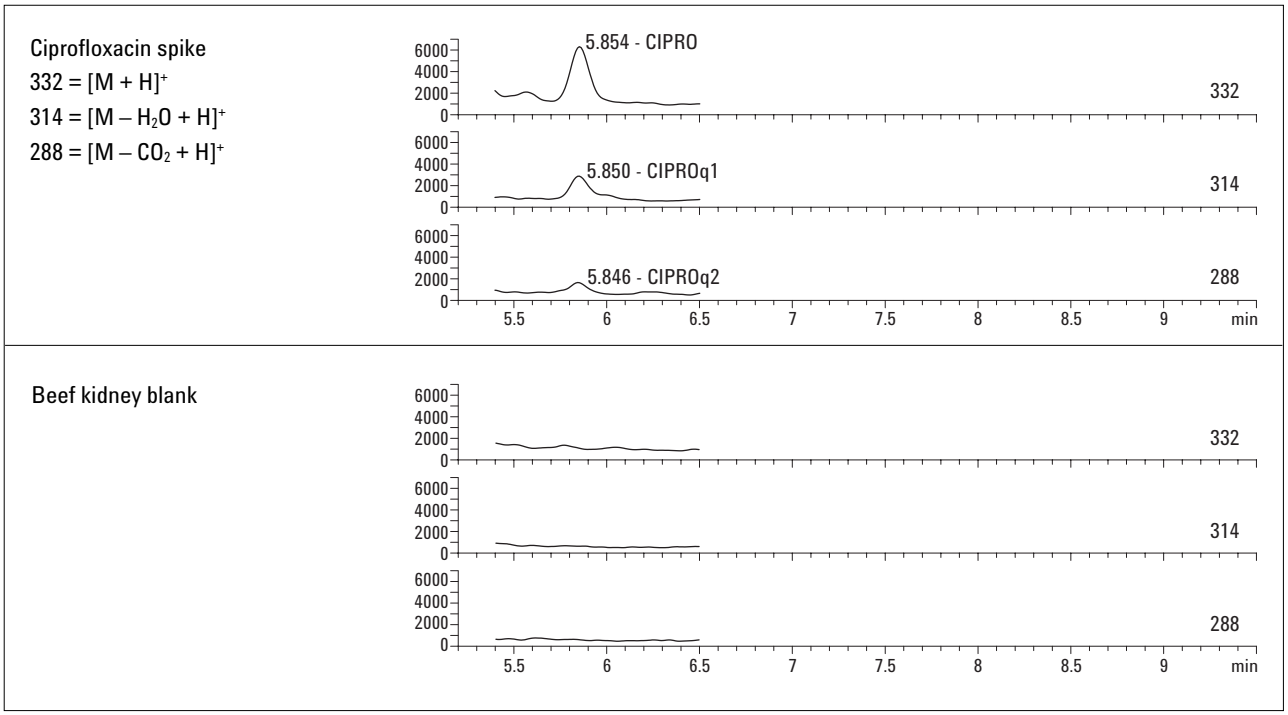


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney.



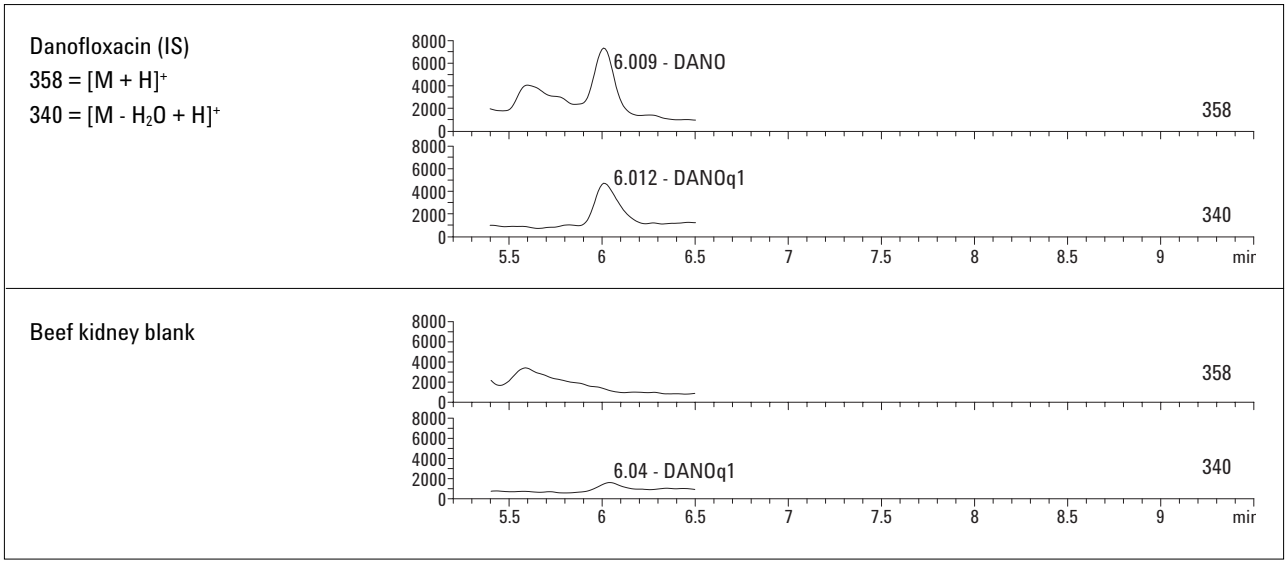
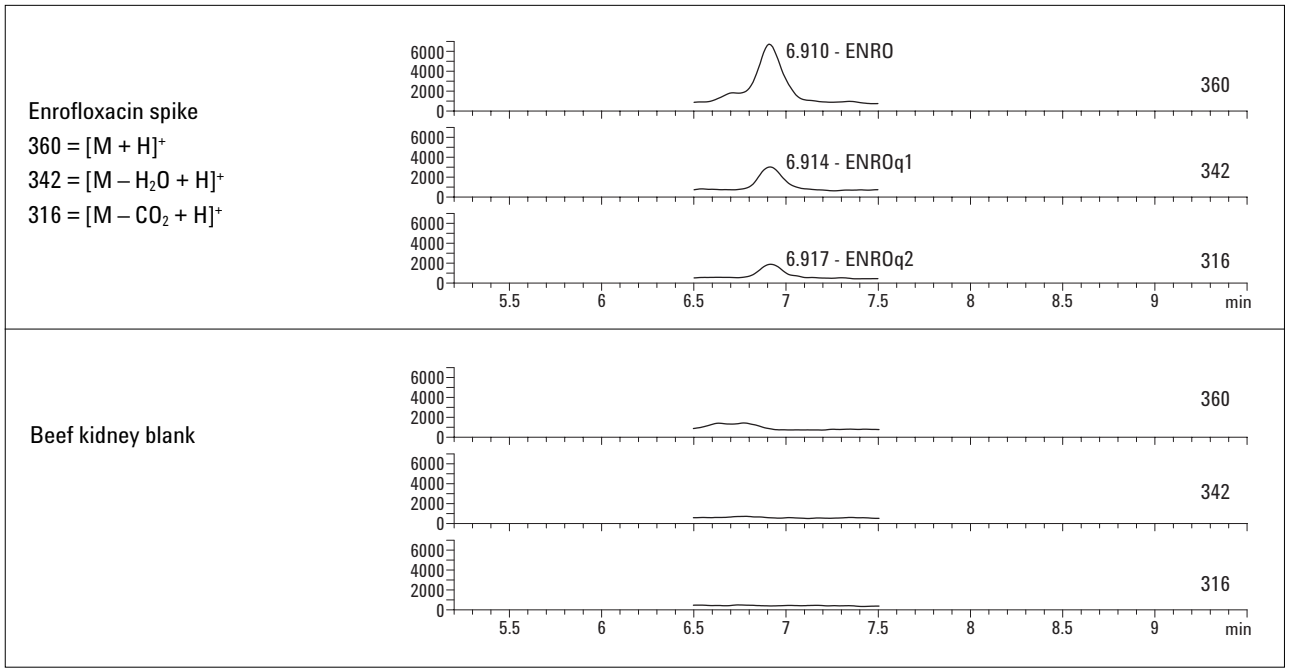


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).



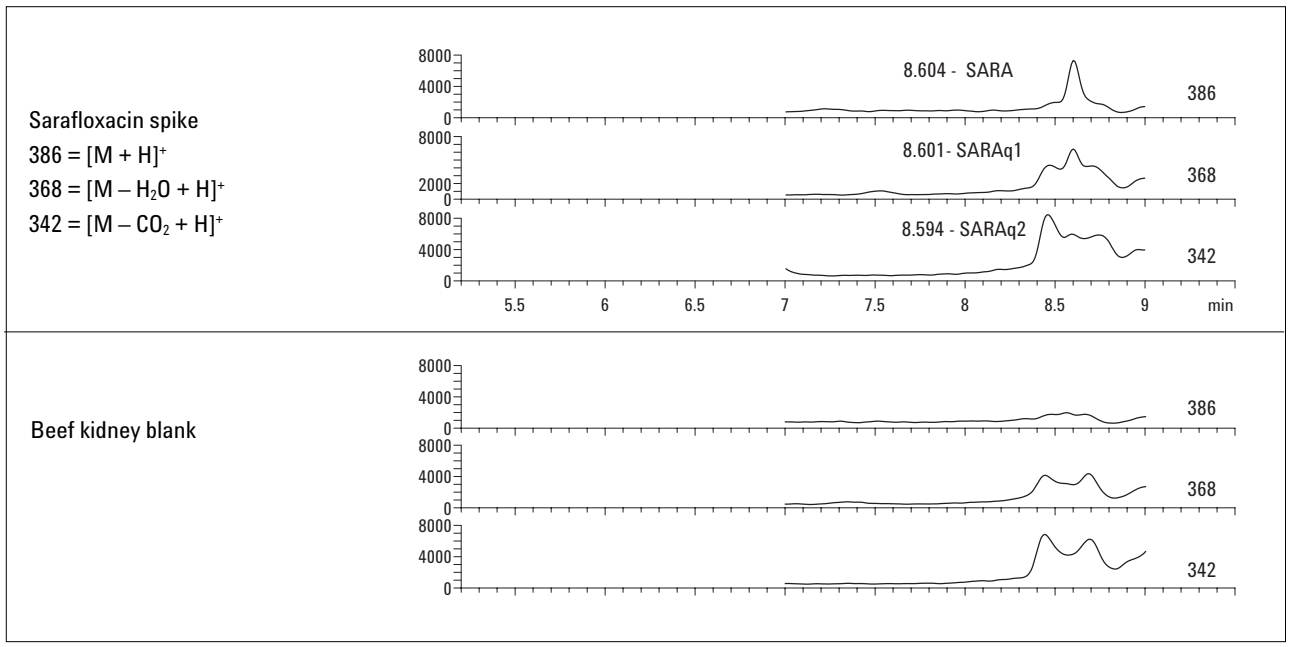


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).

Sarafloxacin elutes from the column in the same region as a number of other co-extractives, making identification and quantitation more difficult. However, as shown in Table 7, the qualifier ions still meet the identification criteria for relative responses of the qualifiers, and so further cleanup of the samples may not be necessary. The effect of these co-extractives will also be reduced at higher incurred residue levels, closer to those permitted by the European Union MRL.

Recoveries

In order to allow results to be corrected for recoveries, where the determined incurred levels are divided by the percent recovered from certified reference materials or spiked samples, Table 2 of the Annex requires that the recoveries for analytes at levels greater than 10 µg/kg be within the range of 80% to 110%. Table 4 shows that recoveries for ciprofloxacin and enrofloxacin meet this requirement, with 96.3% and 86.0%, respectively. However, sarafloxacin fails the requirement, with only 72.6% mean recovery. With a CV of only 8% for this compound, it looks as though the method may still produce acceptable results for screening purposes, but some additional work may be required to produce higher recoveries. Since the work presented here involves spiked samples only, recovery-correction calculations do not apply.

Norfloxacin was added along with danofloxacin as an additional IS. However, examination of the blank beef kidney used in this study shows norfloxacin to be present as an incurred residue, at a concentration approximately one half of the spiking level. Assuming a linear response through the origin, this would mean that norfloxacin was detected at approximately 15–20 µg/kg, which is about 10% of the permitted level for enrofloxacin in bovine kidney. Recoveries for norfloxacin are included in Table 4, even though they were calculated with a single point calibration, and not corrected for incurred residues. However, there is some compensation for this since the standards used for calibration were prepared by addition of the targets to the blank extracts.

Table 4. Recoveries of Fluoroquinolones from Beef Kidney

	Amount recovered (ng)			
Description	**Norfloxacin**	**Ciprofloxacin**	**Enrofloxacin**	**Sarafloxacin**
Kidney spike 1	111.8	96.3	84.9	68.5
Kidney spike 2	93.1	94.0	85.6	64.1
Kidney spike 3	88.0	89.6	83.8	77.6
Kidney spike 4	98.9	95.4	86.2	75.2
Kidney spike 5	82.2	93.8	85.4	82.1
Kidney spike 6	143.0	109.3	87.9	72.9
Kidney spike 7	102.6	101.3	83.3	73.0
Kidney spike 8	110.6	90.8	91.3	67.7
Amount spiked (ng)	100.0	100.0	100.0	100.0
Mean	103.8	96.3	86.0	72.6
SD (Precision) ng	18.9	6.3	2.5	5.8
MDL (SD × t-stat) ng	56.7	19.0	7.6	17.4
LOQ (SD × 10) ng	189.1	63.4	25.4	58.1
CV (SD/Mean) %	18.2	6.6	3.0	8.0
Accuracy (%)	103.8	96.3	86.0	72.6
Linearity (R^2)	0.9895	0.9987	0.9992	0.9987
t-stat (N = 8)	3.00	3.00	3.00	3.00

Compound Identification

For chromatographic separation, Section 2.3.3.1 of the Annex to 2002/657/EC requires that the minimum acceptable RT for the analyte under investigation be at least twice the RT corresponding to the void volume of the column (k'=1). The first compound to elute under these conditions is norfloxacin, with a k' of 2.6, therefore this condition is easily met. The second condition is that the ratio of the RT of the analyte to that of the IS, that is the relative RT, shall correspond to that of the calibration solution at a tolerance of ±2.5% for LC. Table 5 shows the RT times of each analyte in the spiked samples, compared to those of the standards, and that they are well within the allowable tolerance.

Table 5. Relative RTs of Analytes in Samples, Compared to Standards

Compound	Average RRT in standards (N = 15)	CV (%) RRT in standards (N = 15)	RRT in samples, relative to standards (N = 8)
Norfloxacin	0.922	0.12%	99.8%–100.1%
Ciprofloxacin	0.975	0.05%	99.9%–100.1%
Enrofloxacin	1.150	0.16%	99.8%–100.2%
Sarafloxacin	1.439	0.47%	99.5%–100.3%

Compound Confirmation

Section 2.3.3.2 of the Annex to 2002/657/EC gives the maximum permitted tolerances for relative ion intensities, which is reproduced in Table 6.

Table 6. Maximum Permitted Tolerances for Relative Ion Intensities Using a Range of Mass Spectrometric Techniques

Relative intensity (% of base peak)	GC/MS(EI) (relative)	GC/MS(CI), GC/MS^n, LC/MS, LC/MS^n (relative)
>50%	±10%	±20%
>20% to 50%	±15%	±25%
>10% to 20%	±20%	±30%
≤10%	±50%	±50%

Note MS^n equals MS/MS if n = 2

Table 7 shows the relative intensities for each of the qualifier ions for the three target compounds, as well as norfloxacin and danofloxacin (one ion). As expected, norfloxacin meets the criteria in each of the eight spiked samples, even though it had incurred residues. The presence of additional norfloxacin should not negatively affect this qualitative aspect of performance, and it does not. Danofloxacin, however, showed an interference for the single qualifier ion monitored, and so the relative amount of this signal would be expected to vary to a larger degree, depending upon the exact amount of blank extract used in preparing the sample dilutions and standards. As previously mentioned, the standards are prepared by accurately measuring the relative amounts of target and IS compounds into a tube or vial, followed by addition of blank kidney extract and water. The exact proportions of extract and water do not have to be known, since the IS calculations uses amount and response ratios, rather than absolute amount and response, in determining concentrations in unknowns. An accurate measurement of extract and water volumes can, however, reduce interference variability.

Table 7. Relative Intensities of Qualifier Ions for Fluoroquinolones in Beef Kidney, Compared to Permitted Tolerances

	Relative intensities (%) of qualifier ions								
	Norfloxacin		**Ciprofloxacin**		**Danofloxacin**	**Enrofloxacin**		**Sarafloxacin**	
Sample	**Q1 = 302**	**Q2 = 276**	**Q1 = 314**	**Q2 = 288**	**Q1 = 340**	**Q1 = 342**	**Q2 = 316**	**Q1 = 368**	**Q2 = 342**
Spike 1	49	15	47	17	64	44	28	50	15
Spike 2	45	15	48	17	58	46	24	41	17
Spike 3	48	16	46	20	63	44	28	45	14
Spike 4	42	15	46	17	60	43	30	43	13
Spike 5	49	17	45	21	65	44	26	45	11
Spike 6	50	19	39	17	72	45	29	43	12
Spike 7	49	17	41	18	65	46	29	46	13
Spike 8	47	17	42	19	62	39	24	46	12
Average for Stds	49	20	44	20	86	43	26	47	15
Std Dev for Stds	2	1	3	2	22	2	2	6	1
Tolerance(Table 7)	25	30	25	30	20	25	25	25	30
Lower Allowable (calculated)	**37**	**14**	**33**	**14**	**69**	**32**	**19**	**35**	**11**
Upper Allowable (calculated)	**62**	**26**	**55**	**25**	**103**	**53**	**32**	**59**	**20**

Conclusion

A fast and sensitive single quadrupole LC/ESI/MS method was validated for the detection of three fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin, and sarafloxacin) in beef kidney. The detection limits ranged from 8 to 19 µg/kg (ppb), with direct analysis of sample extracts after dilution with water. All qualitative requirements were met with respect to the Annex to EU Directive 2002/657/EC for spiked samples, and recoveries of two of the three compounds met the quantitative requirements. Recovery of sarafloxacin was slightly lower than the level required to allow correction for recoveries in reported results.

References

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4. Annex of Commission Decision 2002/657/EC, Commission Decision of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities, 17.8.2002, L 221/8-36, Table 5, Footnote 4.

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Agilent Technologies

Determining Malachite Green and Leucomalachite Green in Food by LC/MS/MS

Application

Food Safety

Authors

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Abstract

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leucomalachite green in fish with the new Agilent 6410 LC/MS triple quadrupole system. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), qualification and quantification were accomplished without the traditional tedious PbO_2 oxidation process. The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement of 2 µg/Kg set by Japan and the EU.

Introduction

Malachite green (MG) is a metallic-looking crystal. It dissolves in water easily as a blue-green solution. It is a toxic chemical primarily used as a dye and has been found very effective in treating parasites, fungal infections, and bacterial infections in fish and fish eggs.[1] On uptake, MG is rapidly reduced into leucomalachite green (LMG) and deposited in the fatty tissue of the fish with little MG remaining.

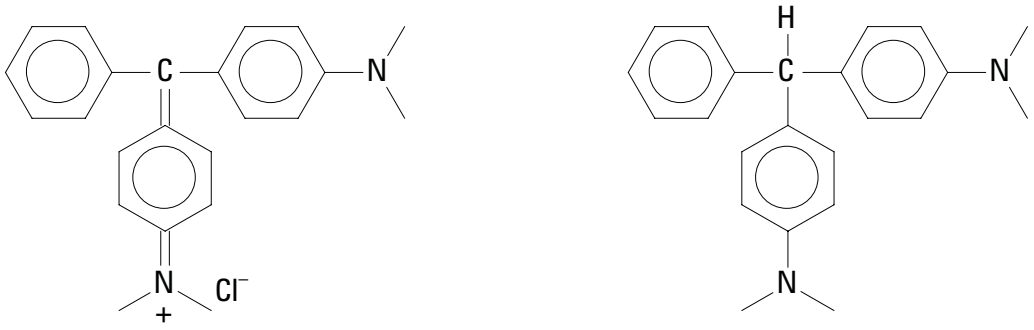
MG can cause significant health risk for humans who eat contaminated fish. For example, it can cause liver tumor formation and is suspected of carcinogenesis.[1] The United States, Japan, China, the European Union, and many other countries have already banned MG in fishery. Due to its low cost and antifungal effectiveness, MG is still being used illegally as indicated in the European Rapid Alert System for Food and Feed.[2]

HPLC with UV detection has been used to analyze MG and LMG. Figure 1 shows the structure of the two compounds. Loss of conjugation by reduction changes the chromaphore of LGM significantly. To obtain the sum of both, the method employs post-column oxidation with PbO_2 to convert LMG to MG, thus providing a sum of both comounds.[3] Most recently, LC/MS has been used to both meet the EU confirmation criteria and provide quantitative results for both compounds without the need for post-column oxidation. In this application, a simple and sensitive method for simultaneously determining MG and LMG is presented.[4, 5] The LC/MS/MS method's LOQ is 0.01 µg/Kg, which easily meets the import requirement set by Japan or the EU.[6]

Experimental

Reagents

MG	Sigma-Aldrich, CAS 569-64-2, USA
LMG	Dr. Ehreastorfer's lab, D-86199, 99% pure, Augsburg, Germany
Acetonitrile	CAS 75-05-8; Burdick & Jackson; Morristown, New Jersey, USA
Acetic acid	Merck, Germany
Ammonium acetate	CAS 631-61-8, Acros Organics, Morris Plains, New Jersey, USA



Malachite green Leucomalachite green

Figure 1. Molecular structure of malachite green and leucomalachite green.

Calibration Solutions

A stock standard solution of MG and LMG in acetonitrile was prepared at 100 µg/mL and stored at −18 °C, avoiding light. The stock solution was diluted in 50:50 acetonitrile:water to make the calibration solutions—10, 50, 100, 500, 1000, 5000, and 10,000 fg/µL.

Sample Preparation

To 5 g tilapia tissue was added 1 mL (0.25 mg/mL) hydroxylamine, 2 mL 1 M toluene sulfonic acid, 2 mL of 0.1 M ammonium acetate buffer (pH 4.5), and 40 mL acetonitrile. The mixture was then homogenized for 2 min. The supernatant was decanted, and to the precipitate was added 20 mL acetonitrile. This was filtered and added to the supernatant. To the combined acetonitrile extracts, 35 mL water and 30 mL methylene chloride were added. The solution was shaken and the methylene chloride layer collected. A second extract of 20 mL methylene chloride was made, and this layer added to the first extract. The methylene chloride was taken to dryness with a gentle stream of nitrogen and the extract reconstituted in 100 µL of acetonitrile

Instrumentation

LC	1100 LC	
Column	C18, 2.1 x 150 mm, 5 µm	
Column temp.	40 °C	
Mobile phase	A − 10 mmol/L ammonium acetate (adjust to pH 4.5 with acetic acid)	
	B − acetonitrile	
Column flow	0.3 mL/min	
Gradient	Time	%B
	0	30
	1	50
	2	95
	8	95
	8.01	30
	13	30
Injection vol.	10 µL	
MS	Agilent 6410 LC/MS Triple Quadrupole	
Ionization	ESI(+)	
Capillary	4000 V	
Nebulizer P.	35 psi	
Drying gas	11 L/min	
Gas temp.	350 °C	
Skimmer	15 V	
OctDc1 (Skim2)	45 V	
Oct RF	500 V	
Q1 resolution	Unit	
Q3 resolution	Unit	
Collision gas	Nitrogen	

The MRM parameters are listed in Table 1.

Table 1. MRM Method Parameters

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Energy (V)
0	MG	329.3	313.3	40	100	40
		329.3	208.2	40	100	40
7	LMG	331.3	316.3	40	100	30
		331.3	239.2	40	100	30

Results and Discussion

To obtain the most sensitive results, optimization of certain fragmentor voltages is important. Figure 2 shows the EICs of both target compounds at fragmentor values of 70 V, 90 V, and 100 V. The results show that the three different fragmentor values have little effect on the intensity of $[M+H]^+$ ions. Thus, 100 V was chosen for this study.

In addition, an optimal collision energy for the MS/MS must be set. Figure 3 shows the MS/MS spectra from three different collisional voltages, (a) 20 V, (b) 30 V, and (c) 40 V. Due to their structural differences, the voltage required for optimum fragmentation of each compound is different. For MG, the optimum fragmentation was observed at 40 V. The ion *m/z* 313 was due to the neutral loss of methane. The ion at *m/z* 208 was due to the neutral loss of N,N-dimethylaniline. For LMG, the optimum fragmentation was observed at 30 V. The ion at *m/z* 316 was due to the loss of a methyl radical. The ion at *m/z* 239 resulted from a subsequent loss of a benzene radical or, more likely, the rearrangement and neutral loss of toluene.

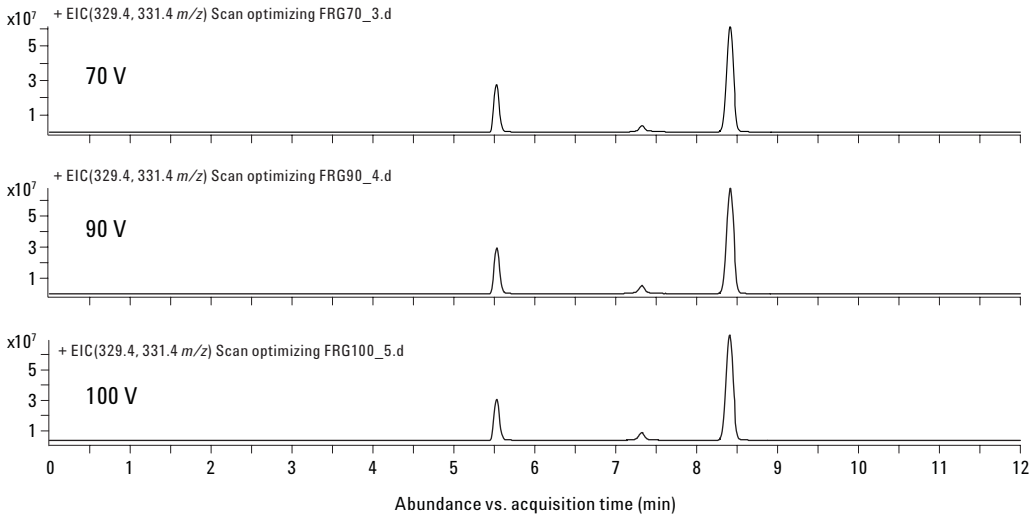


Figure 2. EICs of malachite green and leucomalachite green at fragmentor values of 70 V, 90 V, and 100 V.

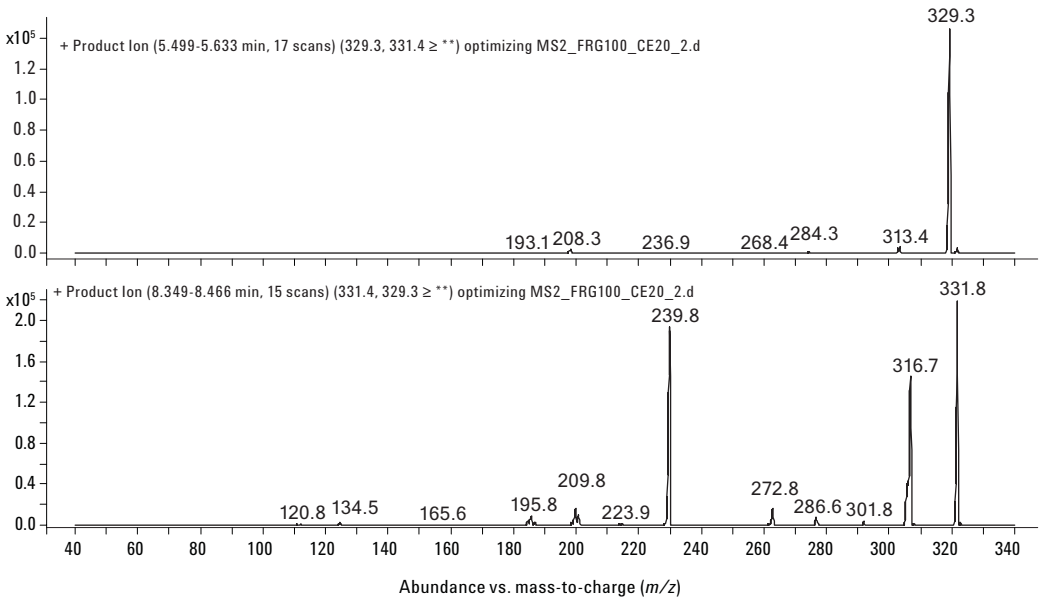


Figure 3a. MS/MS spectra of MG and LMG at collisional voltage of 20 V.

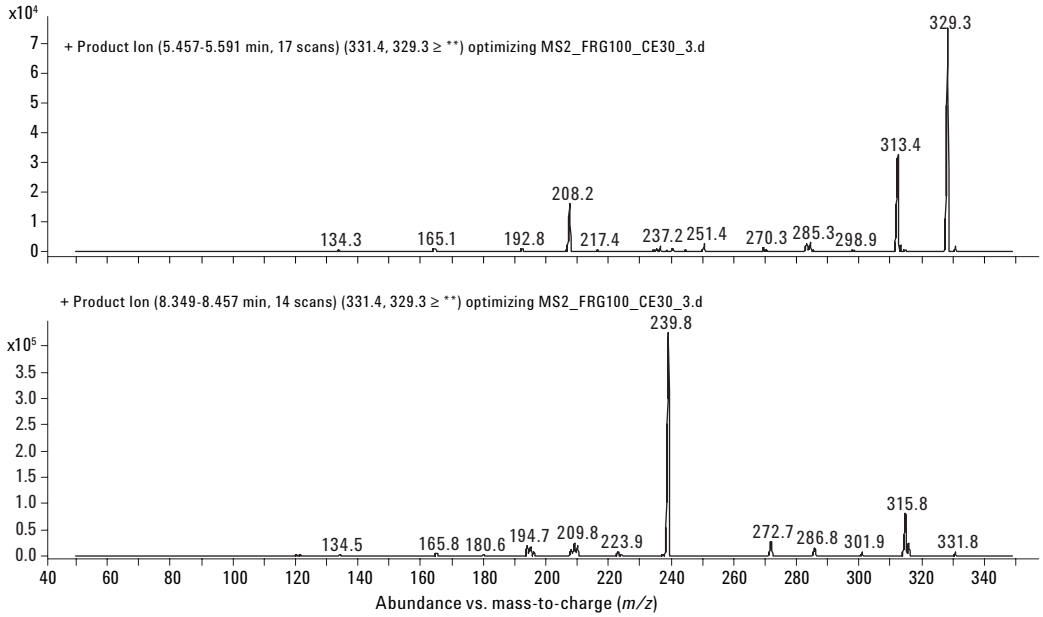


Figure 3b. MS/MS spectra of MG and LMG at collisional voltage of 30 V.

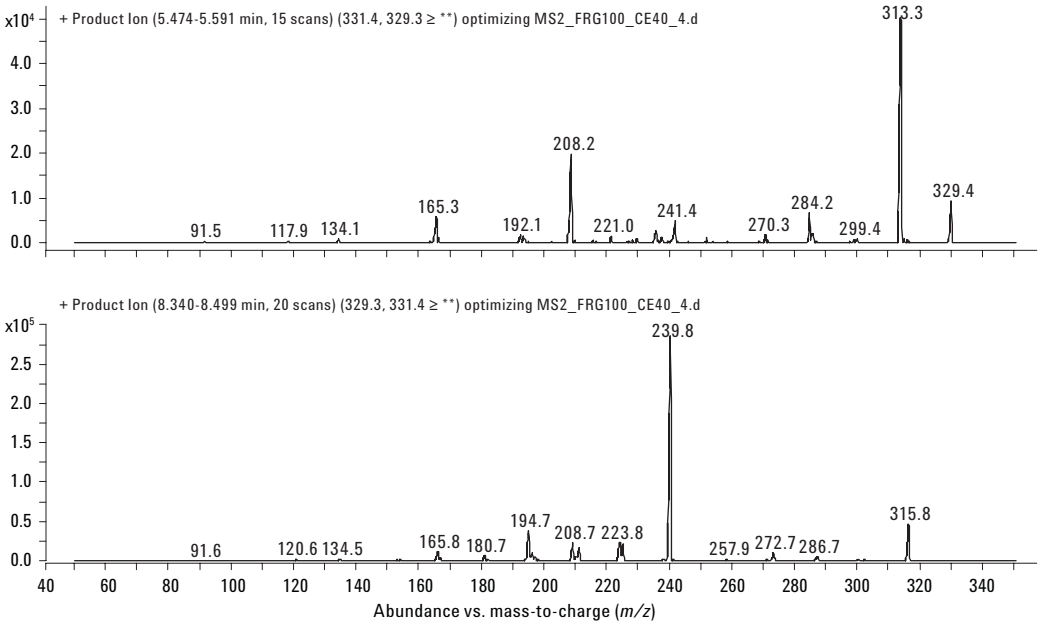


Figure 3c MS/MS spectra of MG and LMG at collisional voltage of 40 V.

Figure 4 shows the calibration curves for both MG (4a) and LMG (4b). Calibration solution concentrations were from 10 to 10,000 fg/µL. The linear calibration range is 100 to 100,000 fg on column for both compounds. The R^2 for both compounds was > 0.999 (origin ignored and no weighting). To demonstrate the sensitivity of the instrument, Figure 5 shows MS/MS spectra of a blank sample extract (5a) and sample extract spiked with 10 ppt of each compound (5b). A sample of tilapia spiked at 100 ppt MG and LMG before extraction was made to demonstrate method performance. The MRM results after extraction and cleanup are shown in Figure 6. The recover-

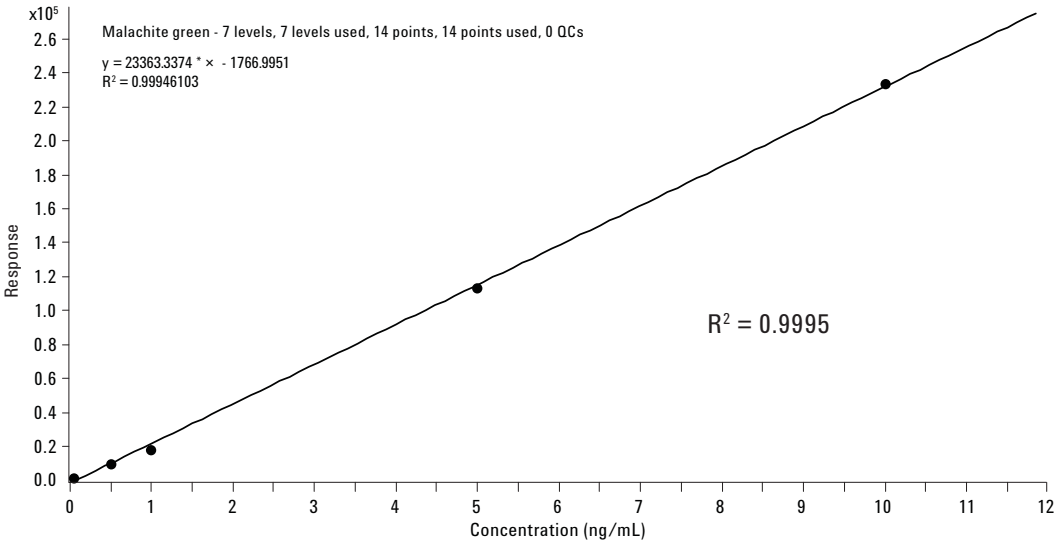


Figure 4a. Calibration curve of malachite green, linear range: 10 ppt to 10 ppb.

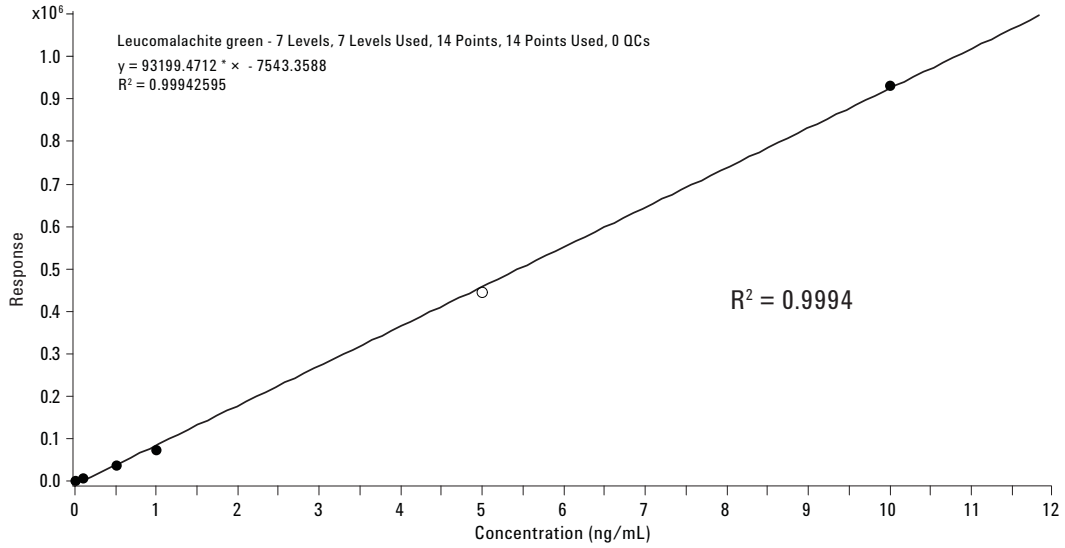


Figure 4b. Calibration curve of leucomalachite green, linear range: 10 ppt to 10 ppb.

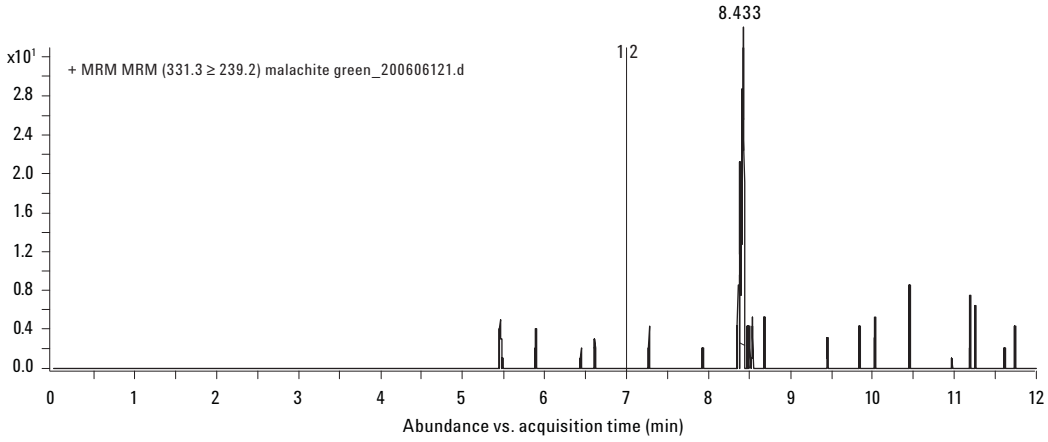
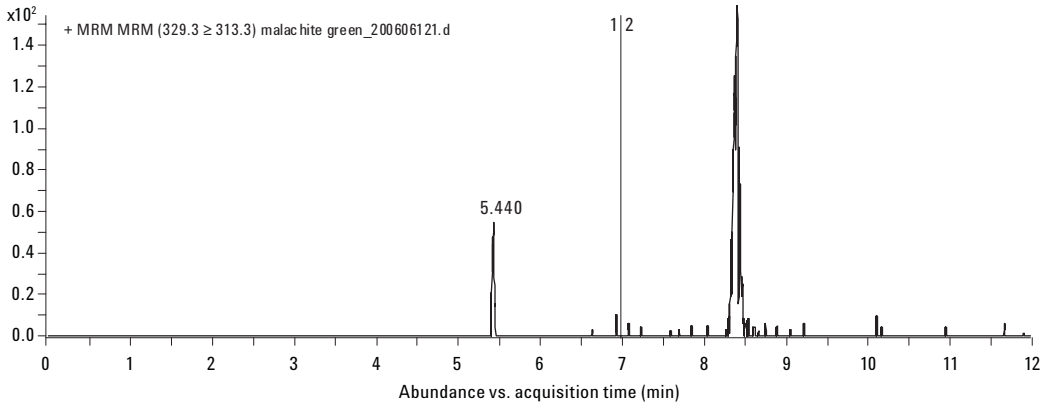


Figure 5a. MG and LMG MRM of a blank sample.



ppt spiked sample.

Figure 5b. MG and LMG MRM of a 10-ppt spiked sample.

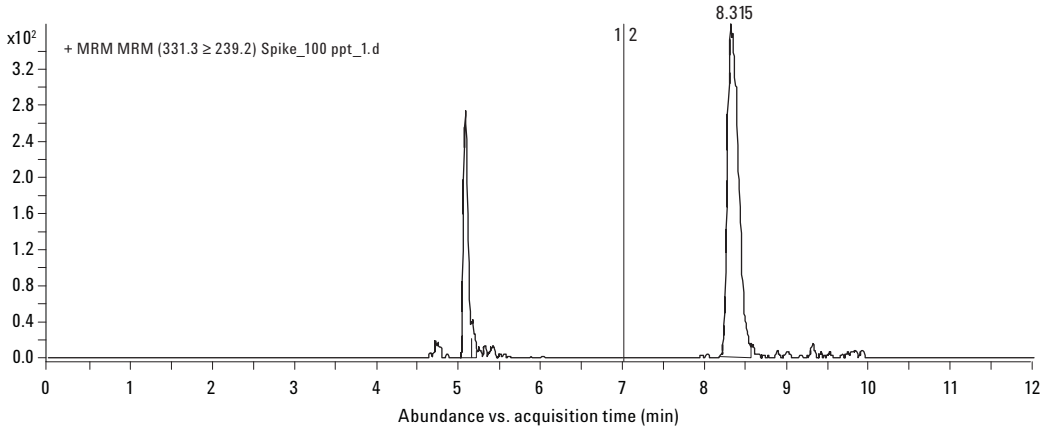


Figure 6. MRM result of talapia extract spiked with 100-ppt MG and LMG.

ies for MG were 48% and 23% for LMG. A mixture of MG and LMG at 100 fg/µL in 50:50 acetonitrile: ammonium acetate was used for the repeatability study for instrument performance. The RSD from eight injections for MG was 3.52% (S/N > 20). The RSD from eight injections for LMG was 2.25% (S/N > 40).

Conclusions

This application note demonstrates a complete method to rapidly and precisely determine residue levels of malachite green and leuco-malachite green in fish. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM) technique, the LC/MS/MS method shows detection limit of 10 ppt, which easily meets the import requirement set by Japan or EU.

