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Determination and Confirmation of Nitrofuran Residues in Honey Using LC-MS/MS

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A method was developed for the determination and confirmation of furazolidone, nitrofurazone, furaltadone, and nitrofurantoin as their side-chain residues in honey using liquid chromatography−tandem mass spectrometry (LC-MS/MS). An initial solid-phase extraction cleanup of the honey samples was followed by overnight hydrolysis and derivatization of the nitrofuran side-chain residues with 2-nitrobenzaldehyde. After pH adjustment and liquid−liquid extraction, the extracts were assayed by LC-MS/MS using electrospray ionization in the positive ion mode. The method was validated at concentrations ranging from 0.5 to 2.0 ppb with accuracies of 92−103% and coefficients of variation of ≤10%. The lowest calibration standard used (0.25 ppb) was defined as the limit of quantitation for all four nitrofuran side-chain residues. The extracts and standards were also used for confirmatory purposes. Honey from dosed beehives was assayed to study the stability of the nitrofuran residues and to demonstrate the effectiveness of the method.

KEYWORDS: Nitrofurans; honey; LC-MS/MS; method

INTRODUCTION

Many countries, including the United States (*1*), have banned the use of nitrofurans in food-producing animals due to the carcinogenicity and mutagenicity of these drugs and their metabolites. Furazolidone, furaltadone, nitrofurantoin, and nitrofurazone are among the most commonly misused nitrofurans. Findings of nitrofuran residues in honey from worldwide origins have been reported by Khong et al. (*2*). Therefore, analytical methods that meet the U.S. Food and Drug Administration (FDA) performance criteria (*3*, *4*) are needed to detect and confirm the presence of nitrofuran residues in honey.

The few methods that have been reported for the detection of nitrofuran residues in honey (*2*, *5*, *6*) are all based on previously published methods for the detection of nitrofuran side-chain residues in animal tissues (*7*−*9* and references cited therein). All of these methods involve a simultaneous acid hydrolysis and derivatization of the released side chains [3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SC), and 1-aminohydantoin (AH)] with 2-nitrobenzaldehyde (**Figure 1**). The difference among the methods resides in the sample cleanup. Our method is a modification of the one reported by Jenkins and Young (*6*). We used the same solid-phase extraction (SPE) cleanup before hydrolysis and derivatization as reported by Jenkins and Young; but instead of using a second SPE cleanup

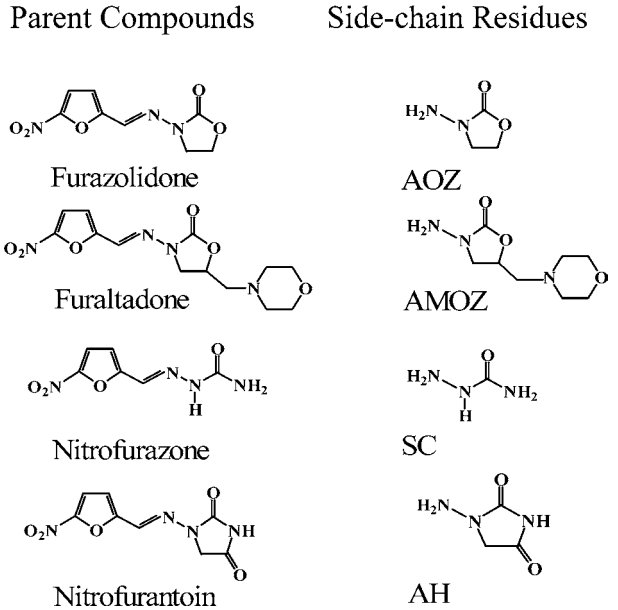

Figure 1. Chemical structures of parent nitrofurans and their side-chain residues: 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SC), and 1-aminohydantoin (AH).

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after the derivatization step, we employed liquid−liquid extractions to further clean up the extracts. During the partitioning steps, NaCl was added to each sample to reduce the emulsion formation and increase the solubility of the analytes in the organic phase (salting-out effect). This approach achieved greater interday accuracy and precision than previously reported by others (*2*, *5*). In the Jenkins and Young method, as well as in ours, the assay is based on the derivatization of the side-chain residues AOZ, AMOZ, SC, and AH, which are not retained in the SPE column.

We describe a liquid chromatography−tandem mass spectrometry (LC-MS/MS) method for the quantitation and confirmation of furazolidone, furaltadone, nitrofurantoin, and nitrofurazone as their side-chain residues in honey at 0.5, 1.0, and 2.0 ng/g levels. The long-term stability in honey of both the parent nitrofurans and their respective side-chain residues was also investigated. In this paper, the letters NP will be added to the abbreviated notation for the side-chain residues when referring to the derivatized moieties (e.g., AMOZ vs NPAMOZ).

