Analysis and Occurrence of 14 Sulfonamide Antibacterials and Chloramphenicol in Honey by Solid-Phase Extraction Followed by LC/MS/MS Analysis

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A method was developed for the analysis of 14 sulfonamide antibiotics and chloramphenicol in honey. These antibiotics have been banned for use in food-producing animals; yet, their residues were found in many samples, illustrating the need for a multiresidue analysis for these antibiotics in honey. The method described here uses an acid hydrolysis step to liberate the sugar-bound sulfonamides followed by a solid-phase extraction to remove potential interferences. Analysis was by liquid chromatography–electrospray ionization–tandem mass spectrometry in negative mode for all 15 analytes. This MRM method generated two structurally significant transitions per compound, and it was designed to conform to U.S. Food and Drug Administration MS confirmation guidelines. It also provides 4-EU identification points. One hundred sixteen samples from 25 countries were analyzed, and 38% were found to contain at least one target antimicrobial. Five different target compounds were found in honey from 13 different countries.

KEYWORDS: Sulfonamide; chloramphenocol; honey; LC/MS/MS; antibiotics

INTRODUCTION

Honeybees are subject to a number of diseases that affect their brood, with two of the most serious being the larval bacterial diseases American and European foulbrood (*1, 2*). Their names do not give an indication of their geographic distribution, as both diseases are found in most regions of the world. American foulbrood is caused by the spore-forming bacterium *Paenibacillus larvae*. Larvae become infected when they consume the *Paenibacillus* spores in their food, which then multiply in the larvae's gut and tissue, ultimately resulting in its death. When a colony is infected, it becomes severely contaminated with resistant spores, killing the entire colony. European foulbrood is a disease caused by the bacterium *Melissococcus plutonius*. It resides in the gut of the larvae, where it competes for food. Death of the larvae is due to starvation. Other bacteria then have the opportunity to feed on these deceased larvae (*3*).

In most countries, few antibiotics are allowed for use in combating these infections, with only tylosin and oxytetracycline approved in the United States. Although oxytetracycline has been effective for years, there are indications that the bacteria that cause foulbrood are developing resistance to it (*4*). Sulfonamides are effective against foulbrood, although they are not permitted in many countries for fear of residues contaminating the honey. The presence of sulfonamides in food products is cause for concern due to the potential toxicity of these compounds, which have been implicated in the development

of thyroid tumors in mice and rats. Although sulfamethazine (SMZ) was the only sulfonamide evaluated in this study, these data initiated a reevaluation of allowable tolerances for the entire class of compounds (*5*). Another issue is the development of microbial resistance to sulfonamides. Overuse and misuse of these antibiotics are possible reasons that various sulfonamide-resistant strains have been identified. By entering the body through the food supply, these drugs could facilitate the generation of resistant strains of a variety of microbes, including pathogenic organisms (*6, 7*). These drugs would then be useless in treating infections caused by these pathogens. Switzerland has set a maximum residue level (MRL) of 50 μg/kg for total sulfonamides in honey (*8*). In much of the European Union (EU), MRLs have not been specifically set for sulfonamides in honey, although they have been set at 0.1 mg/kg for foods of animal origin (*9*).

Sulfonamides are broad-spectrum synthetic antibiotics that competitively inhibit conversion of *p*-aminobenzoic acid (PABA) to dihydropteroate, which bacteria need for folic acid synthesis (*10*). They all contain the common structure of an unsubstituted amine on a benzene ring and a sulfonamide group para to the amine (**Figure 1**). As an antimetabolite, this basic structure is necessary to mimic the structure of PABA. While the amine must remain unsubstituted on the benzene ring, monosubstitutions may be made on the sulfonamide group. These monosubstitutions modulate the pharmokinetic properties of the drug (*11, 12*).

Chloramphenicol (CAP) is a broad-spectrum bacteriostatic originally derived from *Streptomyces venezuelae*. It is one of

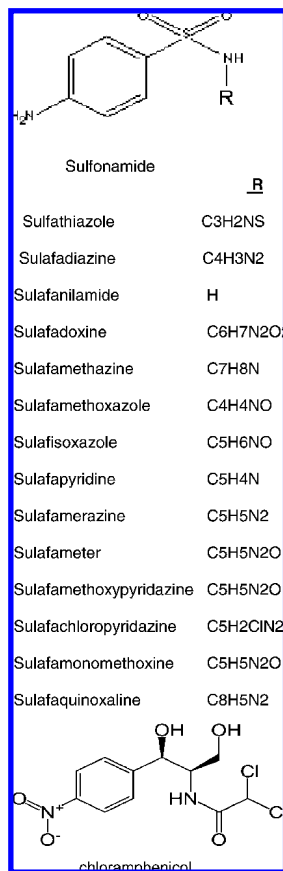**3510** *J. Agric. Food Chem.,* Vol. 56, No. 10, 2008