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Acknowledgement

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Agilent Technologies

Analysis of Nitrofuran Metabolites in Tilapia Using Agilent 6410 Triple Quadrupole

Application

Food Safety

Authors

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Abstract

The metabolites of nitrofuran antibiotics banned in meat and meat products are analyzed by LC/MS/MS with the new Agilent 6410 triple quadrupole. The method is shown to be highly sensitive, to 0.01 ppb (10 ppt), for each of the four analytes. Calibration from 0.1 ppb to 10 ppb is presented with all criteria for confirmation as set by the European Union decisions for analytical method performance. Extracts of tilapia are used to show the performance of the LC/MS/MS method for aquaculture samples.

Introduction

Nitrofurans are inexpensive antibiotics used for Gram positive and Gram negative bacteria. They have been used to treat gastrointestinal and dermatological infections in farm animals and fish. In addition, they have been used to treat bacteria in bees. Because both parent compounds and their metabolites are suspect carcinogens, they have been banned around the world. The Rapid Alert System for Food and Feed Annual report for 2005 [1] shows that these compounds continue to be detected in food samples and remains a major concern for food safety. The four compounds–furazolidone, furaltadone, nitrofurantoin, and nitrofurazone–have been found to metabolize rapidly, and the metabolites bind to muscle tissue. Thus the analytical detection of the metabolites and not the parent compounds are required in samples of animal origin.

The criteria for detection and confirmation of veterinary drugs in animal and animal products established by the European Union (EU) [2] has been accepted in much of the world. This criteria mandates a separation technique combined with a spectrometric technique. For banned substances such as the nitrofurans, no maximum residue limit (MRL) could be set. Therefore a minimum required performance level (MRPL) was set at 1 µg/kg for each metabolite [3]. Only LC/MS could meet these criteria, and very good methods have been reported [4–6]. However, the most widely accepted methodology employs triple quadrupole tandem mass spectrometers. This is the first report showing analysis of these metabolites using the new Agilent triple quadrupole LC/MS system.

Experimental

Chemicals

Derivatized standards of nitrofuran metabolites and all chemicals for sample preparation were received from a food manufacturing company. Acetonitrile was HPLC grade from Merck (Darmstadt, Germany). Formic acid was reagent grade from Merck (Darmstadt, Germany).

Sample Preparation

The accepted procedure for sample preparation was followed. To 2 g of tilapia was added 15 mL 0.125 M HCl and the mixture homogenized.

To this solution, a 50-µL solution of 2-nitrobenzaldehyde (50 mM in DMSO) was added and shaken. The solution was then incubated at 37 °C for 16 hours. This was followed by neutralization to pH ~7 with NaOH and K_2HPO_4. The neutral derivatized sample was then extracted with ethyl acetate, concentrated to dryness, and reconstituted in 100 L of initial LC mobile phase. Standards of the four metabolites were spiked into 0.125 M HCl, derivatized, and extracted for calibration using the same procedure as was used for the samples of tilapia.

LC/MS/MS Method

LC Conditions

Instrument:	Agilent 1100 LC
Column:	C18, 2.1 mm × 150 mm, 3 µm
Column temp.:	40 °C
Mobile phase:	A = 0.1% formic acid in water
	B = acetonitrile
Gradient:	22% B at 0 min
	99% B at 6 min
	99% B at 9 min
Flow rate:	0.3 mL/min
Injection volume:	50 nL

MS Conditions

Instrument:	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	35 psig
Drying gas temp.:	350 °C
V_{cap}:	5000 V

Quantitation

Quantitative analysis was done with the first transition listed in the MRM parameter table. The second transition was used as a qualifier ion for confirmation as per the confirmation criteria. Quantitative results were performed with the new MassHunter quantitative analysis software.

Results and Discussion

The instrument sensitivity is an important performance parameter for this analysis when considering the derivatization and extraction needed to meet the required detection limit of 1 ppb for each metabolite, aminohydantoin (AH), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ), and semicarbazide (SC). To demonstrate this performance, a standard of each 2-nitrobenzaldehyde (2-NBA) derivatized metabolite is shown at 0.01 ppb (10 ppt) in Figure 1. The structure of each derivatized metabolite is given and each is shown with a signal-to-noise ratio of greater than 3:1. Another indicator of performance is linearity. Calibration curves from this concentration (10 ppt) to 10 ppb are displayed in Figure 2 showing the linearity for each compound.

Treatment of fish with nitrofurans is a continual problem for food safety and import into EU member countries. To demonstrate the capability of the Agilent triple quadrupole LC/MS, tilapia samples were spiked with the four metabolites, hydrolyzed, derivatized, and extracted. An analysis of a tilapia extract at 500 ppt is shown in Figure 3 and demonstrates the signal obtained at half the MRPL. In addition to meeting the sensitiv-

MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor voltage (V)	Collision energy (V)	MS2 resolution
AMOZ	335.1 → 291.4	60	100	5	Unit
	335.1 → 262.4	60	100	5	Unit
SC	209.1 → 192.3	60	100	5	Unit
	209.1 → 166.3	60	100	5	Unit
AH	249.1 → 134.2	60	100	5	Unit
	249.1 → 104.2	60	100	5	Unit
AOZ	236.0 → 134.1	60	100	5	Unit
	236.0 → 104.1	60	100	5	Unit

ity requirement, the analysis must also meet the confirmation criteria, including both chromatographic retention time match with the standards and measuring a qualifying ion with a relative intensity ratio within a specified tolerance of the quantitation ion. This tolerance is set by the ratio obtained when analyzing standards and increasing as that ratio decreases. This tolerance ranges from 20% for ions with relative ratio intensities above 0.5 and to 50% for ratios below 0.1.

Table 1 shows tilapia samples spiked with the metabolites, derivatized and extracted. The spikes were used as the calibrants, so the final concentration is obtained from the curve. The table is produced as the batch using the MassHunter software results with outliers highlighted in blue (low) and red (high). The table shows that in the blank a peak is found within the tolerance set for the retention time of AMOZ but the qualifier ratio is low. For AOZ and SC, retention times for suspect peaks are below the specified retention in the same blank. For AH, the 0.5 ppb spike, the qualifier ion ratio is outside the 35% tolerance limit set for this ion (again low).

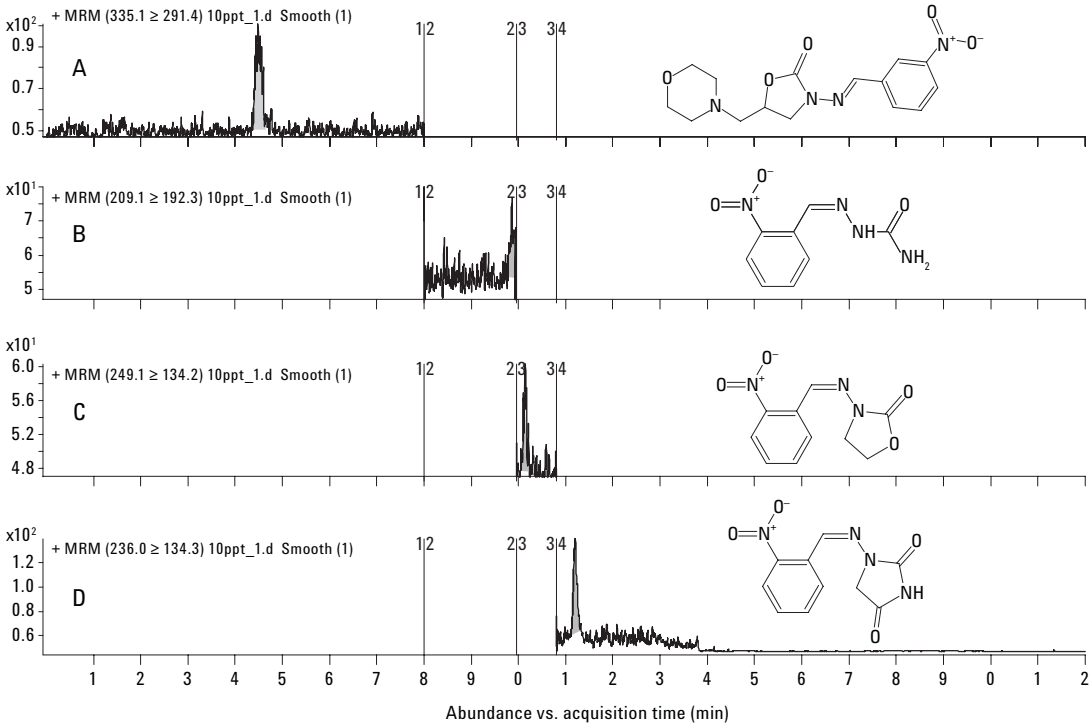

Figure 1. The MRM quant ion chromatogram for each derivatized metabole at 10 ppt of A) 2-NBA AMOZ, B) 2-NBA SC, C) 2-NBA AH, and D) 2-NBA-AOZ

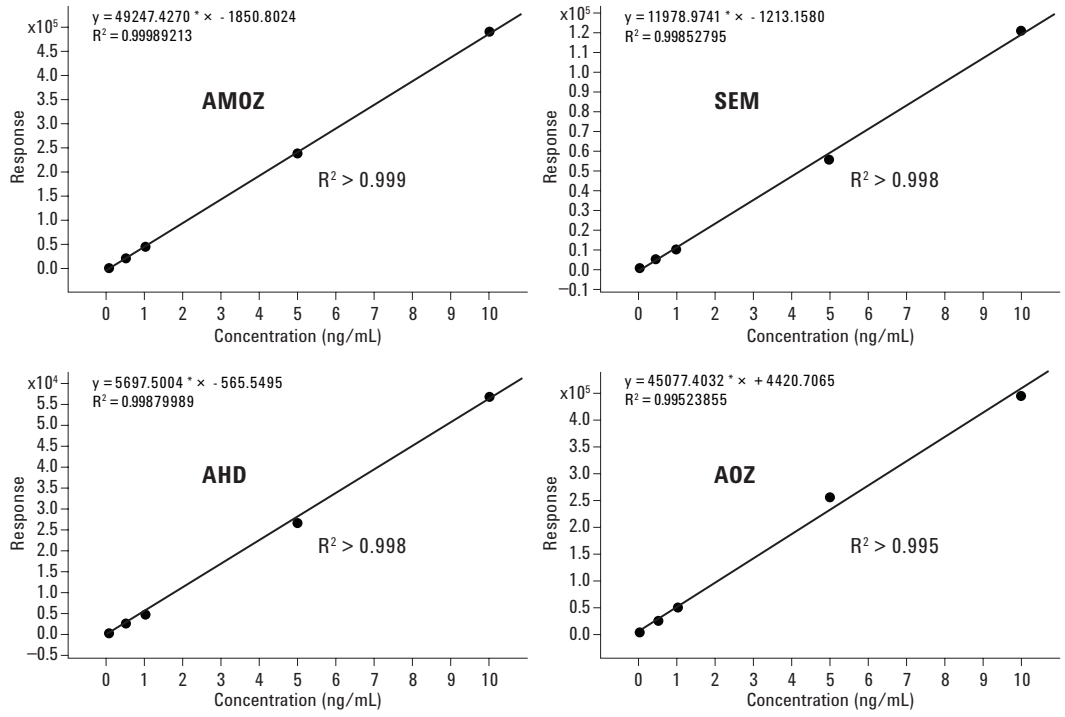

Figure 2. Calibration curves of nitrofuran metabolites linear range from 10 ppt to 10 ppb.

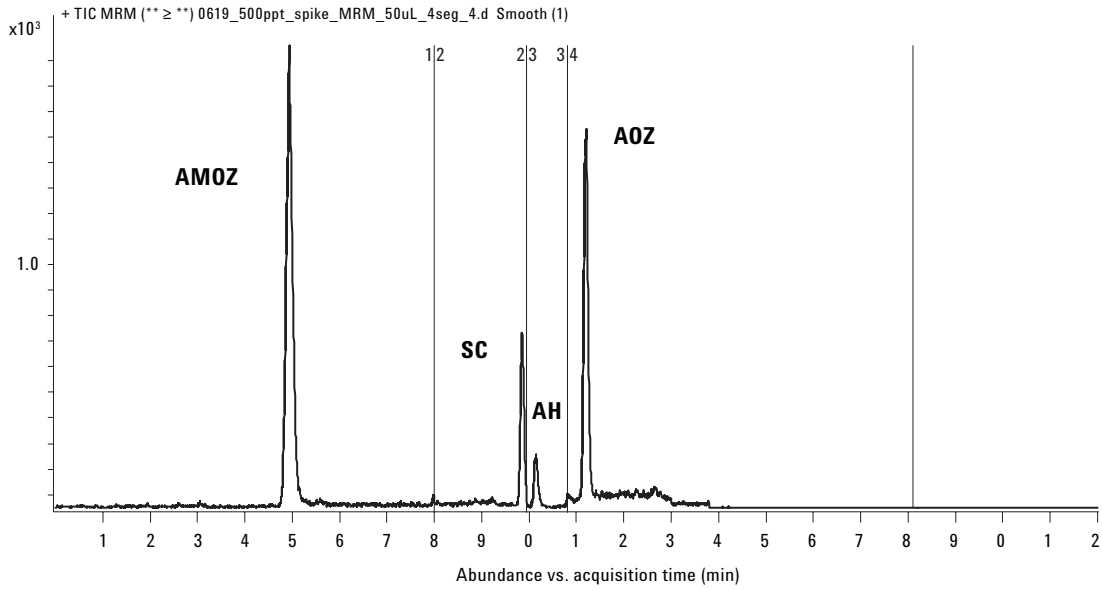

Figure 3. Spiked tilapia sample extract at 500 ppt each metabolite.

Table 1. Analysis of Talapia Spikes Self-Calibrated. Note Qualifier Ratios and Retention Times Reported.

		AH	Qualifier		AMOZ	Qualifier		AOZ	Qualifier		SC	Qualifier
Name	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio
Nitrofuran Blank		0.00		2.72	0.02	8.70	8.71	0.00		7.94	0.00	
Nitrofuran 0.5 ppb	8.66	0.54	6.07	2.62	0.51	17.44	9.19	0.37	7.99	8.44	0.50	65.32
Nitrofuran 1 ppb	8.66	1.07	15.83	2.64	0.92	20.42	9.19	1.06	10.19	8.44	1.05	70.85
Nitrofuran 3 ppb	8.67	3.42	13.78	2.65	3.41	18.67	9.20	3.18	9.51	8.44	2.89	67.47
Nitrofuran 5 ppb	8.66	5.71	14.24	2.66	4.66	19.28	9.19	4.89	8.61	8.44	5.05	69.06

Conclusions

This work shows the high performance of the new Agilent 6410 LC/MS triple quadrupole system for the sensitive analysis of the nitrofuran metabolites in fish samples. The system readily meets the performance requirements and provides advanced quantitation software for calculating and reporting all confirmation parameters specified by the European Commission decision.

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Detection, Confirmation, and Quantification of Chloramphenicol in Honey, Shrimp and Chicken Using the Agilent 6410 LC/MS Triple Quadrupole

Application

Food Safety

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Abstract

This method was developed using the Agilent G6410AA Triple Quadrupole Mass Spectrometer (QQQ) for chloramphenicol in honey, shrimp, and chicken. The sensitivity obtained exceeds the minimum required performance level (MRPL) established by the European Union regulation for food monitoring programs. Using a deuterated internal standard and one simple sample solid phase extraction (SPE) procedure can provide a limit of detection at 10 ppt in sample matrix. The analytical performance of the method was evaluated for three different matrixes and the results show little or no matrix effects. Linearity of response over 2 orders of magnitude was demonstrated (r > 0.99). In addition, good reproducibility of the two required product ion ratios was obtained to meet the EU identification points needed for confirmation. This study is a valuable indicator of the ability of the QQQ for routine quantitative trace analysis of chloramphenicol in honey, shrimp, and chicken.

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic. It was concluded that human exposure to CAP can cause aplastic anemia [1]. Chloramphenicol and other bacterial inhibitors have arguably been the biggest issue facing international seafood trade over the past year. Because chloramphenicol has displayed significant toxicological effects on humans, it has been banned from foods in the European community, Japan, and the United States at levels of 0.3 ppb.

LC/MS has been demonstrated for this analysis by the U.S. Food and Drug Administration[2-4] and others[5]. In this application, the Agilent G6410AA QQQ is used. This method employs negative ion mode with electrospray ionization. An internal standard (IS), CAP-d5, is added at the beginning of the extraction. The use of this IS self-corrects for any extraction variability from sample to sample and response variability caused by the matrix. With the use of this IS, 50 parts per trillion (ppt) CAP levels can be reliably quantified. A solid phase extraction (SPE) procedure is used along with a mobile phase of only methanol and water without salt buffers, which should help minimize MS maintenance.

Experimental

Reagents and Materials

Agilent AccuBond SPE ENV PS DVB Cartridges (P/N 188-3060)
Ethyl acetate from Burdick and Jackson (Morristown, NJ)
Methanol HPLC-Grade from Burdick and Jackson
Water (18 MΩ) from Milli-Q Synthesis System
Chloramphenicol (CAP) from Aldrich Chemical Co. (Milwaulkee, WI)
Deuterated (d5) CAP internal standard from Cambridge Isotope Laboratories (CIL, Andover, MA, U.S.)
Syringe filter (0.2 µm, PTFE) from Agilent (P/N 5185-5843)

Overview of Method

Internal Standard Preparation

1. A 100-µg/mL (100 ppm) stock standard CAP-d5 solution in methanol (MeOH) is purchased from Cambridge Isotope Laboratories, Inc. (Lot SCCE-005)
2. A 1:100 dilution in MeOH of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP-d5
3. A 1:100 dilution in MeOH gives a diluent solution (This diluent solution is used to prepare the samples) concentration of 10 ppb.
4. Every 1-g sample is fortified with 25 µL of CAP-d5 diluent solution for a 0.25 ppb IS (internal standard) concentration

Standard Solution Preparation

1. A 100-µg/mL stock standard CAP solution in methanol (MeOH) is prepared by weighing 5.0 mg CAP std into 50 mL methanol.
2. A 1:100 dilution with methanol of the stock standard gives an intermediate standard concentration of 1 µg/mL (1 ppm) or 1000 ng/mL CAP
3. Add 25 µL CAP-d5 diluent solution into each vial.
4. Prepare standard solutions in these vials: 1 ppb, 0.2 ppb, 0.1 ppb, 0.02 ppb, and 0.01 ppb, with IS at 0.25 ppb level.

Sample Preparation

All SPE cartridges are conditioned with 2 mL of water before use.

1. **Honey**, 1 g of sample is diluted to 5 mL with water and 25 µL 10 ppb IS is added. The solution is loaded onto the SPE cartridge and allowed to stand for 5 min. Elution is performed with 10 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min. The solution is filtered, using a syringe filter, before injection. No additional clean-up of the sample solution is performed.
2. **Shrimp**, 1 g of shrimp is defrosted and mixed in a blender. To the 1 g of the mixed shrimp, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.
3. **Chicken**, 1 g of chicken is defrosted and mixed in a blender. To the 1 g of the mixed chicken, 3 mL of water and 25 µL 10 ppb IS is added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min.; the solution is filtered before injection.

LC/MS conditions

The LC system was the Agilent 1200-SL binary pump with the ALS-SL autosampler. The MS was an Agilent 6410 LC/MS triple quadrupole mass spectrometer. See Table 1 for conditions.

Table1. LC/MS Conditions

HPLC	
Column	ZORBAX SB-C18, 2.1 × 50 mm, 1.8 µm (p/n 827700-902)
Flow rate	0.4 mL/min
Mobile phase	A: water
	B: methanol
Gradient	0-5 min, 30~70% B
	5-6 min, 70~100% B
	8 min, 100% B
Post time	4 min
Temperature	45 °C
Injection	5 µL
MS Source Settings	
Source	ESI
Ion polarity	Negative
Drying gas temperature	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	3500 V
Fragmentor	100 V
Collision energy	10 V for *m/z* 257(qualifier ion)
	15 V for *m/z* 152 (quantitation ion)

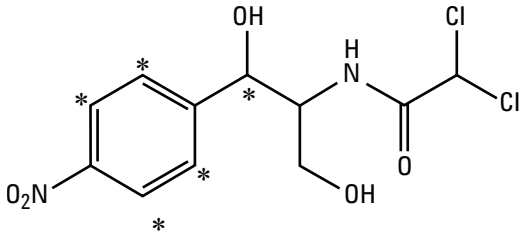
Results and Discussion

Spectral Quality and Sensitivity of Standard

Table 2 lists the structure of the CAP and the fragment ions used for quantitation and confirmation as described by the identification point system.[6] To obtain the most sensitivity, only two or three parameters need to be optimized on this instrument. They are the fragmentor, to provide highest transmission of the precursor ion, the collision energy, to maximize signal for the quantitation and qualifier ion, and possibly the V_{cap} (electrospray voltage), to maximize the number of ions generated.

Table 2. Structure and Fragment Ions of CAP and CAP-d5 (* indicates deuterated positions for the CAP-d5 IS)

Chloramphenicol structure



	m/z	
CAP	257	Qualifier ion
	152	Quantitiation ion
CAP-d5	262	
	157	

Optimization of MS Condition

Figure 1 shows the results of varying the V_{cap}. For this analyte there was little effect from varying this parameter. Only a slight increase in signal is observed at 3,500 V, and this voltage was used. The fragmentor was varied from 90 V to 160 V. Above 120 V, fragment ions are observed and the precursor ion signal drops significantly. At 160 V on the fragmentor almost no *m/z* 321 is observed. This results show that 100 V on the fragmentor provided the highest precursor ion signal. Finally, using a product ion scan of the precursor, m/z 321, the collision energy (CE) was varied from 2 V, 5 V, 8 V, 10 V, 15 V, 18 V to 40 V.

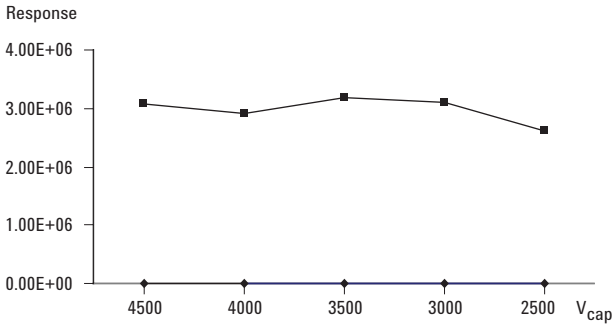


Figure 1. Plot of V_{cap} voltage vs. response of precursor ion at *m/z* 321.

Comparison of extracted ion chromatograms of the quantitation and qualifier ions showed that response maximized at 10 V for *m/z* 257 and at 15 V for *m/z* 152. The product ion spectra for these two collision energy experiments are shown in Figure 2 and Figure 3. As shown in Table 3, the same CE were used for the deuterated internal standard.

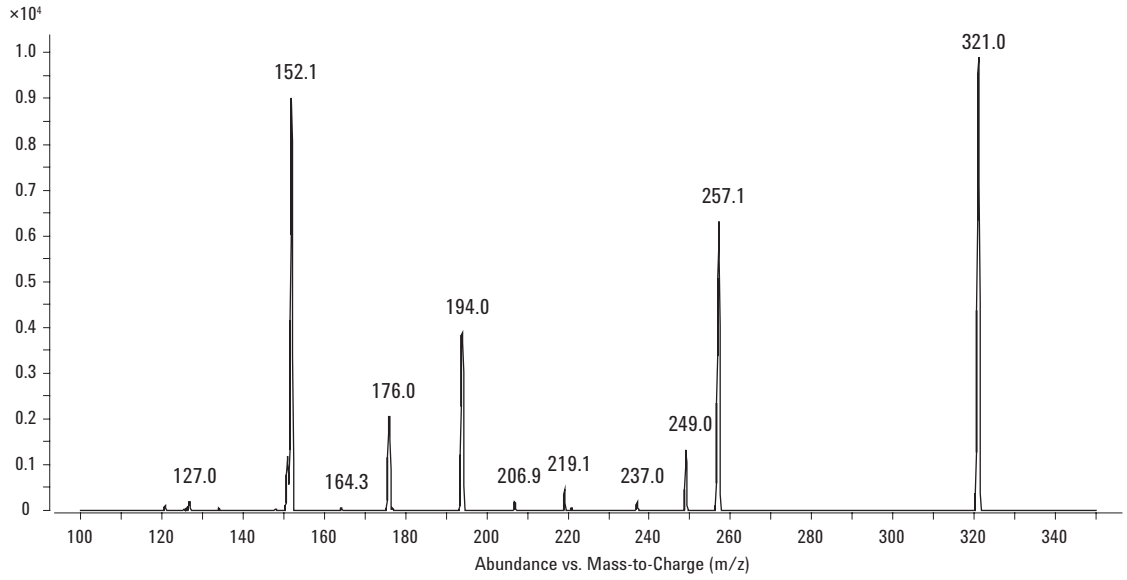


Figure 2. Product ion spectrum of *m/z* 321 at 10 V collision energy.

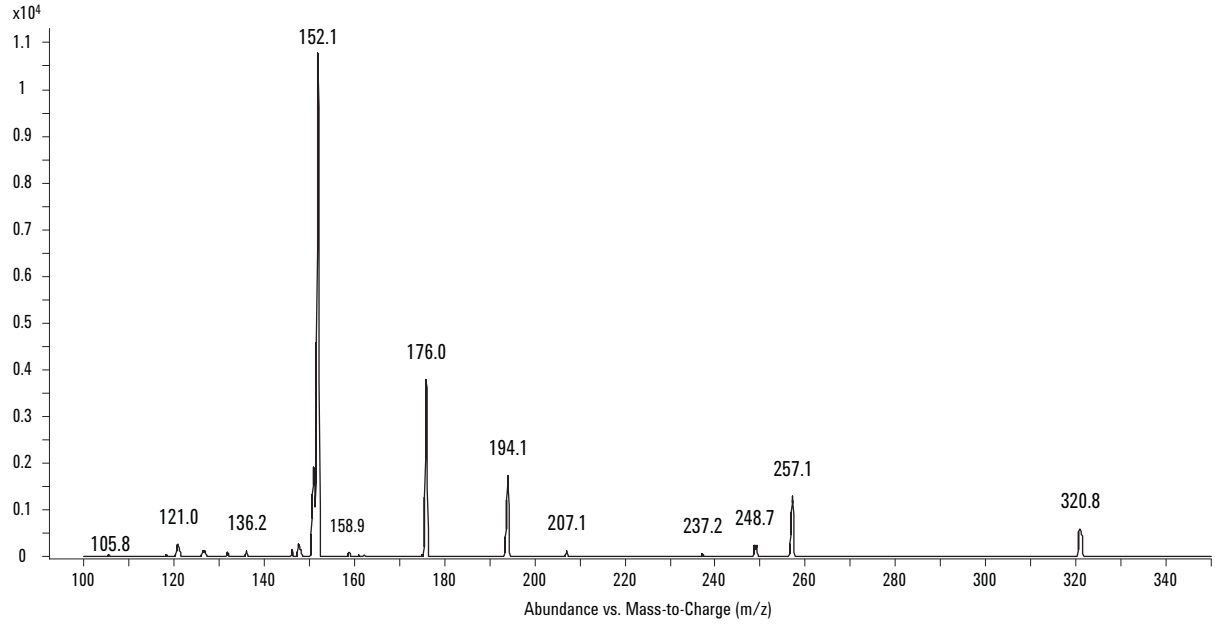


Figure 3. Product ion spectrum of *m/z* 321 at 15 V collision energy.

Table 3. MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor Voltage (V)	Collision) Energy (V)	MS2 resolution
CAP	321–257	200	100	10	Unit
	321–152	200	100	15	Unit
CAP-d5	326–262	200	100	10	Unit
	326–157	200	100	15	Unit

Repeatability

Using honey matrix spiked at 0.1 ppb level as an example, the repeatability was tested by running the extract 15 times. Table 4 shows the area of the qualifier and quantitation ions in both the analyte and the IS. On average the areas of each ion vary about 8% and the ratios 5%, well within the 20% required for ratios 50% and above. Masshunter quantitation software tabulates these results and gives a graphic representation as shown in Figure 4.

Table 4. Integrated Areas of the Quantitation Ion and Qualifier Ion and Their Associated Internal Standard Ion

	Chloramphenicol Quantitative ion (321–152)	Qualifier ion (321–257)	Ratio	d5-chloramphenicol Quantitative ion (326–157)	Qualifier ion (326–262)	Ratio
1	350	165	47.1	262	121	50.4
2	346	157	45.2	258	114	55.3
3	346	5	44.6	259	118	49.4
4	313	164	52.3	267	127	47.6
5	301	154	49.5	261	121	46.4
6	313	168	53.6	253	124	49.0
7	320	160	50.1	228	111	48.6
8	326	145	44.5	225	113	50.4
9	317	141	44.5	241	117	48.6
10	290	135	46.6	226	107	47.1
11	300	138	46.2	253	90	45.7
12	281	136	48.4	240	90	47.6
13	303	143	47.3	220	101	45.9
14	290	140	48.3	214	107	49.8
15	261	131	50.3	217	101	46.6
RSD	8.11%	8.30%	5.91%	7.67%	9.99%	4.83%

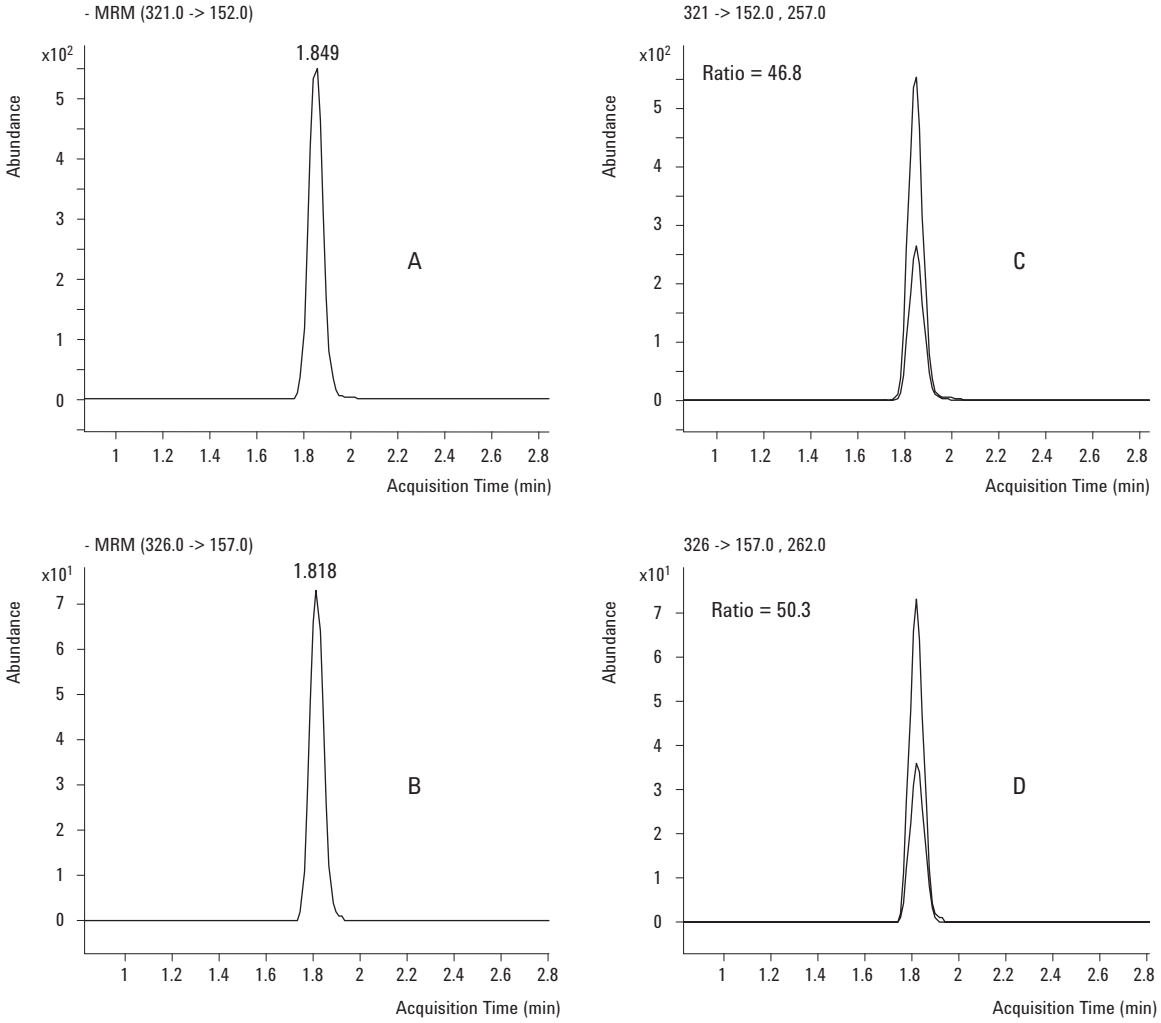


Figure 4. Panels A and B show the CAP and IS peak for the quantitation transition. Panels C and D are the graphic representation of quantitation ion and qualifier ion ratio as shown by MassHunter software.

Linearity

The linearity of the method was determined for CAP in solvent and each of the matrices. This was done from 10 ppt to 1 ppb, well below the minimum required performance level (MRPL) and above that concentration. Figures 5 through 8 show the graphic representation of those results. Each was well above an r^2 value of 0.99.

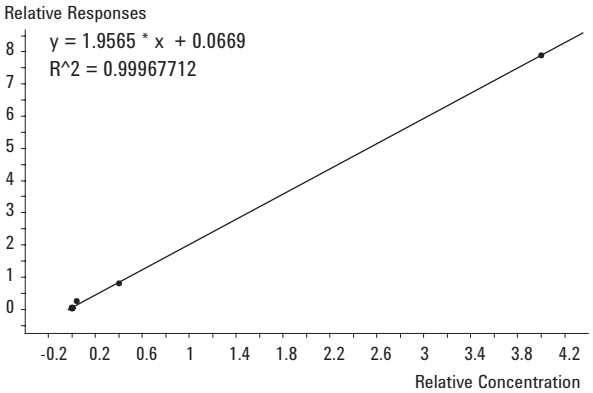


Figure 5. Linearity of CAP in solvent from 10 ppt to 1 ppb.

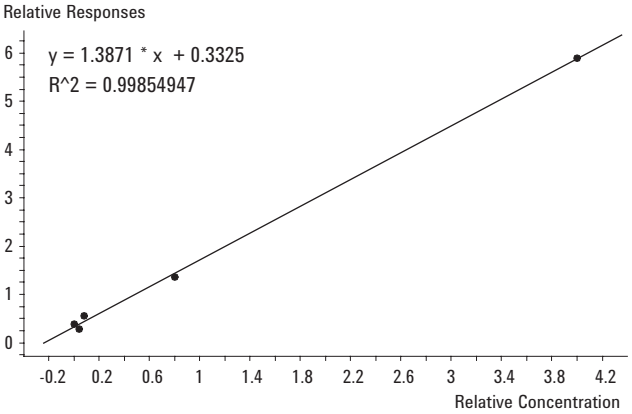


Figure 6. Linearity of CAP in honey from 10 ppt to 1 ppb.

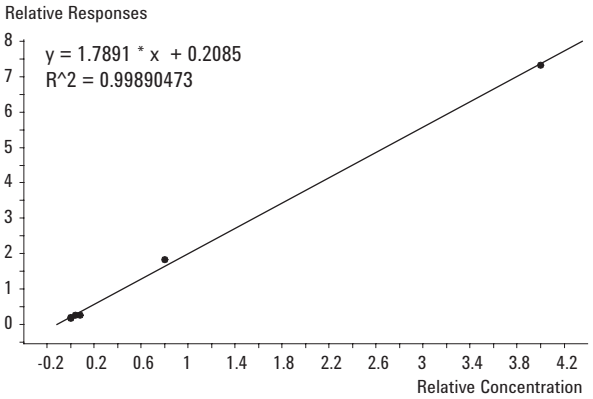


Figure 7. Linearity of CAP in shrimp from 10 ppt to 1 ppb.

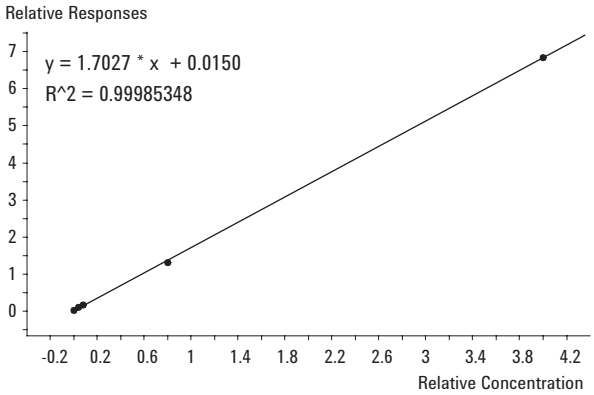


Figure 8. Linearity of CAP in chicken from 10 ppt to 1 ppb.

Sensitivity

The sensitivity of CAP standard in solvent is observed at 10 ppt with an injection volume of 5 µL. The MRM chromatogram is shown in Figure 9. Although this demonstrates the sensitivity of the instrument, it is also important to determine the sensitivity in real sample matrix. This is shown in Figure 10 with a spike concentration of CAP at 10 ppt with a 5-µL injection. Not only is the analyte detectable, but the ratio of the qualifier ion is within the specified tolerance so confirmation can be obtained.

Recovery

Recovery was determined by spiking CAP into three samples of matrix and extracting using the specified SPE. Table 5 shows both the repeatability of extraction and analysis and the mean recovery. Using the internal standard spiked before extraction, recovery is automatically compensated. Thus accuracy of the quantification is very good using this methodology. The recovery results show the overall effectiveness of the method.

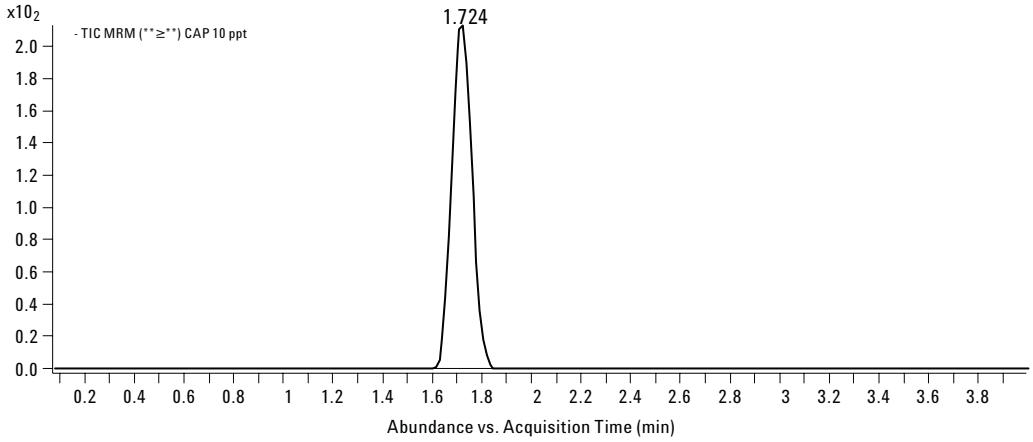


Figure 9. MRM chromatogram of 10 ppt CAP in solvent with injection volume of 5 µL.

Table 5. Recovery of CAP at 0.1 ppb Where Three Sample Aliquots of Each Matrix Were Spiked and Determined

	Honey (n=3)	Shrimp (n=3)	Chicken (n=3)
RSD (%)	6.29	3.93	3.29
Recovery (%)	89.5	85.4	86.4

Conclusions

The method described herein for the analysis of CAP in three important matrices has been shown to be highly effective and meet the criteria for quantitation and confirmation well below the required 0.3 ppb MRPL. Optimization of the method was simple, as few parameters in the mass spectrometer need adjustment. In addition, the requirements for a validated method have been shown. These include sensitivity, repeatability, linearity, and recovery. The Agilent 6410 LC/MS triple quadrupole instrument has been shown to be a highly effective instrument for the analysis of chloramphenicol.

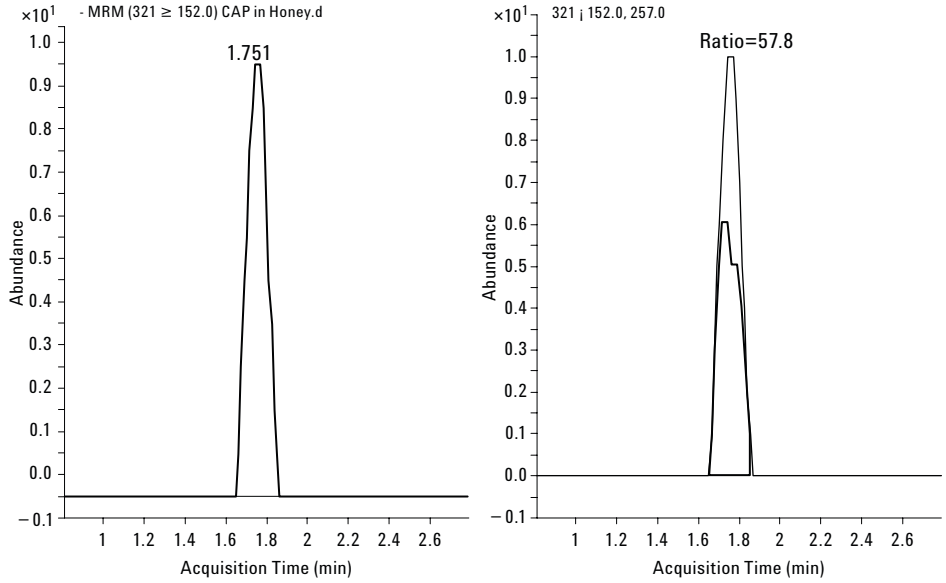


Figure 10. MRM chromatogram of quantitation ion and ratio of qualifier ion for 10 ppt CAP in honey.

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Detection of Recombinant Bovine Somatotropin in Milk by LC-ESI-MS/MS

Application

Food Safety

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Abstract

Recombinant bovine somatotropin (rbST), also called growth hormone, is a protein hormone used in dairy farming to enhance milk production. A method has been developed for the detection of rbST in milk by ESI(+)-LC-MS/MS. This method allowed a detection limit of 20 pg of tryptic N-terminal peptide rbST in standard solution injected on-column and was successfully applied to extracts obtained from milk samples spiked with 50 ng/mL^{-1} (2.3 pmol/mL^{-1}) rbST.

Introduction

Recombinant bovine somatotropin (rbST), also called growth hormone, is used in lactating cows to increase milk production. Different regulations exist regarding its use, but the lack of confirmatory methods [1] for its detection makes it difficult to apply these regulations. It turns out to be an international issue in terms of animal doping and in food safety as well. Indeed, residues of rbST can be present in the milk of dairy animals treated with this hormone.

In order to detect residues of rbST in milk, the choice has been made to focus the analysis on the tryptic N-terminal peptide of the protein, specifically the difference between the endogenous and recombinant forms. The N-terminal amino acid alanine that is present in the endogenous form is replaced by a methionine in the recombinant one [2].

This application describes a method for the detection of rbST by ESI(+) LC-MS/MS. The method was successfully applied to extracts from milk samples spiked with rbST.

Experimental

Standards of Proteins and Peptides

Protein standards of rbST and recombinant equine somatotropin, reST (EquiGen-5), were obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA, USA) and Bresagen Limited (Thebarton, Australia), respectively.