MATERIALS AND METHODS

Reagents and Supplies. The nitrofuran side chains 1-aminohydantoin hydrochloride and semicarbazide hydrochloride were purchased from Aldrich (Milwaukee, WI). 3-Amino-2-oxazolidinone and 3-amino-5-morpholinomethyl-2-oxazolidinone were purchased from WITEGA (Berlin, Germany). The internal standards semicarbazide hydrochloride-$^{13}C,^{15}N_2$ (SC+3), 3-amino-2-oxazolidinone-d_4 (AOZ-d_4), 3-amino-5-morpholinomethyl-2-oxazolidinone-d_5 (AMOZ-d_5), and 2-nitrobenzaldehyde (2-NBA) were also purchased from Sigma-Aldrich (Milwaukee, WI). Furazolidone, furaltadone, nitrofurazone, and nitrofurantoin were purchased from Sigma (St. Louis, MO). HPLC grade methanol, hexane, and ethyl acetate were purchased from Burdick & Jackson (Muskegon, MI). Ammonium acetate, anhydrous potassium phosphate dibasic, and sodium hydroxide (all of ACS reagent grade) were obtained from Sigma-Aldrich. Concentrated hydrochloric acid (ACS reagent grade) and sodium chloride (USP reagent grade) were obtained from Fisher Scientific Co. (Pittsburgh, PA). Distilled deionized water was generated in-house from a Milli-Q-Plus water system.

Oasis HLB SPE (60 mg, 3 mL) columns were obtained from Waters Corp. (Milford, MA). Teflon syringe filters (13 mm, 0.2 μm) were obtained from Gelman Sciences (Ann Habor, MI).

Preparation of Standard Solutions. *Stock Solutions (~1 mg/mL).* Between 10 and 12 mg each of AOZ, SC, AH, and AMOZ was weighed, quantitatively transferred, and brought to the mark with 100% methanol into individual 10-mL volumetric flasks. A 100-mL volumetric flask was used for SC due to its low solubility in MeOH. The concentrations were corrected for the purity and salt form. A final mixed standard solution containing 0.200 ng/μL each of AOZ, SC, AH, and AMOZ was prepared in 50% aqueous MeOH from an intermediate mixed standard stock solution at 2.00 ng/μL prepared in 100% MeOH.

Working Standard Solutions. Working standard solutions of the nitrofuran side-chain residues at 0.00500, 0.0100, 0.0200, 0.0400, and 0.0800 ng/μL were prepared by diluting the final stock solution (0.200 ng/μL) or its diluted solutions with 50% aqueous methanol.

Internal Standard Stock Solutions. Between 2 and 3 mg each of the internal standards AOZ-d_4, SC+3, and AMOZ-d_5 was weighed, quantitatively transferred, and brought to the mark with 100% methanol into individual 10-mL volumetric flasks. The concentrations were corrected for the purity and salt form. A mixed internal standard working solution at 0.0400 ng/μL each of AOZ-d_4, SC+3, and AMOZ-d_5 was prepared in 50% aqueous MeOH from an intermediate mixed standard stock solution at 2.00 ng/μL prepared in 100% MeOH. All standard solutions were stable for a year if stored at < -10 °C.

Calibration Standards. Five-point standard curves for the nitrofuran side-chains AOZ, SC, AH, and AMOZ were prepared at 0.250, 0.500, 1.00, 2.00, and 4.00 ng/g (honey equivalent ppb) by adding 100 μL of the working standard solutions (0.00500, 0.0100, 0.0200, 0.0400, and 0.0800 ng/μL) and 50 μL of the working internal standard (1 ng/g honey equivalent) to blank 50-mL polypropylene centrifuge tubes. These calibration standards were processed along with the honey samples to compensate for the efficiency of the extraction and derivatization reaction.

LC-MS/MS System. The liquid chromatography system consisted of Agilent 1100 model G1312A binary pumps and a model G1329A autosampler (Agilent Technologies, Inc., Wilmington, DE). The liquid chromatography column was an Inertsil ODS-3 5 μm, 150 × 2.1 mm, with a guard column of the same packing (Varian, Walnut Creek, CA). An optional precolumn filter was installed between the autosampler and the guard column (Upchurch, Oak Habour, WA). A Micromass Quattro Micro mass spectrometer equipped with an electrospray ionization (ESI) source was operated in positive ion mode (Waters, Milford, MA).