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Figure 1. Structures for 14 sulfonamides and CAP.

the first antibiotics to be synthesized on a large scale and has been in production since the 1950s. As a bacteriostatic, it functions by inhibiting protein synthesis (*13*). CAP has limited uses in human medicine due to serious side effects such as aplastic anemia (*14*). It is a suspected carcinogen and can result in hypersensitivity in susceptible people. The World Health Organization's committee on food additives (1968) recommended that CAP should not be used for any purpose that might result in the presence of residues in food for human consumption. Following this determination, many countries as well as the U.S. Food and Drug Administration (FDA) banned its use in food-producing animals (*15, 16*). CAP is an inexpensive antibiotic, which is widely produced and used in many developing nations (*16*). Despite international bans on its use in food-producing animals, residues of CAP have been found by the U.S. FDA in Chinese honey (*17*). It is suspected that these residues are the result of treatment of foulbrood. In 2002, Germany's Agriculture and Consumer Protection Ministry found CAP residues in honey imported from China, as did the UK's Food Standard Agency. Canada's Canadian Food Inspection Agency (CFIA) reported CAP residues in Chinese honey at 0.3–34 parts per billion (ppb) (*18*).

Sulfonamide and CAP residue detection has evolved over recent years. Enzyme-linked immunoassays have been proven to be effective in detecting entire classes of compounds in a variety of foods including honey (*19–23*). This technique cannot, however, differentiate between compounds within a given class. Analysis by liquid chromatography (LC) followed by fluorescence or UV detection can give low detection limits and can differentiate between compounds within a given class by retention time (*24–29*). Gas chromatography (GC) analysis can provide good sensitivity and, when coupled to mass spectrometry, can provide instant confirmation, but derivitization is

required to generate a volatile product (*30, 31*). LC/MS has been used to detect CAP and sulfonamides in a variety of food matrices and provides improved selectivity over fluorescence or UV detection (*32–36*). More recently, LC/tandem mass spectrometry (MS/MS) detection methods have been successful in determining CAP and sulfonamides in the low ng/g (ppb) level in a variety of matrices including honey (*37–43*). Nowadays, tandem mass spectrometry is the preferred technique, as it provides improved selectivity and sensitivity over other techniques.

Since 1990, it has been clear that sulfonamides present in honey bind to the sugars and are difficult to detect in this form (*20*). When the sugar-bound sulfonamides are subjected to acid hydrolysis, they are liberated and detectible in their free form. Because the sugar-bound complex is very stable in food, the sulfonamides may only experience strongly acidic conditions in the stomach of a consumer where the biologically active drug will be disassociated from the sugar. Before it was understood that sulfonamides bind to sugars, studies determining sulfonamides in honey most likely underreported the concentrations.

We have developed a method for the extraction and analysis of 14 sulfonamides and CAP in honey and have applied this method for the analysis of 116 honey samples. Simultaneous confirmation and quantification are provided for each antibiotic detected. The extraction included an acid hydrolysis step to release the sugar-bound sulfonamides without negatively affecting CAP stability. Detection and quantification were by LC/electrospray ionization (ESI)-MS/MS, and matrix match standards were used to compensate for matrix suppression. This method has been in use in our laboratory for routine screening of honey samples, and the results from 2005 and 2006 are presented.

MATERIALS AND METHODS

Chemicals and Materials. Sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), sulfameter (SME), sulfamethoxypyridazine (SMP), SMZ, sulfachloropyridazine (SCP), SMX, sulfamonomethoxine (SMM), sulfisoxazole (SSX), sulfadimethoxine (SDM), and sulfaquinoxaline (SQX) standards were purchaced from Sigma (St. Louis, MO). Sulfadiazine (SDZ), sulfadoxine (SDX), and CAP standards were purchased from USP (Rockville, MD). All stock and working standards were prepared in methanol. Methanol, acetone, acetonitrile, and NaOH were purchased from JT Baker (Phillipsburgh, NJ). All deionized water was prepared using a Barnstead Nanopure II (Dubuque, IA). Fifty milliliter disposable centrifuge tubes, vortex, HCl, and NaCl were purchased from Fisher Scientific (Worcester, MA). The solid-phase extraction (SPE) vacuum manifold and SPE cartridge drying manifold were purchased from Supelco (Bellefonte, PA). The waterbath was purchased from Thermo (Waltham, MA). Untreated glass wool was purchased from Krackeler Scientific (Albany, NY). The Titrando automated titrator from Brinkmann (Westbury, NY) was used as an automated technique for adjusting the pH of the diluted honey sample. The Turbovap liquid concentrator was purchased from Caliper Lifie Sciences (Hopkinton, MA). The disposable 0.2 μm syringe filters were purchased from Whatman (Florham Park, NJ). The disposable 3 mL luer lock tip syringes were purchased from Kendall (Mansfield, MA). The Oasis HLB SPE cartridges were purchased from Waters (Milford, MA).