The peptides used as standards, with the following amino acid sequence MFPAMSLSGLFANAVLR (N-terminal tryptic rbST), MFPAMPLSSLFANAVLR (N-terminal tryptic reST), and AFPAMSLSGLFAN-AVLR (N-terminal tryptic bST) were synthesized from Millegen (Labege, France).

Instrumentation

The detail of the instrumentation used for the detection of the N-terminal peptides is described in the following tables.

LC

Instrument	Agilent 1200	
Column	Column Interchrom ModuloCart QS Uptisphere 3HDO 150 mm × 2 mm	
Mobile phase	A: Acetonitrile + 0.1% formic acid B: H_2O + 0.1% formic acid	
Flow rate	0.3 mL/min	
Injection volume	20 µL	
Gradient	Time (min)	%A
	0	10
	5	55
	10	60
	15	100
	17	10
	20	10

MS

Instrument	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode	ESI (+)
Capillary	5000 V
Nebulizer	55 psi
Gas flow	13 L/min
Gas temperature	300 °C

Selected Reaction Monitoring (SRM) Method Parameters

In order to obtain a better specificity, the detection was performed in SRM mode. The transitions monitored are displayed in Table 1.

Table 1. SRM Method Parameters

Compound	RT	Charge	Transitions monitored	Collision energy (V)
Nterm rbST	8.33	z = 2	913.2 → 1047.7	30
			913.2 → 774.1	20
		z = 3	609.3 → 774	10
			609.3 → 643.5	20
Nterm reST	8.39	z = 2	933.2 → 1287.9	30
			933.2 → 794.1	20
Nterm bST	8.20	z = 2	883.2 → 1047.8	20
			883.2 → 774.1	20

Results and Discussion

Application of Triple Quadrupole MS-MS and Electrospray Ionization Mode Methodology

In this method, the choice has been made to use electrospray ionization in positive mode. Indeed, this ionization mode presented as a “soft” ionization technique is optimal for peptides. The ionization of the N-terminal peptide rbST leads to two main forms (z = 2 and z = 3).

This use of a triple quadrupole based methodology enabled very good sensitivity and selectivity and also a possible quantification of the monitored signals.

Separation of the Different Compounds

The detection method was developed for the detection of the tryptic N-terminal peptide of rbST and also for the tryptic N-terminal peptide of endogenous pituitary bovine somatrotropin (bST) and reST as well. Due to the high homology in the amino acid sequence with rbST, reST was used as the internal standard.

The three compounds were separated chromatographically and analyzed utilizing the transitions described in Table 1. The chromatogram corresponding to the injection of 0.2 ng of N-terminal peptide bST, rbST, and reST is shown in Figure 1.

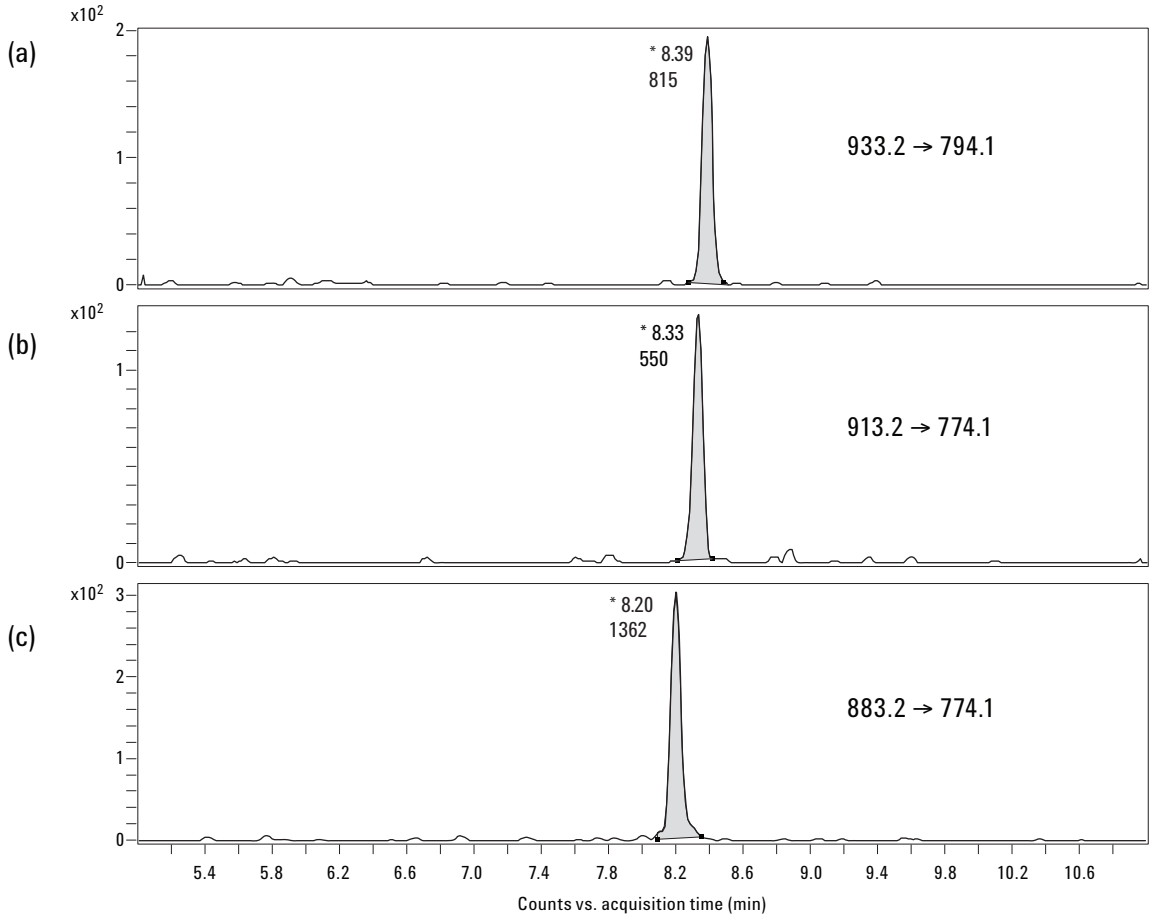

Figure 1. SRM ion chromatograms of standard solutions of tryptic N-terminal peptide of (a) reST, (b) rbST and (c) bST. The injection aliquot used corresponded to 0.2 ng on-column.

Even with an optimized gradient, due to their high homology in terms of sequence, the three compounds eluted with very similar retention times.

Linearity and Sensitivity of the Method

The method described allowed detection of the three peptides with very good sensitivity. A limit of detection of 20 pg injected on-column (~900 femtomole) was reached. Quantification was possible as shown by the good linearity of the calibration curves (Figure 2).

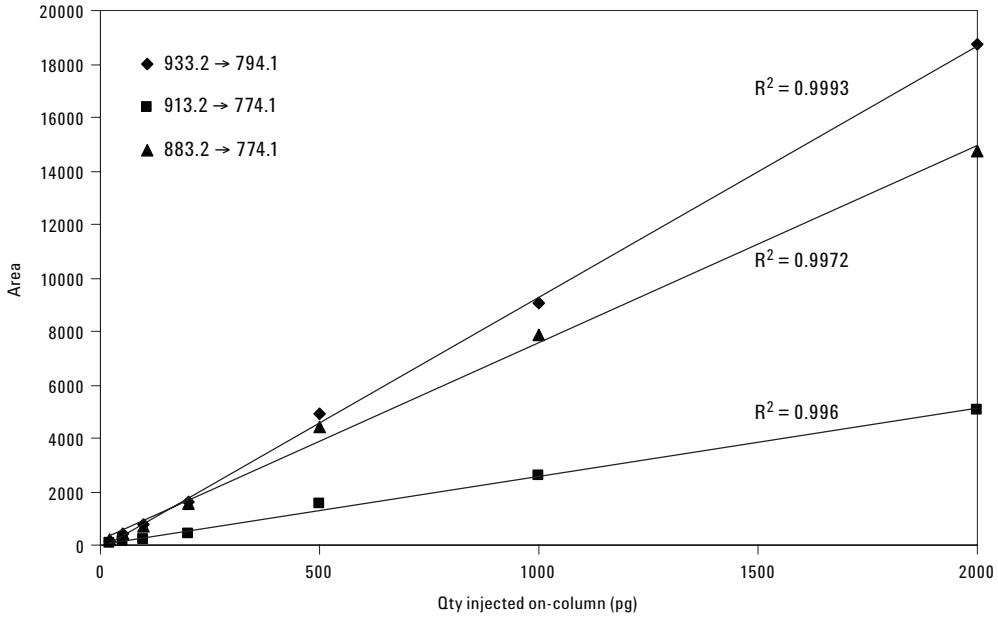

Figure 2. Calibration curve of tryptic N-terminal peptides rbST (913.2 → 774.1), reST (933.2 → 794.1) and bST (883.2 → 774.1).

Results of Spiked Samples

The detection method was applied to extracts obtained from milk samples spiked with rbST. The purification procedure used is described in [3].

Figure 3 shows the chromatogram of a milk sample spiked with 50 ng.mL^{-1} of rbST, in accordance with guidelines for the identification of rbST according to 2002/657 criteria [4]. The chromatogram shows excellent peak shape, and above all, nearly null

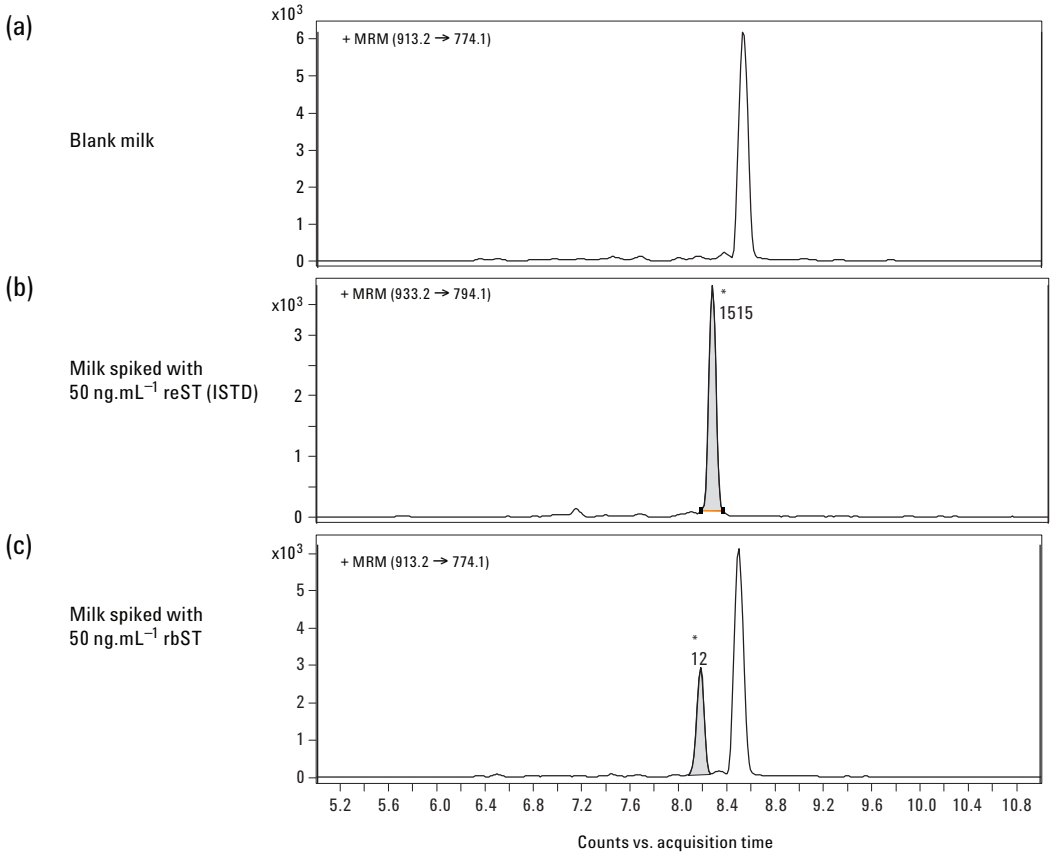

Figure 3. SRM ion chromatograms obtained from milk samples. The different signals correspond to (a) blank milk, (b) the same milk spiked with 50 ng.mL^{-1} reST (internal standard), and (c) 50 ng.mL^{-1} rbST.

background noise, demonstrating the selectivity of the method. The intensity of the signal, although lower than the internal standard, is, however, significant, and shows a clear and distinct signal. The method clearly allows for unambiguous identification of rbST in milk.

Conclusions

The detection of rbST in milk was performed with detection by ESI(+) LC-MS/MS. The method showed very good sensitivity, specificity, and robustness. It was successfully applied to milk samples spiked with rbST at 50 $ng.mL^{-1}$, in accordance with criteria outlined by the 2002/657 Council Directive.

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Development of an LC-MS/MS Method for the Determination of 20-Hydroxyecdysone and Its Metabolites in Calf Urine

Application to the Control of Its Potential Misuse in Cattle

Application Note

Food Safety

Authors

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Abstract

Ecdysteroids, which are steroid hormones present in invertebrates and in plants, could be potentially used as anabolic agents in food-producing animals. The control of ecdysteroid misuse in cattle relies on the development of an efficient method for their detection in biological matrices at trace levels ($\mu g.L^{-1}$). In this context, an analytical procedure dedicated to the identification of 20-hydroxy-ecdysone and its metabolites in urine samples, based on purification on two solid-phase extraction cartridges (SPE C_{18} and SPE SiOH) and LC-(ESI+)-MS/MS measurements has been developed. The performance of tandem quadrupole MS/MS, in terms of sensitivity and specificity, allowed measurements at trace levels in both spiked and incurred samples. Good linearity was observed for all analytes from 0.12 ng to 12 ng on column.

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Introduction

Ecdysteroids are steroid hormones present both in invertebrate species (mainly Arthropods) and plants (belonging to *Asteraceae, Caryophyllaceae,* or *Polypodiaceae*). In arthropods, ecdysteroids act as moulting hormones, whereas these molecules are thought to protect plants against nonadapted phytophagous insects. The archetypal ecdysteroid in both kingdoms is 20-hydroxyecdysone (20E), and several studies have underlined its possible growth-promoting effects in various animal species (rats, mice, and Japanese quail), including humans and cattle [1–3]. Clinical studies demonstrated that 20E is more anabolic than methandrostanolone (dianabol), with no androgenic or other undesirable side effects usually observed with classical steroids [4]. However, despite its growth-promoting properties, only a few methods have been reported for its detection in biological matrices, and no information is available concerning its metabolism in cattle [5]. In this application, the development of a method able to detect and identify 20E and its main metabolites at trace levels (ppb) in calf urine is described [6]. This method was applied to the analysis of calf urine samples after 20E oral administration and used to assess the kinetic of elimination of these substances.

Experimental

Compound Standards

Standard reference 22S,23S-homobrassinolide (belonging to brassinosteroids, vegetable steroid hormones) was from Sigma-Aldrich (St. Quentin Fallavier, France); 20-hydroxyecdysone, 14-deoxy, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone were a kind gift from Pr. Lafont.

Sample Preparation

Twenty-five nanograms of 22S,23S-homobrassinolide were added as internal standard (IS) to 5 mL of calf urine, centrifuged at 3,500 g for 15 min, then purified on SPE C18. The C18-SPE cartridges were conditioned with 10 mL methanol, then 10 mL water, following which the urine samples were applied. The columns were then washed with 6 mL of a water/methanol (80/20) mixture, and the ecdysteroids were subsequently eluted with 10 mL methanol. The eluant was then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 50 µL ethanol and 150 µL cyclohexane before loading onto a SPE SiOH, previously activated with 25 mL cyclohexane. The phase was washed with 6 mL ethyl acetate/cyclohexane (80/20) and the compounds of interest were then eluted with 10 mL of a mixture of chloroform/methanol/acetone (6/2/1). The solvent was evaporated to dryness under nitrogen and the final extract was redissolved in 50 µL of methanol/water (30/70) containing 0.5% acetic acid. From this extract 10 µL was injected onto the HPLC column.

Instrumentation

LC:

Column:	GEMINI C_{18}, Phenomenex (3 µm, 110 Å, 50 × 2 mm)/Agilent equivalent: ZORBAX Extend-C18 3.5 µm, 2.1 mm × 50 mm (p/n 735700-902)	
Column temperature:	40 °C	
Mobile phases:	A: MeOH	
	B: 0.5% acetic acid in water	
Flow rate:	0.3 mL/min	
Gradient:	Time (min)	%B
	0	90
	8	0
	10	0
	12	90
	16	90
Injection volume:	10 µL	
MS:	G 6410A QQQ, Agilent Technologies	
Ionization:	ESI (+)	
Fragmentor:	120 V	
Mass range:	100–500 amu	
Scan time:	300 ms	
Capillary:	4000 V	
Nebulizer:	35 psi	
Drying gas:	11 L/min	
Gas temperature:	325 °C	

The monitored transitions for each target compound are reported in Table 1. The first transition corresponds to the most sensitive signal.

Results and Discussion

Standard solutions of target compounds were analyzed according to the LC-MS/MS parameters described in the Experimental section, which allowed us to obtain the ion chromatograms of 20E, M1, M2, and IS, each at 5 ng on column (Figure 1). All the compounds are eluted within less than 10 min with very good chromatographic resolution and peak shape.

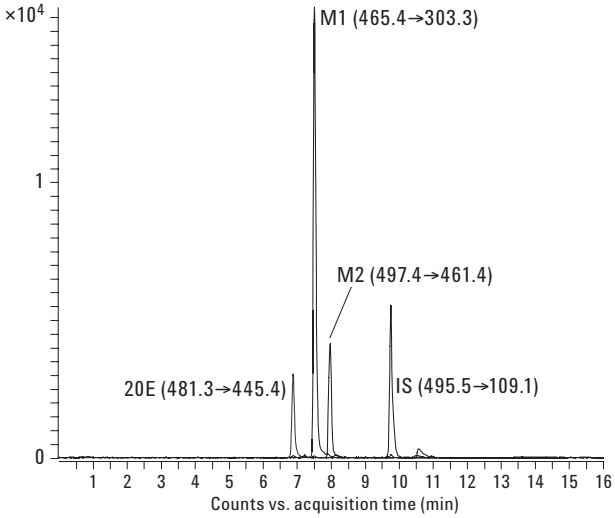

Figure 1. Overlaid extracted ion chromatograms (EICs) for the most sensitive transitions monitored for 20E and its metabolites in positive ion mode.

Table 1. Monitored SRM Transitions for 20E and Its Main Urinary Metabolites and Parameters of Acquisition for Their Analysis by LC-MS/MS (QQQ)

Analytes	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)	Transition 3	Collision energy (eV)	RT (min ± 0.2)
22S,23S-homobrassinolide (IS)	495.5→109.1	20	495.5→127.1	10	495.5→459.1	5	9.8
20-hydroxyecdysone	481.3→445.4	10	481.3→371.4	10	481.3→165.1	20	7.5
14-deoxy,20-hydroxyecdysone (M1)	465.4→303.3	20	465.4→285.3	25	–	–	7.9
20,26-dihydroxyecdysone (M2)	497.3→461.4	5	497.3→351.1	15	497.3→371.2	20	6.8

To assess the specificity of the method, a blank urine and a urine sample fortified with 20E (1 µg.L^{-1}) were analyzed. Figure 2 shows the blank traces without any interference at the expected retention time for 20E, demonstrating the good selectivity of the monitored signals. The target analyte 20E was identified in the spiked urine sample with three SRM transitions. The monitored signals are detected with good sensitivity and show high signal-to-noise (s/n) ratios. These results were in accordance with Decision 2002/657/EC criteria, which require more than four identification points [7] in order to validate an identified compound.

The linearity and the repeatability of the method were assessed with the analysis of a pool of urine samples fortified at different concentration levels: the calibration curve was established with five concentration points (0.2, 0.5, 1, 5, and 20 ng.mL^{-1}). The calibration curve correlation coefficients (R^2) were better than 0.99, thus demonstrating the good linearity of the method for 20E.

The method has been successfully applied to incurred calf urine samples after 20E oral administration over four days. 20-hydroxyecdysone was detected in urine as rapidly as 30 minutes after its administration and up until 24 hours after the last administration. 20E metabolism was investigated and two main metabolites, 14-deoxy,20-hydroxyecdysone (M1) and 20,26-dihydroxyecdysone (M2), could be identified [8]. Both M1 and M2 were monitored by LC-MS/MS (Table 1). Figure 3 presents the ion chromatograms for M1 in the urine samples collected before and two days after the last 20E administration.

As can be observed, M1 was not detected in the urine collected before 20E administration, whereas it was throughout the four-day administration period. Furthermore, it could still be detected and identified (in accordance with the four identification points required) two days after the last administration of 20E. This result is of prime interest in the context of potential misuse of ecdysteroids since it offers the longest period for detection, following administration, and therefore enables a more efficient control mechanism.

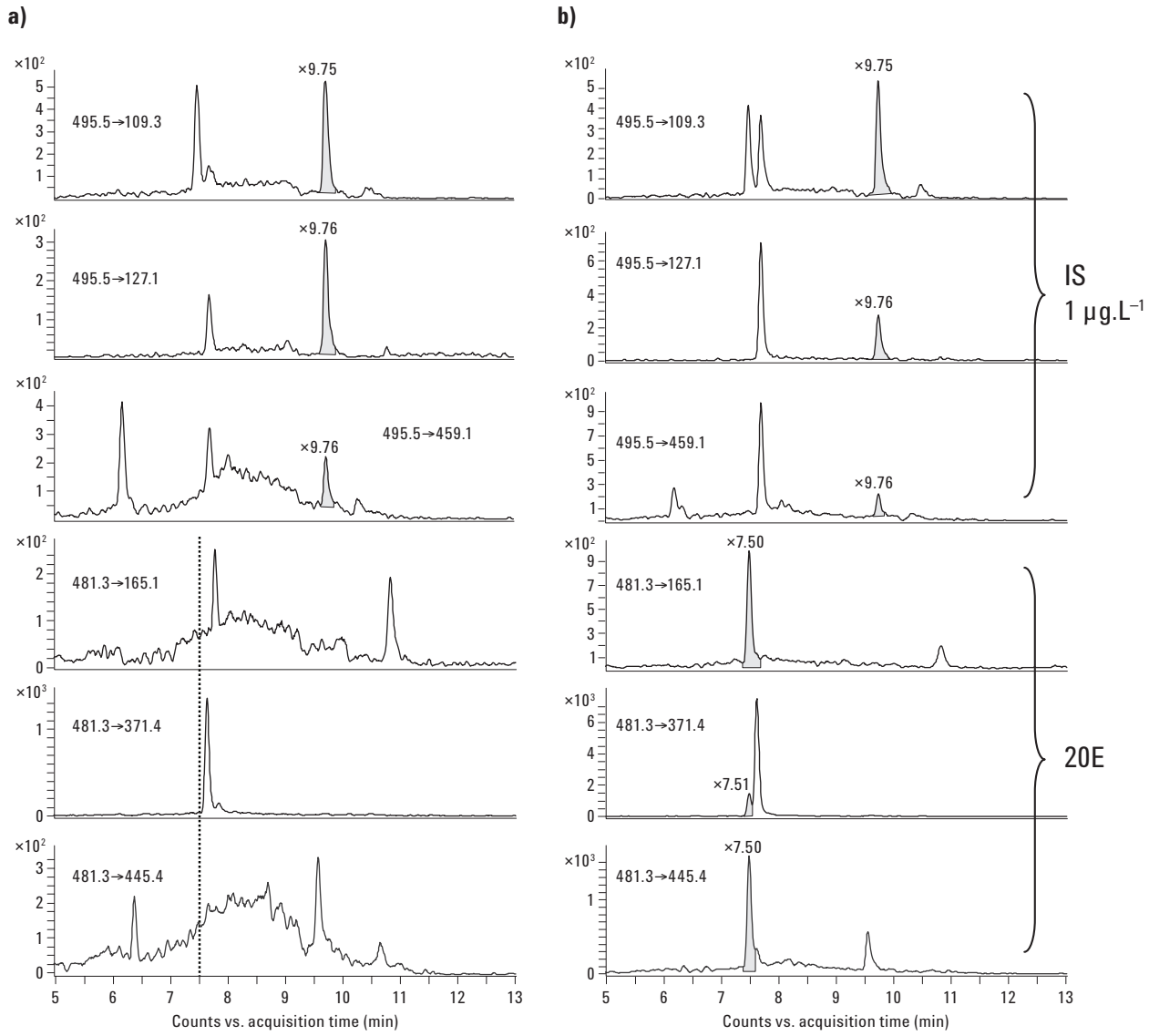

Figure 2. SRM ion chromatograms for a) the blank urine sample and b) the spiked urine sample (1 µg.L-1). LC-(ESI+)-MS/MS measurements.

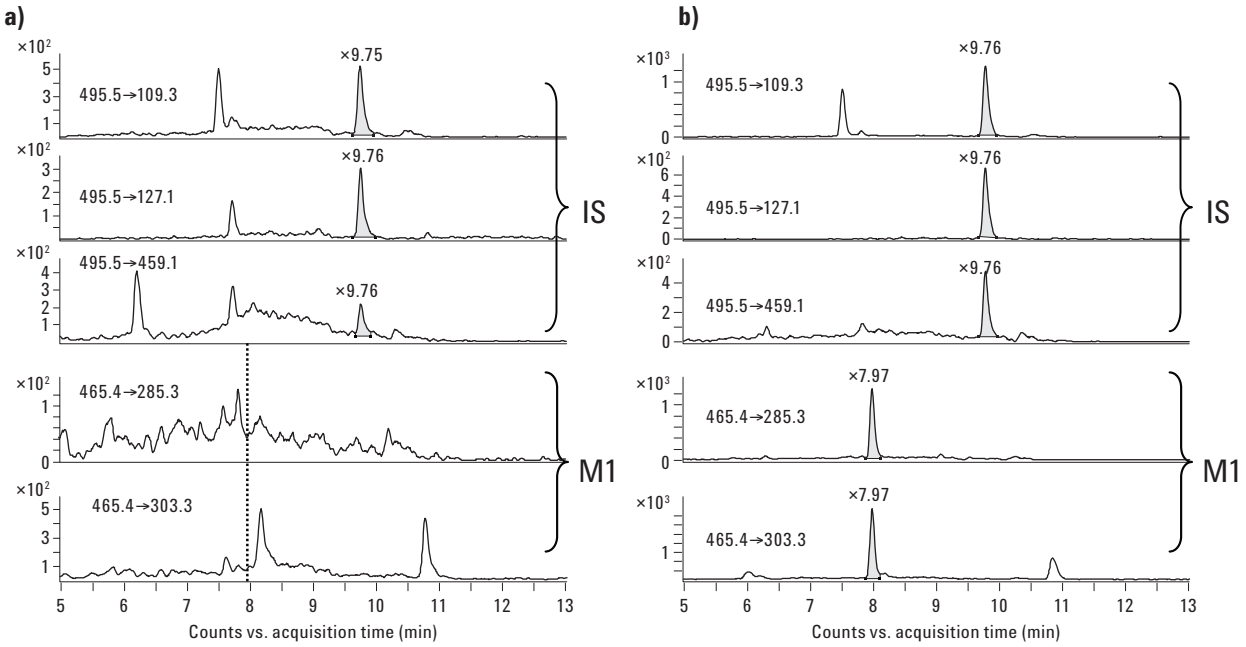

Figure 3. SRM ion chromatograms of IS and M1 in urine sample collected a) before 20E administration and b) two days after the last 20E administration. LC-(ESI+)-MS/MS measurements.

Conclusions

This work demonstrates the performance of LC-MS/MS, which provides efficient identification of 20E and its main metabolites in calf urine. The monitoring of these compounds facilitates the control of the potential misuse of 20E in meat-producing animals. Tandem quadrupole MS/MS is an analytical technique very well suited to this purpose, since it increases confidence in the unambiguous identification of the target compounds, in accordance to the criteria fixed by Decision 2002/657/EC. The successful analysis of the calf urine samples proved the robustness of the developed protocol. Application of this methodology also enabled the determination of the first elimination kinetics and the main metabolites of 20E in calf urine.

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Determination of Tetracyclines in Chicken by Solid-Phase Extraction and High-Performance Liquid Chromatography

Application Note

Food Safety

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Abstract

A method for the simultaneous determination of the seven antibiotic residues of minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, methacycline, and doxycycline in chicken has been developed. In this method, solid-phase extraction (SPE) and HPLC/UV are used consistent with Chinese regulatory methods. Samples are prepared in EDTA-McIlvaine buffer solution (pH 4.0), the clean up is done with an Agilent SampliQ OPT cartridge, and the HPLC separation is performed with an Agilent ZORBAX column (5 µm, 250 mm × 4.6 mm id). The flow rate is 1.5 mL/min, the detector wavelength is 350 nm, and the injection volume is 100 µL. The limits of detection are between 2.5 and 5 µg/kg. Linear calibration curves are obtained over the range of 25 to 500 µg/kg. Overall recoveries range from 59.0 to 99.0%, with RSD values between 1.0 and 6.5%.

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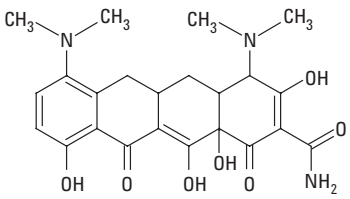
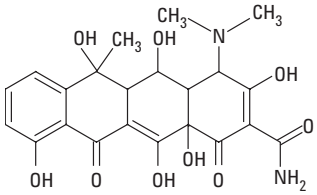
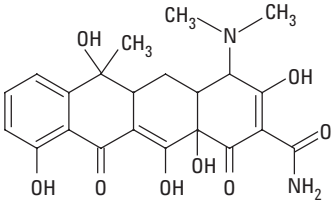
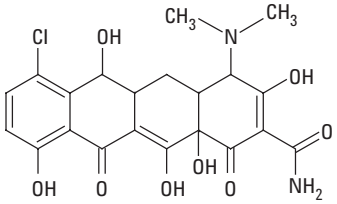
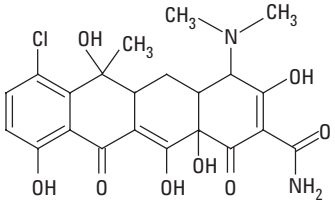
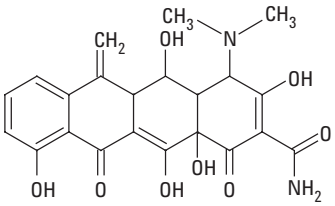
Introduction

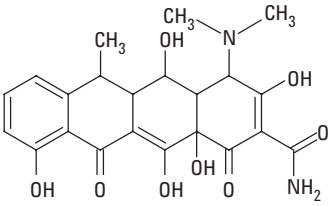
"Tetracyclines" is the common name for a group of antimicrobials with a hydronaphthacene structure (Table 1). Tetracyclines are used against a wide range of gram-negative and gram-positive microorganisms. The Chinese government has set maximum residue limits (MRLs) for tetracyclines in muscle (100 µg/kg) and promulgated a government standard (GB/T 21317-2007) that established a method for the determination of tetracyclines in animal tissues. This application note describes the implementation and optimization of the method described in GB/T 21317-2007 and the results of validation.

Table 1. Tetracyclines Used in This Study

No.	Name	pKa	log P	Structure
1	Minocycline CAS # 10118-90-8	3.3/7.2/9.3	+0.5	
2	Oxytetracycline CAS # 6153-64-6	3.3/7.3/9.1	–0.9	
3	Tetracycline CAS # 60-54-8	3.3/7.7/9.7	–1.3	
4	Demeclocycline CAS # 127-33-3	3.3/7.2/9.3	+0.2	
5	Chlortetracycline CAS # 57-62-5	3.3/7.4/9.3	–0.62	
6	Methacycline CAS # 914-00-1	3.5/7.6/9.2	–0.3	

Continued

Table 1. Tetracyclines Used in This Study (continued)

No.	Name	pKa	log P	Structure
7	Doxycycline CAS# 564-25-0	3.1/7.7/9.3	–0.02	

Experimental

Materials and Chemicals

All reagents and solvents were HPLC or analytical grade. Tetracycline standards were purchased from Sigma-Aldrich or from China's National Institute for the Control of Pharmaceutical and Biological Products (NICPBP).

Stock solution (0.1 mg/mL) was prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with a mixture of methanol/10 mmol/L trifluoroacetic acid solution (1/19). The working solutions were prepared daily.

The SPE cartridges were Agilent SampliQ OPT 3 mL, 60 mg (p/n 5982-3036). The analysis was performed on an Agilent 1200 HPLC with DAD. The analytical column was an Agilent ZORBAX SB-C8 5 µm, 250 mm × 4.6 mm id (p/n 880975-906).

McIlvaine buffer, mix 1000 mL 0.1 mol/L citric acid with 625 mL 0.2 M disodium hydrogen phosphate. Adjust pH to 4.0 ± 0.05 with NaOH or HCl as needed.

Na_2EDTA-McIlvaine buffer (0.1 mol/L), mix 60.5g Na_2EDTA. $2H_2O$ into 1625 mL McIlvaine buffer.

HPLC Conditions

Column:	Agilent ZORBAX SB-C8 250 mm × 4.6 mm, 5 µm
Flow rate:	1.5 mL/min
Column temperature:	30 °C
Injection volume:	100 µL
Detector wavelength:	350 nm
Mobile phase:	Methanol-acetonitrile-10 mmol/L TFA solution, gradient elution

Time (minutes)	% methanol	% acetonitrile	% 10 mmol TFA
0	1	4	95
7.5	6	24	70
13.5	7	28	65
15	1	4	95

Sample Preparation

A 200-g sample of chicken was homogenized with a tissue disintegrator, placed in a clean, sealed container, and stored in a freezer below –18 °C.

A 5-g homogeneous sample (accurate to 0.01 g) was placed into a 50-mL polypropylene centrifuge tube with 20 mL 0.1 mol/L Na_2EDTA-McIlvaine buffer solution and vortex mixed for 1 minute followed by a 10-minute ultrasonic extraction in an ice bath. The sample was then centrifuged at a rotate speed of 3,000 r/min for 5 minutes (below 15 °C). The supernatant was removed and saved in a clean tube. The extraction was repeated twice with 20 mL and 10 mL successively. The combined supernatant fluid was brought to 50 mL with buffer, mixed well, centrifuged at a rotate speed of 4,000 r/min for 10 min (below 15 °C), and filtered with fast filter paper.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. Agilent SampliQ OPT cartridges were preconditioned with 5 mL of methanol, then 5 mL of a 10 mmol/L TFA solution. A 10-mL extract (equivalent to a 1-g sample) was passed through the SampliQ OPT cartridge at a speed of 1 mL/min. After the sample effused completely, the cartridge was washed with 3 mL of water (pH adjusted to 4.5 with TFA). The entire effluent was discarded. The cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, the cartridge was eluted with 10 mL of 10 mmol/L oxalic acid in methanol. The eluent was collected and dried under nitrogen below 40 °C. The resulting residue was dissolved and made to a constant volume of 0.5 mL using the methanol/10 mmol/L TFA solution (1/19). Then the residue was filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed.

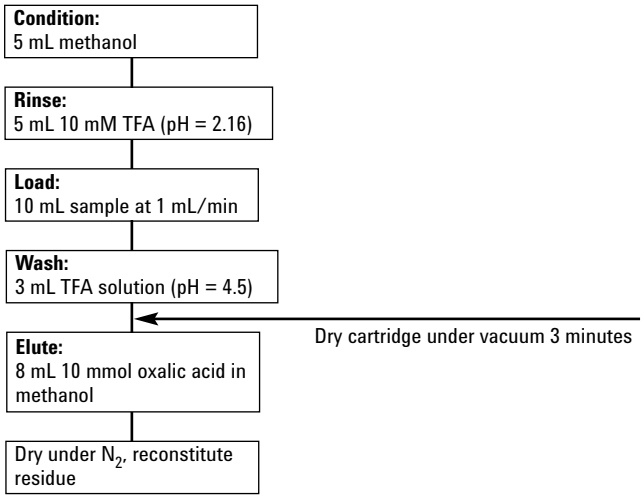


Figure 1. Tetracycline SPE procedure.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the tetracyclines using the areas and the solution concentrations. The limit of detection (LOD) was the injection concentration whose signal-to-noise ratio was between 2 and 3. The linear range was between 25 and 500 µg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Tetracyclines

Compound	Regression equation	Correlation coefficient	LOD (µg/kg)
Minocycline	Y = 86.313 × –0.1491	0.9996	2.5
Oxytetracycline	Y = 95.965 × +0.0261	0.9999	2.5
Tetracycline	Y = 103.97 × –0.4698	0.9999	2.5
Demeclocycline	Y = 68.659 × –0.1172	0.9998	5
Chlortetracycline	Y = 51.752 × –0.0284	0.9999	5
Methacycline	Y = 98.243 × +1.2567	0.9985	2.5
Doxycycline	Y = 76.408 × +1.0756	0.9987	5

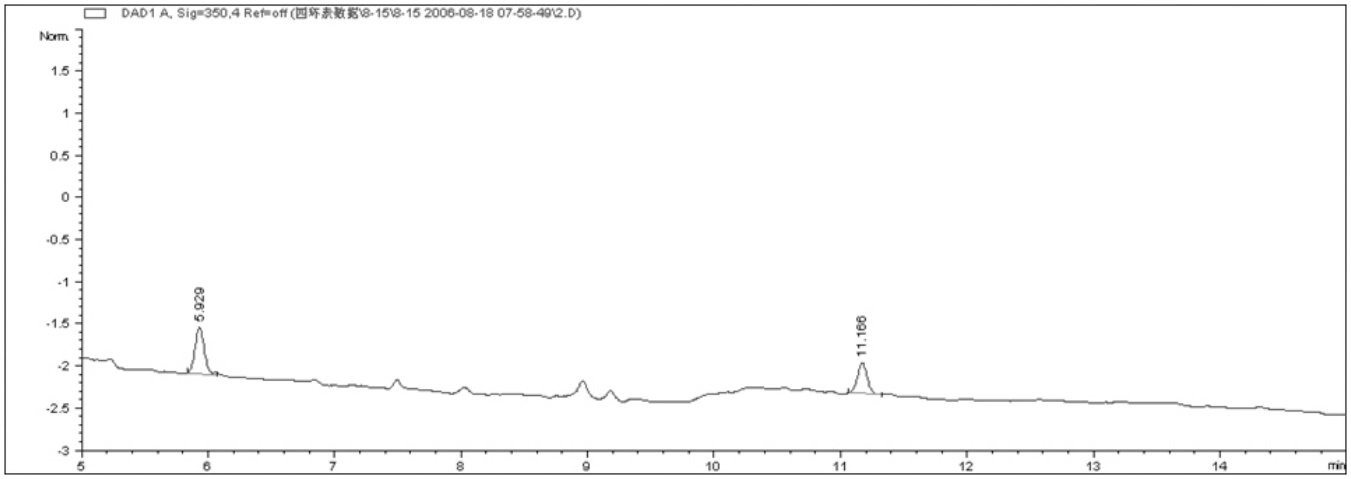


Figure 2. Chromatogram of a chicken blank.

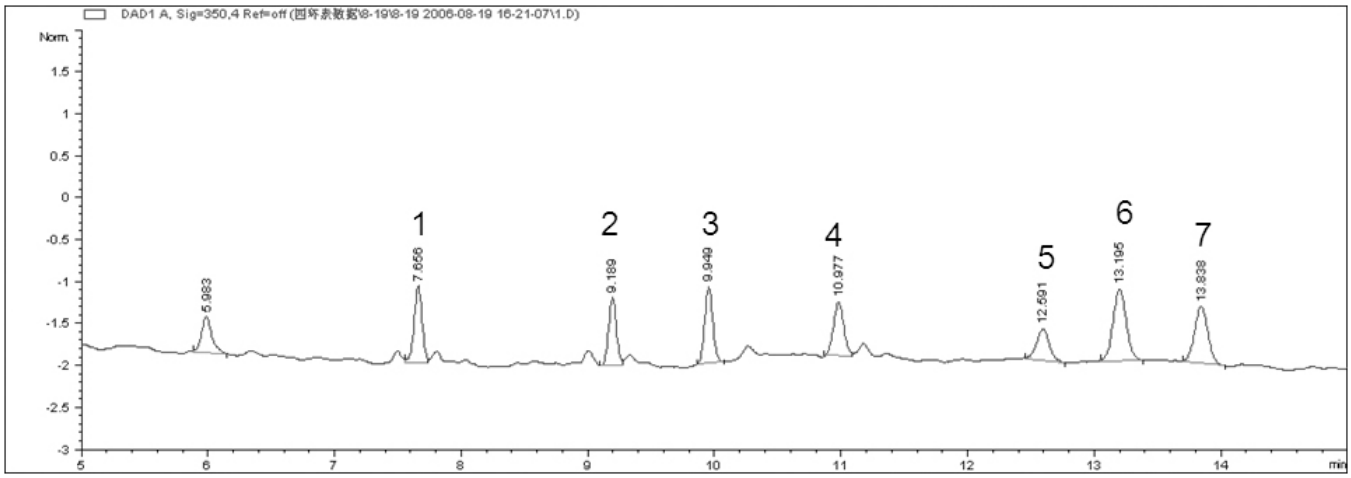


*Figure 3. Chromatogram of a chicken sample spiked at 50 µg/kg. (*1-Minocycline, 2-Oxytetracycline, 3-Tetracycline, 4-Demeclocycline, 5-Chlortetracycline, 6-Methacycline, and 7-Doxycycline)

Recovery and Reproducibility

The precision of the method was determined as recoveries of spiked tetracycline standards in chicken at 50 µg/kg, 100 µg/kg, and 200 µg/kg levels. The analysis was performed in replicates of six at each level. The chromatograms of the blank and spiked standard (50 µg/kg) are shown in Figure 2 and Figure 3. The recovery and reproducibility data are shown in Table 3.

Table 3. Recoveries and RSDs of Tetracyclines in Chicken by SPE

Compound	Spiked level (µg/kg)	Recovery (%)	RSD (%)
Minocycline	50	87.6	4.13
	100	80.8	5.68
	200	81.3	4.19
Oxytetracycline	50	68.8	6.49
	100	63.0	4.87
	200	59.4	4.35
Tetracycline	50	81.0	4.46
	100	70.0	3.47
	200	72.3	4.38
Demeclocycline	50	92.0	2.06
	100	94.8	3.78
	200	92.9	1.92
Chlortetracycline	50	93.3	3.16
	100	92.4	4.01
	200	87.7	2.54
Methacycline	50	93.3	2.89
	100	91.9	2.51
	200	86.6	3.39
Doxycycline	50	95.6	4.38
	100	96.4	1.00
	200	92.0	3.02

Conclusions

Agilent SampliQ provides a simplified and effective single-cartridge method for the purification and enrichment of multiple tetracycline compounds in chicken. The recovery and reproducibility results based on solution standards are acceptable for tetracycline residue determination in chicken under the Chinese regulation. The impurities from chicken were minimal and did not interfere with any of the tetracyclines analyzed. The LODs of the seven tetracyclines were significantly lower than the MRL (of 100 ug/kg).