Other Equipment Used. A TurboVap LV **s**olvent evaporator with a 15-mL test tube rack (Zymark, Hopkinton, MA), an Orion model SA520 pH-meter (Orion Research Inc., Boston, MA), and a model 175D ultrasonic cleaner (Crest Ultrasonics Corp., Trenton, NJ) were also used in this study.

Nitrofuran Beehive Dosing. *Bee Colonies.* Two individual honey bee (*Apis mellifera*) colonies were established on five frames of drawn comb in a five-frame hive body. A second hive body containing five frames of partially drawn comb was place on top of the lower hive body. The queen was confined to the lower hive body by placing a queen excluder between the bottom and top hive bodies, thereby ensuring no bee brood would be reared in the top hive body. The colonies were maintained at the USDA-ARS Bee Research Laboratory in Beltsville, MD. The study was conducted in early May 2005 during the black locust (*Robinia pseudoacacia*) and tulip poplar (*Lirodendroan tulipifera*) nectar flows—two common sources of nectar in this area.

Dosing Nitrofurans to Bees. Furazolidine (23.2 mg), nitrofurazone (25.3 mg), furaltadone (25.8 mg), and nitrofurantoin (25.6 mg) (obtained from Sigma-Aldrich) were mixed with 159.9 g of granulated sugar and 80 g of honey to produce a pliable product that could be formed into a hamburger-like patty. A 50.02 g patty consisting of the nitrofuran−sugar−honey mixture was placed on the top parts of the frames in the upper hive body of one of the experimental colonies. A 25.04 g patty of the same mixture was placed in the same position on the second colony. Two doses were used to ensure an adequate range of drug in the subsequent incurred honey. The honey used in the drug patty was collected from honey bee colonies the prior year and was analyzed to ensure it was drug-free.

Collection of Incurred Honey. The bees in both colonies consumed the respective patties within 24 h. Three and 4 days after the patties were placed on their respective colony, any frames containing honey were removed, labeled, and stored in empty hive bodies in a room at 25 °C. The frames that were removed were replaced with frames containing empty comb. Two additional honey samples were collected from each colony 1 and 2 weeks from the day-4 collections. Honey was extracted by crushing the comb with stainless steel spatulas and allowing gravity to filter the honey through sterilized cheesecloth into methanol-rinsed individual jars. Depending on the colony and the number of days between collections, the bees produced between 300 mL and >1 L of incurred honey for each sample period.

Incurred Honey for Method Validation. Each sample of incurred honey received from the Bee Research Laboratory was subdivided for storage in the dark at three different temperatures as follows: room temperature, −20 °C, and −80 °C. These samples were assayed to study the stability of the parent nitrofurans and their side-chain residues in incurred honey stored at room temperature. Honey stored at −80 °C was used as the reference point for zero time at room temperature. Periodically, samples were taken from the incurred honey at room temperature and stored at −80 °C to slow any degradation reaction taking place. Degradation of the parent nitrofurans and their side-chain residues while the honey was kept in the beehive or at room temperature waiting to be filtered after collection was not studied. Incurred honey stored at −20 °C was used for method validation. Control honey received from the Bee Research Laboratory and honey purchased at local grocery stores were used to prepare fortified samples, conduct interference studies, and test the performance of the method on honey

with different physical characteristics (crystallized, dark, clear, and containing large amounts of comb or pollen).

Extraction Procedure for Side-Chain Residues of Nitrofurans. Five standards and 12 samples can be comfortably assayed in a 2-day period. Homogenized control honey (2.00 ± 0.1 g) was weighed into 15-mL polypropylene centrifuge tubes. After weighing, 100 μL of the appropriate side-chain working standards was added to prepare fortified samples at three levels (0.500, 1.00, and 2.00 ng/g). Fortification of five calibration standards (0.250, 0.500, 1.00, 2.00, and 4.00 ng/g) was done also at this step by adding 100 μL of the side-chain working standards to individual 50-mL polypropylene centrifuge tubes. A negative control and incurred samples were assayed concurrently. To all samples and standards were added 50 μL of the working internal standard mixture to give an equivalent concentration in honey of 1.00 ng/g.

After the addition of 5 mL of 10% NaCl, the samples and standards were capped and vortex-mixed until the honey dissolved. Honey samples (but not the standards) were centrifuged at room temperature for 5 min and 4000 rpm (3400 RCF). The supernatants were loaded using Pasteur pipets onto Oasis HLB SPE columns previously conditioned with 2 mL of methanol and 2 mL of water. The eluates were collected using 50-mL polypropylene centrifuge tubes. The nitrofuran side-chain residues were not retained on the SPE column; they passed through and were collected with the eluate. The sample tubes were vortexed twice with 1 mL of water. These water washes were decanted sequentially into the respective SPE column and collected in the same tube as the sample eluate.