Standard Solutions and Reagents. All individual standard solutions were prepared by dissolving the appropriate amount of neat standard in methanol. Each individual stock standard was prepared to 1000 μg/mL and combined in a 15 compound mixed standard solution. The mixed standard was diluted to 10 times the LOQ using 20% methanol/80% deionized water (v/v). Further dilutions were obtained by dilution in the same solvent.

The phosphate buffer solution, pH 8.5, was generated by dissolving 28.4 g of dibasic sodium phosphate in 2 L of deionized water. The pH

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Table 1. MS/MS Conditions for 14 Sulfonamides and CAP

analyte	RT		ion	cone voltage (V)	dwell time (s)	collision energy (eV)	function no.
SDZ	9.25	quant ion	248.85 > 184.80	45.00	0.10	20.00	1
		qual ion	248.85 > 91.70	45.00	0.10	37.00	
STZ	10.15	quant ion	253.80 > 155.70	30.00	0.10	18.00	1
		qual ion	253.80 > 97.55	30.00	0.10	25.00	
SPD	10.77	quant ion	247.90 > 183.80	45.00	0.10	18.00	1
		qual ion	247.90 > 92.60	45.00	0.10	27.00	
SMR	11.68	quant ion	262.90 > 198.90	45.00	0.10	18.00	1
		qual ion	262.90 > 107.70	45.00	0.10	32.00	
SME	13.01	quant ion	278.90 > 263.90	45.00	0.10	17.00	2
		qual ion	278.90 > 195.80	45.00	0.10	30.00	
SMP	13.65	quant ion	278.90 > 155.70	40.00	0.10	25.00	2
		qual ion	278.90 > 263.90	45.00	0.10	17.00	
SMZ	13.44	quant ion	276.90 > 105.70	55.00	0.10	35.00	2
		qual ion	276.90 > 121.70	55.00	0.10	30.00	
SCP	14.04	quant ion	282.80 > 155.70	45.00	0.10	27.00	3
		qual ion	282.80 > 91.60	45.00	0.10	32.00	
SMX	14.22	quant ion	251.80 > 155.70	30.00	0.10	20.00	3
		qual ion	251.80 > 91.60	30.00	0.10	25.00	
SMM	14.40	quant ion	278.90 > 131.70	50.00	0.10	30.00	3
		qual ion	278.90 > 65.60	50.00	0.10	27.00	
SSX	14.69	quant ion	265.90 > 170.70	35.00	0.10	20.00	4
		qual ion	265.90 > 238.90	35.00	0.10	20.00	
SDX	14.78	quant ion	308.90 > 155.70	30.00	0.10	35.00	4
		qual ion	308.90 > 250.80	30.00	0.10	25.00	
SDM	16.19	quant ion	309.00 > 65.60	50.00	0.08	34.00	4
		qual ion	309.00 > 121.70	50.00	0.08	36.00	
SQX	16.62	quant ion	298.90 > 143.80	50.00	0.08	35.00	4
		qual ion	298.90 > 116.60	45.00	0.08	30.00	
CAP	15.86	quant ion	320.90 > 151.70	35.00	0.15	19.00	4
		qual ion	320.90 > 256.90	35.00	0.15	12.00	

was then adjusted to 8.5 using the Titrando autotitrator. High-performance liquid chromatography mobile phase A was produced by filling a 2 L volumetric flask approximately half-full with deionized water and adding 3.0 mL of glacial acetic acid. The volumetric flask was then brought to volume with deionized water. Mobile phase B was produced by filling a 2 L volumetric flask approximately half-full with methanol and adding 3.0 mL of glacial acetic acid. The volumetric flask was then brought to volume with methanol.