Part number	Description
5982-3013	OPT Polymer - Box, 100 × 1 mL tubes, 30 mg
5982-3036	OPT Polymer - Box, 50 × 3 mL tubes, 60 mg
5982-3067	OPT Polymer - Box, 30 × 6 mL tubes, 150 mg
5982-3096	OPT Polymer - 96 Well Plate, 10 mg

References

GB/T 21317-2007, Determination of tetracyclines residues in food of animal origin-LC-MS/MS method and HPLC method.

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Agilent Technologies

Determination of Penicillins in Meat by High Performance Liquid Chromatography (HPLC/UV) and HPLC/MS/MS

Application Note

Food

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Abstract

Penicillins are antibiotics widely used to treat diseases in animals. They are occasionally found in animal products destined for human consumption. In this paper, a solid phase extraction method with a high performance liquid chromatograph tandem mass spectrometer (HPLC/MS/MS) is shown for the simultaneous determination of six antibiotic residues: azlocillin, penicillin G, oxacillin, cloxacillin, nafcillin, and dicloxacillin in animal tissues (porcine muscle). In the method, the reversed phase column Agilent ZORBAX Eclipse Plus C18 (3.5 µm, 100 mm × 2. 1 mm) and an Agilent mixed mode polymer solid phase extraction cartridge (Agilent SampliQ OPT) were combined to give a total solution to the analysis of residual penicillins. The performance of the solid phase extraction procedure on trace residues is quantitatively evaluated by HPLC/MS/MS.

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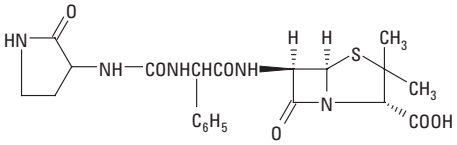
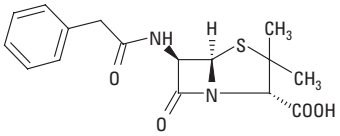
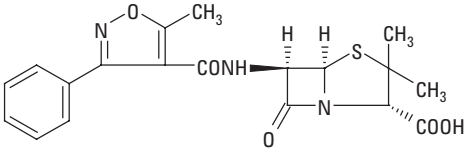
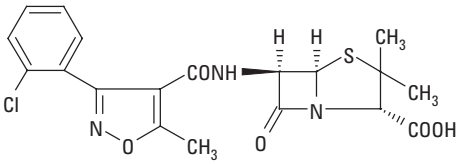
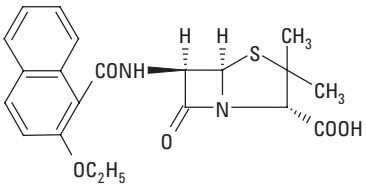
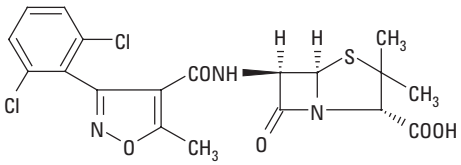
Introduction

ß-lactam antibiotics (penicillins and cephalosporins) represent some of the most important antibacterial agents used in animals. However, serious reactions are known to occur in some individuals exposed to penicillins and, as a result, these compounds are carefully monitored in foods. Maximum residue limits (MRLs) for pencillins in a variety of foods are established worldwide and are generally in the range of 1ng/g. These regulations require detection and quantification by HPLC/MS/MS. This application will show the development of a sample extraction and cleanup method and the quantification by LC/MS/MS

The structures and chemical constants for the compounds used in this study are shown in Table 1. ß-lactam antibiotics are readily decomposed in acid or base [1] and experimentally show a 20 percent loss in 70 hours at 4 °C (data not shown). Thus, it is necessary to perform the extractions, cleanup, and analysis within 36 hours.

Table 1. The Compounds in This Study

No.	Name	log P.	pKa	Structure
1	Azlocillin	0.2	2.8	
2	Penicillin G	1.5	2.74	
3	Oxacillin	2.4	2.72	
4	Cloxacillin	2.6	2.78	
5	Nafcillin	3.3	2.65	
6	Dicloxacillin	3.7	2.8	

Experimental

Reagents and Chemicals

Water, acetonitrile, and methanol are all HPLC grade (Honeywell Burdick & Jackson). The standards and other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO).

Phosphate buffer (pH = 8.5), 0.05 mol/L: dissolve 8.7 g of potassium phosphate dibasic in HPLC grade water to make 1 L.

Standard stock solutions (1 mg/mL) were made fresh daily in methanol. Spiking solutions were made by appropriate dilution of the stock solutions in phosphate buffer.

Equipment

Agilent 1100 HPLC with diode array detector (Agilent Technologies, Inc., Santa Clara, CA, USA)

Agilent 6410 triple quadrupole LC/MS system with electrospray ionization source (Agilent Technologies, Inc., Santa Clara, CA, USA)

Polytron homogenizer (Brinkman Instruments, Inc., PT10-35, USA)

Refrigerated centrifuge (Sorvall Instruments, RC-5B, rotor SA-600)

Rotary evaporator (BÜCHI, Switzerland/USA)

Sample Preparation

Extraction

Weigh 5 g of raw ground pork (accurate to 0.01 g) into a 50 mL capped polypropylene centrifuge tube, add 15 mL of acetonitrile/water (15:2). Homogenize completely using the Brinkman Polytron (1 minute). Centrifuge at 4,000 rpm and 4 °C for 5 minutes. Save the supernatant. Add 10 mL acetonitrile/water (15:2) to pellet, mix with a spatula to resuspend the ground meat. Homogenize for 1 minute. Centrifuge at 4,000 rpm and 4 °C for five minutes. Combine supernatants. Repeat one additional time.

Take the combined supernatants and place into a round bottom flask and evaporate the acetonitrile at 37 °C. There should be approximately 6 mL of water remaining in the flask. Bring the total volume to 20 mL using pH 8.5 phosphate buffer. Filter with a regenerated cellulose, 25 mm, 45 µm syringe filter (Agilent p/n 5185-5831). Load 10 mL of extract onto the Agilent SampliQ OPT 6 mL/150 mg SPE cartridge (Agilent p/n 5982-3067).

Purification

The procedure for SPE extraction is shown in Figure 1. Load 10 mL of the extract onto the conditioned and equilibrated cartridge. The cartridge is washed with 0.1 percent formic acid in water and then pH 8.5 potassium phosphate buffer. Finally, the sample is eluted with 3 mL acetonitrile. The sample is filtered with a 13 mm, 45 µm PTFE syringe filter (Agilent p/n 5185-5836). The eluent is dried under nitrogen at room temperature. The residue is resuspended in mobile phase to 1.0 mL. The sample is vortexed for 2 minutes and then transferred to a 2 mL autosampler vial (Agilent p/n 5182-0864).

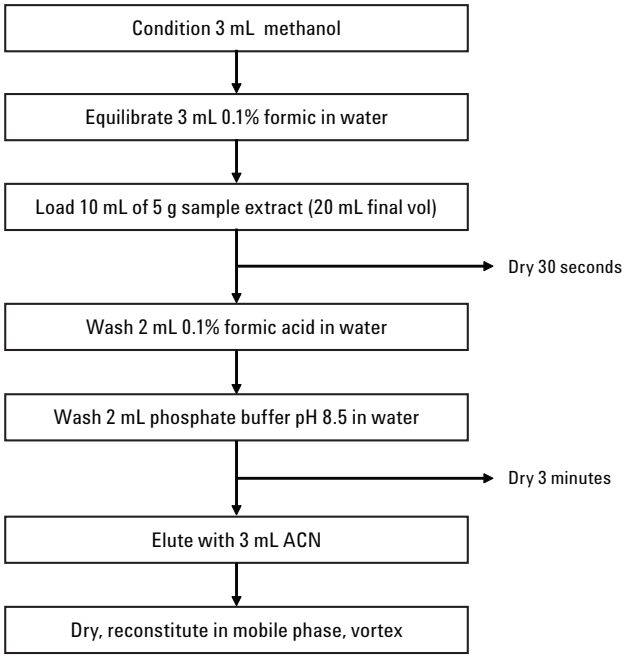


Figure 1. Agilent SampliQ OPT solid phase extraction of penicillins from pork.

Instrument Setting

The HPLC conditions are shown in Tables 2 and 3.

Table 2. HPLC Conditions

HPLC	
Column	Agilent ZORBAX Eclipse Plus, 2.1 mm × 100 mm, 3.5 µm (p/n 959793-902)
Flow rate	0.6 mL/min
Mobile phase	A: water/10 mM ammonium acetate B: acetonitrile
Run time	12 minutes
Post run	3 minutes
Temperature	30 °C
Injection	10 µL

Table 3. HPLC Gradient

Time	%B
0	2
1.2	2
2.0	10
6.0	30
8.0	40
8.5	80
11.9	80
12.0	2

Results and Discussion

Figure 2 shows the chromatograms of the meat spike sample at the limit of quantification (LOQ), 1.0 ng/g (2a), the matrix spiked blank at the LOQ (2b) and the meat extract blank (2c). The cleaned-up pork extract does not show any interferences with the target analytes. HPLC/UV is a significantly less specific detector than HPLC/MS/MS so the impurities remaining after cleanup are more visible using the general UV detector. As shown in Figure 3, the HPLC/UV chromatogram demonstrates that the sample is extremely clean after the sample extraction and SPE cleanup.

Matrix blank material is prepared by taking the meat through the entire extraction and sample cleanup procedure. External standard calibration curves in spiked matrix blanks are made at concentrations of 0.2, 1.0, 10, and 20 ng/g. Table 4 shows the calculated recoveries of spiked meat taken through the entire sample preparation and SPE procedures. All data were calculated automatically with the Agilent MassHunter Quantitative Data Analysis software. Figure 4 shows these results graphically. All of the compounds show acceptable recovery and low relative standard deviation (%RSD).

Conclusions

The results of this study show that the Agilent SampliQ OPT cartridge provides an effective method for cleaning up complex food samples such as porcine muscle. This is demonstrated using penicillins as target compounds. HPLC/UV is

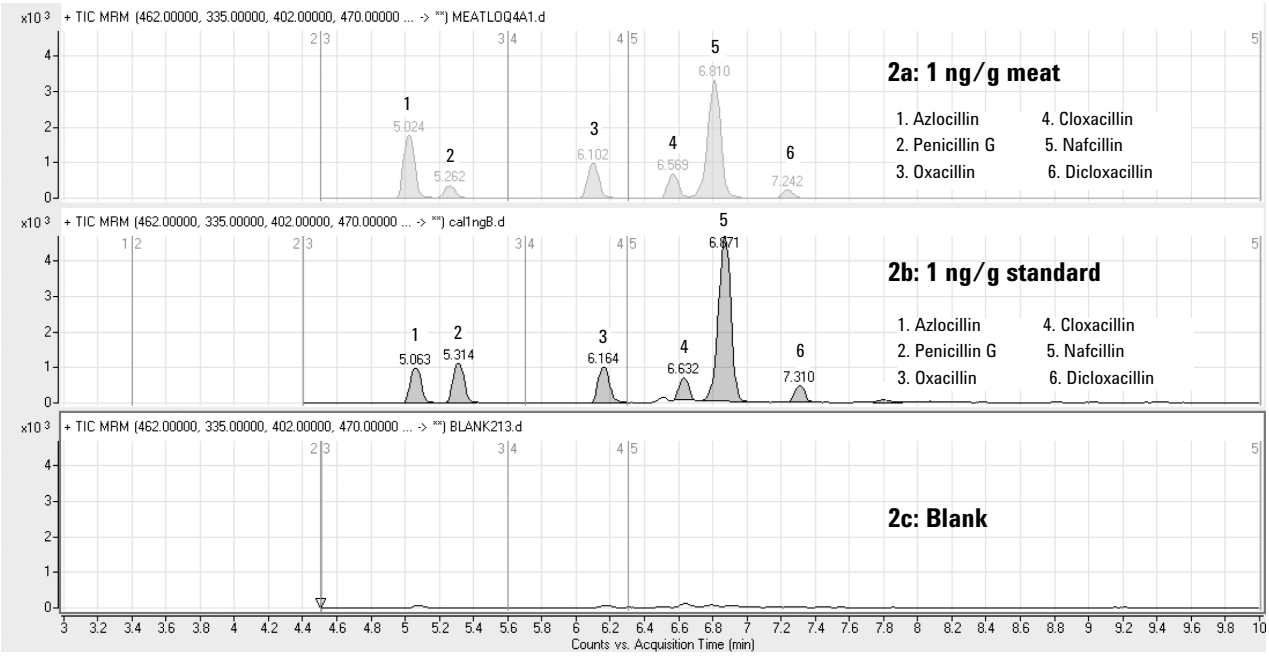


Figure 2. Meat spiked at 1 ng/g taken through extraction and SPE clean-up (2a), meat taken through extraction and clean-up then spiked at 1 ng/g (2b), and unspiked meat taken through extraction and cleanup (2c).

Table 4. Calibration Results for Spiked Meat Blanks

			1.0 ng/g		20 ng/g	
Name	**Linear regression**	**R^2**	**% recovery n = 6**	**%RSD n = 6**	**% recovery n = 6**	**%RSD n = 6**
Azlocillin	y = 6089x –1283	0.9590	77.8	24.2	75.5	6.7
Penicillin G	y = 2690x –513	0.9924	43.6	6.3	48.8	6.8
Oxacillin	y = 2319x +1487	0.9794	96.5	6.0	102.8	8.9
Cloxacillin	y = 2229x +128	0.9848	86.3	7.3	95.3	8.1
Nafcillin	y = 16654x –1264	0.9891	98.5	6.6	101.9	3.1
Dicloxacillin	y = 1899x –113	0.9870	105.7	13.5	103.9	3.6

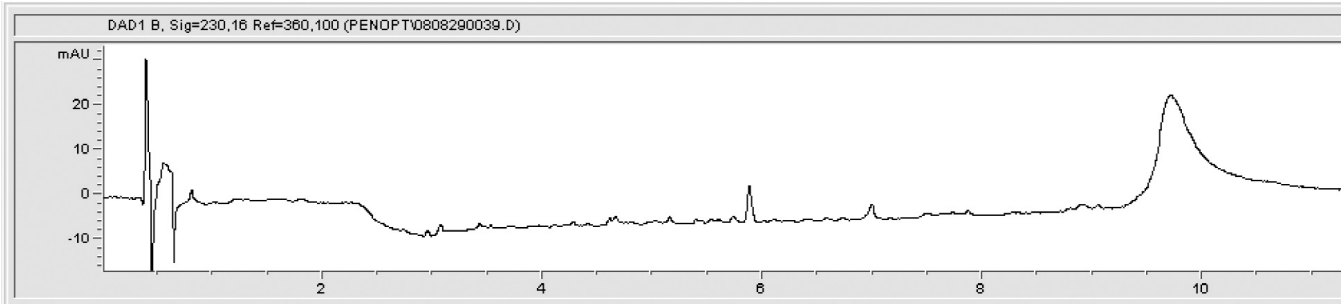


Figure 3. HPLC/UV chromatogram of an unspiked meat sample taken through extraction and SPE cleanup. Wavelength 230 nm.

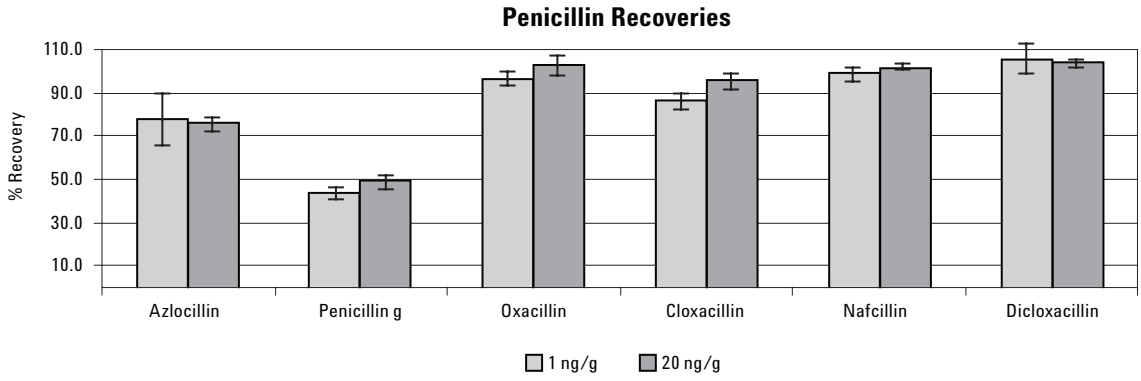


Figure 4. Recovery data for meat extracts at 1.0 and 20 ng/g.

used as a detector to demonstrate the extent of cleanup, which is found to be excellent. The LC/MS/MS is used to demonstrate the recovery of the penicillins at trace level. Even with extensive extraction and SPE cleanup, recoveries are acceptable and reproducibilities are excellent.

Reference

1. Xinbo Lu, Huabin Xing, Baogen Su, and Qilong Ren, Effect of Buffer Solution and Temperature on the Stability of Penicillin G, *J. Chem Eng.* 53 (2), 543–547, 2008

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Determination of Chloramphenicol, Florfenicol, and Thiamphenicol in Honey Using Agilent SampliQ OPT Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Food Safety

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Abstract

A method for the simultaneous determination of three antibiotic residues of chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) in honey has been developed and validated. The analytes are purified by liquid/liquid extraction and solid-phase extraction (SPE) and are quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in negative ion multiple reaction monitoring (MRM) mode. Chloramphenicol-D_5 is used as the internal standard. The method is validated by achieving reproducible, satisfactory, quantitative results. The method provides a sub-ng/g to ng/g level of limit of quantitation (LOQ) for all three antibiotics in honey. The overall recoveries range from 74.9 to 107% with RSD values between 0.5 and 9.7%. The dynamic calibration ranges for chloramphenicol and florfenicol are obtained over 0.1 to 20.0 ng/g and 1.0 to 20.0 ng/g for thiamphenicol. The method is demonstrated to be fast, simple, and efficient for monitoring chloramphenicol, florfenicol, and thiamphenicol residues in honey.

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Introduction

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic antibiotic, obtained originally from the bacterium *Streptomyces venezuelae*. Due to potential side effects in humans, the drug is not recommended for the treatment of minor diseases, but is reserved for the treatment of serious infections. In veterinary medicine, CAP has been shown to be a highly effective, well-tolerated antibiotic; the potential side effects observed in humans have not been reported in animals. However, because of its toxicity in humans, the use of CAP in animal-derived foods, including honey from honeybees, has been strictly regulated. The European Union (EU) has defined a maximum residue limit (MRL) for CAP in food of animal origin at a level of 0.3 µg/kg [1], while China has an MRL level of 0.5 µg/kg [2]. Thiam-phenicol (TAP) and florfenicol (FF) are the analogue compounds of CAP. They can be used as a replacement veterinary antibiotic for CAP in many countries. The MRLs have been set for TAP (50 ng/g) and FF (100 ng/g) in food to date [3]. Table 1 shows the chemical structure and properties of these three compounds. This application note describes a method for the simultaneous determination of three phenicols in honey, and the results of validation.

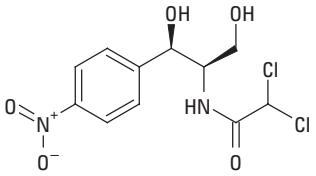
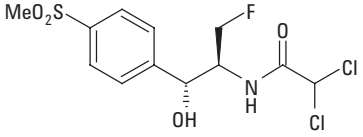
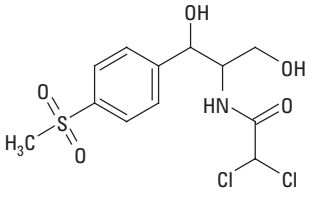
Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile and methanol were from Honeywell, Burdick & Jackson (Muskegon, MI); ethyl acetate was from J.T.Baker (Phillipsburg, NJ). Dimethyl sulfoxide was from Sigma-Aldrich (St. Louis, MO). The standards and other chemicals were purchased from Sigma-Aldrich.

Water (pH 8.5) was prepared by pH adjustment of Milli-Q water with 0.05% NH_4OH in water solution monitored by a pH meter. A solution of 20:80 methanol/ethyl acetate was prepared by combining 40 mL of methanol and 160 mL of ethyl acetate and mixing well. A solution of 20:80 acetonitrile/H_2O was prepared by adding 40 mL of acetonitrile into 160 mL of Milli-Q water.

Table 1. Chemical Structure and Properties of Target Analytes

Compounds	Log P	pKa	Structure
Chloramphenicol	1.02	9.61	
Florfenicol	–0.12	9.03	
Thiamphenicol	–0.27	9.76	

Standard stock solutions (1.0 mg/mL) were made in dimethyl sulfoxide (DMSO) individually, and stored in the refrigerator at 4 °C. A combined working solution (2,500 ng/mL) was made weekly in 20:80 ACN/H_2O, and also stored at 4 °C. The spiking solutions were then made daily by appropriate dilution of the combined working solution in Milli-Q water or 20:80 ACN/H_2O.

Internal standard (IS) stock solution (0.1 mg/mL) was made in DMSO and stored in the refrigerator at 4 °C. An IS spiking solution (50 ng/mL) was made weekly by appropriate dilution of stock solution into Milli-Q water, and stored at 4 °C.

Equipment and Materials

Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent 6410 Triple Quadrupole LC/MS/MS system with electrospray ionization source (Agilent Technologies Inc., Santa Clara, CA, USA)

Agilent SampliQ OPT solid-phase extraction cartridges, 50 × 3 mL tubes, 60 mg (p/n 5982-3036) (Agilent Technologies Inc., Wilmington, DE, USA)

CentraCL3R centrifuge (Thermo IEC, Needham Heights, MA, USA)

N_2 dryer (Glas-Col, Terre Haute, IN, USA)

Sample Preparation

Liquid-Liquid Extraction

5g of honey (± 0.05 g) was weighed into a 50 mL capped polypropylene tube. 0.5 mL of IS spiking solution (50 ng/mL) was added to the tube and vortexed until mixed. This was followed by the addition of 5 mL of Milli-Q water and vortexing for 3 minutes to mix the sample thoroughly. 5 mL of ethyl acetate was then added, capped tightly, and the tubes shaken for 5 minutes. The tubes were then centrifuged at 3,200 rpm for 5 minutes, before the upper organic layer was carefully transferred to another tube. Ethyl acetate addition, shaking, centrifuging, and organic layer transfer was repeated two more times with all supernatants combined. Samples were evaporated to dryness with a controlled N_2 flow drier at 50 °C before being reconstituted into 5 mL of Milli-Q water, vortexed, and sonicated to completely dissolve the residue. The sample was then ready for SPE purification. Figure 1 shows the extraction procedure flowchart.

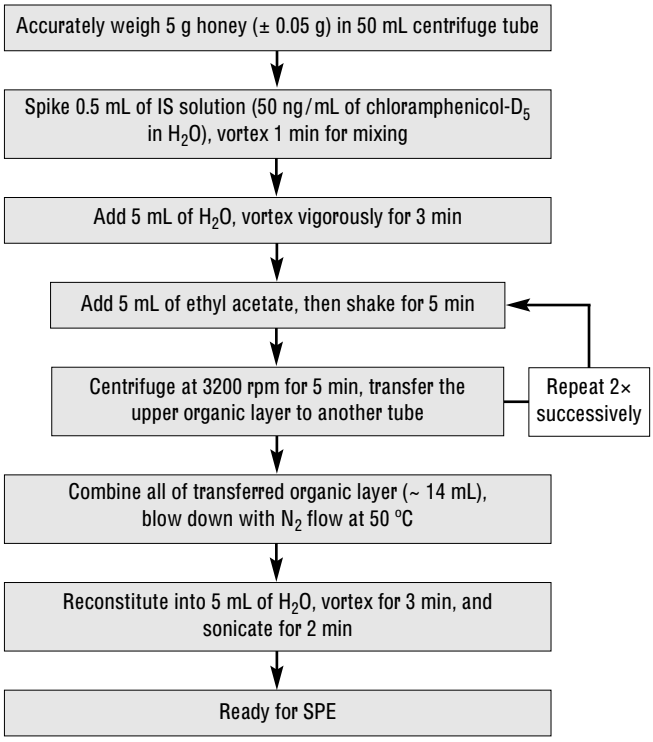


Figure 1. Sample preparation – liquid liquid extraction of phenicols in honey.

Solid-Phase Extraction

The procedure for SPE extraction is shown in Figure 2. Agilent SampliQ OPT cartridges were preconditioned with 3 mL of MeOH, and then equilibrated with 5 mL of water. The 5 mL sample extract was then loaded onto a cartridge and passed through the cartridge slowly by gravity (0.5 mL/min). The tubes were rinsed with 5 mL of Milli-Q water twice. Repeat the above wash procedure once. The entire effluent was discarded. Apply full vacuum to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 20:80 MeOH/ethyl acetate (2.5 mL × 2) at a rate of 1 mL/min. The eluent was collected into clean tubes and dried under N_2 flow at 50 °C. The residue was reconstituted in 0.5 mL of 20:80 AcN/H_2O. The sample was vortexed and sonicated to completely dissolve the residue in the tubes. The sample was transferred to a centrifuge tube and centrifuged at 3,200 rpm for 2 minutes. The samples were then transferred to 2 mL autosampler vials for analysis.

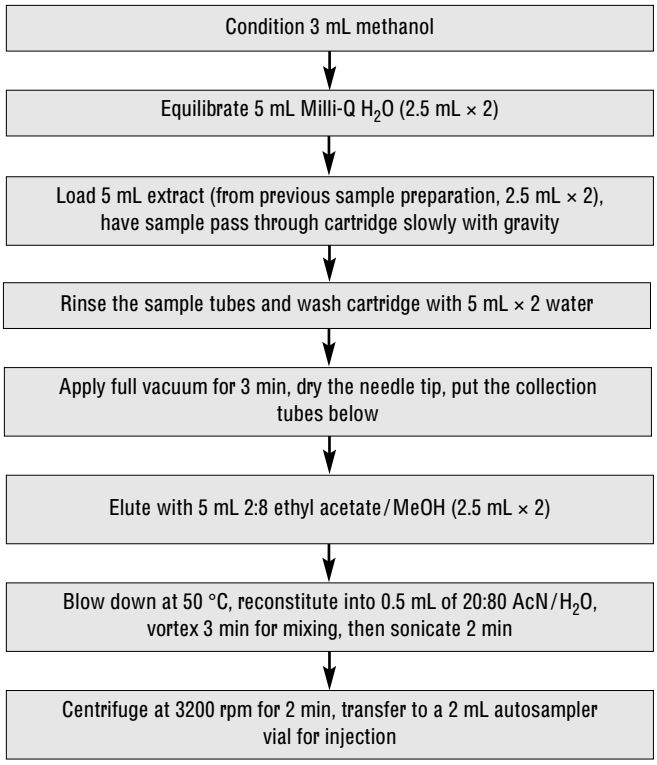


Figure 2. Sample clean-up – Agilent SampliQ solid-phase extraction.

Instrument Conditions

HPLC Conditions

Column:		Agilent ZORBAX Eclipse Plus 150 mm × 2.1 mm, 5 µm (PN: 959701-906)	
Flow rate:		0.3 mL/min	
Column temperature:		30 °C	
Injection volume:		20 µL	
Mobile phase:		pH 8.5 H_2O (A), Acetonitrile (B)	
Gradient:	Time	% Acetonitrile	Flow rate (mL/min)
	0	20	0.3
	0.5	20	0.3
	6.0	80	0.3
	6.01	100	0.5
	6.50	100	0.5
	6.51	20	0.3
	7.00	STOP	

MS Conditions

The three compounds were monitored in the negative ionization mode. The multiple reaction monitoring channels are shown in Table 2.

Table 2. Masses Monitored in the Multiple Reaction Monitoring (MRM) Experiment

Analyte	MRM (*m/z* → *m/z*)	Dwell time (ms)
Thiamphenicol	354.0 → 184.9 (quantifier)	50
	354.0 → 290.0 (qualifier)	25
Florfenicol	355.8 → 185.0 (quantifier)	50
	355.8 → 336.0 (qualifier)	25
Chloramphenicol	320.9 → 152.0 (quantifier)	50
	320.9 → 176.0 (qualifier)	25
Chloramphenicol-D_5 (IS)	325.9 → 156.8	25

Results and Discussion

Linearity, Limit of Detection

The extracted ion chromatograms of fortified honey at a concentration of 0.2 ng/g are shown in Figure 3. The extracted honey blank was clean and free from any analytes, indicating that the cleaned-up honey extract does not contribute any interference with the target analysis.

The concentration ranges studied here are significantly below the limit of quantitation (LOQ) defined by the MRL for TAP (50 ng/g) [3]. In this study the limit of quantitation (LOQ) found for TAP is 1.0 ng/g, and the linear calibration range used for TAP is 1.0 to 20.0 ng/g. The linear calibration range for CAP (LOQ 0.1 ng/g, MRL 0.3 ng/g) and FF (LOQ 0.1 ng/g, MRL 100 ng/g) was 0.1 to 20.0 ng/g.

Calibration curves spiked in matrix blanks were made at levels of 0.1, 0.2, 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g for CAP and FF. While for TAP they were spiked at a level of 1.0, 5.0, 10.0, 15.0, and 20.0 ng/g. The chloramphenicol-D_5 was used as internal standard at 5 ng/g level. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte/peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The limit of detection (LOD) was determined with a signal-to-noise ratio between 4 and 5. Table 3 shows the linearity equation, correlation coefficient (R^2) and LOD. The calibration curve for chloramphenicol is shown in Figure 4.

Table 3. Linearity and LODs of Phenicols

Analytes	Regression equation	R^2	LOD (ng/g)
Chloramphenicol	Y = 0.5643X – 0.0001	0.9957	0.02
Florfenicol	Y = 0.8790X + 0.0006	0.9932	0.02
Thiamphenicol	Y = 0.1510X – 0.0018	0.9953	0.20

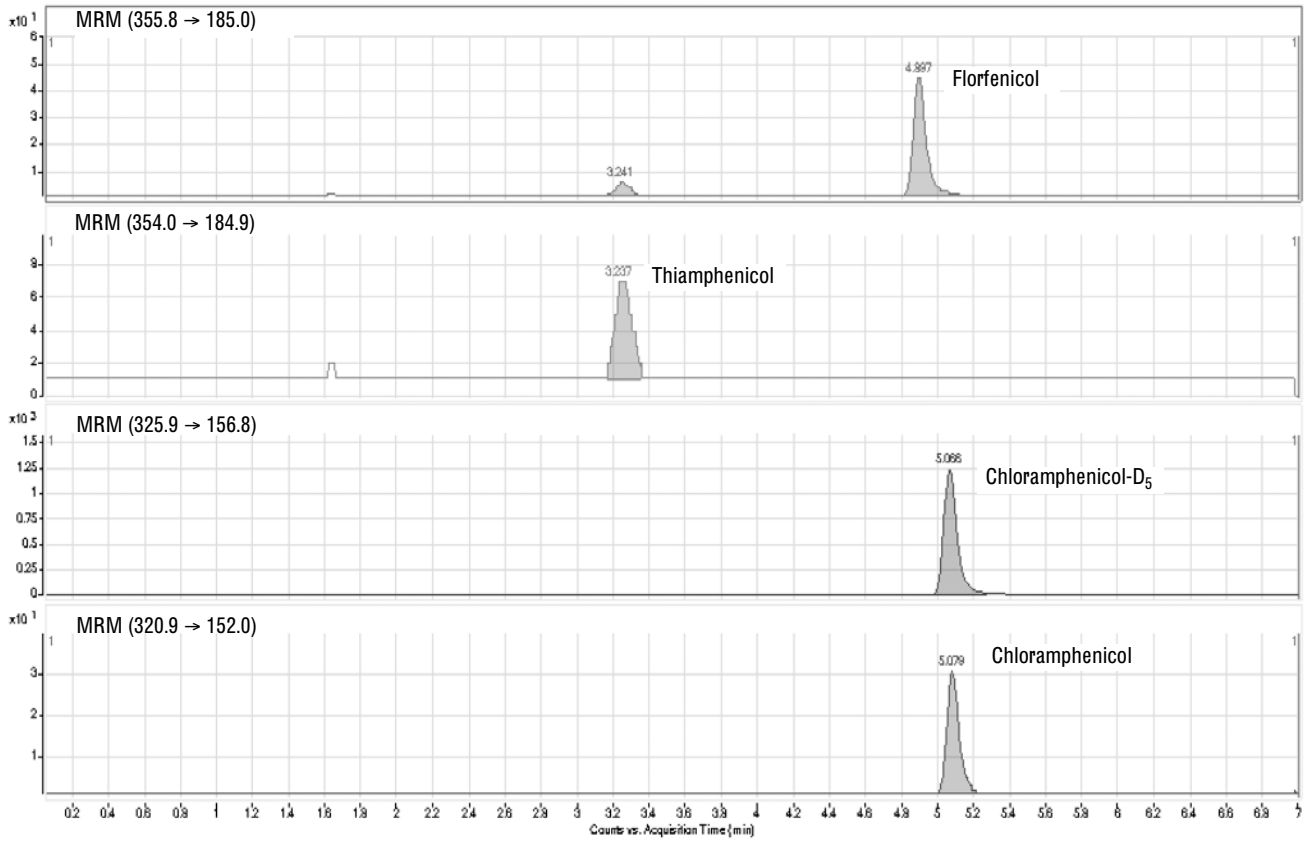


Figure 3. Chromatograms of 0.2 ng/g fortified honey extract.

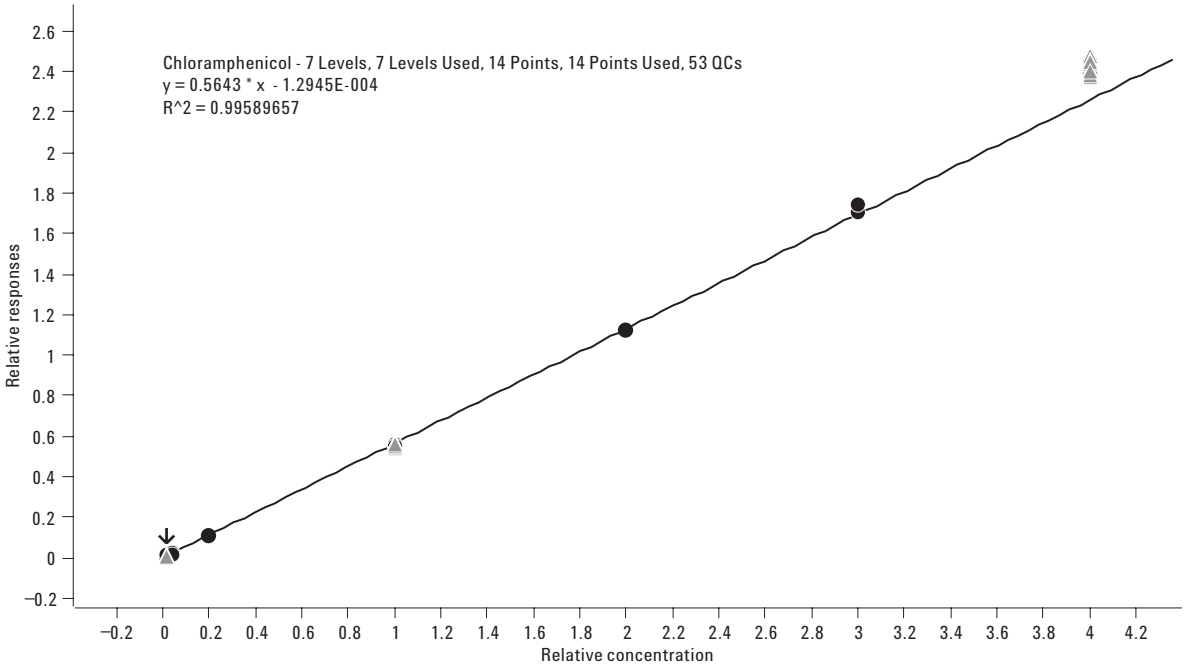


Figure 4. Calibration curve of chloramphenicol (0.1 to 20.0 ng/g). Dots (•) indicate sample results of calibration curve points, and trianges (Δ) indicate sample results of quality controls.

Recovery and Reproducibility

The recovery and reproducibility were evaluated by spiking phenicol standards in honey at levels of 0.1 ng/g (1.0 ng/g for TAP), 5.0 ng/g and 20.0 ng/g as quality control samples (QCs), and quantifying those QCs against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level, except four replicates for TAP low level. The recovery and reproducibility (shown as %RSD) data are shown in Table 4. CAP and FF show excellent recovery and reproducibility at all QC levels. The recovery of TAP is adequate at all concentrations and the reproducibility is excellent.

Table 4. Recoveries and Reproducibility of Phenicols in Fortified Honey

Analytes	Spiking Level (ng/g honey)	Recovery (%)	RSD (%) n = 6
Chloramphenicol	0.10	96.94	3.51
	5.00	98.88	0.87
	20.00	107.32	0.46
Florfenicol	0.10	100.67	9.77
	5.00	100.28	2.84
	20.00	107.49	2.55
Thiamphenicol	1.00	76.00	4.39*
	5.00	74.89	2.34
	20.00	89.81	3.83

* The experiment was done in replicates of four.

Conclusions

Agilent SampliQ OPT SPE cartridges provide a simple and effective method for the purification and enrichment of chloramphenicol, florfenicol, and thiamphenicol in honey. The recovery and reproducibility results based on matrix spiked standards are acceptable for chloramphenicol residue determination in honey under EU or Chinese regulations. The impurities and matrix effect from honey are minimal and do not interfere with the quantitation of any target compound. The LOQs of the three phenicols are significantly lower than the MRLs.

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1. GB/T 18932.19-2003, "Determination of Chloramphenicol Residues in Honey -LC/MS/MS Method."
2. "Commission Decision" 2003/181/ED of 13 March 2003, Off. J. Eur. Commun. L71/17 (2003).
3. "Handbook of Food Analysis: Residue and Other Food Component Analysis," CRC Press, 2004, pgs 937, 940.

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Determination of Sulfonamides in Milk Using Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Pharmaceuticals

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Abstract

The extraction of trace levels of nine nitrogen-containing sulfa drugs (sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine) in milk samples by solid-phase extraction was studied using Agilent SampliQ polymeric strong cation exchange (SCX) cartridges. An Agilent 6410 triple quadrupole LC/MS-MS System was used for the separation and determination of the sulfa drugs. For reversed-phase chromatography, an Agilent ZORBAX Eclipse Plus column (C18, 3.0 mm × 50 mm, 1.8 µm) with a 0.1% formic acid/acetonitrile gradient was used. Overall recoveries from the milk samples ranged from 73 to 99%, with %RSD values less than 10%. Limits of detection ranged from 0.2 to 2.0 ng/mL in milk (S/N = 3) depending on the sulfa drug, below the U.S. Food and Drug Administration acceptable levels in milk.

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Introduction

Since the discovery of the effective antimicrobial properties of sulfonamides in the early 1900s, they have been used to treat a variety of diseases. In the dairy farming industry, sulfa drugs are administered to dairy cattle to prevent infection. This leads to the possibility of the drugs being excreted in the milk and passed on to the consumer. This ingestion can cause a drug resistance, making the drugs ineffective in later uses to treat illness [1,2].

Sulfonamides, commonly known as sulfa drugs, have proven to be effective antimicrobial agents since their discovery in 1929 by Gerhard Domagk. Today, β-lactam antibiotics are much more commonly used to prevent infection than sulfonamides, but sulfonamides are still routinely used in different parts of the world due to their low cost. Over the years, many microorganisms have become resistant to these compounds. Of growing concern are drug-resistant bacteria that may be passed from animals to humans. One major cause of the resistance to these compounds is that feed animals are being fed antimicrobial drugs at low levels to treat diseases. In the 1990s, the United States Food and Drug Administration (FDA) began conducting tests on several milk supplies [3]. If dairy cattle were given sulfa drugs, low levels of these compounds could be found in the milk, leading to allergic reactions in some consumers, as well as an increase in drug-resistant organisms. This application demonstrates a complete solution to the analysis in milk of nine important sulfa drugs: sulfamethoxazole, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, and sulfadimethoxine (see Figure 1 for structures and chemical properties).

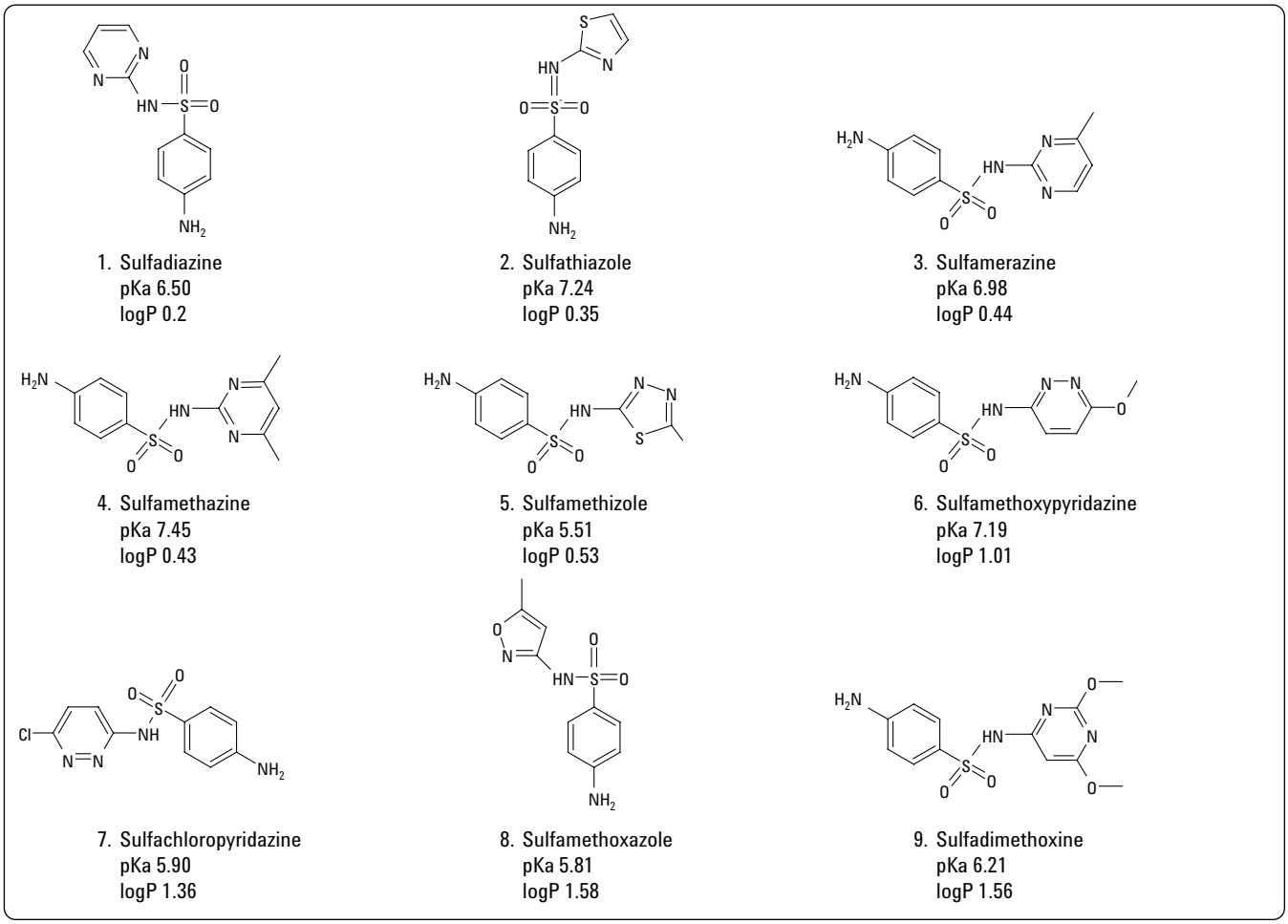

Figure 1. Structures and chemical constants for sulfa drugs used in this study.