The final volume of the standards was made equal to that of the sample eluates by adding 3 mL of Milli-Q water to the standards. To both sample eluates and standards were added 100 μL of freshly prepared 2-nitrobenzaldehyde solution (50 mM in methanol) followed by the addition of 5 mL of aqueous 0.125 M HCl. Samples and standards were vortex-mixed and placed in a 37 °C water bath overnight (~14−16 h) with moderate shaking.

Samples and standards were cooled to room temperature, and 3 mL of 0.1 M K_2HPO_4 buffer was added. One at a time, the pH of samples and standards was adjusted between 7.15 and 7.25 by adding 0.8 M aqueous NaOH or 0.125 M HCl as needed. A fourth of a teaspoon (~1.25 mL) of granular NaCl and 5 mL of hexane were added. After gentle hand shaking and centrifugation at 4000 rpm (~3400 RCF) for 10 min at 15 °C, the hexane layer was discarded along with any interface layer. The aqueous layer was partitioned twice with 6 mL of EtOAc. Samples and standards were vortex-mixed vigorously for 15 s. After centrifugation at 4000 rpm (~3400 RCF) for 5 min at 15 °C, the top EtOAc layers were collected into clean 15-mL polypropylene tubes leaving behind any interface layer. The EtOAc was washed twice with 2 mL of H_2O and evaporated to dryness using the Turbo Vap LV set at 50 °C. To obtain a high degree of accuracy and reproducibility (particularly for NPAMOZ) as soon as the extracts were dry, 2 mL of methanol was added to wash the inside wall of the tubes. The tubes were vortex-mixed vigorously for 10 s, and the evaporation was continued to dryness.

Reconstitution of the extracts was done in two steps. First, 40 μL of methanol was added, and the extracts were vortex-mixed for 15 s and sonicated for 2 min. Next, 160 μL of water was added followed by vortexing for 15 s and filtering through 0.2-μm PTFE filters into autosampler vials with 300-μL inserts.

Extraction Procedure for Parent Nitrofurans. The above procedure was modified to concurrently determine the parent nitrofurans present in incurred honey samples being assayed for the nitrofuran side-chain residues. After the aqueous SPE eluate containing the side-chain residues was collected, the SPE columns were vacuum-dried for 1 min, washed with 3 mL of hexane, and vacuum-dried for another minute, and then the parent nitrofurans were eluted with 3 mL of acetonitrile. The acetonitrile was evaporated using the Turbo Vap LV set at 50 °C; 5 mL of 10% NaCl was added and the procedure continued at the ethyl acetate partition. In brief, to detect the parent nitrofurans the derivatization step with 2-NBA and the pH adjustment were omitted. The LC-MS/MS analysis of furaltadone and furazolidone was done using electrospray ionization in the positive ion mode, whereas nitrofurazone and nitrofurantoin were assayed in the negative ion mode. The concentrations for all four parent nitrofurans found in the second elution of the SPE columns were corrected for procedural losses and compared to the concentrations of the side-chain residues found in the first elution of the SPE columns.

Table 1. SRM Transitions

derivative	precursor ion (*m/z*)	product ion (*m/z*)	cone (V)	collision energy (eV)
NPAH	249	104	29	21
		134	29	12
		178	29	13
NPAOZ	236	104	28	21
		134	28	12
		149	28	13
NPSC	209	134	24	11
		166	24	10
		192	24	12
NPAMOZ	335	128	21	22
		262	21	16
		291	21	12
int std NPAOZ-d_4	240	134	28	12
int std NPSC+3	212	168	24	10
int std NPAMOZ-d_5	340	296	21	12

Chromatographic Conditions. The mobile phase components were aqueous 10 mM NH_4Ac (A) and 100% methanol (B). Extracts were analyzed using a 20-min gradient at a constant flow of 0.200 mL/min: 80 A/20 B v/v for 2 min, a 1-min ramp, 50 A/50 B v/v for 14 min, a 1-min ramp, and 80 A/ 20B v/v for 2 min. The injection volume was 10 μL with the autosampler temperature set at 4 °C and the column compartment at 25 °C. The LC column was equilibrated with the first step of the gradient for at least 1 h prior to injection of samples. During this time the collision gas and the instrument electronics were also equilibrated. Three injections of a standard were made to equilibrate the gradient and to test the instrument response. A typical injection sequence was as follows: a solvent blank, the standards, a solvent blank, negative and positive controls, fortified samples, a solvent blank, incurred residue samples, a solvent blank, and the standards again to bracket the samples. After each batch, the column was flushed for 1 h with 75% MeOH/25% H_2O at a flow of 0.200 mL/min to remove the NH_4Ac and other retained materials from the column.