Extraction Method. The extraction of honey samples entailed the following procedure: Five grams of honey was weighed into a 50 mL disposable centrifuge tube, and the actual weight was recorded to the nearest 0.001 g. Any spikes were added at this time, and the spiked sample was allowed to remain at room temperature for at least 3 h in order for the sulfonamides to sufficiently bind to the sugars in the honey. Ten milliliters of 2 M HCl was added and vortexed well. The mixed sample was placed in a 50 °C water bath for 1 h. A 200 mg Oasis HLB SPE cartridge was prepared by placing approximately 2 cm of glass wool in the SPE cartridge. Three milliliters of acetone was added and allowed to pass through with gravity. Similarly, 3 mL of acetonitrile was added followed by 5 mL of phosphate buffer, and about 2 mL of phosphate buffer was left in the cartridge. A slight vacuum may be necessary to pass the phosphate buffer through the cartridge. The bottom of the SPE cartridge was capped and set aside. The sample was transferred from the centrifuge tube to a 30 mL beaker and a small magnetic stir bar was added. The sample was stirred, and 1.0 mL of 50% NaOH was added while the pH was measured. The pH was continually measured, and NaOH diluted 1:10 was added until the pH was 8.5, which typically required less than 3 mL of this solution. An automated titrator such as a Metrohm Titrando can be used to avoid raising the pH too high. A 75 mL reservoir was attached to the top of the SPE cartridge, and the cartridge was attached to a vacuum manifold. The sample was loaded on the cartridge at a flow rate of about 2–5 mL/min. The cartridge was washed with about 1 mL of phosphate buffer. The glass wool was removed, and the cartridge was washed with an additional 1 mL of phosphate buffer. The cartridge was dried with N₂ for about 30 min. The cartridge was eluted with 10 mL of acetonitrile by gravity and concentrated dry in a turbovap concentrator. To the dried extract, 200 µL of methanol was added and vortexed. An

800 µL amount of deionized water was added, vortexed, and transferred to an autosampler vial. The extract should now be ready for analysis by LC/MS/MS.

LC/MS/MS Analysis. LC separation was performed with a Waters (Milford, MA) Alliance 2695 LC binary pump system with column heater, in line degasser, and autosampler. The LC system was coupled to a Waters Quattro Premier triple quadrupole mass spectrometer. Samples were held at 15 °C in the autosampler. The sample injection volume was 10 µL, and the analytical column was a Waters Xterra 2.1 mm × 150 mm MS C18 with 3.5 µm particle size. A guard column containing the same stationary phase was connected prior to the analytical column to prolong column life. Both the guard column and the analytical column were held at 42 °C. Mobile phase A consisted of 0.15% acetic acid, and mobile phase B was 0.15% acetic acid in methanol. The flow rate was held at 0.2 mL/min throughout the entire method. The LC gradient began with mobile phase A at 97% and mobile phase B at 3% with these conditions held for 0.1 min. At 5.0 min, A was 86%, and at 17.0 min, A is 100%. At this point, the mobile phase was returned to the initial conditions so that the system could re-equilibrate before the next injection. The LC transfer line was connected to the input port of the MS/MS divert valve, which allowed much of the coextracted matrix interference to be sent to waste. This helped to keep the sample cone clean and extended the number of samples that could be analyzed before a reduction in sensitivity was observed. LC flow was diverted to waste for the first 7 min when flow was then sent to the MS source. SDZ was the first analyte to elute at 9.2 min, and SQX eluted last at 16.7 min. At 17.8 min, the divert valve again switched to the waste position for the remainder of the method. **Table 1** describes the MS/MS conditions used to collect the data including cone voltage, parent > daughter transitions, dwell time, and collision energy.

RESULTS AND DISCUSSION

Extraction. It has been well-established that an acid hydrolysis step is necessary to disassociate sugar-bound sulfonamides in order for them to be extracted (*20, 37*). When conducting spiking experiments, it is necessary to allow the

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Table 2. Method Validation Results for 14 Sulfonamides and CAP

analyte	LOD (ng/G)	LOQ (ng/G)	mean % recovery	curve correlation coefficient
STZ	0.5	1.5	68	0.9995
SDZ	1.0	3.0	64	0.9982
SPD	2.0	6.0	63	0.9997
SMR	2.0	6.0	67	0.9987
SME	0.5	1.5	70	0.9999
SMP	2.0	6.0	61	0.9999
SMZ	2.0	6.0	75	0.9994
SCP	2.0	6.0	66	0.9989
SMX	0.5	1.5	85	0.9992
SMM	5.0	15.0	45	0.9991
SSX	1.0	3.0	70	0.9999
SDX	2.0	6.0	70	0.9999
SDM	5.0	15.0	66	0.9995
SQX	5.0	15.0	52	0.9991
CAP	0.2	0.6	78	0.9979

Table 3. Analysis Results from 116 Honey Samples Originating from Various Countries

antibiotic	% of total samples containing antibiotic	countries of origin of honey containing antibiotic	maximum concentration (ng/G)
CAP	9	China Russia Georgia Moldova	91
STZ	19	Poland Russia Moldova Bulgaria Slovakia Switzerland Israel unknown	132
SMX	6	China Malaysia Russia Ukraine United States	7.5
SMZ	4	Slovakia Hungary Poland	5.9
SMP	1	unknown	0.70
all other compounds not detected 116 total samples			

Figure 2. Percent of samples analyzed found to contain antibiotics by geographical region.