Experimental

Materials and Chemicals

Water (EMD Chemicals, Gibbstown, NJ), acetonitrile, and methanol (Burdick and Jackson, Muskegan, MI) were HPLC grade. Sulfa drugs were analytical grade and purchased from Sigma-Aldrich (Saint Louis, MO). The stock solution (~1.19 mg/mL) was prepared in 25 mL of methanol and kept refrigerated for up to 14 days. Working solutions were made daily by dilution of the stock solution in water.

The SPE cartridges were Agilent SampliQ SCX, 3 mL/60 mg p/n 5982-3236), a polymeric cation exchanger with a 30-µm average particle size. The analysis was performed on an Agilent 1200 Series HPLC coupled to a 6410 triple quadrupole mass spectrometer with electrospray ion source. The analytical column was an Agilent ZORBAX Eclipse Plus C18, 3.0 mm × 50 mm, 1.8 µm (p/n 959941-302). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ) (Baker PCS reagent, 90%) for use in mobile phase preparation and for precipitation of proteins and lipids in the milk.

Sample Preparation

20 µL of a 45% solution of formic acid in water (prepared by mixing 10 mL of 90% formic acid with 10 mL of water) solution was added to each 1 mL of whole milk to precipitate proteins and lipids. The milk samples were then centrifuged at 8000 rpm for 10 minutes (Eppendorf 5810R 15 amp, Westbury, NY). Alternatively, the samples may be centrifuged at 3500 rpm for 20 minutes. An aliquot of the supernatant (prepared whole milk extract) was removed and used to load onto SampliQ SCX cartridges.

SPE Purification

After the SPE cartridge was conditioned and equilibrated as described in Figure 2, 5 mL of prepared whole milk extract was loaded onto the column. Care was taken that the flow rate during the load step did not exceed 1.5 mL/min. During both drying steps, the cartridge was dried under vacuum at 15 in Hg for the time indicated. The eluate was dried under nitrogen and then reconstituted in 1 mL of solvent (9:1 water:methanol). The samples were then sonicated (Branson 1200, Danbury, CT) for 5 minutes and analyzed using the Agilent 6410 Triple Quad LC/MS-MS system.

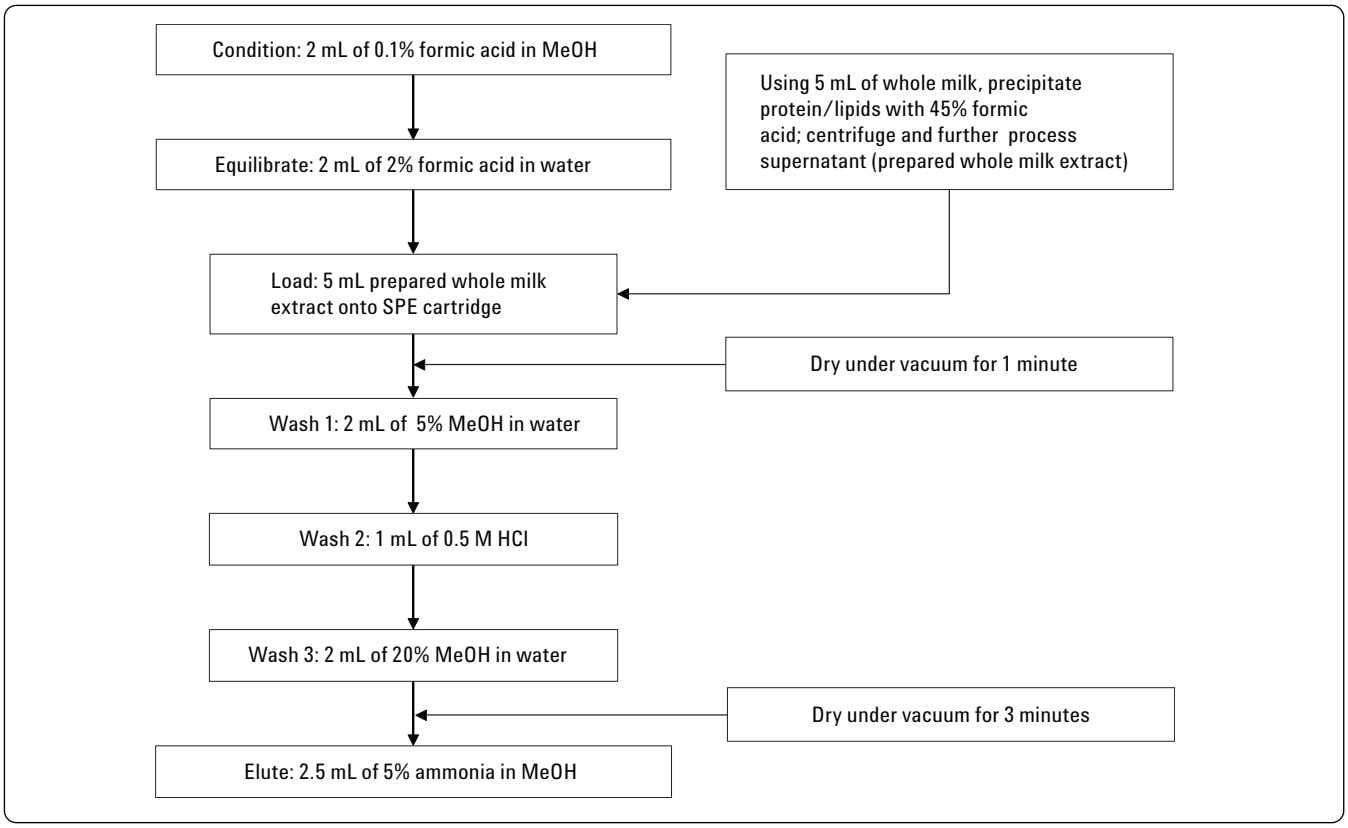

Figure 2. SPE procedure.

Due to the low concentrations of sulfa drugs being analyzed, and the very low LOD that can be achieved with Agilent 6410 Triple Quad LC/MS-MS system, extra care must be taken to keep the SPE manifold system clean to prevent contamination of samples. The manifold system must be thoroughly cleaned between uses or, if the option is available, needle inserts may be newly installed.

Separation and Analysis

The chromatographic and MS/MS experimental setup is shown in Tables 1, 2, and 3.

Table 1. HPLC Setup

Column	Agilent ZORBAX Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959941-302)			
Flow rate	0.42 mL/min			
Column temperature	35 °C			
Injection volume	1.7 µL w/ needle wash; wash for 30 s in flush port with MeOH/H_2O (5:1)			
Mobile phase	A: H_2O/acetonitrile (9:1) w/ 0.1% formic acid B: Acetonitrile w/ 0.1% formic acid			
Run time	8 min			
Post time	3 min			
Gradient	Time	0	3.5	8
	%B	0	0	65

Table 2. MS/MS Conditions

T_R (min)	Compound	Precursor ion	Product ion
1.69	Sulfadiazine	251.2	156.0 108.0
1.93	Sulfathiazole	256.1	156.0 108.0
2.41	Sulfamerazine	265.2	156.0 108.0
3.44	Sulfamethazine	279.2	186.1 124.1
3.89	Sulfamethizole	271.1	156.0 108.0
4.10	Sulfamethoxypyridazine	281.2	156.0 108.0
5.99	Sulfachloropyridazine	285.1	156.0 108.1
6.42	Sulfamethoxazole	254.2	156.1 108.1
7.17	Sulfadimethoxine	311.2	156.0 108.0

Table 3. Conditions for Electrospray Ionization Source

Gas temperature	350 °C
Gas flow	12 L/min
Nebulizer	40 psi
Capillary	4000 V

Results and Discussion

Linearity and Limits of Detection

Solutions used to create external calibration curves were prepared by using a stock solution to spike matrix blanks. Matrix blanks were created by taking the milk through the entire procedure, including the precipitation, centrifugation, and SPE procedures. The results for the calibration curves are summarized in Table 4. The regression results were used to calculate the recoveries. The limits of detection were chosen as the concentration of each drug that gave a signal-to-noise (S/N) ratio greater than 3:1. The limits of detection are given in Table 4.

Table 4. Calibration Curve Regression Analysis for Sulfa Drugs

Compound	Regression equation	R^2	LOD in milk (ng/mL)
Sulfadiazine	y = 282.62x + 225.59	1.0000	0.4
Sulfathiazole	y = 440.38x + 246.43	0.9996	1.0
Sulfamerazine	y = 358.34x + 485.54	0.9998	1.0
Sulfamethazine	y = 539.09x + 576.81	0.9989	1.0
Sulfamethizole	y = 499.57x + 333.03	0.9994	2.0
Sulfamethoxypyridazine	y = 494.61x + 139.66	0.9970	1.0
Sulfachloropyridazine	y = 343.78x + 92.808	0.9999	2.0
Sulfamethoxazole	y = 260.05x – 351.97	0.9901	2.0
Sulfadimethoxine	y = 956.97x + 1420.9	0.9973	0.2

Recovery and Reproducibility

The recoveries and precision for the method were determined at two levels, milk spiked to a concentration of 5 ng/mL and 10 ng/mL. Since the procedure for the SPE used 5 mL of prepared whole milk extract to load the SPE cartridge and the extract was reconstituted in 1 mL of mobile phase, the sample analyzed is five times as concentrated as the milk samples. The analyzed solutions are therefore 25 ng/mL for the samples that were spiked with 5 ng/mL in milk, and 50 ng/mL for the samples that were spiked with 10 ng/mL. The analysis was performed with five replicates at each level. The recovery and reproducibility data are shown in Table 5. The chromatograms for the blank and spiked milk extracts (5 ng/mL) are shown in Figure 3.

Table 5. Recovery and Precision Data for Nine Sulfa Drugs Used in This Study

Compound	Level spiked in milk (ng/mL)	Recovery	RSD (%)
Sulfadiazine	5	74.2	8.3
	10	99.7	5.7
Sulfathiazole	5	76.8	4.4
	10	83.2	4.7
Sulfamerazine	5	73.2	6.3
	10	84.8	0.6
Sulfamethazine	5	78.3	7.5
	10	89.0	3.1
Sulfamethizole	5	78.4	7.0
	10	94.5	5.3
Sulfamethoxypyridazine	5	76.3	6.2
	10	86.9	2.2
Sulfachloropyridazine	5	78.3	9.4
	10	84.3	6.0
Sulfamethoxazole	5	74.0	4.3
	10	87.7	6.4
Sulfadimethoxine	5	75.4	3.1
	10	82.5	5.4

Conclusions

The results of this study show that Agilent SampliQ SCX cartridges can be used as an effective method of purification and enrichment of multiple sulfonamides in complex samples such as whole milk. The impurities remaining after the SPE cleanup step were minimal and did not interfere with the quantitation of the sulfonamides. The levels at which the quantitation was performed are below the levels of sulfonamides that are considered by the FDA as safe in milk for consumption (10 ng/mL). The LOD for the method was also well below these levels (3 ng/mL in milk).

References

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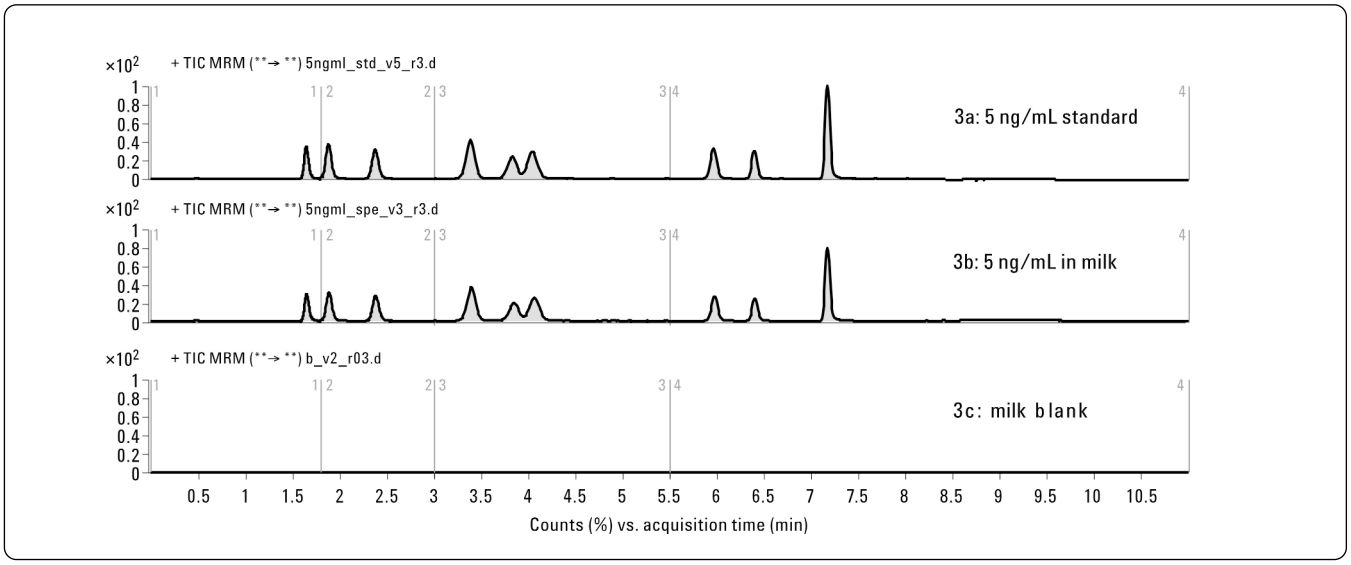

Figure 3. Total ion chromatograms of (3a) milk taken through extraction and cleanup, then spiked with sulfa drugs; (3b) milk spiked at 5 ng/mL, then taken through extraction and SPE cleanup; and (3c) milk blank.

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Determination, Confirmation, and Quantification of Trace ß-Lactam Antibiotics in Milk by LC/MS/MS

Pei-Bin Hu, Xin Ma, and Tao Bo

Application Brief

Food Safety

The ß-lactam antibiotics are widely used in veterinary medicine for the treatment and prevention of disease. This use can result in the presence of residues in milk and edible tissues which can lead to problems in the fermentation processes and to health problems for individuals who are hypersensitive to ß-lactams.

To protect the consumer, MRL values were laid down in EU regulation 2377/90. For milk these values range from 4 µg/kg for penicillin G, ampicillin, and amoxicillin to 30 µg/kg for dicloxacillin, cloxacillin, and oxacillin.

In practice, screening is performed by microbiological and immunological methods. In this way it is possible to give, for a positive sample, the specification of the group to which the residue belongs. Confirmation, however, has to be performed by an independent physicochemical technique. Therefore, a highly sensitive LC/MS/MS method was developed for the detection of penicillins, etc., in milk samples.

Highlights

- Ease of use for method optimization
- Good linearity $R^2 \geq 0.98$ in real milk samples
- Good separation to get almost all the compounds separated well within 9 minutes

Experimental

Sample Preparation

An aliquot of 2 mL of milk (half skimmed consumption milk or raw milk) was mixed with 4 mL of acetonitrile for protein precipitation. The sample was vortexed and then centrifuged for 10 minutes at 3500 rpm. After filtration, 20 µL of the supernatant was injected into the LC/MS/MS system.

LC Conditions

Instrument	Agilent 1200SL
Column	Agilent ZORBAX SB-C18, 2.1 × 150 mm, 3.5 µm (p/n 830990-902)
Mobile phase	A: Water/0.3% acetic acid B: Acetonitrile/0.3% acetic acid
Flow rate	0.3 mL/min
Gradient	0–2 min/A 90%; 2.01–8 min/A 35%; 8.01–9/A 5%
Column compartment temperature	30 °C
Stop time	9 min
Post time	6 min
Injection volume	10 µL

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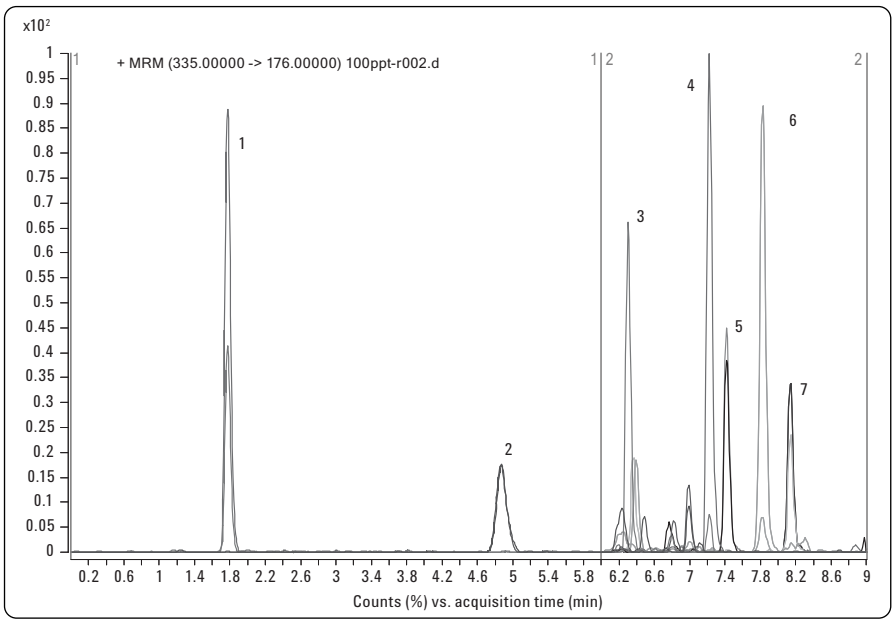
MS Conditions

Instrument	Agilent 6410A triple quadrupole LC/MS system
Source	ESI +
Drying gas temperature	350 °C
Drying gas flow	10 L/min
Nebulizer pressure	45 psi

MRM Setting

TS	Compound	Precursor	Product	Dwell (ms)	Frag (V)	CE (V)
0	Amoxicillin	366	114	100	110	15
			208	100	110	5
	Ampicillin	350	160	100	100	5
			192	100	110	10
6	Dicloxacillin	470	311	70	110	10
			160	70	110	10
	Nafcillin	415	256	70	110	15
			160	70	110	10
	Oxacillin	402	243	70	110	10
			160	70	110	10
	Penicillin V	351	192	70	100	5
			160	70	100	10
	Penicillin G	335	176	70	100	10
			160	70	100	5

Results



1. Amoxicillin
2. Ampicillin
3. Dicloxacillin
4. Nafcillin
5. Oxacillin
6. Penicillin V
7. Penicillin G

Compounds	S/N (Conc = 0.1 pppb)	R^2
Amoxicillin 366-114	224	0.992
Ampicillin 350-160	61.6	0.984
Dicloxacillin 470-160	48.5	0.981
Nafcillin 415-199	52.6	0.998
Oxacillin 402-160	70.9	0.993
Penicillin V 351-160	225.9	0.998
Penicillin G 335-160	33.2	0.981

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Using LC/MS/MS 6410 for Analysis of Chloramphenicol, Thiamphenicol, and Florfenicol in Fish Samples

Application Brief

Xiaorong Ran, Tao Bo

Chloramphenicol is a banned compound in the EU. It is a zero tolerance compound, and some methods have already been developed for this analysis. The LC/MS/MS is often used for its greater sensitivity and higher selectivity.

In many cases, not only chloramphenicol but also thiamphenicol and florfenicol are also found. This method uses a simple method for detecting all three compounds within less than 6 minutes. Furthermore, the results gave the good results, showing the performance at the negative mode.

Experimental

LC Conditions

Column	Agilent ZORBAX Eclipse Plus, 2.1 mm × 50 mm, 1.8 µm
Mobile phase	A: Water B: Methanol
Flow rate	0.4 mL/min
Gradient	0–2 min/B 10% to 90%; 2–3 min/B 90%; 3.01/B 10%
Stop time	6 min
Column compartment temperature	45 °C
Injection	5 µL

MSD Condition

Instrument	Agilent 6410A triple quadrupole LC/MS system
Source	ESI –

Highlights

- Using a simple RRLC method can separate the compounds well within 6 minutes
- Quite high sensitivity in negative mode
- The ISTD method can remove the matrix effect and minimumize the sample preparation interference

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Sample Preparation

All SPE cartridges are conditioned with 2 mL of water before use.

1. **Honey**, 1 g sample is diluted to 5 mL with water and 25 µL 10 ppb IS is added. The solution is loaded onto the SPE cartridge and allowed to stand for 5 min. Elution is performed with 10 mL ethyl acetate. The eluate is collected and the solvent is evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min. The solution is filtered, using a syringe filter, before injection. No additional cleanup of the sample solution is performed.
2. **Shrimp**, 1 g of shrimp is defrosted and mixed in a blender. To the 1 g of the mixed shrimp, 3 mL of water and 25 µL 10 ppb IS are added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.
3. **Chicken**, 1 g of chicken is defrosted and mixed in a blender. To the 1 g of the mixed chicken, 3 mL of water and 25 µL 10 ppb IS are added. The portion is centrifuged for 5 min (8,000 rpm). The supernatant is loaded on the cartridge and allowed to stand for 5 min. Elution is performed with 5 mL ethyl acetate. The eluate is collected and the solvent evaporated under a nitrogen stream at 40 °C. The residue is redissolved in 1 mL methanol and put in an ultrasonic bath for 1 min; the solution is filtered before injection.

MRM Setting

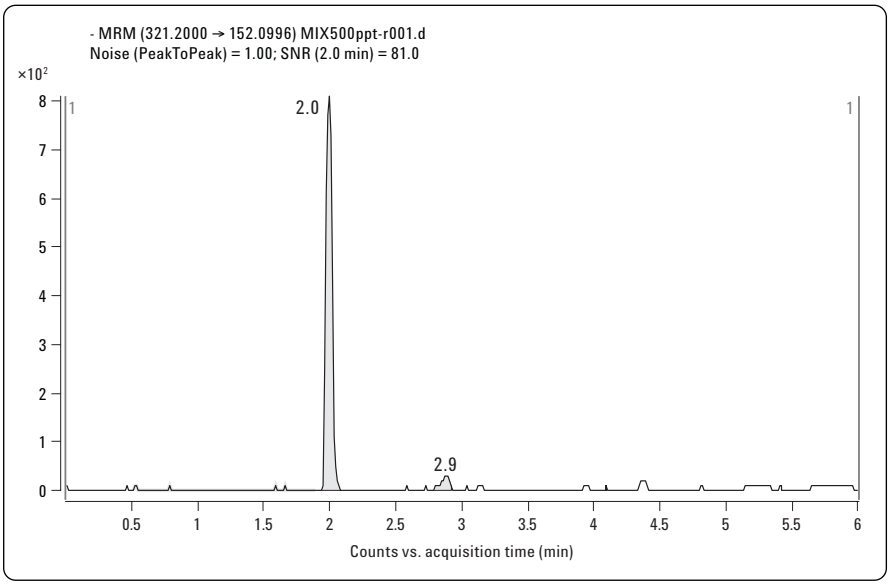
Name	Precursor ion	Product ion	Frag (V)	CE (V)	Dwell (ms)
TAP	354.1	185.1*	120	20	60
	354.1	289.9	120	10	60
FF	356	185.1*	120	20	60
	356	335.8	120	5	60
CAP	321.2	152.1*	120	10	60
	321.2	257.1	120	5	60
D5-CAP (ISTD)	326.2	157.2	130	15	60

Results

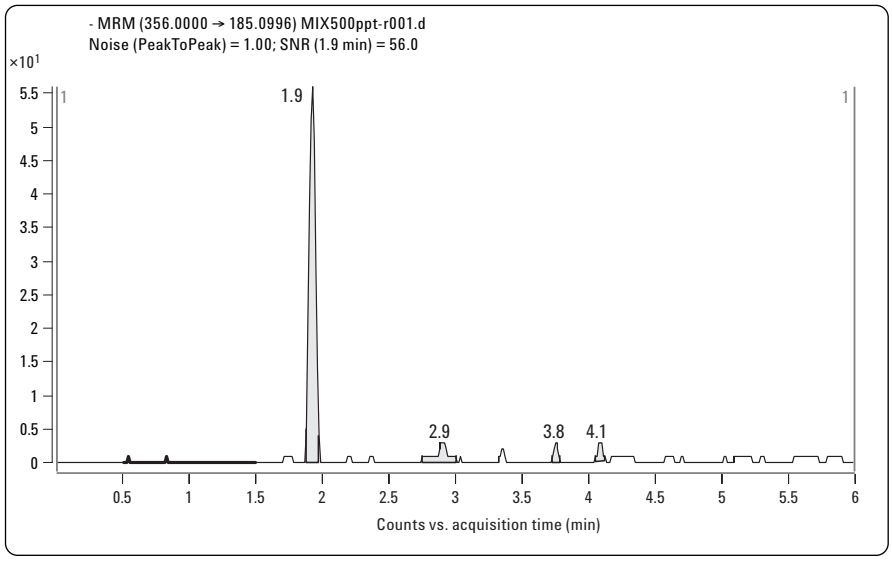
Name	**Linearity** (0.5–20 ppb)
TAP	0.994
FF	0.992
CAP	0.994

Sensitivity

1. CAP: 0.5 ppb S/N = 81



2. FF: 0.5 ppb S/N = 56



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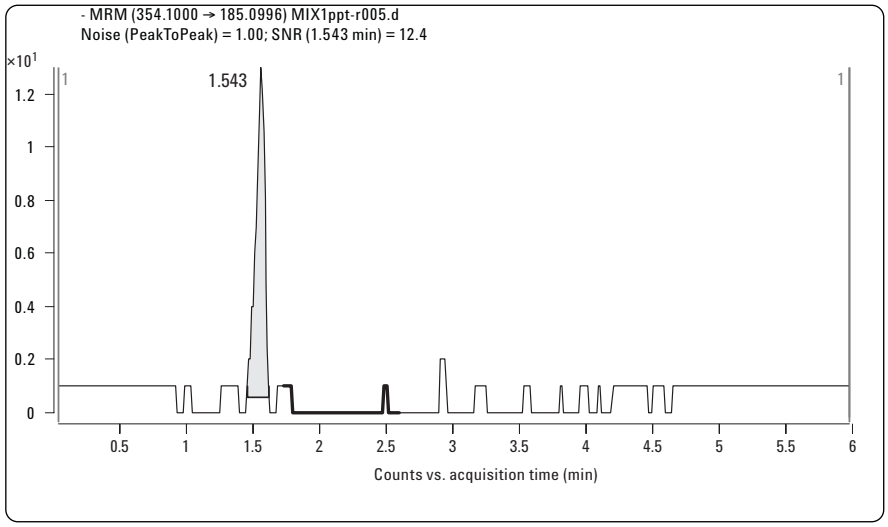
3. TAP 1 ppb, S/N = 12



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Agilent Technologies

Detection, Monitoring, and Quantitation of Trace Sulfonamides in Pork Muscle Using the Agilent 6410A LC/MS/MS

Application Brief

Jian-qiu Mi

Sulfonamides are the one of the oldest groups of veterinary medicines in use today. All sulfonamide drugs are currently included in Annex 1 of the Council Regulation 2377/90. The existing EU maximum residue level (MRL) for all drugs of the sulfonamide group is 100 µg/kg in all food-producing species.

A variety of methods have been used to measure sulfonamide residue in biological materials, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), and enzyme-linked immunosorbent assay (ELISA). Now, the LC/MS/MS method is used more widely.

In this study, a multiresidue analysis was performed to simultaneously determine sulfonamides in pork by the Agilent 6410A LC/MS/MS. This multiresidue analysis for sulfonamides can detect different kinds of sulfonamides within one run. Compared with the classic methods, this method can achieve greater sensitivity and be used for screening, confirmation, and quantification.

Highlights

- The pH of the mobile phase played an important role in the LC separation because the retention behaviors of the drugs were dependent on the ionization of the sulfonamides.
- Different kinds of sulfonamide drugs can be analyzed within one run.
- Using the RRLC can get all 14 compounds to elute within 10 minutes.
- High sensitivity easily meets the EU requirements.

Experimental

Sample Preparation

1. **Weigh** – 3-g samples of pork muscle were weighed directly into 50-mL polypropylene centrifuge tubes.
2. **Homogenize** – The samples were homogenized for 3 minutes with 10 mL acidified methanol.
3. **Centrifuge** – The samples were then centrifuged for 10 minutes.
3. **Extract** – 10 mL acidified methanol was extracted, filtered, and injected

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Instrument Settings

LC Conditions

LC	Agilent 1200 Series LC
Column	Agilent ZORBAX SB-C18 (2.1 × 50 mm, 1.8 µm)
Mobile phase	A: 0.1% TFA, B: Acetonitrile 0 min: 5% B 6 min: 23% B 9 min: 23% B 9.01 min: 90% B
Stop time	10 min
Column temperature	30 °C
Injection volume	1 µL
Flow rate	0.3 mL/min

MSD Conditions

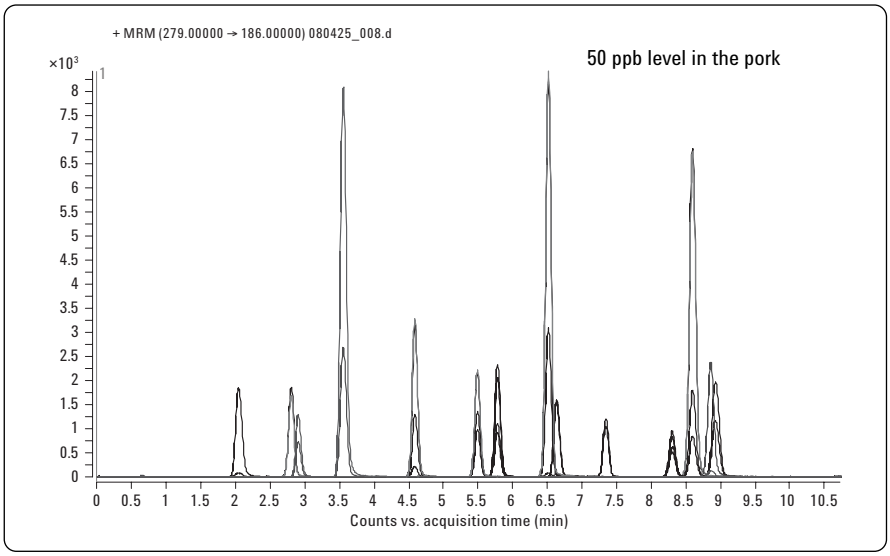
Ionization	ESI (positive)
Scan range	*m/z* 100 to 450
Drying gas	7 L/min at 350 °C
Nebulizer gas	30 psi

MRM setting

Compound	MRM	Frag	CE (V)
Sulfachloropyridazine (SCP)	285–156 285–108	100	15 20
Sulfadiazine (SD)	251–156 251–185	120	10 10
Sulfamethazine (SDM)	311–156 311–218	140	15 15
Sulfamethoxypyridazine (SMP)	281–156 281–215	120	10 15
Sulfamerazine (SM1)	265–156 265–172	120	15 15
Sulfamethazin (SM2)	279–156 279–204	140	15 15
Sulfalmethoxazole (SMZ)	254–156 254–147	120	15 20
Sulfamonomethoxine (SMM)	281–156 281–126	120	10 20
Sulfathiazole (ST)	256–156 256–107	120	15 15
Sulfaquinoxaline (SQX)	301–156 301–208	140	15 15
Sulfadoxine (SDM)	311–156 311–108	140	15 20
Sulfaphenazole (SPP)	315–156 315–160	140	20 20
Sulfaclozine	285–156 285–131	100	15 20
Sulfafurazole (SIZ)	268–156 268–113	120	5 10

Results

Good separation and response



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Agilent Technologies

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.

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Introduction

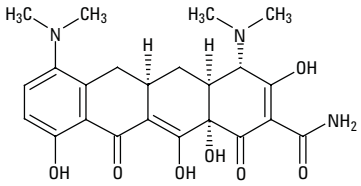
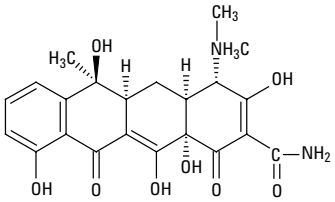
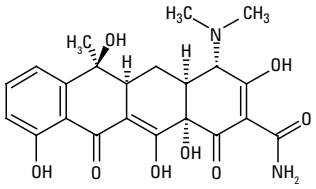
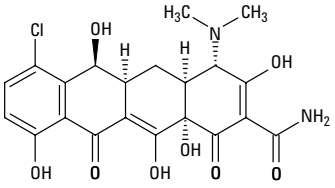
Antibiotics are used worldwide to control bacterial infection and promote healthy farm animals for milk production. Tetracyclines are broad-spectrum antibiotics, so they are widely used. However, it is undesirable to have them in the milk supply.

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.

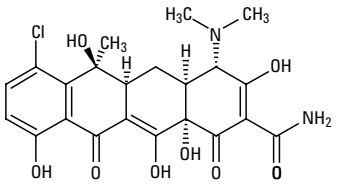
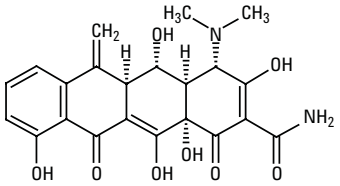
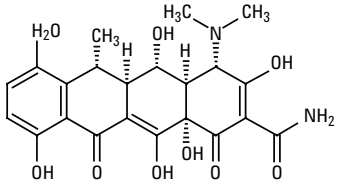
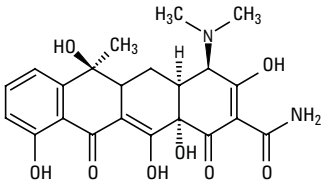
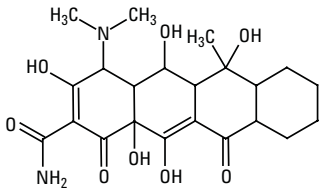
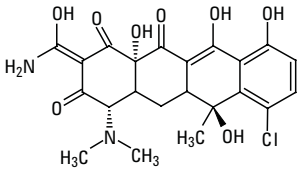
The purpose of this study is to develop a method for the Agilent 6410 LC/MS/MS to determine the presence of tetracyclines and their metabolite residues in milk. The method is rapid and easy to use. The tetracyclines and their metabolites are given in Table 1.

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.

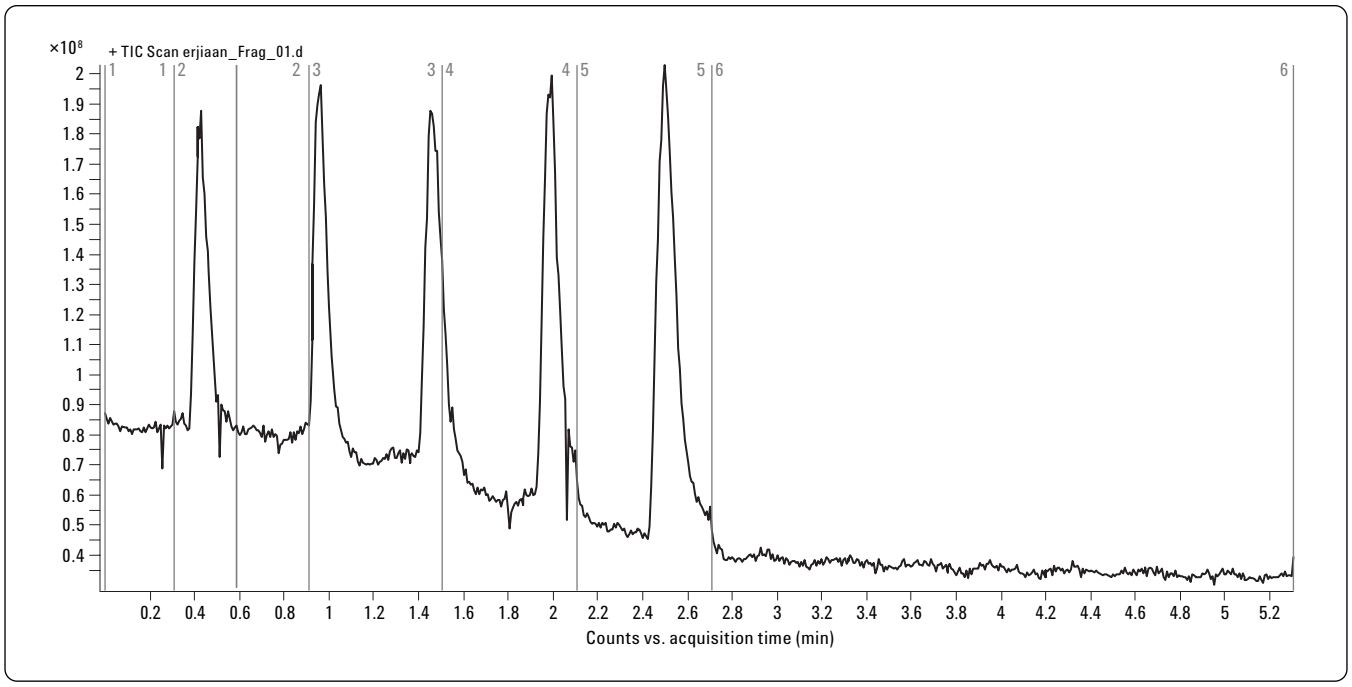


Figure 1. Optimization of fragmentor voltage for minocycline from 60-160 by steps of 20 V.

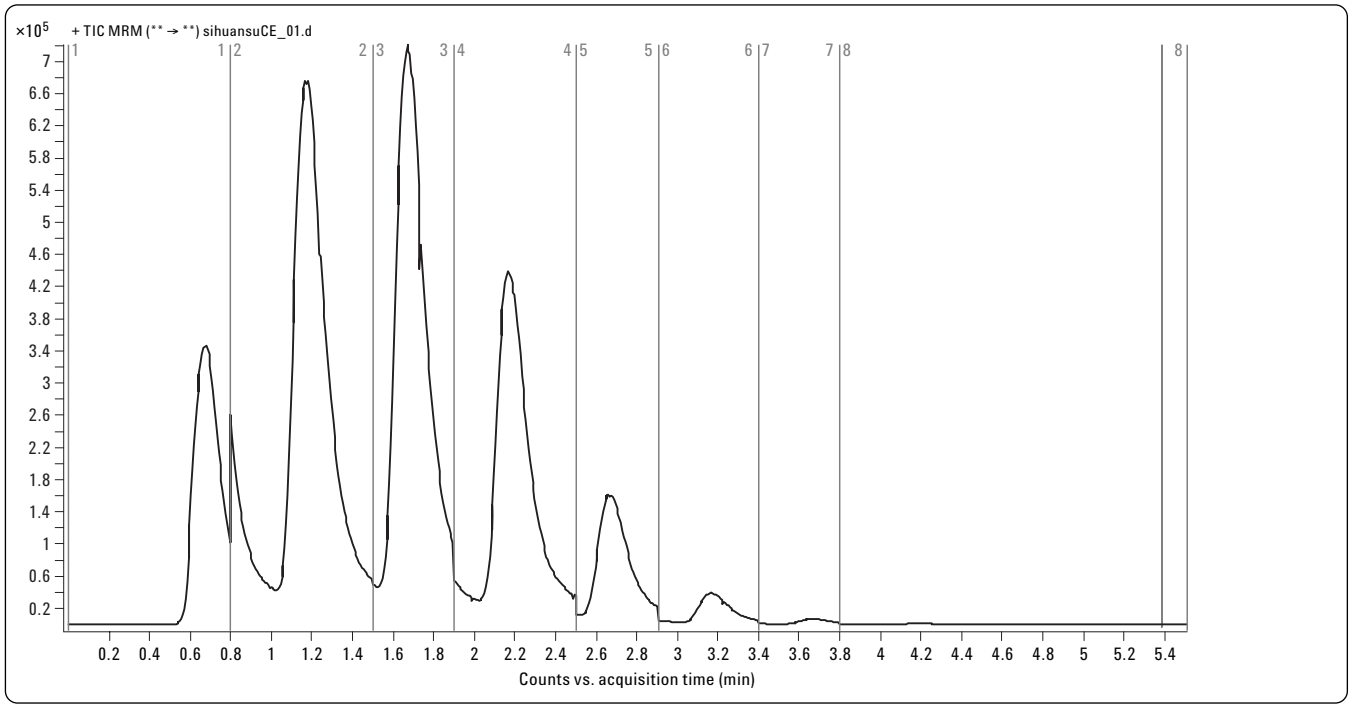


Figure 2. Manual collision energy optimization of tetracycline.

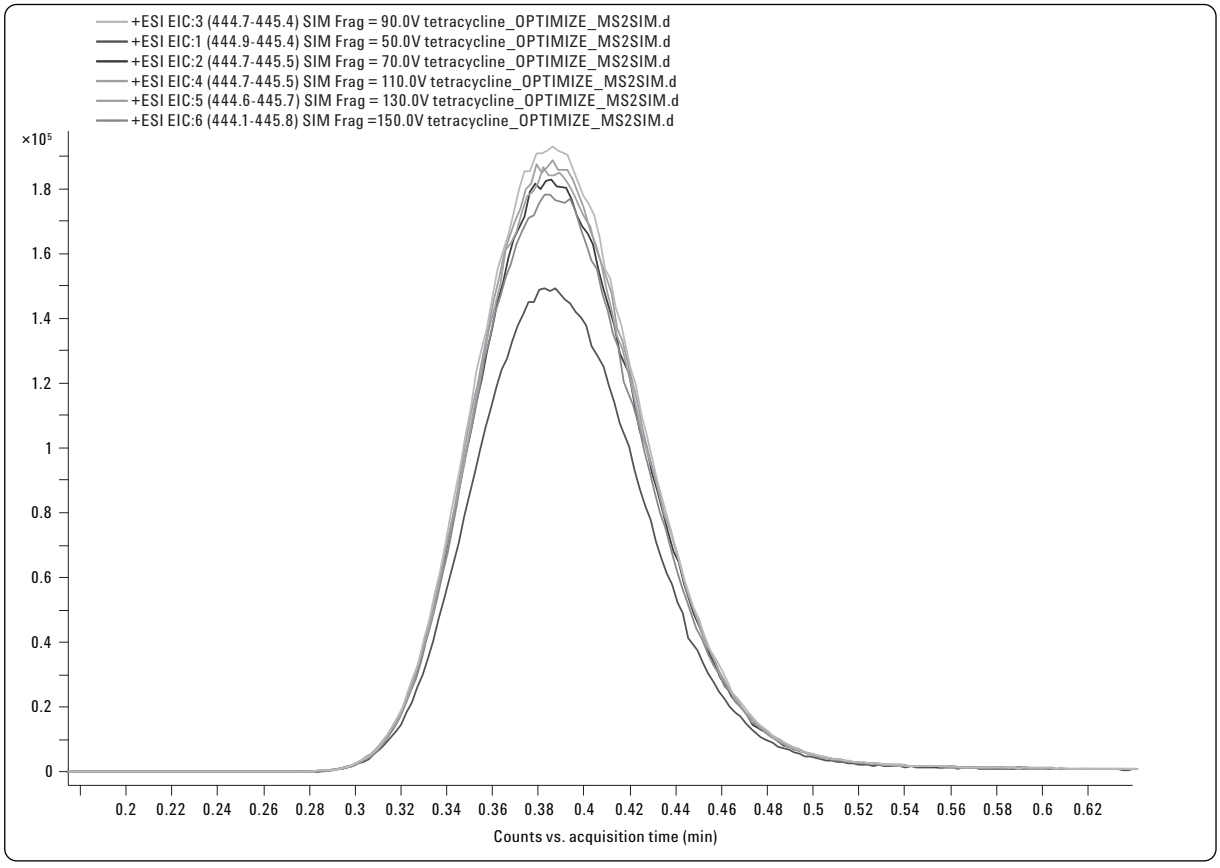


Figure 3. Single injection automatic determination of fragmentor voltage for tetracycline using the Optimizer program.