MS/MS Analysis. The protonated ions, $[M + H]^+$, for the derivatives at *m/z* 249 (NPAH), 209 (NPSC), 236 (NPAOZ), and 335 (NPAMOZ) were selected as the precursor ions for collision-induced dissociation (CID). **Table 1** lists the selected-reaction-monitored (SRM) transitions used for MS/MS analysis. The dwell time for each transition was 0.500 s. Relevant instrument parameters were as follows: capillary, 0.31 kV; extractor, 2.0 V; RF lens, 0.2 V; source block, 125 °C; desolvation heater, 400 °C; desolvation gas, 600 L/h; and multiplier, 650 V.

Quantitation. The three ion chromatograms specific for each analyte were summed and integrated by the mass spectrometer processing software (Target Lynx). Calibration curves were prepared by plotting the peak area ratio between the analyte and the internal standard versus the concentration of the standards (nanograms per gram). NPAMOZ-d_5 was used as the internal standard for the quantitation of NPAH because deuterated NPAH was not commercially available at the time this study was conducted. The concentration of the analytes in the samples was interpolated from the equations of the linear-regression graphs. Correlation coefficients (r^2) for each of the calibration curves were >0.99. The experimental analyte concentrations (except for controls and fortified samples) were corrected for the mass difference of the individual samples to the nominal 2-g sample used to calculate the concentration of the analytes in the calibration standards.

Confirmation. The mass spectrometer processing software was used to integrate the individual product ion chromatograms for each analyte and to check that the signal-to-noise ratio for each product ion was >3:1. An analyte was confirmed to be positive if the retention time of the individual product ions in the sample matched the mean retention

Table 2. Method Performance on Fortified Honey ($n = 5$)

fortification (ng/g)	found (ng/g)	standard deviation	CV (%)	accuracy (%)
		AMOZ		
0.500	0.49	0.01	3	99
1.00	1.01	0.03	3	101
2.00	1.98	0.04	2	99
		AH		
0.500	0.46	0.03	6	92
1.00	0.97	0.10	10	97
2.00	2.00	0.12	6	100
		AOZ		
0.500	0.51	0.01	3	101
1.00	1.03	0.02	2	103
2.00	2.06	0.03	1	103
		SC		
0.500	0.49	0.03	6	98
1.00	0.99	0.04	4	99
2.00	1.96	0.04	2	98

time of the product ions in the standards within 3% and if the two area ratios obtained from the three product ions of the analyte in the sample were arithmetically within 10% of their mean in the standards (*4*).

RESULTS

Fortified Honey Samples. Table 2 presents the validation results collected on four separate assay sets done by the same operator using the same equipment over a 2-week period. For all four nitrofuran side-chain residues, the reproducibility errors were ≤10% and the accuracies were between 92 and 103%. The average absolute recoveries using matrix-matched standards without internal standards were 76% for AMOZ, 72% for AH, 60% for AOZ, and 45% for SC. Matrix-matched standards were prepared by spiking the final control honey extract with the derivatized nitrofuran side chains commercially available.

No interference was observed in the negative controls at the retention time of the analytes. The limit of quantitation (LOQ) for each analyte was defined as the lowest calibration standard used (0.25 ppb). Much lower LOQs could have been achieved for NPAOZ and NPAMOZ due to their much greater response in the LC-MS/MS analysis. Lower LOQs were not pursued because a more straightforward preparation procedure for standards and easier quantitation algorithm were considered to be more important.

The method was tested using control honey from five different sources. These samples had different physical characteristics (crystallized, dark, clear, and containing large amounts of comb or pollen). The method performed well with all five types of honey. Fortified samples had accuracies of >90% and CVs of <10% for all four nitrofuran side-chain residues.