The SPE step allows for retained potentially interfering coextractives to be removed during the SPE wash procedure. In honey samples, the high sugar content presents unique challenges. If the extracted sample is concentrated along with the coextracted sugars, the sulfonamides could recombine to the unextractable complex. Unremoved sugars also contribute to matrix suppression during LC/MS ionization. This was the case in our initial attempts of liquid/liquid extractions, where signal abundance was much lower in matrix matched standards as compared to standards in solvent. A wash solution of 5 mL of buffer, pH 8.5, proved much more effective than deionized water alone in removing signal-suppressing interferences. To determine this, blank honey matrix was loaded onto duplicate HLB SPE cartridges. For the final cartridge rinse, one cartridge was rinsed with deionized water, and the duplicate cartridge was rinsed with a phosphate buffer. Following the rinse, the SPE cartridges were spiked with 1.0 mL of a low level standard and eluted with acetonitrile. Using this technique, we were able to monitor the effect of coextracted matrix on signal suppression. A signal abundance improvement of 2−5 times was observed with the phosphate buffer wash with an improvement in peak shape as well.

During method development, the HLB SPE cartridge would become clogged during sample loading. This was due to the high wax content in some honey samples that would not pass through the top frit of the HLB cartridge. When the frit became clogged, it was impossible to completely load the diluted sample. Two centimeters of glass wool placed on the top frit effectively kept much of the wax off of the top frit and allowed the entire sample to be loaded without reducing the flow rate.

LC/MS/MS Optimization. LC conditions were chosen to provide good peak shape within a short run time. It was not essential to chromatographically separate each peak since unique MRM transitions correspond to each analyte. However, peaks that can be baseline resolved provided the opportunity to divide the method into more MRM functions with fewer MRM transitions in each function. This allowed for each MRM transition to have a longer dwell time and consequently better sensitivity. Fewer transitions per function also resulted in more scans per transition, which translated to better peak shape and reproducibility.