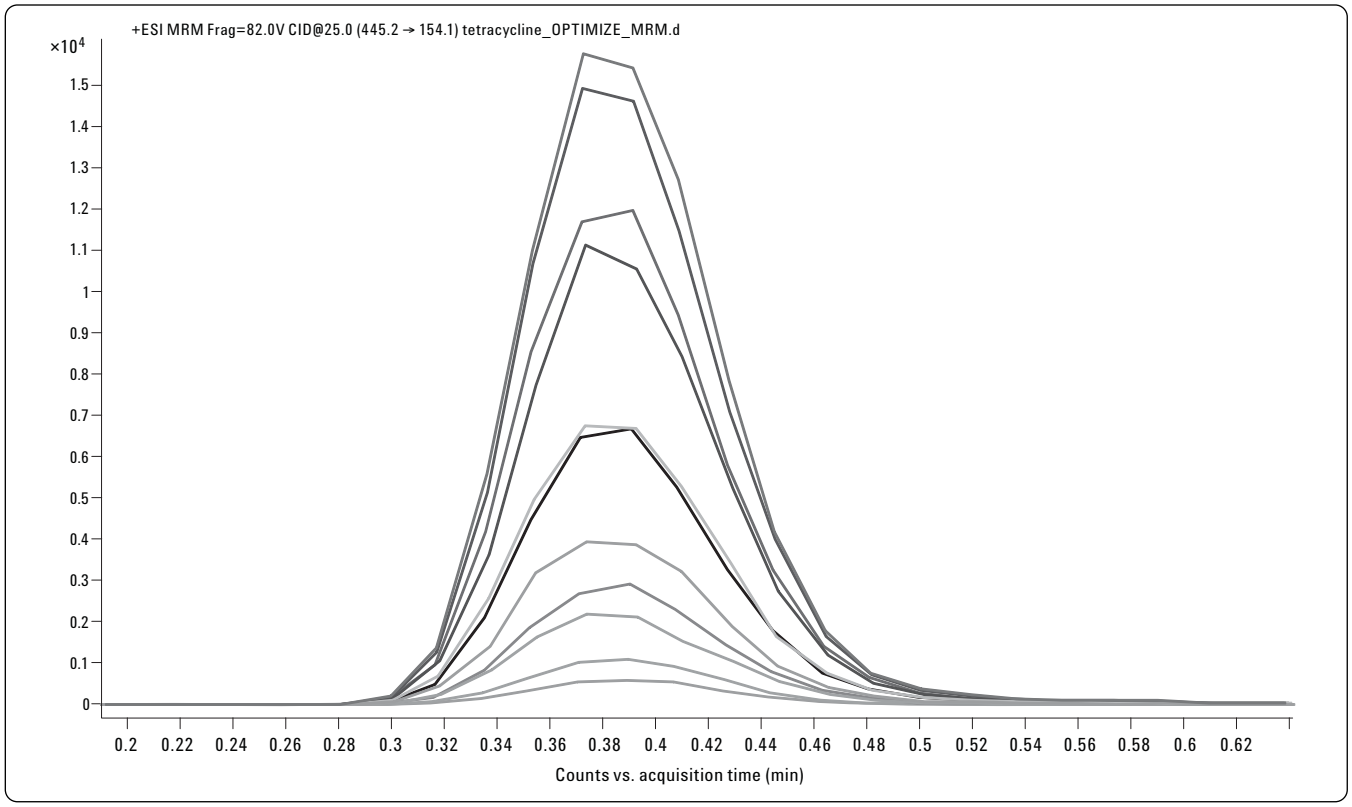


Figure 4. Single injection automated collision energy determination using Optimizer program for tetracycline.

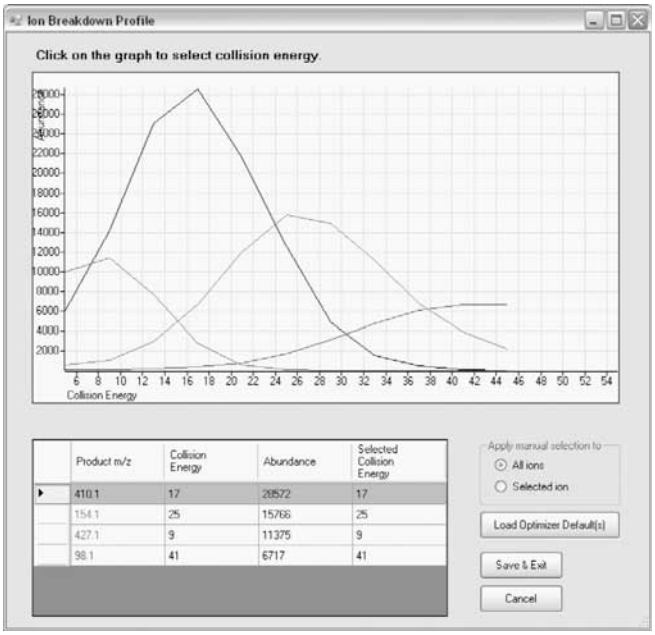


Figure 5. Ion breakdown profile for tetracycline as determined by the Optimizer program.

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:

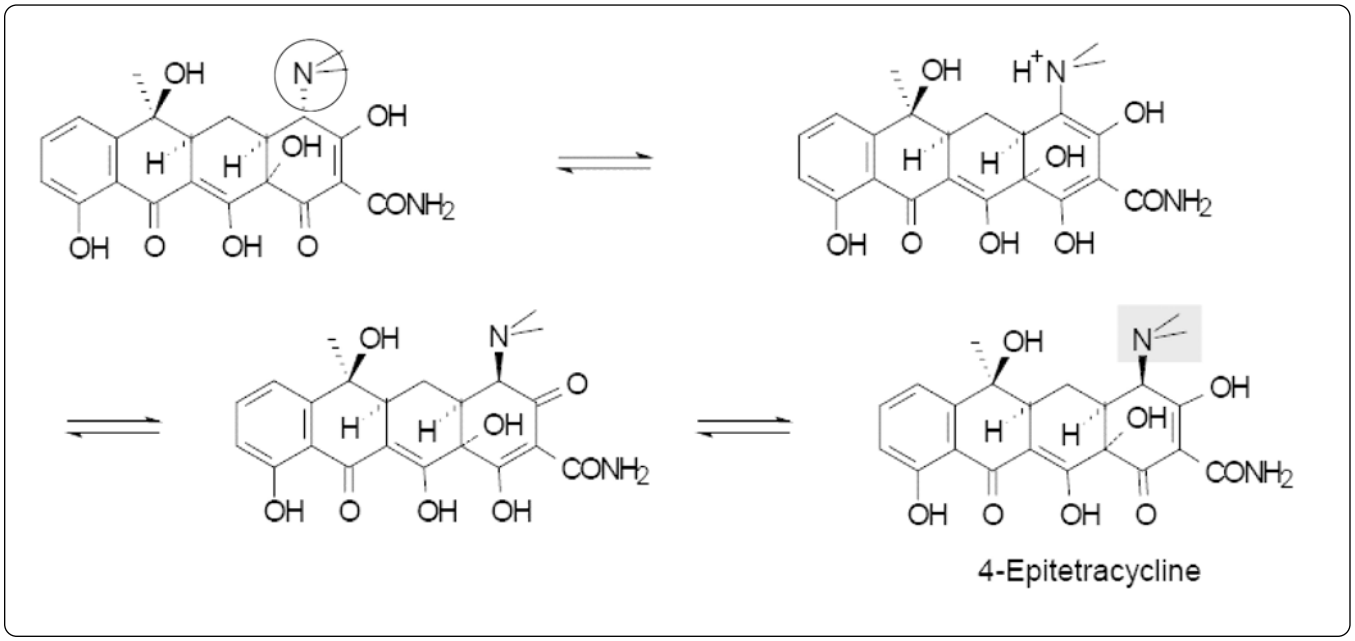


Figure 6. The degradation of tetracycline to 4-epitetracycline.

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.

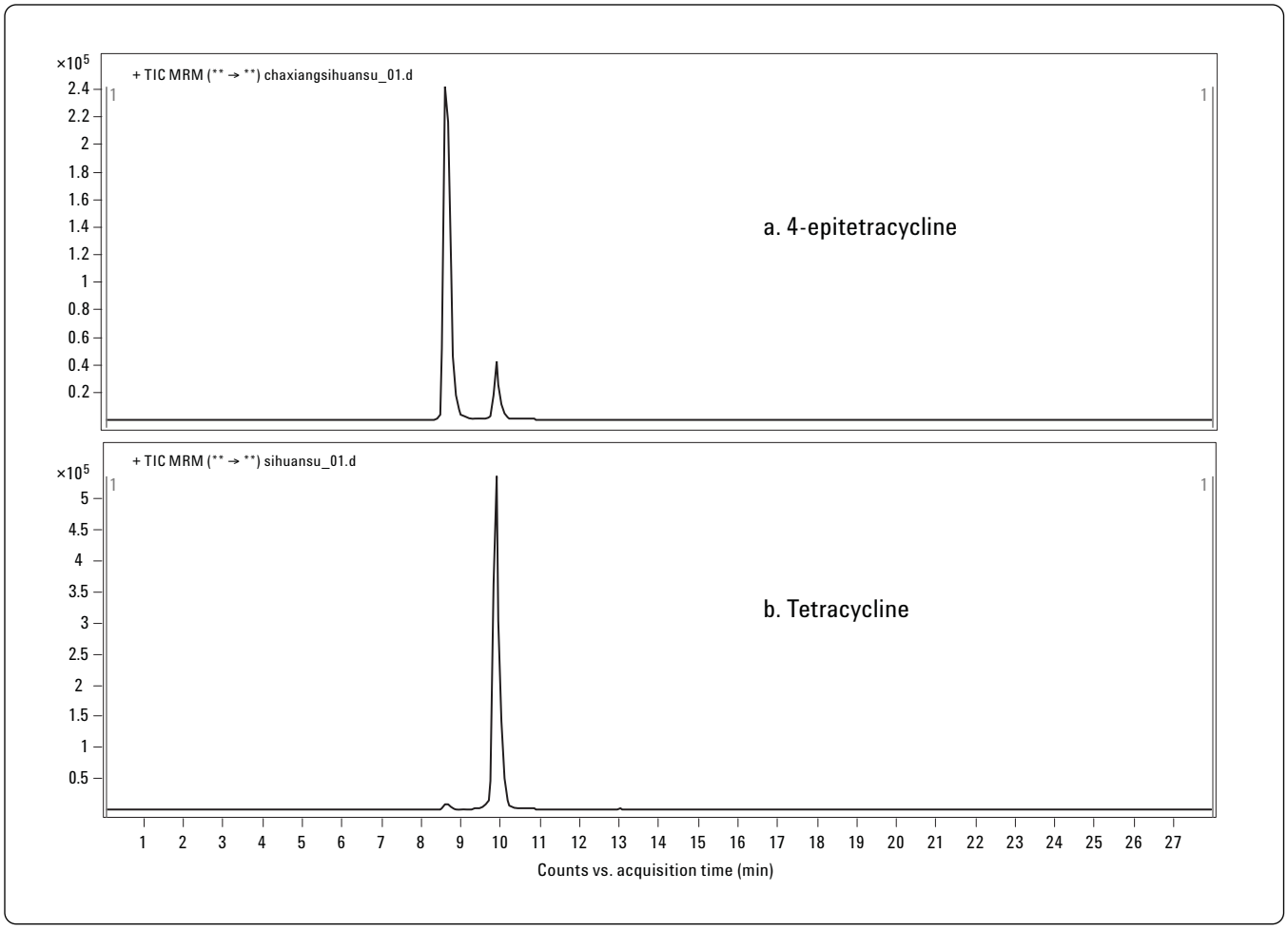


Figure 7. The separation of tetracycline and its degradation product 4-epitetracycline.

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

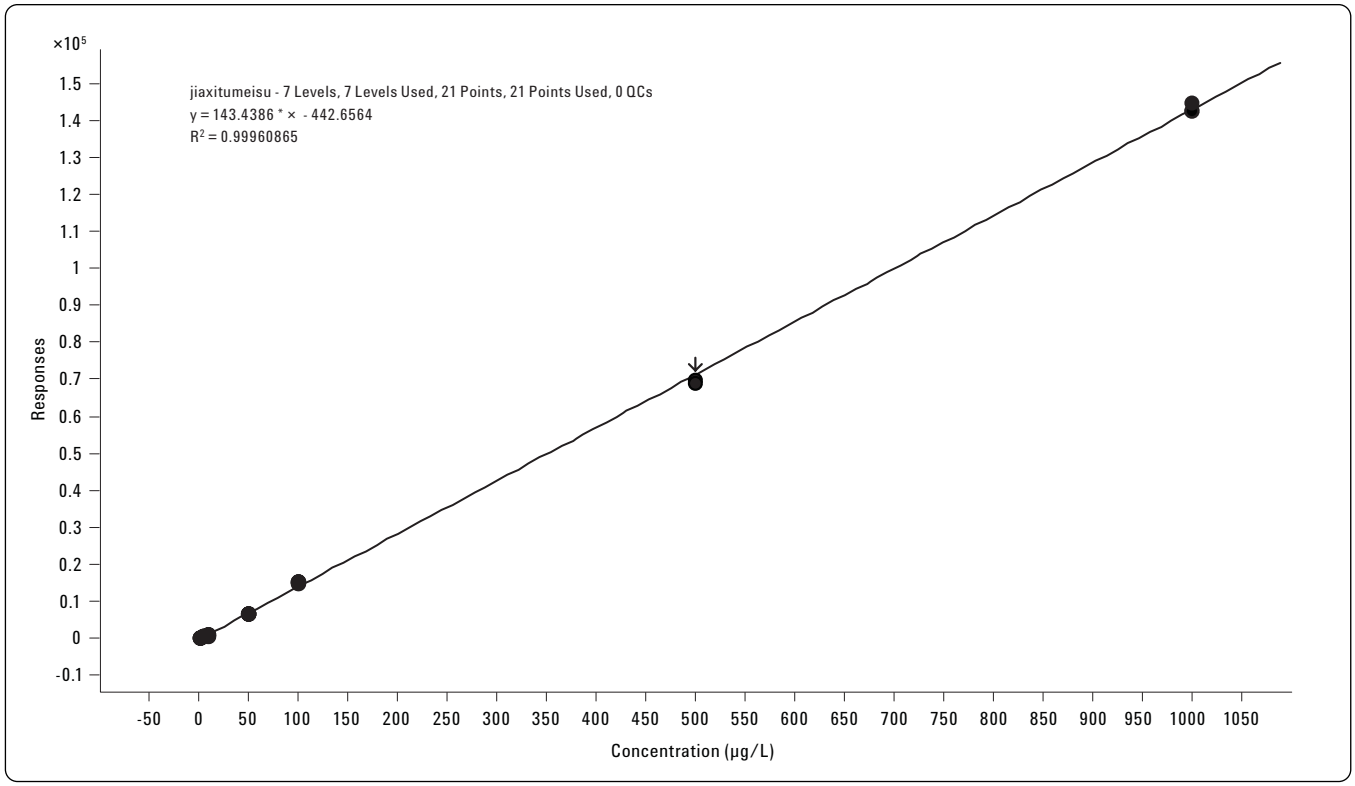


Figure 8. Tetracycline calibration curve from 1 ppb to 1000 ppb.

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

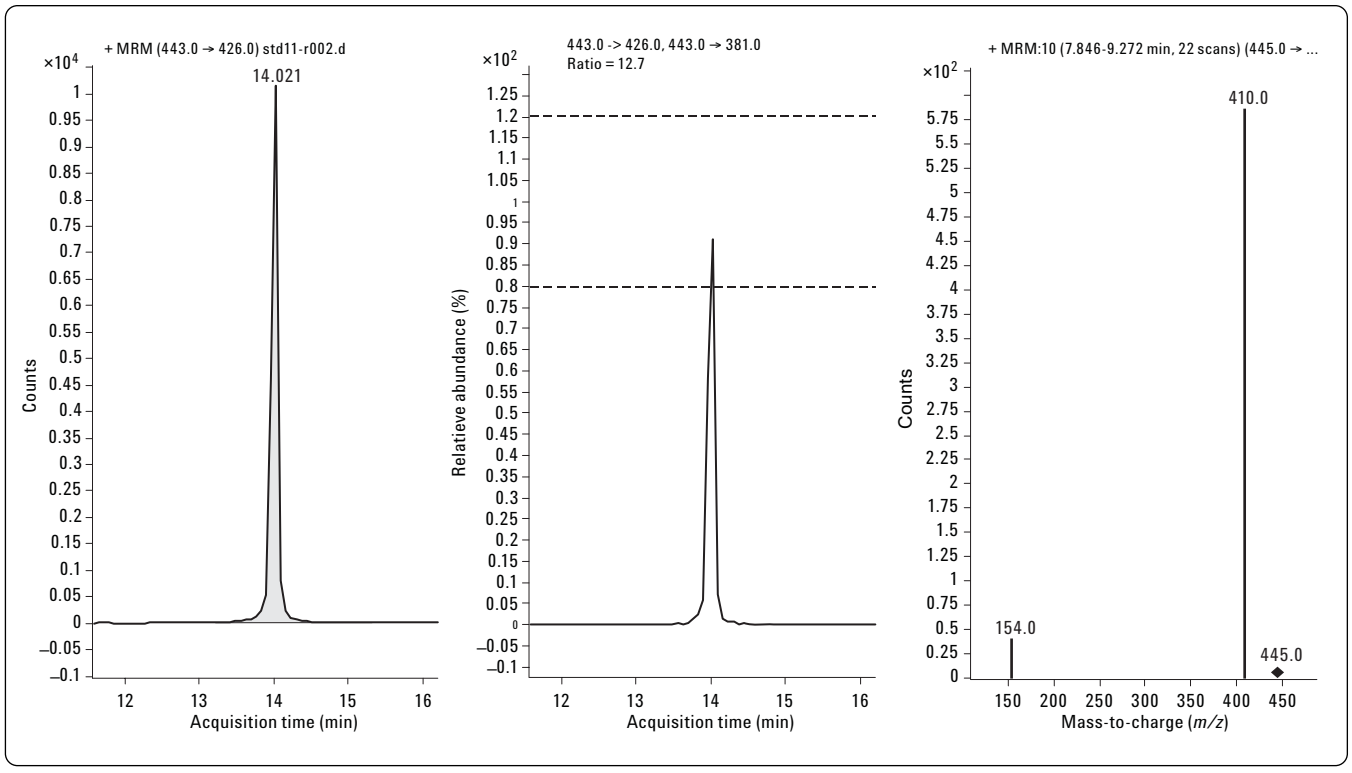


Figure 9. Shows the ion ratios for qualifier ion and the quantitation ion of methacycline.

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.

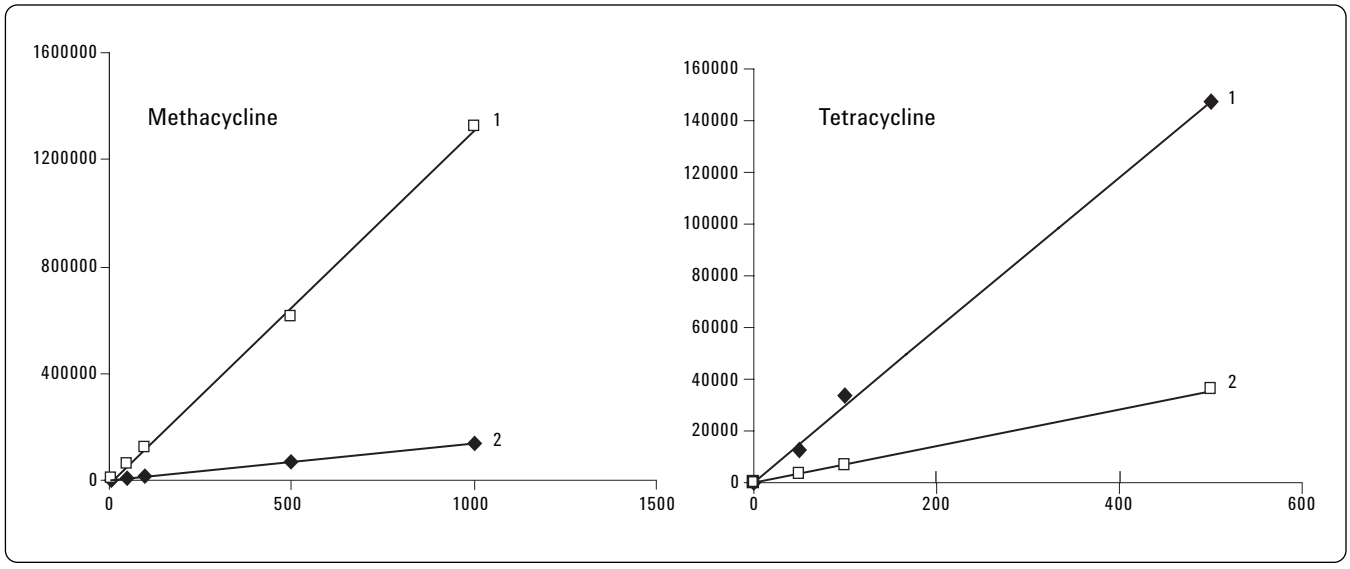


Figure 10. Ion suppression of two of the tetracyclines in milk; 1) response in solvent, 2) response in milk.

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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Agilent Technologies

Determination of Hormones in Fish (*Carassius Carassius*) by SampliQ-OPT Solid Phase Extraction with High Performance Liquid Chromatography

Application Note

Food Safety

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Abstract

Solid-phase extraction (SPE) coupled with high performance liquid chromatography (HPLC) was optimized for the extraction and determination of sixteen hormones (estriol, prednisolone, hydrocortisone, prednisone, methylprednisolone, betamethasone, dexamethasone, triamcinolone acetate, gestrinone, prednisolone acetate, hydrocortisone acetate, prednisone acetate, estradiol, cortisone acetate, methyltestosterone, estrone) in crucian carp (*Carassius carassius*) meat. Results indicate that SPE using an Agilent SampliQ OPT (60 mg, 3 mL) and HPLC using an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 250 mm, 5 µm) is suitable for extraction of these compounds. Recoveries ranged from 76.2 to 106.1 % with relative standard deviations (RSDs) between 1.7 and 8.9 %.

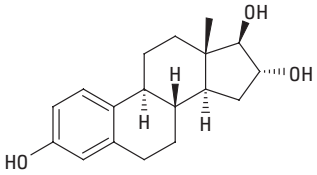
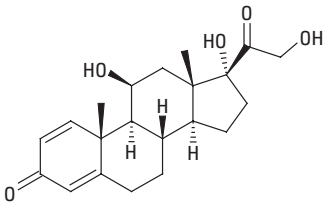
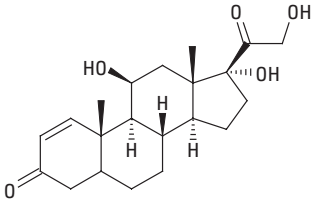
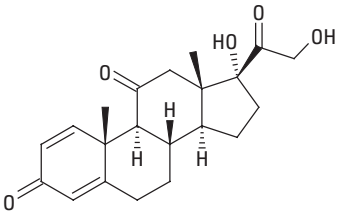
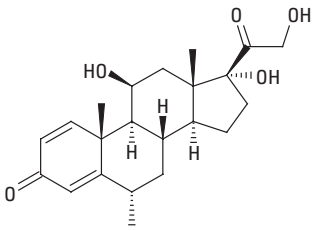
Agilent Technologies

Introduction

Food safety has increasingly become an important concern of people worldwide. Many chemicals added to food create potential hazards to human health. Hormones are a common food additive. Long-term consumption of glucocorticoid can lead to hyperglycemia, osteoporosis, birth defects, and immune function decline. Other hormones such as estrogen, androgen, and progesterone are carcinogenic and can lead to breast cancer, ovarian cancer and cell carcinoma. Many countries' regulations clearly define residual limits for these compounds in food.

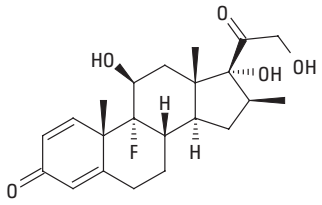
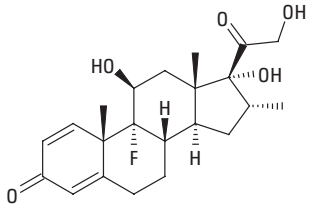
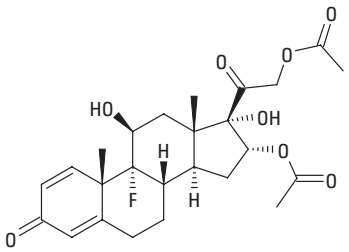
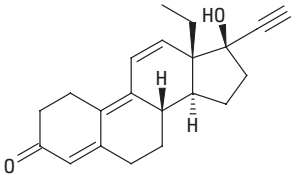
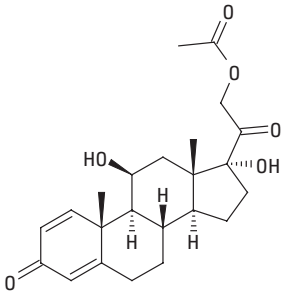
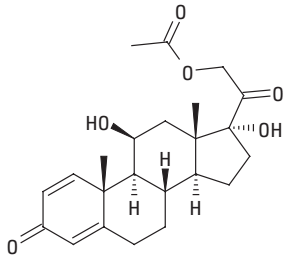
An Agilent SampliQ OPT SPE cartridge was used to extract 16 kinds of hormones (Table 1) from crucian meat and an HPLC method was established to detect these 16 compounds.

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
1	Estriol	50-27-1	2.45	
2	Prednisolone	50-24-8	1.66	
3	Hydrocortisone	50-23-7	1.79	
4	Prednisone	53-03-2	2.07	
5	Methylprednisolone	83-43-2	2.06	

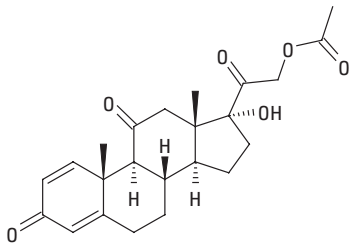
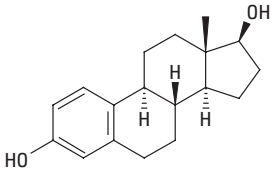
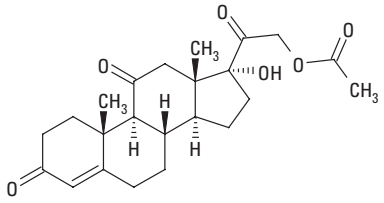
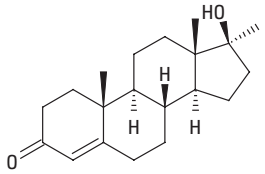
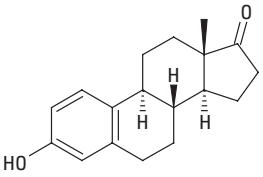
(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
6	Betamethasone	378-44-9	1.93	
7	Dexamethasone	50-02-2	1.93	
8	Triamcinolone acetate	67-78-7	1.9	
9	Gestrinone	16320-04-0	NA	
10	Prednisolone acetate	52-21-1	NA	
11	Methylprednisolone	83-43-2	NA	

(Continued)

Table 1. Hormones Used in this Study

No.	Name	CAS No.	Log P	Structure
12	Prednisone acetate	125-10-0	NA	
13	Estradiol	50-28-2	3.57	
14	Cortisone acetate	50-04-4	2.35	
15	Methylestosterone	58-18-4	NA	
16	Estrone	53-16-7	4.03	

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Hormone standards were purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products). Crucian was purchased from a local market.

Stock solutions (1 mg/mL) were prepared in methanol and kept in the freezer (–20 °C). Working solutions were prepared using the stock solution diluted with methanol. The working solutions should be prepared every week and need to be stored below 4 °C.

The SPE cartridges were Agilent SampliQ OPT (3 mL, 60 mg, p/n 5982-3036). The analysis was performed on an Agilent 1200 Series HPLC with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 (5 µm 250 mm × 4.6 mm id, p/n 959990-902). An Agilent 0.45-µm PTFE Premium Syringe Filter (p/n 5185-5836) was used to filter the sample solution before HPLC.

HPLC conditions

Column:	ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm		
Flow rate:	1.0 mL/min		
Injection volume:	5 µL		
Column temperature:	18 °C		
Detection wavelength:	230 nm		
Mobile phase:	Water-Acetonitrile Gradient		
	Time (minutes)	% Water	% Acetonitrile
	0	70	30
	10	65	35
	23	50	50
	30	20	80

Separation

1. Weigh 200 grams of crucian meat, homogenize, and store in a clean, sealed container at –18 °C.
2. Place 1 g of homogeneous sample (accurate to 0.01 g) into a 10-mL polypropylene centrifuge tube with 5 mL of methanol.
3. Vortex for 1 minute.
4. Extract ultrasonically for 10 minutes in an ice bath.
5. Centrifuge the sample at a speed of 4000 r/min for 5 minutes and remove the 3 mL of supernatant.
6. Save in a clean tube and evaporate with N_2 below 40 °C.
7. Reconstitute the residue in 5 mL of 5 % methanol in water.

SPE Purification

The procedure used for the SPE extraction is shown in Figure 1. Agilent SampliQ OPT cartridges are preconditioned with 3 mL of methanol then 5 mL of water. The 5-mL extract (equivalent to 0.6 g sample) is passed through the SampliQ OPT cartridge at a speed of 1 mL/min. After it effuses completely, the cartridge is washed with 5 mL of 30% methanol in water and the entire effluent is discarded. The cartridge is dried under negative pressure (below 2.0 kPa) for 3 minutes. The sample is then eluted with 6 mL of methanol, and the eluent is collected and dried under nitrogen below 40 °C. The residue is dissolved and brought to a constant volume of 1.0 mL using methanol, filtered through a 0.45 µm PTFE filter membrane, and analyzed by HPLC.

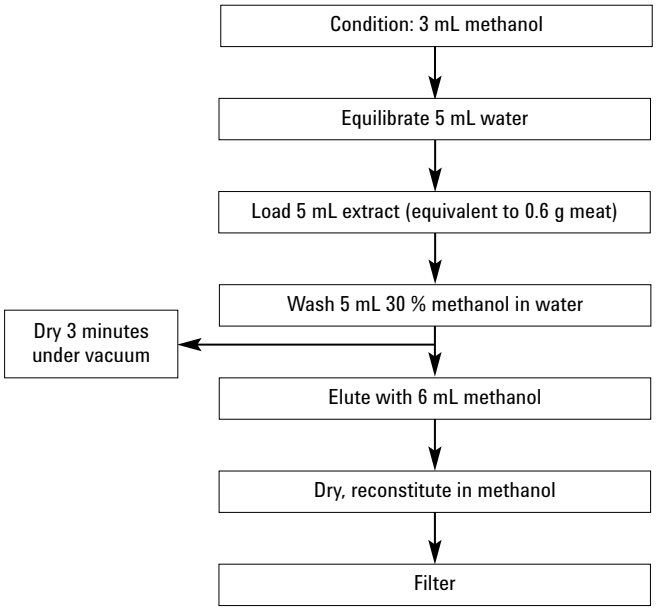


Figure 1. Hormones in crucian meat SPE procedure.

Results and Discussion

Linearity, Limits of Detection

Stock solutions were diluted to different concentrations and analyzed by HPLC. Linear regressions were calculated for the hormones based on the areas and the solution concentrations. Limit of detection (LOD) signifies the injection concentration at which the signal to noise ratio was between 2 and 3. Linear range was between 1–100 mg/kg. The linearity and LOD are shown in Table 2.

Table 2. Linearity and LODs of Hormones.

No.	Compound	Regression equation	Correlation coefficient	LOD (mg/kg)
1	Estriol	Y = 8.096 × –0.824	0.9998	0.5
2	Prednisolone	Y = 17.418 × –2.088	0.9999	0.2
3	Hydrocortisone	Y = 15.746 × –1.518	0.9999	0.3
4	Prednisone	Y = 20.192 × –2.152	0.9998	0.2
5	Methylprednisolone	Y = 16.986 × –1.894	0.9999	0.4
6	Betamethasone	Y = 20.439 × –1.106	0.9997	0.2
7	Dexamethasone	Y = 20.176 × –2.176	0.9999	0.2
8	Triamcinolone acetate	Y = 16.374 × –1.558	0.9997	0.4
9	Gestrinone	Y = 6.370 × –0.668	0.9998	1.0
10	Prednisolone acetate	Y = 15.589 × –1.627	0.9999	0.4
11	Hydrocortisone acetate	Y = 15.051 × –1.584	0.9999	0.4
12	Prednisone acetate	Y = 24.106 × –2.401	0.9997	0.2
13	Estradiol	Y = 8.709 × –0.635	0.9999	0.8
14	Cortisone acetate	Y = 19.826 × –2.336	0.9996	0.4
15	Methyltestosterone	Y = 19.980 × –2.209	0.9996	0.3
16	Estrone	Y = 10.701 × –0.847	0.9999	0.4

Recovery and Repeatability

The precision of the method was determined in terms of the recovery of spiked hormone standards in crucian meat at 2, 5, and 10 mg/kg levels. The analysis was repeated six times at each level. The chromatograms of the blank, the standards, and the spiked standard (2 mg/kg) sample are shown in Figures 2 through 4. The recovery and reproducibility data are shown in Table 3.

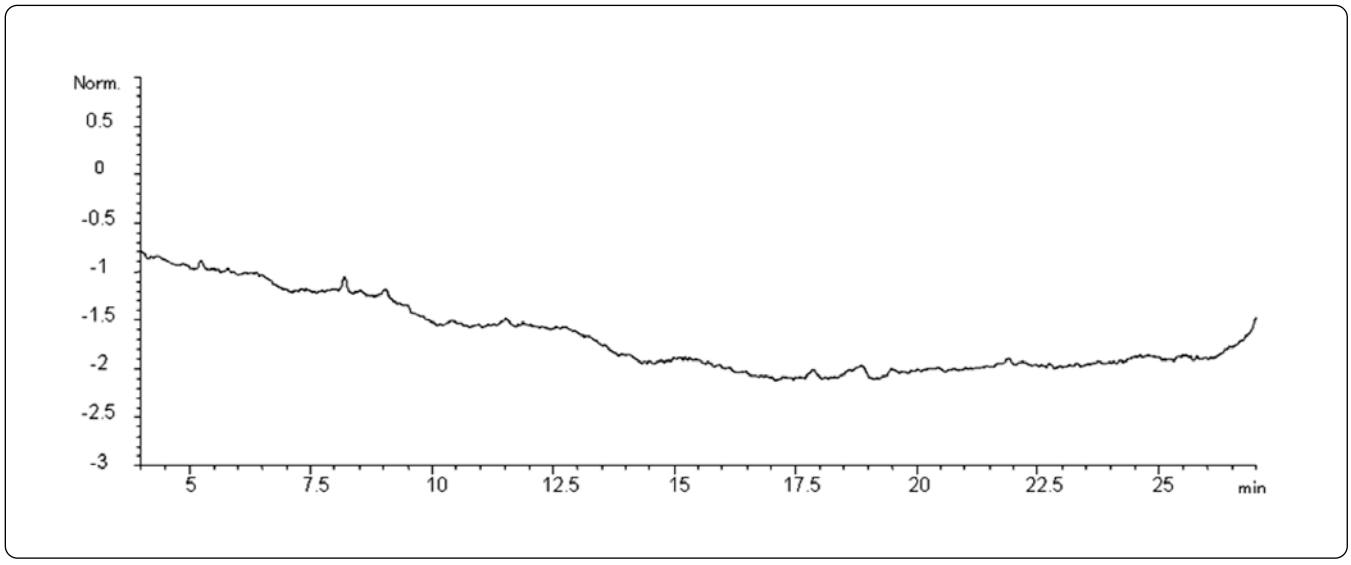
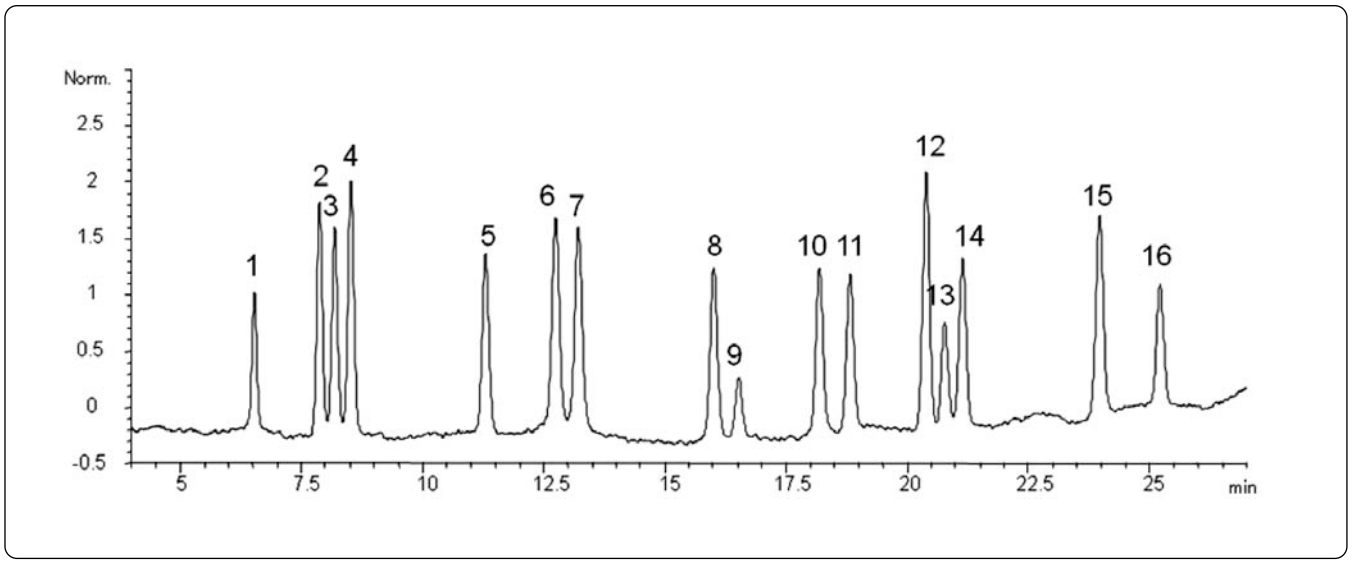
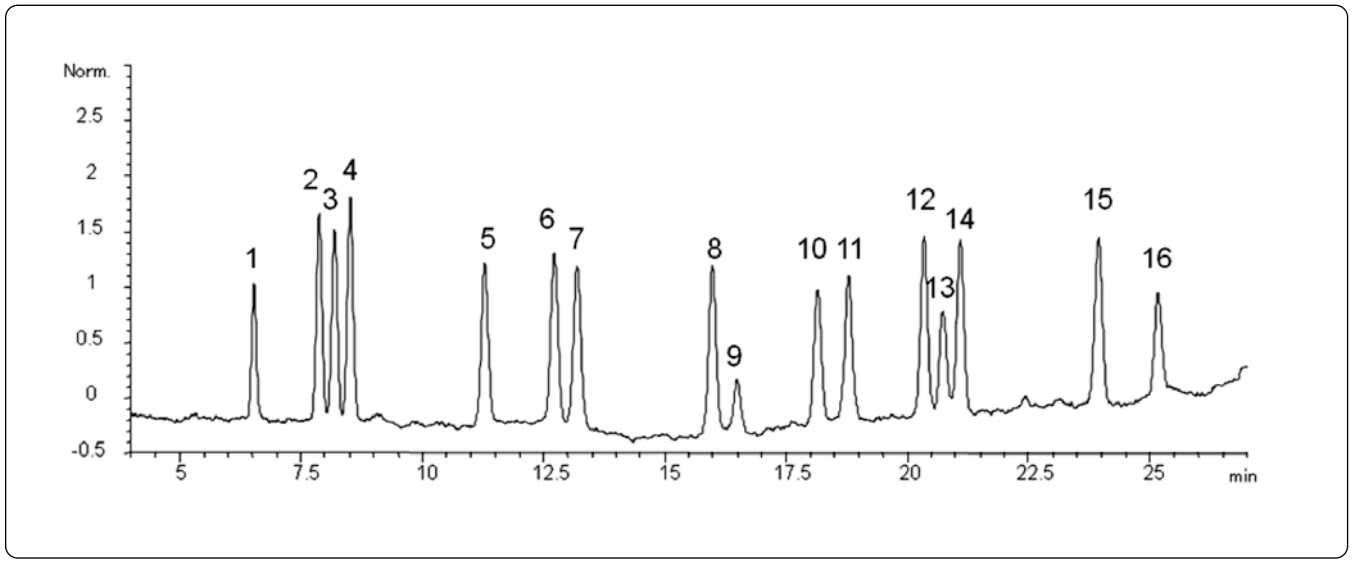


Figure 2. Chromatogram of crucian meat blank.



1	Estriol	5	Methylprednisolone	9	Gestrinone	13	Estradiol
2	Prednisolone	6	Betamethasone	10	Prednisolone acetate	14	Cortisone acetate
3	Hydrocortisone	7	Dexamethasone	11	Hydrocortisone acetate	15	Methyltestosterone
4	Prednisone	8	Triamicinolone acetate	12	Prednisone acetate	16	Estrone

Figure 3. Chromatogram of hormone standards at 2 mg/kg.



1	Estriol	5	Methylprednisolone	9	Gestrinone	13	Estradiol
2	Prednisolone	6	Betamethasone	10	Prednisolone acetate	14	Cortisone acetate
3	Hydrocortisone	7	Dexamethasone	11	Hydrocortisone acetate	15	Methyltestosterone
4	Prednisone	8	Triamicinolone acetate	12	Prednisone acetate	16	Estrone

Figure 4. Chromatogram of crucian meat sample spiked hormone standards at 2 mg/kg.