To test the specificity of the method, two control honey samples were fortified with 38 veterinary drugs and extracted using the nitrofuran side-chain procedure. The chromatograms showed no interference peaks at the retention time of the nitrofuran analytes. The drugs tested were sulfadimethoxine, sulfamerazine, sulfadiazine, ormetoprim, trimethoprim, oxytetracycline, chlortetracycline, tetracycline, doxycycline, flumequine, oxolinic acid, difloxacin, danofloxacin, enrofloxacin, sarafloxacin, ciprofloxacin, penicillin G, amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, cephalexin, erythromycin,

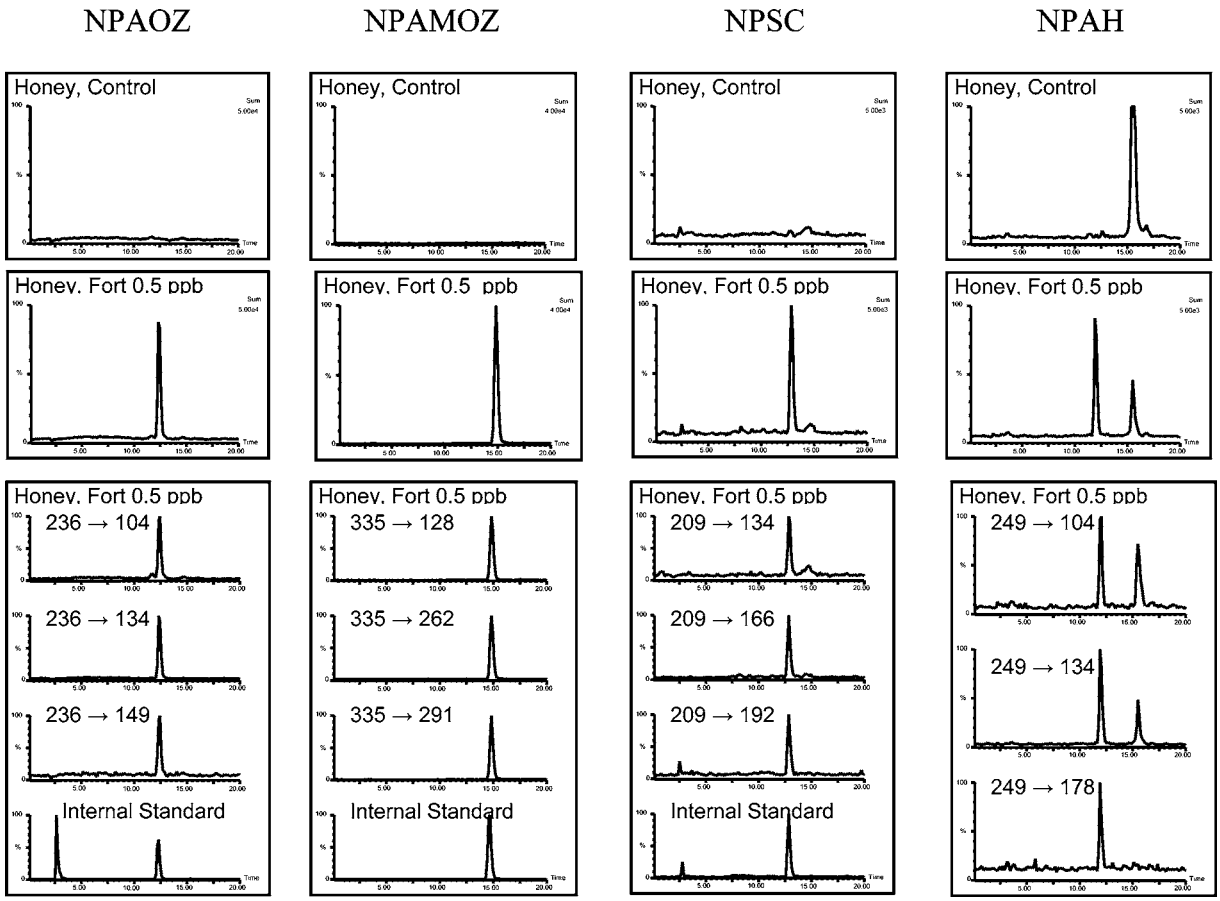

Figure 2. Representative chromatograms for derivatized (NP) AOZ, AMOZ, SC, and AH: (**top**) RIC of control honey; (**middle**) RIC of honey fortified at 0.5 ng/g of the nitrofuran side chains; (**bottom**) SRM ion chromatograms of honey fortified at 0.5 ng/g of the nitrofuran side chains and 1 ng/g of the internal standards.