Optimal MS conditions were obtained by infusing diluted analyte directly into the ion source while optimizing MS parameters. MS conditions are presented in **Table 1** for the negative ionization of the selected analytes. CAP ionizes well in the negative mode with the parent $m/z = 321$ producing daughters of $m/z = 257$ and 152 under the proper conditions. An alternative or additional choice of fragments can include
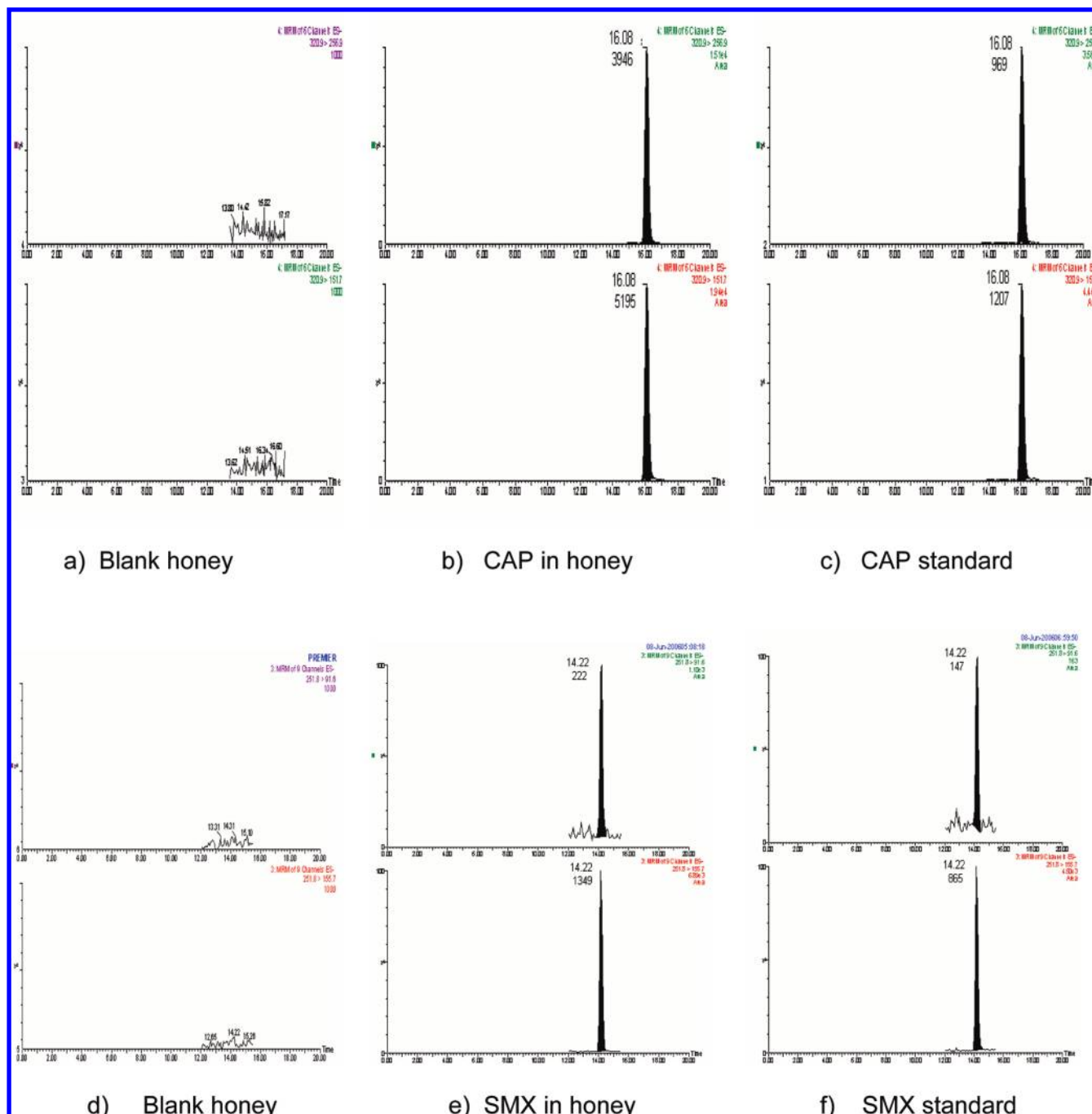
spiked honey sample to sit for at least 1 h to allow the sulfonamides to bind to the available sugars; omitting this step can give artificially high recoveries not representative of actual incurred sulfonamide residues (*38*).

Sample pH greatly affects the retention of analytes to the SPE stationary phase. To determine the optimal pH value, a series of honey spikes were brought through the hydrolysis procedure. They were then adjusted to a range of pH values and loaded onto a 200 mg HLB cartridge. After spikes at pH values between 6 and 11 were analyzed, it was found that some compounds were very sensitive to a small change in pH. Most analytes recovered best when loaded at pH > 7, and recoveries began dropping after the pH > 8.5. SPD with a relatively high pK_a of 8.5 recovered best at a pH of 10, but most analytes recovered most efficiently at a pH of ~8.5. Because the pH values for all 14 sulfonamides vary, it is impossible to optimize for each analyte. Fortunately, CAP remained intact after the acid hydrolysis and recovered well from the SPE cartridge.

Figure 3. Chromatograms of blank honey (a), incurred CAP in honey (b), CAP standard (c), blank honey (d), SMX in honey (e), and SMX standard (f).

the corresponding Cl^{37} CAP parent of $m/z = 323$, giving daughters of $m/z = 259$ and 154.

Historically, sulfonamides have been analyzed in positive mode in a variety of applications (*36–41*). However, these compounds have also been found to ionize well with ESI negative. Because negative ionization is essential for the detection of CAP, the use of ESI^- for sulfonamide detection simplifies the generation of an MRM method. If positive ionization were chosen for the sulfonamides, CAP would have to be chromatographically separated and the corresponding ESI^- MRM conditions kept in its own function. Because it is not possible to perform negative and positive ionization in the same function with the instrumentation described here, the only other option would be to adjust the function time setting so that positive and negative functions overlap, although this is not

recommended, as this would require longer interchannel delay times, which would result in fewer data points collected. Alternatively, two injections per sample could be made to collect positive and negative data. Negative ionization also reduces the possibility of sodium adduct formation, which, in some cases, can be more abundant than the M + 1 signal.