Table 3. Recoveries and RSDs of Hormones in Crucian Meat by SPE

Compund	Spiked level (mg/kg)	Recovery (%)	RSD (n = 6, %)
Estriol	2	100.4	2.2
	5	106.1	1.9
	10	102.4	4.4
Prednisolone	2	89.4	3.8
	5	90.9	7.6
	10	100.7	2.9
Hydrocortisone	2	85.3	6.7
	5	91.4	7.6
	10	101.4	3.4
Prednisone	2	82.5	7.2
	5	92.1	5.2
	10	100.7	2.9
Methylprednisolone	2	83.2	8.3
	5	93.6	3.2
	10	97.4	1.7
Betamethasone	2	88.3	8.9
	5	99.6	4.9
	10	100.8	3.8
Dexamethasone	2	79.1	4.3
	5	98.4	5.3
	10	98.4	3.9
Triamcinolone acetate	2	86.7	8.4
	5	97.6	5.9
	10	97.9	4.1
Gestrinone	2	78.0	6.6
	5	78.8	8.1
	10	85.3	8.0
Prednisolone acetate	2	86.9	7.3
	5	101.2	4.3
	10	101.9	5.7
Hydrocortisone acetate	2	87.3	6.8
	5	102.7	5.1
	10	101.5	7.9
Prednisone acetate	2	76.7	7.7
	5	94.1	3.5
	10	97.7	4.3
Estradiol	2	78.7	4.2
	5	94.7	3.5
	10	97.4	4.8
Cortisone acetate	2	82.8	6.9
	5	87.8	6.5
	10	94.4	4.1
Methyltestosterone	2	82.9	3.4
	5	91.9	4.9
	10	93.6	4.6
Estrone	2	76.2	6.4
	5	90.0	8.7
	10	93.9	5.9

Conclusions

Agilent's SampliQ OPT, a polymeric sorbent with combined hydrophilic and lipophilic characteristics that allows retention of both polar and non-polar compounds, provides a simplified and effective single cartridge method for the purification and enrichment of multiple hormone compounds in crucian carp. Recovery and reproducibility (routinely below 10%) based on solution standards are acceptable for hormone residue determination in crucian meat. Impurities from crucian were minimal and did not interfere with any of the hormones analyzed.

Product Information

Part number	Description
5982-3013	OPT Polymer - Box, 100x 1 mL tubes, 30 mg
5982-3036	OPT Polymer - Box, 50x 3 mL tubes, 60 mg
5982-3067	OPT Polymer - Box, 30x 6 mL tubes, 150 mg
5982-3096	OPT Polymer - 96 Well Plate, 10 mg
95990-902	Agilent ZORBAX Eclipse Plus C18 250 mm × 4.6 mm, 5 µm
5185-5836	Agilent PTFE 0.45 µm Premium Syringe Filter

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Agilent Technologies

Determination of β2-Agonists in Pork Using Agilent SampliQ SCX Solid-Phase Extraction Cartridges and Liquid Chromatography-Tandem Mass Spectrometry

Application Note

Food Safety

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Abstract

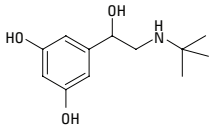
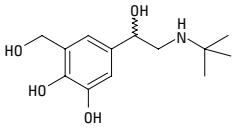
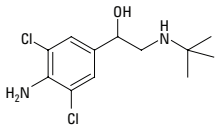
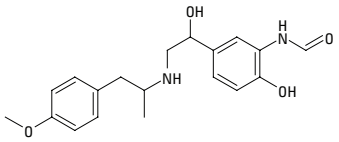
A method for simultaneous determination of four β2-agonist residues of terbutaline, salbutamol, clenbuterol and formoterol in pork has been developed and validated. The analytes are purified by liquid-liquid extraction (LLE) and solid-phase extraction (SPE) and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The method provides a sub-ng/g level of limit of detection (LOD) for all four β2-agonists in pork. The dynamic calibration ranges for these compounds are obtained from 0.25 to 5 ng/g. The overall recoveries range from 78 to 101% with RSD values between 1.8 and 7.2%.

Agilent Technologies

Introduction

The β2-agonists have been used worldwide as illegal growth promoters in pork production. Recent incidences of poisoning have occurred due to high levels of the β-agonist (clenbuterol) in pork. This application note used Agilent's new SPE products to extract and enrich four β-agonists from pork and analysis by LC-MS/MS. Table 1 shows the name and structure of the four β-agonist compounds.

Table 1. β2-Agonist Compounds Used in this Study

Compound	Log P	Structure
Terbutaline	0.55	
Salbutamol	0.44	
Clenbuterol	2.94	
Formoterol	1.91	

Experimental

Reagents and Chemicals

All reagents were MS, HPLC or analytical grade.

Acetonitrile and water were from Scharlau. Ethyl acetate and isopropanol were from Fisher. The standards were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Pork was purchased from a local market.

Standard solutions (1.0 mg/mL) were made in methanol individually, and refrigerated at 4 °C. A combined working solution (10 µg/mL) was made in methanol-water (10:90) and also stored at 4 °C. The spiked solutions were then made weekly by appropriately diluting the combined working solution in water.

Equipment and Materials

Agilent 1200 HPLC system

Agilent 6460 Triple Quadrupole LC-MS/MS system

Agilent SamliQ SCX Polymer cartridges, 50 × 3 mL tubes, 60 mg (p/n: 5982-3236)

Agilent ZORBAX Eclipse Plus C18, 50 × 2.1 mm, 1.8 µm (p/n: 959741-906)

Agilent Vaccum Manifold processing station (p/n: 5982-9120)

Sample Preparation

Liquid-Liquid Extraction

A 2 g amount of pork (±0.01 g) was weighed into a 15 mL capped polypropylene tube. To the pork, 8 mL of 0.2 M sodium acetate (pH 5.2) solution were added and mixed in a vortex. Next, 100 µL β-glucuronidase (1000 U/mL) were added and the tube vortexed thoroughly for 2 minutes. The sample was hydrolyzed at 37 °C for 16 hours.

The hydrolysate was shaken for 15 minutes and centrifuged at 4000 rpm for 10 minutes. A 4 mL amount of supernatant was transferred to another centrifuge tube. A 5 mL amount of 0.1 M perchloric acid solution was added and the pH was adjusted to 1 ± 0.3. The tubes were then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to another tube, and the pH was adjusted to 11 with 10 M sodium hydroxide.

Ten milliliters each of a saturated sodium chloride solution isopropanol-ethyl acetate (60:40) were added to the tubes. The tubes were shaken for 5 minutes. The tubes were centrifuged at 4000 rpm for 5 minutes before the organic layer was carefully transferred to another tube. Isopropanol-ethyl acetate addition, shaking, centrifuging and organic layer transfer were repeated twice, and all supernants were combined.

Samples were evaporated to dryness with nitrogen at 40 °C. The residue was dissolved in 5 mL of 0.2 M sodium acetate (pH 5.2). The sample was then ready for SPE purification.

Solid-Phase Extraction

The SPE procedure is shown in Figure 1. Agilent SampliQ SCX cartridges were preconditioned with 3 mL of methanol and then equilibrated with 3 mL water. Five milliliters of the sample solution were then loaded onto a cartridge and passed through the cartridge by gravity (about 1 mL/min). The tubes were rinsed with 2 mL of water and 2 mL 2% formic acid in water. The entire effluent was discarded. Full vacuum was

applied to the cartridge for 3 minutes to completely dry the resin. Finally, the compounds were eluted with 5 mL of 5% ammonia solution in methanol at a rate of 1 mL/min. The eluent was dried with nitrogen flow at 40 °C. The residue was reconstituted in 1 mL of 0.1% formic acid in water/acetonitrile (90:10). The sample was vortexed and ultrasonicated to completely dissolve the residue. The sample was transferred to a 1.5 mL tube and centrifuged at 3000 rpm for 5 minutes. The sample was transferred to a 2 mL chromatography vial for analysis.

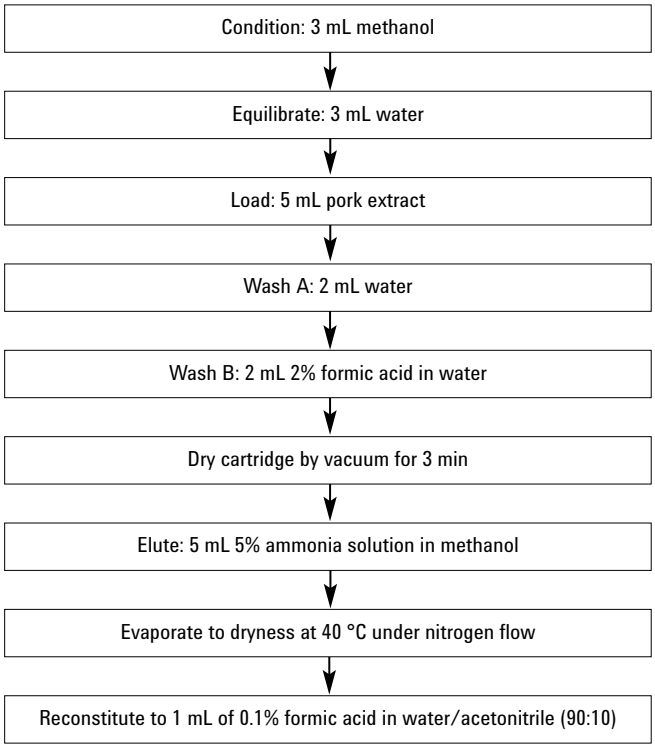


Figure 1. Pork clean up and enrichment – SPE procedure.

Instrument Conditions

HPLC Conditions

Column:	Agilent ZORBAX Eclipse Plus C18 2.1 mm × 50 mm 1.8 µm (p/n: 959741-906)		
Flow rate:	0.4 mL/min		
Column temperature:	40 °C		
Injection volume:	5 µL		
Mobile phase:	Water (0.1% FA+2 mM NH_4Ac, A), Acetonitrile (0.1% FA, B)		
Gradient:	Time (min)	%A	%B
	0	90	10
	0.5	90	10
	1.8	20	80
	2	90	10
	3.5	90	10

MS Conditions

These four compounds were monitored in the positive mode. The source conditions are shown in Figure 2 and the MRM channels are shown in Table 2.

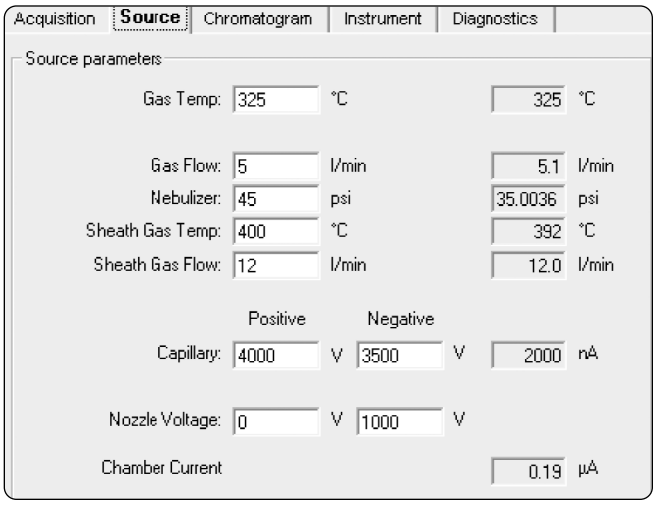


Figure 2. MS source parameters for these four compounds.

Table 2. Masses Monitored in the MRM

Compound	MRM for quantification	MRM for confirmation
Terbutaline	226.1 → 152.1	226.1 → 125
Salbutamol	240.1 → 148.1	240.1 → 222.1
Clenbuterol	227 → 203	227 → 259.1
Formoterol	345.1 → 149.1	345.1 → 327.1

Results and Discussion

Linearity and Limit of Detection

Solutions used to create external calibration curves were prepared by using a combined working solution to spike matrix blanks (0.25, 0.5, 1.0, 2.0 and 5.0 ng/g). Matrix blanks were created by taking pork through the hydrolysis, LLE and SPE procedures. The results for the calibration curves are shown in Table 3. The limits of detection (LOD) were chosen as the concentration of each compound that gave a signal to noise (S/N) ratio greater than 3:1. The LODs are also shown in Table 3.

Table 3. Linearity and LODs of β2-Agonists

Compound	Regression equation	R^2	LOD in pork (ng/g)
Terbutaline	Y = 3470x + 1325.4	0.9972	0.05
Salbutamol	Y = 13099x + 2900.3	0.9921	0.05
Clenbuterol	Y = 27028x + 1143.7	1	0.02
Formoterol	Y = 23251x + 487.44	0.9983	0.02

Recovery and Reproducibility

The recovery and reproducibility of the method were determined at three levels: pork spiked to a concentration of 0.5, 1.0, and 2.0 ng/g. The analysis was performed with six replicates at each level. The recovery and reproducibility data is shown in Table 4. The chromatograms of spiked pork extracts (1.0 ng/g) are shown in Figure 3.

Table 4. Recoveries and Reproducibility of β2-agonists in Pork After SPE Employing Agilent's SampliQ SCX; (p/n: 5982-3236), Recovery 90% and RSD 4.4% on Average

Compound	Spiked level (ng/g pork)	Recovery (%)	RSD (n=6)
Terbutaline	0.5	88.7	5.4
	1	98.0	7.2
	2	100.8	5.9
Salbutamol	0.5	100.6	1.8
	1	92.9	2.1
	2	97.4	3.9
Clenbuterol	0.5	82.3	5.0
	1	91.5	6.3
	2	90.6	4.3
Formoterol	0.5	85.1	1.9
	1	83.0	4.0
	2	77.9	2.5

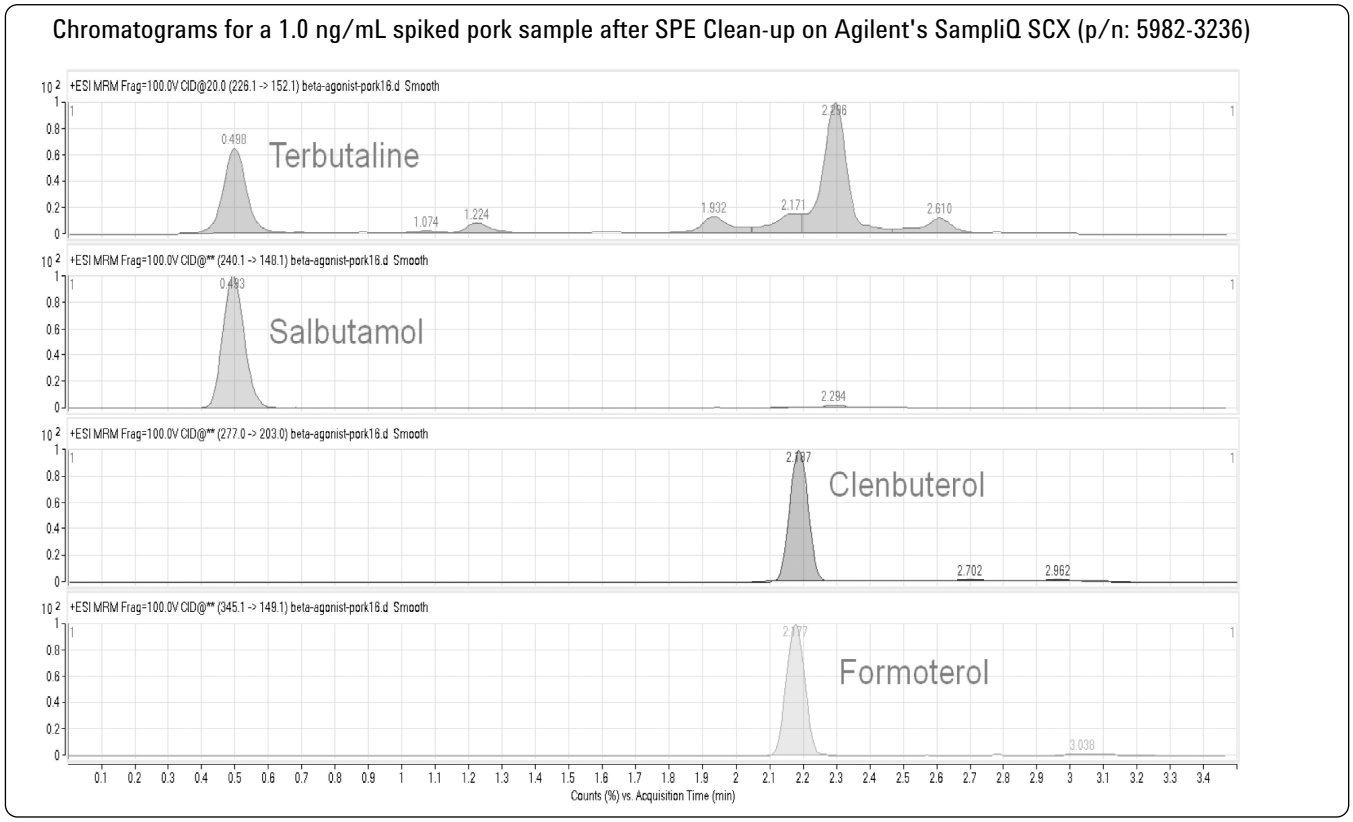


Figure 3. Chromatograms of 1.0 ng/g spiked pork sample extract.

Conclusions

The result of this study show that Agilent SampliQ SCX can be used as an effective method for purification and enrichment of multiple β2-agonists in a complex matrix such as pork. The recovery and reproducibility results based on matrix spiked standards are acceptable for β2-agonists residue determination in pork under Chinese regulations. The impurities and matrix effects are minimal and do not interfere with the quantification of any target compound. The LOQ are significantly lower than the MRLs [1,2].

References

1. GB/T 21313-2007 "Analysis of β2-agonists in Foods of Animal Origin by High Performance Liquid Chromatography Tandem Mass Spectrometry"
2. SN/T 1924-2007 "Determination of Clenbuterol, Ractopamine, Salbutamol and Terbutalin Residues in Foodstuffs of Animal Origin for Import and Export -HPLC-MS/MS Method."

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Agilent Technologies

Profiling medications for decorative or aquarium fish using the Agilent 1290 Infinity LC system and Agilent ZORBAX Poroshell 120 2.7 µm columns

Application Note

Consumer Products

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Abstract

The Agilent 1290 Infinity LC system has significant capabilities for a wide range of HPLC and UHPLC applications. It exhibits a broader power range (for example, the combination of pressure and flow capabilities), and the flexibility to operate a wide range of column dimensions and particle sizes than any other commercially available system. Advanced optical design in the diode array detector allows a wide dynamic range and high sensitivity, both of which are critical in the monitoring of small impurities in fine chemicals.

The combined benefits are demonstrated by a separation of primary components and related impurities including sulfa drugs, nitrofurans and malachite green found in samples of fish medications. A broad range of products for treating ailments in decorative or pet fish are available. Many of these medications are banned or restricted for use in edible fish. If present in edible fish, the levels would be very low or undetectable by HPLC with UV based detection. These examples show a few medications and detail the rapid method development used to establish a rapid MS-compatible separation environment. Many fish medications advertise the use of "pharmaceutical quality" ingredients, and may imply pharmaceutical quality manufacturing and quality control procedures. When profiling these products one should expect to see very low levels of related impurities, consistent with the goals of pharmaceutical quality manufacturing

The high pressure capability of the system allows the use of methanol, and acetonitrile, to explore the selectivity of the two solvents. At 1 mL/min, using a simple 3 minute gradient and a 3.0 mm x 50 mm Poroshell 120 column, the analysis time is only less than 5 minutes including the late eluting phthalate artifact. The separation of the main components of a medicated powder with acetonitrile and methanol is shown in Figure 1, and the extraction of a medicated feed is shown in Figure 2.

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The speed, resolution and flexibility of the system are further demonstrated by a separation of a sulfa standard mix using solvent, gradient and temperature optimization with a 100 mm length Poroshell 120 column (see Figure 3).

After further optimization of the sulfa mix using methanol with the elevated temperature, all of the samples were run with the final method configuration, as shown in Figure 4.

Configuration

- Agilent 1290 Infinity Binary Pump with Integrated Vacuum Degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1200 Diode Array Detector (G1315C)

Conclusion

Taking advantage of flexible solvent and column selection features, and high pressure capability, of the system allows one to use highly efficient columns to rapidly develop separations with remarkable resolution while conserving solvent over the use of 4.6 mm id columns.

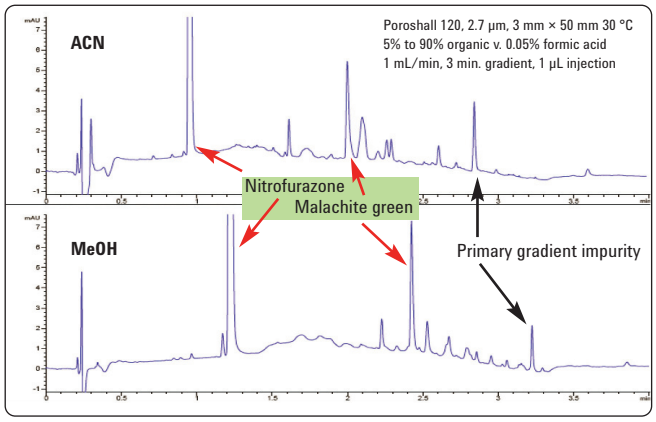

Figure 1
"Super Ick" medicated powder.

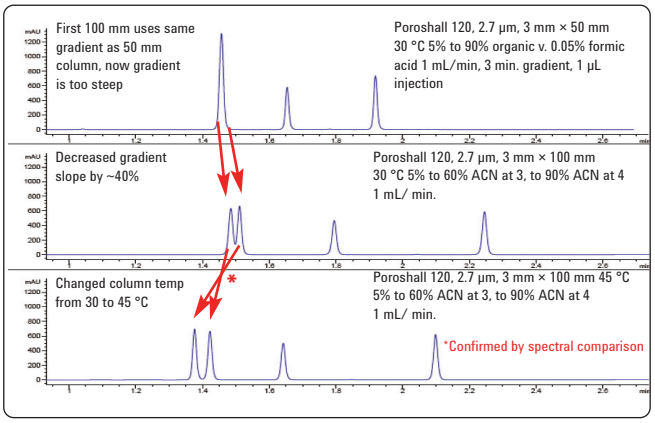

Figure 3
Sulfa standard mixture (Agilent p/n 59987-20033).

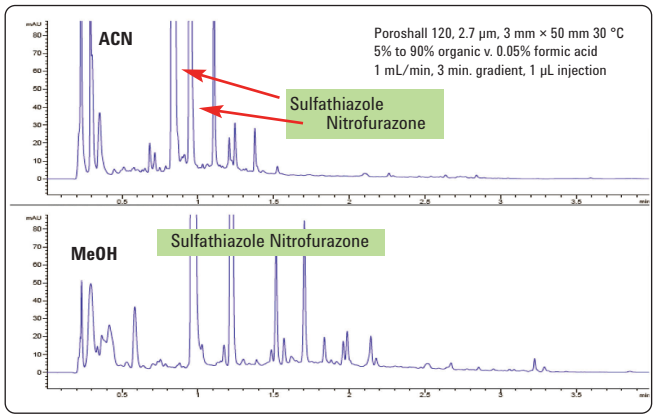

Figure 2
Separation of "Jungle" medicated fish food after methanol/water/formic acid extraction.

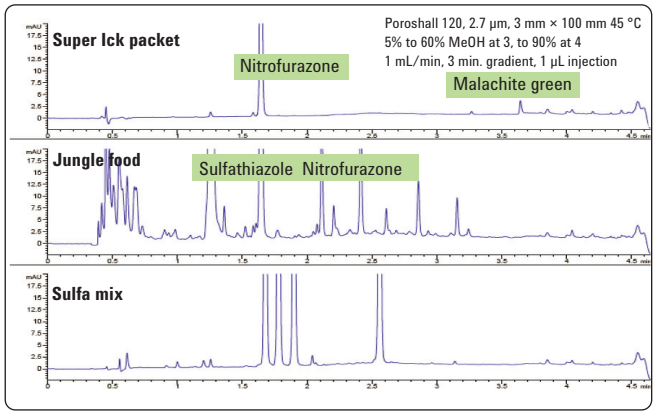

Figure 4
Final method configuration.

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Agilent Technologies

Determination of Sulfonamide Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS EN Kits by LC/MS/MS

Application Note

Food

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Abstract

This paper presents an analytical method for the determination of nine sulfonamide antibiotic residues in bovine liver: sulfadizine, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, and sulfadimethoxin. The procedure involves a rapid and efficient pretreatment with SampliQ QuEChERS kits. The homogenized liver sample was initially extracted in a buffered aqueous/1% acetic acid acetonitrile system with an extraction and partitioning step after the addition of salts. Finally, the sample was cleaned up using dispersive solid-phase extraction (dispersive-SPE). The final extracts were analyzed by the sensitive and selective determination of all compounds in a single run using LC-ESI-MS-MS operating in positive ion multiple reaction monitoring (MRM) mode. Sulfapyridine was selected as the internal standard. The accuracy of the method, expressed as recovery, was between 53 and 93%. The precision, expressed as RSD, was between 2.1 and 16.8%. The established 5 ng/g limits of quantification (LOQ) were much lower than the respective Maximum Residue Limit (MRL) for sulfonamide in animal food products (20-100 ng/g).

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Introduction

Sulfonamides (SAs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic, prophylactic, and growth-promoting purposes. Residues of SAs may remain in animal tissues if adequate withdrawal time is not observed or if the SAs have been improperly administered. The maximum residue limit (MRL) in the European Union countries and United States for SAs in animal muscle tissue is 100 ng/g, while in Japan it is 20 ng/g. [1]

The QuEChERS EN method is an important variation, which has been officially accepted by the European Committee for Standardization and is widely applied to pesticide analysis in foods of plant origin [2]. The original EN buffered method was designed mostly for multiresidue pesticide analysis in plant food products. In summary, the method uses acetonitrile extraction followed by the salting out of water from the sample using anhydrous magnesium sulfate ($MgSO_4$), NaCl, and buffering citrate salts to induce liquid-liquid partitioning. For cleanup, a dispersive solid-phase extraction (dispersive-SPE) is conducted using a combination of primary secondary amine (PSA) to remove fatty acids from other components and anhydrous $MgSO_4$ to reduce the remaining water in the extract. After mixing and centrifuging, the upper layer is ready for analysis. Fatty dispersive-SPE, which contains 25 mg C18EC per mL of ACN extract, is employed to remove more lipids from the matrix when using fruits and vegetables with fats and waxes.

Food matrices from animal origin contain a substantial amount of proteins and lipids. Therefore, they are very different from food matrices of plant origin such as fruits and vegetables. In this study, a SampliQ QuEChERS EN buffered extraction kit (p/n 5982-5650) and EN fatty dispersive-SPE 15 mL kit (p/n 5982-5165) were tested for the analysis of sulfonamide antibiotics in bovine liver. Because of the differences in food matrices and the chemical properties of the target analytes, modifications to the method were also investigated to achieve efficient extraction and cleanup.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO), and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The sulfonamides standards and the internal standard were from Sigma (St Louis, MO, USA).

Solutions and Standards

A 1 M ammonium acetate stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate solution in water, pH 3, was made by adding 5 mL of 1 M ammonium acetate stock solution into 1 L of Milli-Q water. The pH was then adjusted to 3 by adding glacial acetic acid and monitoring with a pH meter. A 1:1 MeOH/ACN solution was made by combining 500 mL of MeOH and ACN, and mixing well. A 1% acetic acid in ACN solution was prepared by adding 5 mL of glacial acetic acid to 500 mL of ACN, and mixing well. A 1:1 ACN/H_2O solution with 0.1% FA was prepared by combining 50 mL of ACN and Milli-Q water, then adding 100 µL of formic acid. A 1:9 MeOH/H_2O solution with 0.1% FA was prepared by combining 10 mL of MeOH and 90 mL of Milli-Q water, then adding 100 µL of formic acid.

Standard and internal standard (IS) stock solutions (1.0 mg/mL for all) were all made in DMSO and stored at 4 °C. Six of the sulfonamides are light-sensitive, so the stock

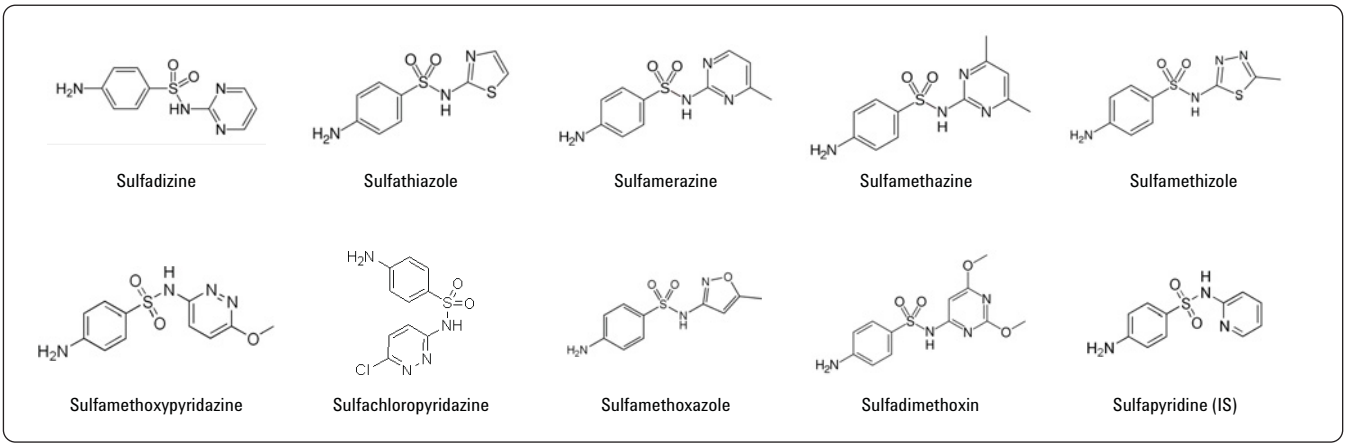

Figure 1. Chemical structures of the quinolone antibiotics investigated in this study.

solutions were kept in amber vials and wrapped in aluminum foil. Three combined QC spiking solutions of 0.2, 4, and 16 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 20 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract. Due to light sensitivity of certain sulfonamides, all of the combined solutions were kept in amber vials and wrapped in aluminum foil. A 20 µg/mL IS spiking solution of Sulfapyridine was made in 1:1 ACN/H_2O containing 0.1% FA.

Equipment and Material

- Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA)
- Agilent 6410 Triple Quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA)
- Agilent SampliQ QuEChERS EN Extraction kit p/n 5982-5650 (Agilent Technologies Inc., DE, USA)
- Agilent SampliQ QuEChERS EN fatty dispersive-SPE kit for 15 mL p/n 5982-5156 (Agilent Technologies Inc., DE, USA)
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- 2010 Geno Grinder (Spex SamplePrep LLC, Metuchen, NJ, USA)
- Multitube Vortexer (Henry Troemner LLC, Thorofare, NJ, USA)

Instrument conditions

HPLC conditions

Column	Agilent ZORBAX Solvent Saver HT Eclipse Plus C18 50 × 3.0 mm, 1.8 µm (p/n: 959941-302)		
Flow rate	0.3 mL/min		
Column Temperature	30 °C		
Injection volume	10 µL		
Mobile Phase	A: 5 mM ammonium acetate, pH 3.0 in H2O		
	B: 1:1 MeOH/ACN		
Needle wash	1:1:1:1 ACN/ MeOH/ IPA/ H2O with 0.2% FA.		
Gradient	Time	% B	Flow rate (mL/min)
	0	15	0.3
	0.2	15	0.3
	6.0	60	0.3
	6.01	100	0.3
	7.0	STOP	
Post run	3.5 min		
Total cycle time	~11 min.		

MS conditions

Polarity	positive
Gas Temperature	325 °C
Gas Flow	8 L/min
Nebulizer	50 Psi
Capillary	4000 V

Other conditions relating to the analytes are listed in Table 1.

Table 1. Instrument Acquisition Data for the Analysis of 9 Sulfonamide Antibiotics by LC/MS/MS.

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)
Sulfadizine	1) 251.1 → 108.0	100	25	2.1
	2) 251.1 → 156.0		13	
Sulfathiazole	1) 256.0 → 156.0	94	13	2.3
	2) 256.0 → 92.1		29	
Sulfamerazine	1) 265.1 → 92.1	125	29	2.9
	2) 265.1 → 108.1		25	
Sulfamethizole	1) 271.0 → 156.0	112	9	3.7
	2) 271.0 → 92.1		29	
Sulfamethazine	1) 279.1 → 124.0	116	21	3.8
	2) 279.1 → 92.1		33	
Sulfamethoxypyridazine	1) 281.1 → 156.0	128	13	3.9
	2) 281.1 → 92.1		29	
Sulfachloropyridazine	1) 285.0 → 156.0	106	9	4.5
	2) 285.0 → 92.1		29	
Sulfamethoxazole	1) 254.1 → 92.1	113	25	4.8
	2) 254.1 → 108.0		21	
Sulfadimethoxin	1) 311.1 → 156.0	141	17	6.0
	2) 311.1 → 92.1		37	
Sulfapyridine (IS)	250.1 → 92.1	113	29	2.7

1) Quantifier transition channel 2) Qualifier transition channel

Sample preparation

Sample preparation includes sample homogenization, extraction and partitioning, and dispersive-SPE cleanup. Since the main focus of existing QuEChERS methodology has been the extraction of pesticides from plant and vegetable matrices, certain modifications were adapted in order to optimize results for the determination of sulfonamides in bovine liver. These modifications will be discussed in detail later.

Bovine liver was purchased from a local grocery store. It was then washed and chopped into small pieces. The chopped liver sample was homogenized thoroughly with a food grinder, then stored at -20 °C. Two gram (±0.05 g) amounts of homogeneous sample were placed into 50 mL centrifuge tubes. Sample tubes were centrifuged 30 s to move any sample sticking to the inside wall of tube to the bottom. Samples were then fortified with appropriate QC spiking solutions (50 µL) when necessary. Then 50 µL of IS spiking solution (20 µg/mL of sulfapyridine) were added. After vortexing sample for 30 s, 8 mL of Milli-Q water were added. Tubes were then vortexed another 10 s for mixing. Ten milliliters of 1% AA in ACN were added to each tube. Tubes were capped and shaken by a 2010 Geno Grinder for 30 s. An Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650) was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min by the 2010 Geno Grinder at 4 °C.

A 6 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ EN QuEChERS fatty dispersive-SPE 15 mL tube (p/n 5982-5156). This 15 mL dispersive-SPE tube contained 150 mg of PSA, 150 mg of C18EC, and 900 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 2 min. The 15 mL tubes were centrifuged at 4000 rpm for 5 min. A 4 mL amount of extract was then transferred into another tube and dried by N_2 flow at 40 °C. Samples were reconstituted into 800 µL of 1:9 MeOH/H_2O solution with 0.1% FA. After vortexing and sonicating for 10 min, the sample was filtered by a 0.22 µm Cellulose Acetate Spin Filter (p/n 5185-5990). The clear filtered sample was transferred into an autosampler vial. The samples were capped and vortexed thoroughly, in preparation for LC/MS/MS analysis.

See Figure 2 for the flow chart of the extraction procedure for a bovine liver sample.

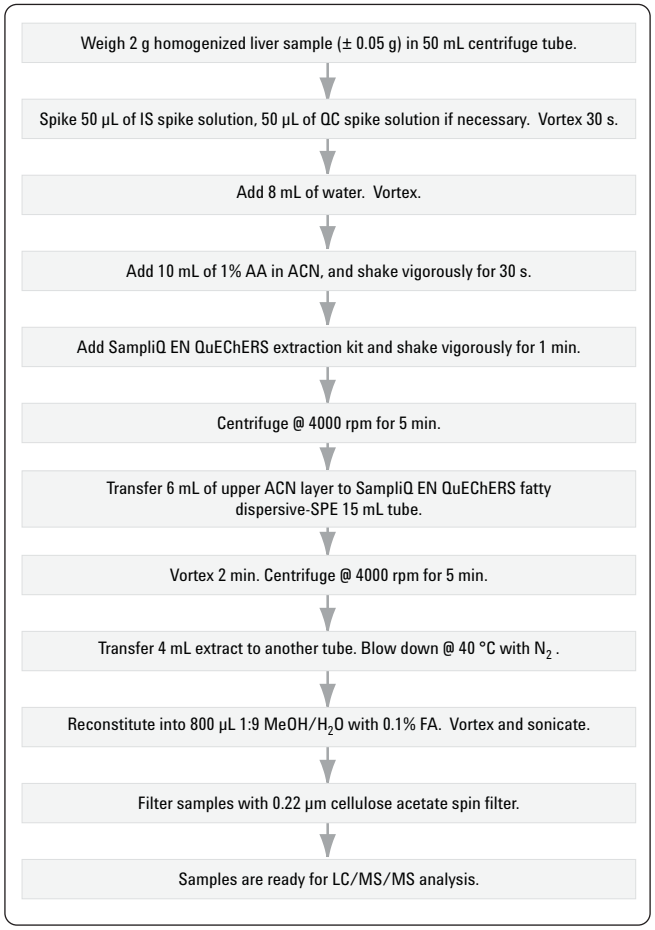

Figure 2. Flow chart of QuEChERS procedure for the determination of sulfonamides in bovine liver.

Results and Discussion

Method Optimization in the Liver Matrix

As mentioned previously, modifications of the QuEChERS EN method were investigated relative to extraction efficiency. An EN buffered extraction system provides a solution with a pH of 5.0 – 5.5, which illicits neutral sulfonamide analytes (pKa ~ 6-7). PSA sorbent used in dispersive-SPE can strongly interact with acid compounds and remove various co-extractive interferences, such as polar organic acids, sugars, and fatty acids. However, it may also interact with target compounds and cause the loss of analytes. Therefore, the QuEChERS fatty EN dispersive-SPE kit with PSA was compared to the dispersive-SPE kit without PSA. A previous study [4] showed that the addition of acid to acetonitrile can inhibit the absorption of PSA, weakening the attraction of PSA to the compounds of interest. Therefore, a 1% AA in ACN was evaluated in addition to standard ACN in the first partitioning step.

To evaluate the original EN and modified method, a 50 ng/g of fortified liver sample was extracted with the following procedures:

1. EN buffered extraction kit with ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL)
2. EN buffered extraction kit with ACN and dispersive-SPE kit without PSA (25 mg C18EC per mL)
3. EN buffered extraction kit with 1% AA in ACN and dispersive-SPE kit with PSA (25 mg PSA and C18EC per mL).

The corresponding matrix blanks were extracted at the same time, then post-spiked with the same amount of sulfonamide standards. Neat solution post-spiked at the same concentration was also compared to the matrix post-spiked samples.

The results are shown in Table 2. The results of method 1 and method 2 show that PSA does contribute to the matrix cleanup during the dispersive-SPE step. The matrix effect values for all of the analytes in method 1 were lower than those in method 2 indicating that the sample processed by method 1 was cleaner than the sample processed by method 2. This was also demonstrated by the color of the ACN extract. After the extraction step, the ACN extract was a brownish-red color. Using PSA in the dispersive step produced an ACN extract that was light yellow in color. Removing PSA from the dispersive step maintained the previously observed brownish-red ACN extract. Unfortunately, the presence of PSA in the dispersive step also caused the loss of certain analytes and produced very low recovery for sulfachloropyridazine (30%) and sulfamethizole (15%). Method 3 produced substantially better recoveries for all the analytes relative to methods 1 or 2. The matrix effect values show that the sample processed by method 3 is as clean as the sample processed by method 2, but not as clean as the sample processed by method 1. The addition of acid in the ACN partitioning step impedes the performance of PSA in the dispersive step preventing the loss of analytes. It also decreases the interaction of PSA with other matrix interferences, producing a greater matrix effect. This is also shown by the color of the ACN extract. Although PSA was used in the dispersive step, the presence of acidified ACN extract elicited a light brown-red color, rather than the light yellow extract in method 1.

Table 2. Method Optimization Results for the Analysis of Sulfonamides in Bovine Liver

	Method 1		Method 2		Method 3	
Analytes	**Recovery**	**Matrix effect**	**Recovery**	**Matrix effect**	**Recovery**	**Matrix effect**
Sulfadiazine	91.9	-33.0	85.2	-65.2	85.6	-57.9
Sulfathiazole	39.9	-35.9	42.0	-57.3	87.7	-65.7
Sulfamerazine	77.0	-19.3	43.9	-23.9	89.0	-51.7
Sulfamethizole	15.3	-33.6	46.5	-46.9	63.2	-49.8
Sulfamethazine	85.7	-23.1	51.4	-31.6	87.3	-42.0
Sulfamethoxypyridazine	76.6	-32.6	49.0	-31.7	86.1	-49.1
Sulfachloropyridazine	29.6	-38.5	51.1	-42.3	84.8	-50.6
Sulfamethoxazole	60.0	-40.9	53.4	-46.9	87.5	-54.5
Sulfadimethoxin	67.4	-35.3	56.9	-43.0	89.6	-51.9

Method 1 — EN buffered extraction kit + ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL)

Method 2 — EN buffered extraction kit + ACN + Dispersive-SPE kit without PSA (25 mg C18EC + 150 mg $MgSO_4$ per mL)

Method 3 — EN buffered extraction kit + 1% AA ACN + Fatty dispersive-SPE kit (25 mg PSA + 25 mg C18EC + 150 mg $MgSO_4$ per mL)

$$\% \text{ Recovery} = \frac{Response_{\text{extracted sample}}}{Response_{\text{post-extracted spiked sample}}} \times 100$$

$$\% \text{ Matrix Effects} = \left(\frac{Response_{\text{post-extracted spiked sample}}}{Response_{\text{non-extracted neat sample}}} - 1 \right) \times 100$$

Method 3 was selected for the final study. In this study, liver sample was extracted by the EN buffered extraction kit (p/n 5982-5650) with 1% AA in ACN. After centrifuging, the ACN extract was further cleaned by EN fatty dispersive-SPE 15mL tube (p/n 5982-5156). Figure 3 shows the MRM chromatograms of the liver control blank and 100 ng/g fortified liver extract. The liver control blank chromatogram indicated that the target analytes were free from any interference.

Linearity and limit of quantification (LOQ)

The linear calibration range for all the sulfonamide antibiotics was 5 - 400 ng/g. Matrix blanks were prepared for evaluation. Calibration curves, made from spiked matrix blanks, were made at levels of 5, 10, 50, 100, 200, 300 and 400 ng/g for each analyte. The sulfapyridine was used as an internal standard at 200 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) versus the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g limits of quantification (5 ppb) of the sulfonamides is far below the MRLs for residues of these antibiotics in animal food products (20 - 100 ng/g). Table 3 shows the regression equation and correlation coefficient (R^2). Linear regression fit was used with $1/x^2$ weight. Results indicated excellent linearity for all of analytes calibration curves over a broad quantification range.