Table 3. Method Performance in Incurred Honey ($n = 5$)

incursion	found (ng/g)	standard deviation	CV (%)
	AMOZ		
level 1	0.76	0.03	4
level 2	1.50	0.05	3
	AH		
level 1	0.52	0.01	2
level 2	1.41	0.09	6
	AOZ		
level 1	0.65	0.03	5
level 2	1.16	0.03	3
	SC		
level 1	0.72	0.03	4
level 2	1.28	0.04	3

Table 4. Comparison of Accuracy and Precision[a]

method	AMOZ	AH	AOZ	SC
Khong (*2*)	83–94 (9)	94–97 (16)	88–93 (4)	102–108 (12)
Matus (*5*)	71.2–105.8 (6.5)	71.2–105.8 (6.5)	84.2–118.5 (5.6)	70.6–117.8 (8.3)
Lopez	98.9–101.2 (3)	92.2–100.1 (10)	101.3–103.4 (3)	98.0–98.8 (6)

[a] Maximum coefficients of variation reported in parentheses.

tilmicosin, tylosin, emamectin (Bla), invermectin, metronidazole, albendazole, fenbendazole, ketoconazole, malachite green, leuchomalachite green, lincomycin, florfenicol amine, diflubenzuron, and teflubenzuron. However, honey samples that are packed in jars closed with metal lids sealed with a plastic gasket may confirm positive for nitrofurazone because semicarbazide gradually leaks from the gasket (*10*).

Incurred Honey Samples. The performance of the method was evaluated with incurred honey obtained from beehives treated with furazolidone, furaltadone, nitrofurantoin, and nitrofurazone. All four parent nitrofurans and their respective side-chain residues were detected in the incurred honey. For each nitrofuran side-chain residue, two levels within the validated concentration range were assayed. The results are presented in **Table 3**. The reproducibility errors for all four nitrofuran side-chain residues in the incurred honey were ≤6%.

Confirmation. The confirmation criteria described earlier are in accordance with Guidance 118 of the U.S. Food and Drug Administration (*4*). Upon application of the confirmation criteria, the presence of all analytes was confirmed in all fortified and incurred samples, but was not confirmed in all negative controls. Typical reconstructed ion chromatograms (RIC) of control and fortified controls are shown in **Figure 2** along with the individual SRM chromatograms used for confirmation purposes. Khong et al. (*2*) proposed structures for the precursor and product ions for all four nitrobenzaldehyde derivatives of the nitrofuran side-chain residues.

DISCUSSION

The initial sample cleanup described in this method was a modification of the one developed by Jenkins and Young (*6*) for the determination of nitrofuran side-chain residues in honey. In their method, the honey was initially dissolved in 0.12 M HCl and then passed through an Oasis HLB column. In our method, the honey sample was dissolved in 10% NaCl before the Oasis HLB column. This approach gave higher absolute recoveries than those obtained when the samples were dissolved in 0.12 M HCl. The derivatization procedure was adapted from that initially reported by Hoogenboom et al. (*9*) as modified later by others (*7*) to detect nitrofuran metabolites in a variety of matrices (*8* and references cited therein). After derivatization and pH adjustment, NaCl was added to each sample to reduce the emulsion formation and increase the solubility of the analytes in the organic phase during the partitioning steps (salting-out effect). A hexane partition was included to remove bee wax and excess 2-NBA. These modifications gave a highly sensitive method with better interday accuracy and precision than those reported by others (**Table 4**). Jenkins and Young did not report the details of their method interday accuracy and precision.

A preliminary evaluation of the reaction time needed for hydrolysis and derivatization of nitrofuran side-chain residues in incurred honey was conducted. As honey contains very small amounts of proteins, we were expecting much shorter reaction times than those needed for the determination of protein-bound nitrofuran residues in animal tissues (usually between 14 and 16 h). Nevertheless, 14−16 h was still required to get maximum derivatization for all four nitrofuran side-chain residues. In