The observed CAP fragments of $m/z = 321 > 152$ and $321 > 257$ have been suggested elsewhere (*14, 42, 43*). The ion $m/z = 257$ represents $C_{10}N_2O_4ClH_9$, showing the isotopic pattern indicative of one Cl. Few descriptions of sulfonamide fragmentation patterns have been described. However, Heller et al. provided detailed proposed fragments for 16 sulfonamides using an ion trap positive ionization (*40*). Interestingly, many of the ions observed using positive ionization were also visible here using negative ionization. Using positive ionization, products
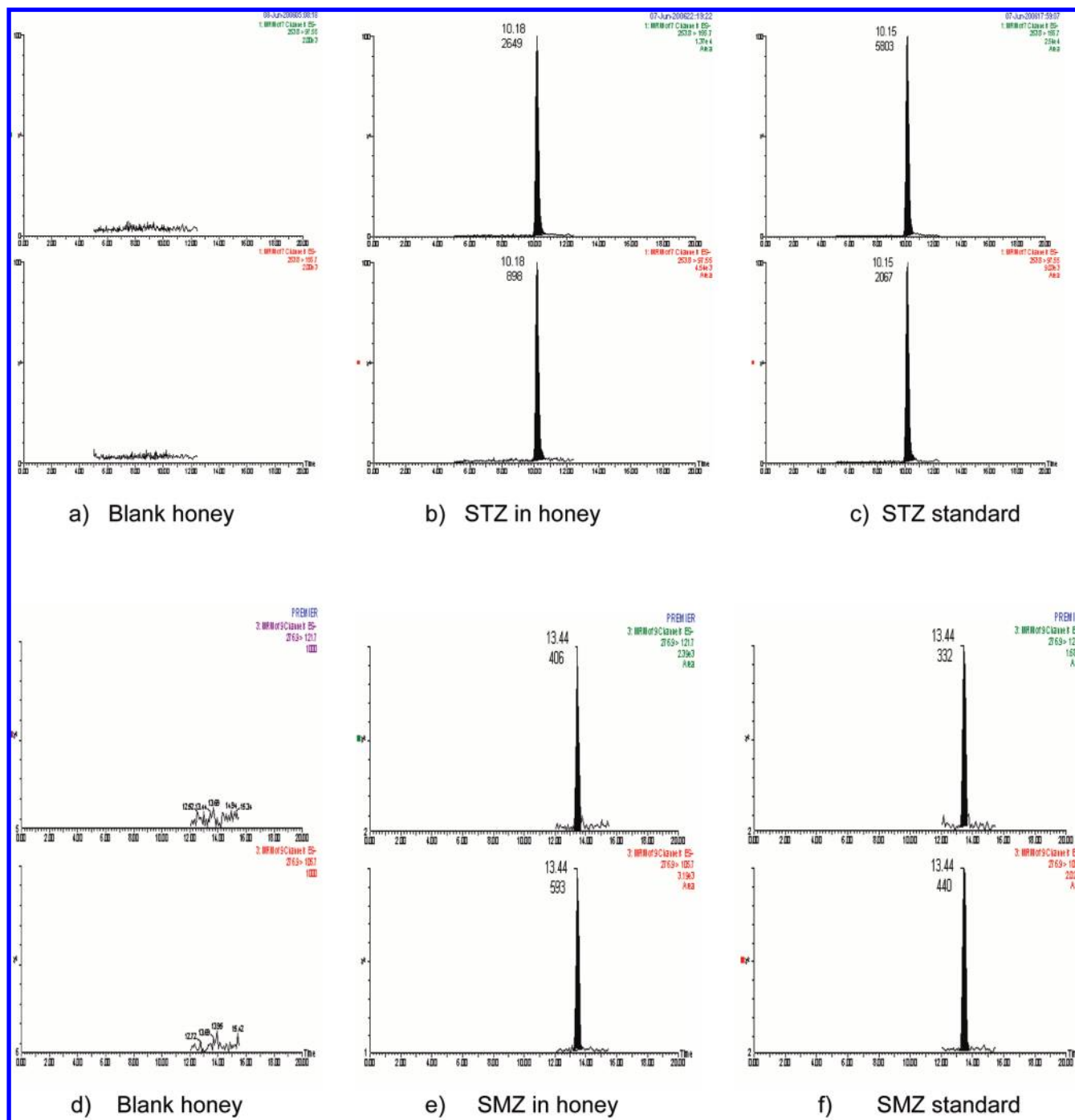
Figure 4. Chromatograms of blank honey (a), incurred STZ in honey (b), STZ standard (c), blank honey (d), SMZ in honey (e), and SMZ standard (f).

of SPD of $m/z = 250 > 184$ and 92 can also be seen by negative ionization $(M - 1)$ 248 > 184 and 92. SMR was also seen to produce fragments of $+ (M + 1)$ 265 > 108 and 199 as well as $- (M - 1)$ 263 > 108 and 199. Other ions common to both techniques include $m/z = 156$ and 144. A complete description of the remaining negative ionization fragments requires further attention.