Table 3. Linearity of Sulfonamide Antibiotics in Bovine Liver

Analytes	Regression Equation	R^2
Sulfadiazine	Y = 1.6585X + 0.0002	0.9963
Sulfathiazole	Y = 1.3899X + 0.0002	0.9942
Sulfamerazine	Y = 3.5019X – 0.0001	0.9962
Sulfamethizole	Y = 2.3064X + 0.0001	0.9963
Sulfamethazine	Y = 4.3780X – 0.0004	0.9977
Sulfamethoxypyridazine	Y = 4.4044X + 0.0003	0.9941
Sulfachloropyridazine	Y = 1.5869X – 0.0005	0.9971
Sulfamethoxazole	Y = 1.9047X – 0.0001	0.9936
Sulfadimethoxin	Y = 4.5106X + 0.0020	0.9922

Recovery and Reproducibility

The recovery and reproducibility were evaluated by fortifying sulfonamides standards in homogenized liver sample at levels of 5, 100, and 400 ng/g. These QC samples were quantified against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data are shown in Table 4. The results show that all of the sulfonamides were somewhat low but still at acceptable recoveries (average of 77.8%) and good precision (average of 7.2% RSD). Samples were concentrated during the procedure to optimize sensitivity, which also caused additional matrix effects that possibly contributed to a higher RSD of target compounds at low levels.

Table 4. Recovery and Repeatability of Sulfonamides in Fortified Liver Homogenate

	5 ng/g fortified QC		100 ng/g fortified QC		400 ng/g fortified QC	
Analytes	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Sulfadiazine	73.9	15.6	90.0	13.7	81.9	5.3
Sulfathiazole	62.9	16.8	75.3	8.4	67.9	5.8
Sulfamerazine	77.6	11.5	92.8	6.6	82.0	4.2
Sulfamethizole	62.8	4.7	60.7	6.5	53.0	2.1
Sulfamethazine	87.4	6.9	90.0	10.7	83.4	3.4
Sulfamethoxy-pyridazine	81.8	9.4	84.8	8.1	76.4	2.9
Sulfachloro-pyridazine	84.2	10.0	78.6	6.3	73.8	3.6
Sulfamethoxazole	85.9	7.6	82.3	5.9	78.1	3.3
Sulfadimethoxin	77.8	8.4	80.9	4.9	75.6	3.3

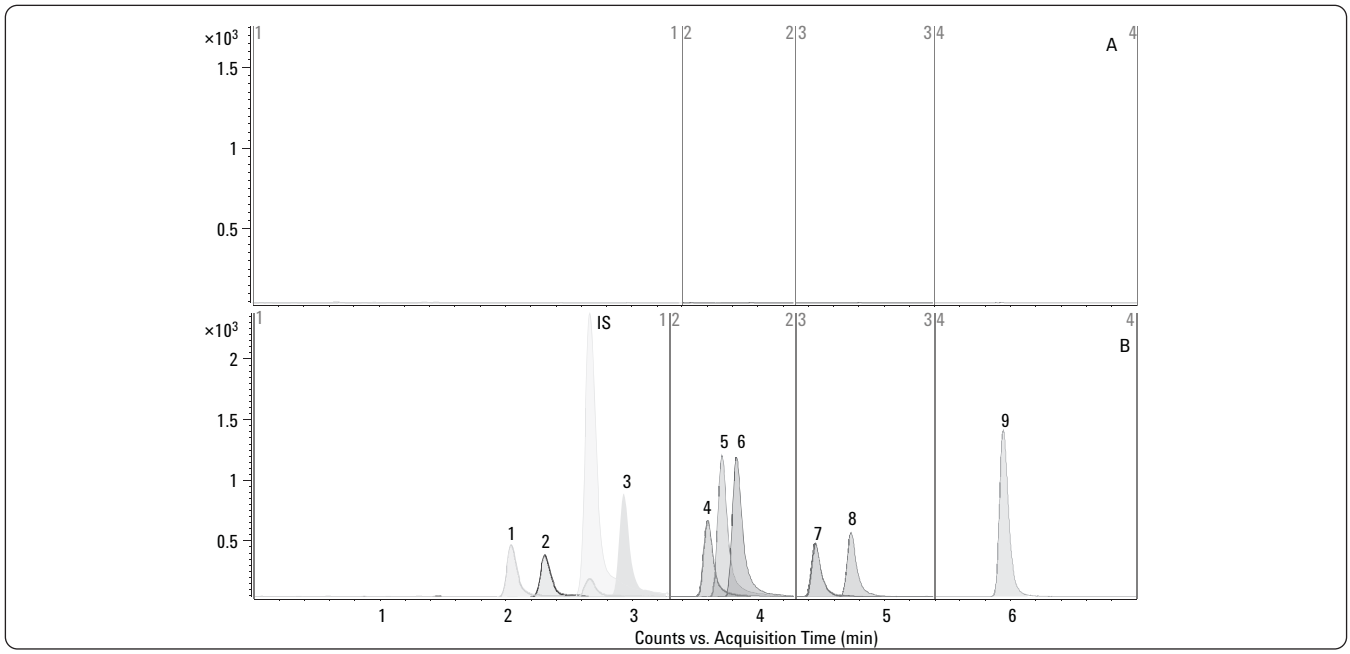

Figure 3. LC/MS/MS Chromatograms of A) liver blank extract, and B) 100 ng/g fortified liver extract. Peaks identification: 1. sulfadizine, 2. sulfathiazole, 3. sulfamerazine, 4. sulfamethizole, 5. sulfamethazine, 6. sulfamethoxypyridazine, 7. sulfachloropyridazine, 8. sulfamethoxazole, 9. sulfadimethoxin, IS (internal standard), sulfapyridine.

Conclusions

The Agilent SampliQ EN Buffered Extraction kit and SampliQ EN fatty dispersive-SPE kit provide a simple, fast, and effective method for the purification of sulfonamide antibiotics in bovine liver. When compared to other sample preparation methods, such as LLE and SPE, QuEChERS methodology is easy, fast, low cost and does not require automation. In addition, it is labor saving and a "greener" technology. The recovery and reproducibility, based on matrix-spiked standards, were acceptable for multiresidue sulfonamide determination in bovine liver. The impurities and matrix effects from liver were minimal and did not interfere with the quantification of any target compound. The LOQs of the quinolones were much lower than their regulated MRLs in animal food products (20-100 ng/g). This modified QuEChERS procedure is a very promising methodology for the quantitative analysis of sulfonamides in food products of animal origin. This application demonstrates great potential of SampliQ QuEChERS extraction and dispersive-SPE kits, and extend far beyond plant matrices to bio-matrices, such as animal food products and bio-fluid.

References:

[1] Sun H., Ai L., Wang F., Quantitative Analysis of Sulfonamide residues in Natural Animal Casings by HPLC, Chromatographia 2007, 66, Sept, (no. 5/6), 333-337.

[2] European Council Regulation 2377/90/EC 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, OJ L, 1990, p. 224.

[3] European Committee for Standardization/Technical Committee CEN/TC 275 (2007), Foods of plant origin: Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE-QuEChERS method. European Committee for Standardization, Brussels.

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Determination of Quinolone Antibiotics in Bovine Liver Using Agilent SampliQ QuEChERS Kits by LC/MS/MS

Application Note

Food

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Abstract

This paper presents an analytical method which allows the determination of 11 quinolone antibiotic residue in bovine liver: pipemidic acid, ofloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, cinoxacin, oxolinic acid, nalidixic acid, and flumequine.

The procedure involves a rapid and efficient pretreatment by SampliQ QuEChERS kits. The homogenized liver sample was initially extracted in a buffered aqueous, 5% formic acid acetonitrile system. An extraction and partitioning step was performed after the addition of salts. Cleanup was done using dispersive solid phase extraction (dispersive-SPE). The final extracts allowed determination of all compounds in a single run using LC-ESI-MS-MS operating in positive ion multiple reaction monitoring (MRM) mode. Norfloxacin was selected as the internal standard. The accuracy of the method, expressed as recovery, was between 62 and 113%. The precision, expressed as RSD, was between 2.2 and 13.4%. The established limit of quantification (LOQ) was 5 ng/g and is significantly lower than the respective Maximum Residue Limit (MRL) for quinolones in food producing animals.

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Introduction

Quinolones are a family of synthetic broad-spectrum antibiotics. They prevent bacterial DNA from unwinding and duplicating. There is evidence that quinolones in food animals lead to the emergence of quinolone-resistant bacteria in animals. The resistant organisms are transmitted to humans via direct contact with the animal or through the consumption of contaminated food and water. Quinolone-resistant campylobacter is an example of animal-to-human transmission and has been observed in many European countries since the early 1990s [1]. Therefore, public health agencies in many countries such as the EU commission [2], the USA FDA administration [3], and the Chinese Ministry of Agriculture [4] have established maximum residue limits (MRLs) of veterinary drugs in food-producing animals. Given the different drugs in different food origins and in different countries, the MRLs of quinolones in food products of animal origin are usually at the level of 100 µg/kg or higher.

As animal food origins, such as muscle, liver, and eggs, are complicated matrices, it is critical to use an efficient sample pretreatment method for analyte extraction and concentration, and matrix cleanup. The established sample pretreatment methods used for determination of quinolones include traditional solvent extraction, solid phase extraction (SPE), or a combination of both. Although they have been widely used, these traditional methods have inherent limitations. Traditional methods are labor intensive, time consuming, require a large amount of solvent and waste disposal. In 2003, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method for pesticide residue analysis in fruit and vegetable matrices was introduced. [5] There are two validated QuEChERS methodologies: the AOAC and EN versions. Both are widely accepted and effective for the multiresidue analysis of pesticides in fruit, vegetables and other plant food matrices. The QuEChERS method contains significant advantages over traditional methods, including high recoveries for a wide range of pesticides, high sample throughput, minimal labor, time savings, limited solvent usage, and low waste. In addition, the method is manually accommodating which has made QuEChERS a very popular methodology for the analysis of pesticide residues in fruits and vegetables in recent years.

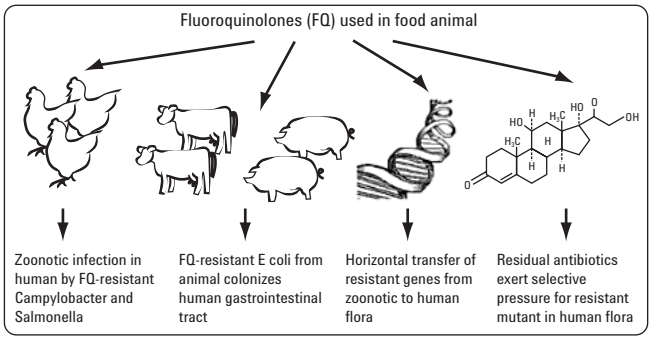

Figure 1. Animal to human transmission of resistant bacteria [1].

Although the current QuEChERS methodology has been designed for removing matrix interferences in food products of plant origin, such as polar organic acids, sugars, and lipids, it also has potential for other food matrices such as meat. Based upon the chemical properties of the compounds of interest and food matrices, some modifications of the original method might be necessary to obtain accurate and precise results. The purpose of this work is to extend the QuEChERS methodology to veterinary drug residues in food-producing animals. Agilent SampliQ QuEChERS EN buffered extraction kits (p/n 5982-5650) and dispersive-SPE 2 mL kits for drug residues in meat (p/n 5982-4921) were used for the analysis of 11 quinolone antibiotics in bovine liver: pipemidic acid, ofloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, cinoxacin, oxolinic acid, nalidixic acid and flumequine (Figure 2). The method was validated in terms of recovery and reproducibility.

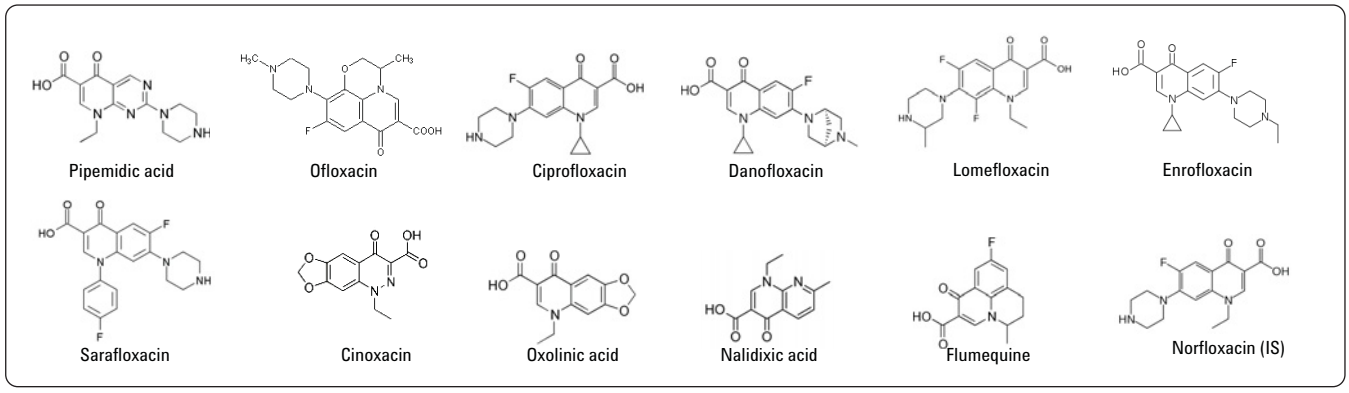

Figure 2. Chemical structures of the quinolone antibiotics investigated in this study.

Experimental

Reagents and Chemicals

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) was from Honeywell (Muskegon, MI, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and glacial acetic acid (HAc) were from Sigma-Aldrich (St Louis, MO, USA). Ammonium acetate (NH_4OAc) was from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid (FA) was from Fluka (Sleinheim, Germany). The quinolone standards and internal standard were purchased from Sigma-Aldrich (St Louis, MO, USA). Potassium phosphate, monobasic (KH_2PO_4), was from J.T. Baker (Phillipsburg, NJ, USA).

Solutions and Standards

1M ammonium acetate stock solution was made by dissolving 19.27 g NH_4OAc powder in 250 mL Milli-Q water. The solution was stored at 4 °C. A 5 mM ammonium acetate in water solution with pH 3 was made by adding 5 mL of 1M ammonium acetate stock solution into 1 L of Milli-Q water, then adjusting the pH to 3 with glacial acetic acid. A 1:1 MeOH/ACN solution was made by combining 500 mL of MeOH and ACN, then mixing well. A 5% formic acid solution in ACN was made fresh daily by adding 10 mL of formic acid to 190 mL of ACN, then mixing well. A 30 mM KH_2PO_4 buffer, pH 7.0, was made by dissolving 4.08 g KH_2PO_4 powder into 1 L Milli-Q water and adjusting the pH to 7.0 with 1 M KOH solution. A 1:1 ACN/H_2O with 0.1% FA was prepared by combining 50 mL of ACN and Milli-Q water, then adding 100 µL of formic acid. A 1:9 MeOH/H_2O solution with 0.1% FA was prepared by combining 10 mL of MeOH and 90 mL of Milli-Q water, then adding 100 µL of formic acid.

Standard and internal standard (IS) stock solutions (1.0 mg/mL for all, except 0.25 mg/mL for ciprofloxacin) were made in DMSO and stored at 4 °C. Due to the solubility of quinolones, it is essential to sonicate stock solutions to ensure they completely dissolve. Three combined QC spiking solutions of 0.2, 8 and 16 µg/mL were made fresh daily in 1:1 ACN/H_2O containing 0.1% FA. A 10 µg/mL standard spiking solution in 1:1 ACN/H_2O containing 0.1% FA was made for the preparation of calibration curves in the matrix blank extract. A 20 µg/mL IS spiking solution of norfloxacin was made in 1:1 ACN/H_2O containing 0.1% FA.

Equipment and Material

- Agilent 1200 Series HPLC with Diode Array Detector (Agilent Technologies Inc., CA, USA).
- Agilent 6410 Series triple quadrupole LC/MS system with Electrospray Ionization (Agilent Technologies Inc., CA, USA).
- Agilent SampliQ QuEChERS EN Extraction kits, p/n 5982-5650, and SampliQ QuEChERS dispersive-SPE kits for Drug Residues in Meat, 2 mL, p/n 5982-4921 (Agilent Technologies Inc., DE, USA).
- CentraCL3R Centrifuge (Thermo IEC, MA, USA)
- Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY, USA)
- 2010 Geno Grinder (Spex SamplePrep LLC, Metuchen, NJ, USA)
- Multi-tube Vortexer (Henry Troemner LLC, Thorofare, NJ, USA)

Instrument conditions

HPLC conditions

Column	Agilent ZORBAX Solvent Saver Eclipse Plus Phenyl-Hexyl 150 × 3.0 mm, 3.5 µm (p/n 959963-312)		
Flow rate	0.3 mL/min		
Column Temperature	30 °C		
Injection volume	10 µL		
Mobile Phase	A: 5 mM ammonium acetate, pH 3.0 in H_2O B: 1:1 MeOH/ACN		
Needle wash	1:1:1:1 ACN/ MeOH/ IPA/ H_2O with 0.2% FA.		
Gradient	Time	% B	Flow rate (mL/min)
	0	15	0.3
	0.2	15	0.3
	8.0	75	0.3
	9.0	100	0.3
	11.5	STOP	
Post run	4 min		
Total cycle time	~16 min.		

MS conditions

Polarity	positive
Gas Temp.	325 °C
Gas Flow	8 L/min
Nebulizer	50 Psi
Capillary	4000 V
Solvent cut	5 min

Other conditions relating to the analytes are listed in Table 1.

Table 1. Instrument Acquisition Data for the Analysis of 11 Quinolone Antibiotics by LC/MS/MS

Analyte	MRM channels (m/z)	Fragmentor (V)	CE (V)	RT (min)
Pipemidic acid	1) 304.1 → 286.1	128	17	5.9
	2) 304.1 → 215.1		37	
Ofloxacin	1) 362.2 → 318.1	150	17	6.7
	2) 362.2 → 344.1		21	
Ciprofloxacin	1) 332.1 → 314.1	131	21	6.8
	2) 332.1 → 231.0		41	
Danofloxacin	1) 358.2 → 340.2	159	25	6.9
	2) 358.2 → 82.1		49	
Lomefloxacin	1) 352.2 → 265.2	144	21	7.0
	2) 352.2 → 334.1		21	
Enrofloxacin	1) 360.2 → 342.2	159	21	7.3
	2) 360.2 → 316.2		17	
Sarafloxacin	1) 386.1 → 368.1	144	21	7.9
	2) 386.1 → 348.2		33	
Cinoxacin	1) 263.1 → 217.1	103	21	8.8
	2) 263.1 → 189.0		29	
Oxolinic acid	1) 262.1 → 216.0	106	29	9.2
	2) 262.1 → 160.0		41	
Nalidixic acid	1) 233.1 → 104.1	94	45	10.3
	2) 233.1 → 159.1		33	
Flumequine	1) 262.1 → 202.0	106	33	10.8
	2) 262.1 → 126.0		50	
Norfloxacin (IS)	320.1 → 302.1	134	17	6.6

1) Quantifier transition channel 2) Qualifier transition channel

Sample preparation

The sample preparation procedure includes sample homogenization, extraction/partitioning, and dispersive-SPE cleanup. As mentioned previously the QuEChERS methods were designed for pesticides analysis in fruit and vegetable matrices; therefore modifications were necessary to optimize the results for the determination of quinolones in bovine liver.

Bovine liver was purchased from a local grocery store. It was washed and chopped into small pieces. The chopped liver was homogenized thoroughly with a food grinder and stored at -20 °C. Two-gram (±0.05g) samples of homogenized liver were placed into 50 mL centrifuge tubes. The tubes were centrifuged for 30 s to move the sample from the inside tube wall to the bottom of the tube. Samples were then fortified with appropriate QC spiking solutions (50 µL) when necessary, then 50 µL of IS spiking solution (20 µg/mL of norfloxacin). After vortexing the sample for 30 s, 8 mL of 30 mM KH_2PO_4 buffer, pH 7.0, were added. Tubes were then vortexed for 10 s to mix. A 10 mL volume of 5% FA in ACN was added to each tube. Tubes were capped and shaken by a 2010 Geno Grinder for 30 s. An Agilent SampliQ QuEChERS EN extraction salt packet (p/n 5982-5650) was added to each tube. Sample tubes were capped tightly and shaken vigorously for 1 min by a 2010 Geno Grinder. Tubes were centrifuged at 4,000 rpm for 5 min at 4 °C.

A 1 mL aliquot of the upper ACN layer was transferred into an Agilent SampliQ QuEChERS dispersive-SPE 2 mL tube for Drug Residues in Meat (p/n 5982-4921). This 2 mL dispersive-SPE tube contained 25 mg of C18 and 150 mg of anhydrous $MgSO_4$. The tubes were tightly capped and vortexed for 1 min. The 2 mL tubes were centrifuged with a microcentrifuge at 13,000 rpm for 3 min. An 800 µL volume of extract was transferred into another tube and dried by N_2 flow at 40 °C. Samples were reconstituted into 800 µL of 1:9 MeOH/H_2O with 0.1% FA. After vortexing and sonicating for 10 min, the sample was filtered by a 0.22 µm Cellulose Acetate Spin Filter (p/n 5185-5990). The clear filtered sample was transferred into an autosampler vial. The samples were capped and vortexed thoroughly in preparation for LC/MS/MS analysis. Figure 2 shows the flow chart of the entire extraction procedure for bovine liver sample.

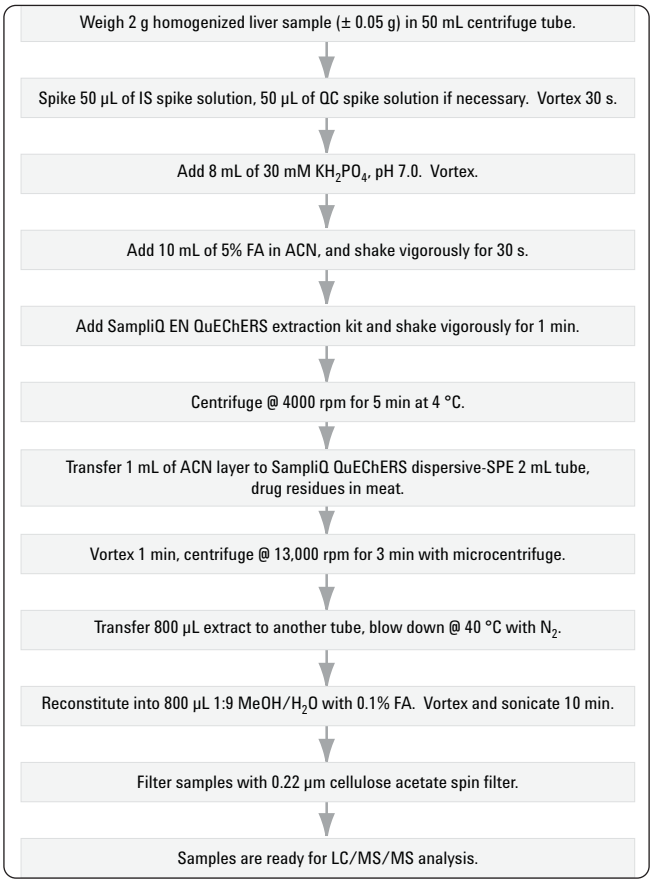

Figure 3. Flow chart of QuEChERS procedure for the determination of quinolones in bovine liver.

Results and Discussion

Feasibility Test

Quinolones are a group of relatively new antibacterials synthesized from 3-quinolone carboxylic acid. As shown in Figure 2, they all contain the carboxylic group, and are weakly acidic (pKa 4-6). Since this is the first time for quinolones determination by the QuEChERS method, the feasibility test was done by extracting 50 ng/mL of neat quinolone solution (prepared in water) with different SampliQ QuEChERS kits, including the SampliQ AOAC extraction kit, SampliQ EN extraction kit, and SampliQ Original extraction kit. In addition, bovine liver is a very different matrix than fruit and vegetables. Therefore, the cleanup was followed by the corresponding fatty dispersive-SPE kit (AOAC and EN fatty dispersive-SPE kit) because these fatty dispersive-SPE kits contain C18 which is critical for removing lipids from liver matrix.

However, the test results were initially very disappointing. All of the analytes had extremely low or nonexistent recoveries. The ACN extracts were tested at two points in the procedure to investigate where the analytes were being lost. The first test was made after the extraction step. The second test was made after both the extraction and the dispersive-SPE steps. Figure 3 shows the chromatogram comparison for the neat ACN extracts after the extraction step using different extraction kits. The ACN extracts using the EN extraction kit (p/n 5982-5650) showed much higher responses than those using the AOAC extraction kit (p/n 5982-5755) and the original extraction kit (p/n 5982-5550). The buffer system in the extraction/partitioning step provided by the addition of salts plays a key role in the extraction efficiency. The pH when the acidic analytes exist in their neutral forms facilitates the extraction. Both the EN and AOAC extraction kits provide a buffer system of approximately pH 5.0 [6, 7], which is the point where most quinolones are neutral. Therefore, these extraction kits generate better extraction efficiency than the original nonbuffered extraction kit. However, it is unknown why the neat extract from the EN extraction buffer system produced higher responses than that from the AOAC extraction buffer system, especially for the early eluted analytes. From these results, the SampliQ EN buffered QuEChERS extraction kit was selected for future work.

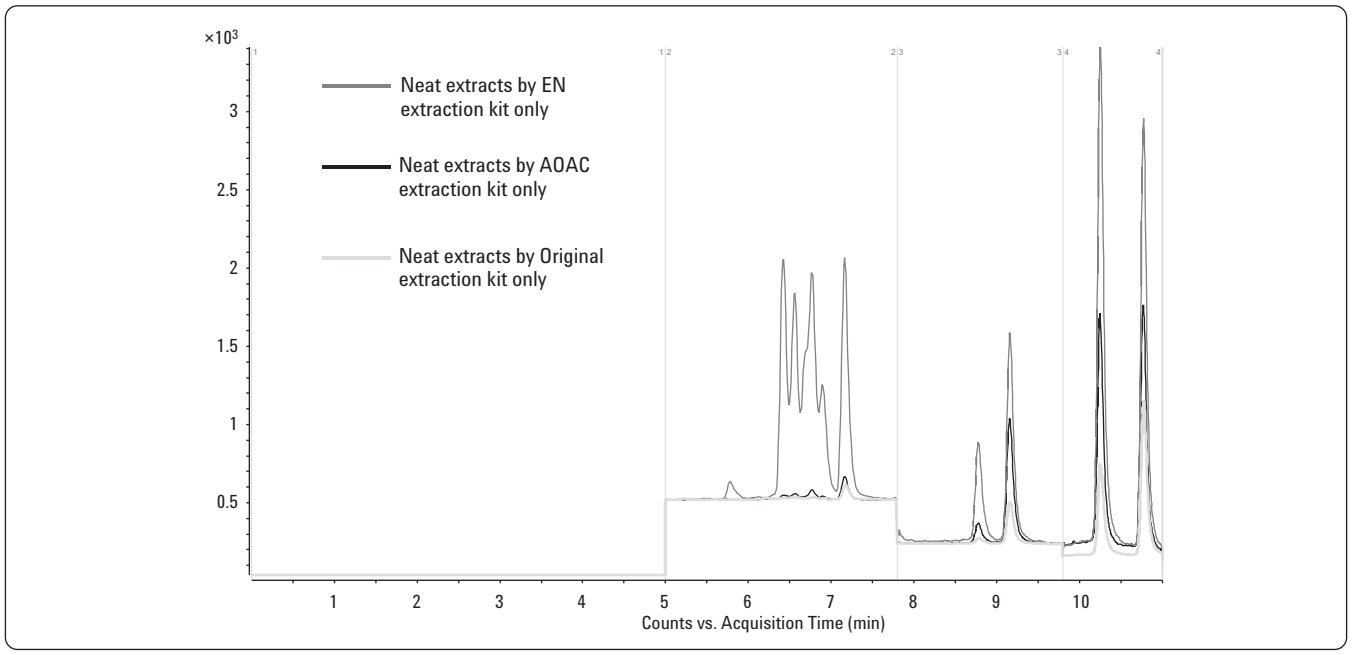

Figure 4. Feasibility test results 1: chromatogram comparison of the neat extracts (no dispersive-SPE) obtained by SampliQ QuEChERS EN extraction kit , AOAC extraction kit, and original extraction kit.

The addition of acid to acetonitrile during the extraction/partitioning step was also investigated. Acetonitrile only, used in the original EN method, and acidified acetonitrile with 5% formic acid were evaluated for their efficiency. As demonstrated in Figure 5 by comparing the results from columns A and D, better analyte recoveries were achieved (10-30% higher) with the acidified acetonitrile. The addition of formic acid into solvent extraction inhibits the acid dissociation for quinolones. Therefore, their protonated neutral form can be extracted easily into the solvent phase [8]. Furthermore, the addition of acid into acetonitrile greatly decreased the negative impact caused by PSA in the dispersive-SPE step (Figure 5, columns C and E). The formic acid in ACN extract interacts with PSA in the dispersive-SPE step, greatly decreasing the binding of PSA with the target quinolones. From these results, 5% (vol/vol) formic acid in acetonitrile was chosen as an extraction solvent for further study.

Although the EN extraction kit generated better recovery, the cleanup using the fatty dispersive-SPE kit in step two significantly lowered extraction efficiency (Figure 5). The selected fatty dispersive-SPE kit contains PSA (primary secondary amine), C18, and $MgSO_4$; however the loss of quinolones was mostly due to the PSA. In the QuEChERS method, PSA is used in all dispersive-SPE kits, because it acts as a weak anion exchanger. It strongly interacts with acidic interferences from fruits and vegetables such as polar organic acids, sugars, and fatty acids. However, it can also strongly interact with the target analytes, the quinolones, leading to the loss of analytes. When acetonitrile was used in the extraction step, PSA from the dispersive-SPE kit caused almost total loss of all of analytes (Figure 5, columns D and E). When acidified acetonitrile was used in the extraction step, the existence of PSA in the dispersive-SPE kit still caused a 10-40% loss of analytes (Figure 5, columns A and C). Because of these results, a brand new SampliQ dispersive-SPE kit for Drug Residues in Meat (p/n 5982-4921) was used for this study. This new SampliQ dispersive-SPE kit contains 25 mg C18 and 150 mg $MgSO_4$ per mL of ACN extract. The new dispersive-SPE kit's effect on the analytes recovery is negligible (Figure 5, columns A and B).

According to the above feasibility test results, a QuEChERS method was developed and applied for the subsequent study in the liver matrix. This method uses the SampliQ EN buffered extraction kit and 5% FA in ACN for the extraction/ partitioning step as well as the new SampliQ dispersive-SPE kit for drug residues in meat for the following cleanup.

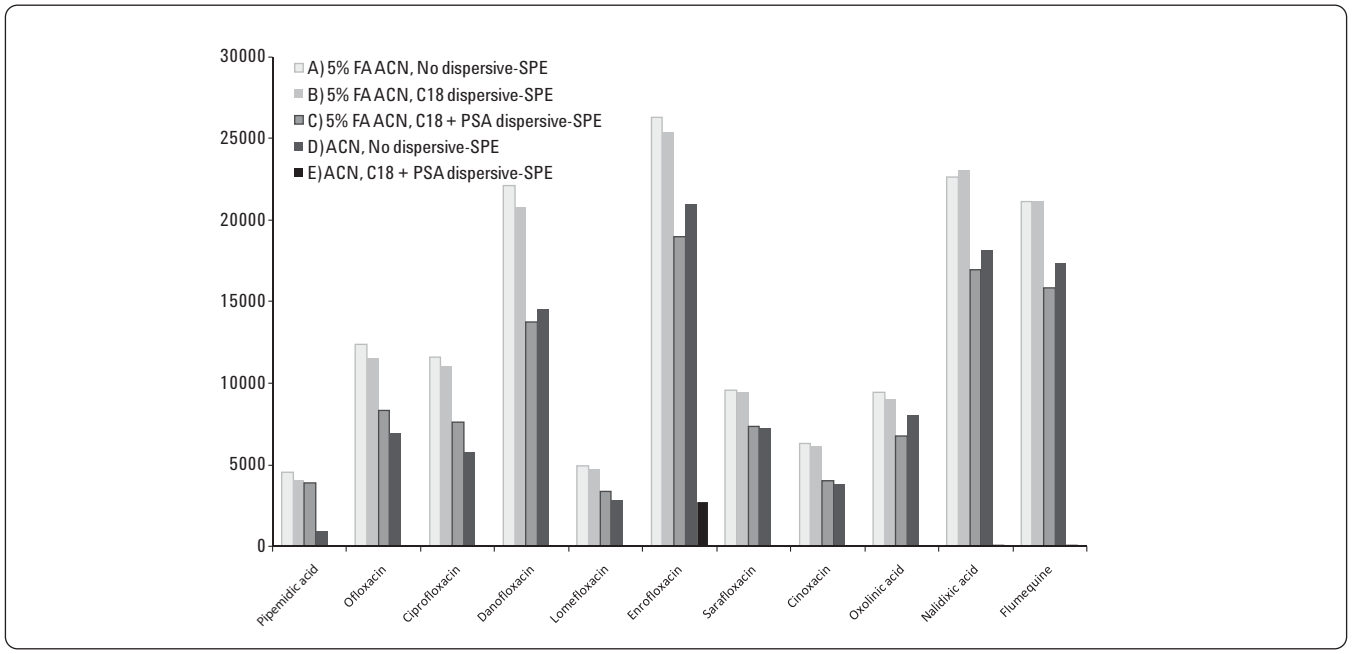

Figure 5. Feasibility test 2. Analytes peak area comparison for the neat extract processed by different procedures. Comparisons include pure ACN and acidified ACN, with and without PSA dispersive-SPE.

Method Optimization in the Liver Matrix

The QuEChERS method established from the results of the feasibility test was applied to the determination of quinolones in bovine liver.

The homogenized liver sample was very thick and could not be used directly for the extraction. Therefore, it was necessary to dilute the liver sample with an aqueous buffer (30 mM KH_2PO_4 in water, pH 7.0) before the extraction. Different sample/buffer ratios including 1:4, 3:7, 1:1, were investigated by adding 8 mL, 7 mL and 5 mL of buffer to 2 g, 3 g, and 5 g of homogeneous liver sample. After dilution, 10 mL of 5% FA in ACN was added. Visually, the more sample used, the more foam was generated during the extraction/partitioning step resulting in a darker red ACN extract. Although more sample should lead to a lower detection limit, it simultaneously introduced more matrix interferences and higher matrix effect. Since the addition of 5% FA ACN to the liver sample is also a protein precipitation procedure, a sample/ACN ratio of 1:4 to 1:5 usually provides the best precipitation effect and sufficient cleanup for proteins. Therefore, a sample/buffer ratio of 1:4 (2 g of liver sample and 8 mL of buffer) was employed.

After the extraction/partitioning step, the sample was centrifuged at 4,000 rpm and 4 °C for 5 min. The low temperature helped to remove lipids from the ACN extracts. After centrifuging, a thin layer of lipids might show up on the surface of the ACN layer. Additional lipids will be removed by C18 in the dispersive-SPE step. A 1 mL amount of ACN extract was transferred into a 2 mL dispersive-SPE tube containing 25 mg C18 and 150 mg $MgSO_4$ for cleanup. An 800 µL amount of upper solvent was transferred into another tube by vortexing and centrifuging. This was the final extract after the QuEChERS extraction and cleanup. It appeared light brown to red in color and was transparent. In order to get sufficient sensitivity and integrity of peak shape, the sample was dried under N_2 flow and reconstituted into 800 µL 1:9 MeOH/H_2O with 0.1% FA. The reconstituted sample was cloudy and filtration was necessary, which was done by a 0.22 µm cellulose acetate spin filter. The sample became colorless and clear after filtering, and was ready for LC/MS/MS injection.

Figure 6 shows the MRM chromatograms of liver control blank and 5 ng/g fortified liver extract (LOQ). The liver control blank chromatogram indicated that it was free from any interference to the target analytes. The 5 ng/g fortified liver extract chromatogram demonstrated that the 5 ng/g limits of quantitation (LOQ) for all of analytes were well established with a signal-to-noise ratio (S/N) greater than 5.

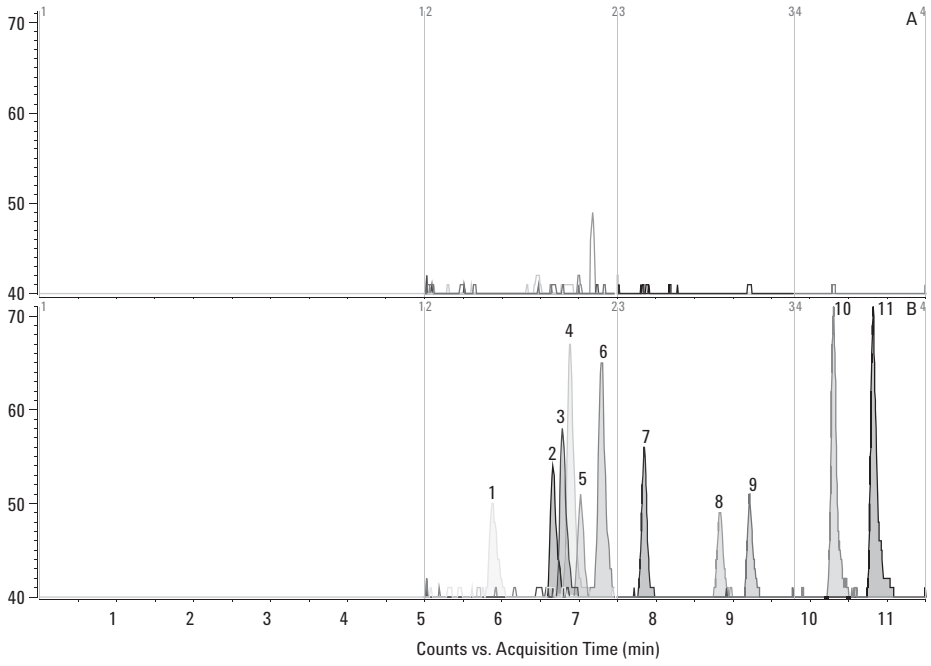

Figure 6. LC/MS/MS chromatograms of A) liver blank extract, and B) 5 ng/g fortified liver extract (LOQ). Peaks identification: 1. Pipemidic acid, 2. Ofloxacin, 3. Ciprofloxacin, 4. Danofloxacin, 5. Lomefloxacin, 6. Enrofloxacin, 7. Sarafloxacin, 8. Cinoxacin, 9. Oxolinoc acid, 10. Nalidixic acid, 11. Flumequine.

Linearity and limit of quantification (LOQ)

The linear calibration range for all of the quinolone antibiotics was 5 – 400 ng/g and matrix blanks were prepared for evaluation. Calibration curves spiked in matrix blanks were made at levels of 5, 10, 50, 100, 200, 300, and 400 ng/g for each analyte. The norfloxacin was used as an internal standard at 200 ng/g. The calibration curves were generated by plotting the relative responses of analytes (peak area of analyte / peak area of IS) to the relative concentration of analytes (concentration of analyte/concentration of IS). The 5 ng/g limit of quantification LOQ (5 ppb) established for all of the quinolones is far below the MRLs for residues of these antibiotics in animal food products. Table 2 shows the regression equation and correlation coefficient (R^2). Linear regression fit was used with $1/x^2$ weight. Results indicated excellent linearity for all of the analytes calibration curves over a broad quantification range.

Table 2. Linearity of Quinolone Antibiotics in Bovine Liver.

Analytes	Regression equation	R^2
Pipemidic acid	Y = 0.2081X – 0.00002	0.9966
Ofloxacin	Y = 0.2221X + 0.00001	0.9964
Ciprofloxacin	Y = 0.2971X – 0.00005	0.9975
Danofloxacin	Y = 0.6861X – 0.0039	0.9957
Lomefloxacin	Y = 0.1702X – 0.00003	0.9958
Enrofloxacin	Y = 0.6530X – 0.0020	0.9962
Sarafloxacin	Y = 0.2132X – 0.0004	0.9937
Cinoxacin	Y = 0.0933X – 0.0004	0.9959
Oxolinic acid	Y = 0.1043X + 0.0003	0.9939
Nalidixic acid	Y = 0.3223X + 0.0005	0.9974
Flumequine	Y = 0.3232X + 0.0003	0.9966

Recovery and Reproducibility

The recovery and reproducibility were evaluated by fortifying quinolone standards in homogenized liver sample at levels of 5, 200 and 400 ng/g. These QC samples were quantified against the matrix spiked calibration curve. The analysis was performed in replicates of six at each level. The recovery and reproducibility (shown as RSD) data are shown in Table 3. It can be seen from the results that all of quinolones except pipemidic acid gave excellent recoveries (average of 95.9%) and precision (average of 6.6% RSD). Pipemidic acid gave lower recovery (average of 66.7%) but great precision (average of 5.7% RSD). Additionally, it still meets the 5 ng/g LOQ requirement. Therefore, the results are acceptable.

Table 3. Recovery and Repeatability of Pesticides in Fortified Liver with 2 mL Dispersive-SPE Tube (p/n 5982-4921)

	5 ng/g fortified QC		200 ng/g fortified QC		400 ng/g fortified QC	
Analytes	Recovery	RSD (n=6)	Recovery	RSD (n=6)	Recovery	RSD (n=6)
Pipemidic acid	71.6	8.1	62.0	6.8	66.4	2.2
Ofloxacin	72.9	9.7	101.0	7.7	102.4	5.7
Ciprofloxacin	108.2	8.3	101.4	4.2	98.9	2.3
Danofloxacin	88.2	7.9	109.3	7.8	114.0	6.1
Lomefloxacin	82.6	13.4	96.8	8.5	97.8	5.3
Enrofloxacin	88.6	7.5	109.5	8.3	113.1	5.8
Sarafloxacin	99.6	9.0	97.7	8.4	97.0	4.6
Cinoxacin	92.3	9.3	95.1	7.9	93.5	2.6
Oxolinic acid	95.1	9.8	92.7	4.3	87.6	2.9
Nalidixic acid	92.7	6.0	90.2	5.3	87.7	3.5
Flumequine	91.6	6.6	93.3	5.3	89.9	2.9

Conclusions

The Agilent SampliQ Buffered Extraction EN kit and the SampliQ dispersive-SPE kit for Drug Residues in Meat provide a simple, fast and effective method for the purification of quinolone antibiotics in bovine liver. Compared to the other sample pretreatment methods, such as LLE and SPE, the QuEChERS method is easier to handle, faster, labor-saving, and cheaper. The recovery and reproducibility, based on matrix spiked standards, were acceptable for multiresidue quinolone determination in bovine liver. The impurities and matrix effects from liver were minimal and did not interfere with the quantification of any target compound. The LOQs of the quinolones were much lower than their regulated MRLs in animal food products. On the whole, the QuEChERS procedures presented here appear to be a promising reference method for the quantitative analysis of quinolones in food products of animal origin. This method also has the potential to extend the applications of SampliQ QuEChERS extraction and dispersive-SPE kits to the quantitative analysis in other bio-matrices, such as animal food products and bio-fluids, rather than just plant matrices.

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