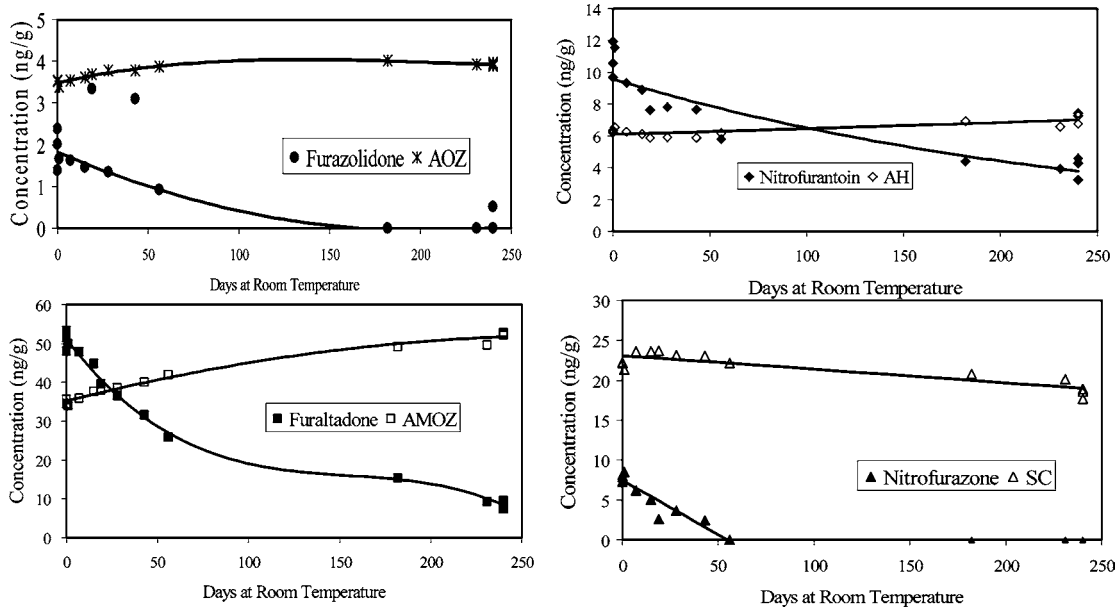

Figure 3. Stability of nitrofurans and their nitrofuran side-chain residues at room temperature.

honey, the nitrofuran side chains might have formed Schiff base adducts with the carbohydrates, aldehydes, or ketones present. The hydrolysis of the side-chain adducts during the derivatization with 2-NBA might explain the long reaction time needed.

To assay for the parent nitrofurans that were also present in the incurred honey, the SPE columns were eluted with 3 mL of acetonitrile after the aqueous eluate containing the nitrofuran side-chain residues was collected. The method to extract the parent nitrofurans is similar to the one used to extract the nitrofuran side-chain residues except that the derivatization step with 2-NBA and the pH adjustment are omitted. The modified method to detect the parent nitrofurans is less sensitive and less precise than the nitrofuran side-chain method. All four parent nitrofurans were found in incurred honey stored at room temperature; however, they gradually decomposed (**Figure 3**). As observed in **Figure 3**, with time, the nitrofuran side-chain residues were found at a higher concentration than the parent nitrofurans. Only the concentration of semicarbazide (the side chain of nitrofurazone) was observed to slightly decompose at room temperature. Honey is a commodity that is usually stored at room temperature for weeks or months before it reaches the consumer or the testing laboratory. Lower detection levels and better precision are achievable for the nitrofuran side-chain residues than for the parent nitrofurans. Therefore, the determination of the presence of nitrofurans in honey was done by assaying for the side-chain residues rather than for the parent nitrofurans.

To avoid clogging of the SPE columns, the samples were loaded onto the conditioned SPE columns using Pasteur pipets. First, the middle portion of the sample was added and allowed to percolate through the SPE column before the floating solids on top of the sample or the pollen at the bottom of the tube were added. This was a critical step in the method as even samples apparently free from debris clogged the SPE columns when they were loaded by pouring them onto the columns.

The reconstitution of the extract before injection was done in two steps. First, 40 μL of MeOH was added to dissolve the residues followed by 160 μL of water for a total of 200 μL of 20% MeOH as the reconstitution solvent. During the last solvent evaporation, a thin layer of residues on the wall of the sample tube traps the analytes. These residues are soluble in 100% MeOH but not in 20% MeOH. The analytes are freed by dissolving the residue in 100% MeOH while the solid residues are eliminated during the filtration step. Reconstitution of the extracts in two steps gave better recoveries and precision for all side-chain derivatives, in particular for NPAMOZ. Aqueous 20% MeOH is also the composition of the first step of the mobile phase gradient.

Injecting a small volume of extract (10 μL) into the LC-MS/MS had more impact in reducing matrix effects than increasing the reconstitution volume of the extract and injecting larger volumes. The use of a small injection volume and the mobile phase gradient were essential in obtaining linear standard curves with $r^2 > 0.99$.

For confirmation, solutions of commercially available derivatized standards can be used instead of standards derivatized in situ, because the ion ratios remain the same despite matrix effects during the MS/MS analysis. The use of "off-the-shelf" derivatized standards can increase the number of samples assayed per day and also facilitates tuning the mass spectrometer for optimal analyte sensitivity. However, quantitation is affected by matrix effects that suppress the response for all four analytes in the extracts, thus giving rise to apparently low recoveries if "off-the-shelf" derivatized standards are used to prepare the standard curve. The use of standard curves prepared with standards derivatized in situ and the use of internal standards eliminate this problem and are indeed needed for quantitation.

The method complies with the performance criteria of the Center for Veterinary Medicine for the analysis of veterinary drug residues in animal products (*3*, *4*). Therefore, the method can be used for regulatory purposes and to monitor the presence of nitrofuran side chains in honey.

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