Limits of detection (LOD) were determined by spiking blank honey at a variety of levels. Sensitivities were found to vary among analytes, and a standard mix was made based on the relative sensitivity of each. An LOD spike could therefore be made by the addition of the mix at the appropriate volume. Analyte detection was based on several criteria highlighted by the U.S. FDA in the mass spectrometry confirmation guidelines

(*44*). Retention times of an analyte must match that of the standard within 5% with the signal abundance being at least three times signal-to-noise. Also, the relative abundance of two structurally significant ions must match that of the standard within 10%, which provides the required four EU identification points. Fulfilling these requirements of mass spectrometry confirmation is recommended whenever possible for regulatory purposes.

All LODs were found to be <10 ng/G with CAP being the lowest at 0.2 ng/G. This fulfills the EU and U.S. FDA minimum required performance limit of 0.3 ng/G for the detection of CAP residues in food. These levels were confirmed by extracting a blank honey spiked at the LOD concentrations. Each analyte must fulfill requirements of confirmation, which include a

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retention time match as well as an ion ratio match within 10% of that of the standard. The mean % recovery reported in **Table 2** reflects the results of spikes at 1, 5, and 10 times the LOQ for each analyte. Each level of concentration was extracted in triplicate and quantified using a calibration curve produced with matrix-matched standards to compensate for signal suppression. Most recoveries were >60%, with SMM being the poorest at 45%. Calibration curve correlation coefficients were all >0.997, covering 1 order of magnitude for quantification.

Honey Data. One hundred sixteen honey samples collected from 2005 to 2007 originating from 25 different countries have been analyzed using the described method. Thirty-eight percent of these were found to contain at least one unapproved antibiotic. **Table 3** indicates which antibiotics were detected, the percent of samples analyzed that contained the antibiotic, and the countries where the honey originated. Although the EU has not established a MRL, individual member countries have set limits from 10 to 50 ng/g for sulfonamides in honey (*26*). The U.S. FDA has also not set a tolerance limit for sulfonamide residues in honey; however, New York has established a limit of 20 ng/ G. Using these criteria along with the zero tolerance for CAP, 12% of analyzed samples were found to be in violation. Of these violative samples, all were imported into the United States, and when just imported honey is considered, 43% contained at least one unapproved antibiotic. Fourteen percent of imported honey contained unapproved antibiotics above the tolerance level. Although all geographical regions are not equally represented by a similar number of samples, there does appear to be some general trends (**Figure 2**). Forty-seven percent of honey from Eastern Europe was found to contain STZ, including several that contained over 100 ng/G of STZ. Twenty-six percent of honey from the Eastern Europe region also contained CAP. It is also evident that three unapproved antibiotics were found at the highest concentrations in honey originating from Eastern Europe. Unfortunately, it is impossible to determine the country of origin for some honey. These samples were included in the "other" category in **Figure 2**.

Figures 3 and **4** show typical chromatograms of detected unapproved antibiotics in extracted honey samples as well as chromatograms of a standard and matrix blank. **Figure 3a** shows the two CAP ion transitions with no response above baseline noise. **Figure 3b** represents a honey sample where the two ions produced a ratio of 76%. **Figure 3c** represents a CAP standard prepared in honey matrix, which gives an ion ratio of 80%. Similarly, **Figure 3d**−f represents sulfamethoxazole (SMX) in a honey blank, sample, and standard, respectively. The ion ratio for SMX in the sample in this case was 16%, whereas the ratio in the standard was 17%. **Figure 4a**−c shows the ion transitions for STZ in a honey blank, sample, and standard, respectively, with ion ratios of 34% for the sample and 36% for the standard. **Figure 4d**−f shows the ion transitions for SMZ in a honey blank, sample, and standard, respectively, with ion ratios of 68% for the sample and 75% for the standard. In all cases, the RT and ion ratios are within the acceptance limits, and all signals are well above 3× noise.

CONCLUSION

A method for the determination of 14 sulfonamides and CAP in honey has been developed and applied to 116 samples. Good sensitivity and recovery have been achieved with the use of SPE sample preparation followed by LC/MS/MS detection. Even though CAP has been banned for use in food-producing animals for many years due to its association with aplastic anemia and carcinogenesis, it is clearly still being used in certain countries

producing and exporting honey. Sulfonamides are also not allowed for use on food-producing animals in most countries due to the possible link with thyroid cancer as well as the concerns over the development of resistant strains of animal and human pathogens. However, several compounds from this class have been found in imported and domestic honey. STZ was the most commonly detected sulfonamide with the highest concentration being 132 ng/G. The results of the on going monitoring program indicate continuing misuse of antibiotics in the production of honey.

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