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Determination of Nitrofuran Residues in Milk of Dairy Cows Using Liquid Chromatography−Tandem Mass Spectrometry

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An analytical method has been developed for the determination of total bound and extractable residues of the nitrofuran drugs furazolidone, nitrofurazone, furaltadone, and nitrofurantoin in milk of dairy cows. The method involves overnight acid hydrolysis and simultaneous derivatization of the released side chains with 2-nitrobenzaldehyde. During hydrolysis, the bound metabolites are hydrolyzed to the side chains. After pH adjustment and solid-phase extraction cleanup, the derivatives are detected and quantitated using a liquid chromatography−tandem mass spectrometry system with an atmospheric pressure chemical ionization interface. Validation of the method is accomplished by fortifying control milk with a mixture of side chains at 1, 2, and 4 ng/g. Internal standards are added at the beginning of the procedure to compensate for matrix effects and recovery losses. Method accuracies range from 83 to 104% with coefficients of variation less than 13% for all four analytes. The limits of detection are ≤0.2 ng/g for the side chains. In the milk of a dosed cow, nitrofurantoin exhibits the lowest level of residues among the four nitrofurans. Seventy-two hours after dosing, side-chain residue levels in milk drop below 0.2 ng/g.

KEYWORDS: Nitrofurans; milk; LC-MS/MS; method; residues

INTRODUCTION

Nitrofurans (NFs; **Figure 1**) are broad-spectrum antibacterial drugs effective in the treatment of protozoan and bacterial infections in both humans and animals (*1*). Nitrofurazone (NFZ) was the first NF approved for veterinary use by the U.S. Food and Drug Administration (FDA) in 1948 (*2*, *3*). This was followed by the approval of furazolidone (FZD) in 1953 to treat fowl typhoid, paratyphoid, and pollorum in turkeys and chickens. In 1962, furaltadone (FTD) was also permitted for mastitis treatment in dairy cows administered via intramammary infusion (*2*, *3*). Nitrofurantoin (NFT) is a human drug and was permitted for treatment of urinary tract infections and tracheobronchitus in dogs, cats, and horses (*4*).

Despite their therapeutic values, NFs have been shown to be mutagenic and carcinogenic (*2*). For this reason, the FDA withdrew the approvals of FZD and NFZ for antiprotozoal use in poultry and swine in 1991 (*5*). Veterinarians were still allowed to use NFs but only in nonfood animals and in food-producing animals for topical and ophthalmic uses. In 2002, the FDA banned all extralabel uses of NFs in food-producing animals (*6*). The decision was based on findings of an FDA-sponsored radiolabel study, in which residues were detected in edible tissues (including milk) of cows dosed with NFZ by intramammary, intrauterine, and ocular routes (*7*). In spite of the ban, NFs continue to be of regulatory concern, as many countries have detected NF residues in their imported food products (*8*).

Several methods have been described for the determination of parent NFs in milk. For example, Long and collaborators described a method for FZD in milk using matrix solid-phase dispersion (*9*). Galeano Díaz et al. reported a method for the determination of NFT, FZD, and FTD in milk using HPLC with electrochemical detection (*10*). Using on-line dialysis and column switching liquid chromatography, Aerts et al. developed a method capable of detecting and quantitating NFZ, NFT, FZD, and FTD in milk (*11*).

Studies have shown that NFs are metabolized rapidly and extensively to protein-bound metabolites, which persist in edible tissues for a prolonged time after treatment (*12*, *13*). Therefore, newer analytical methods have been directed to the detection and determination of the side-chain residues instead of the parent NFs. Despite differences in sample cleanup, these methods typically involve an overnight acid hydrolysis and simultaneous derivatization of the released side chains to their nitrophenyl derivatives followed by liquid chromatography−tandem mass spectrometry (LC-MS/MS) determination (*14*−*16*). To date, methods have been reported for animal tissues (*17*−*19*), honey (*8*), shrimp (*20*), and eggs (*21*). Methods for determining the side-chain residues in milk, however, are scarce. In this article, we describe an LC-MS/MS method capable of determining the total bound and extractable side-chain residues of four NFs in milk of dairy cows.

MATERIALS AND METHODS

Reagents. LC grade water was purified in-house with a Milli-Q Plus water system and was used in the preparation of all solutions.

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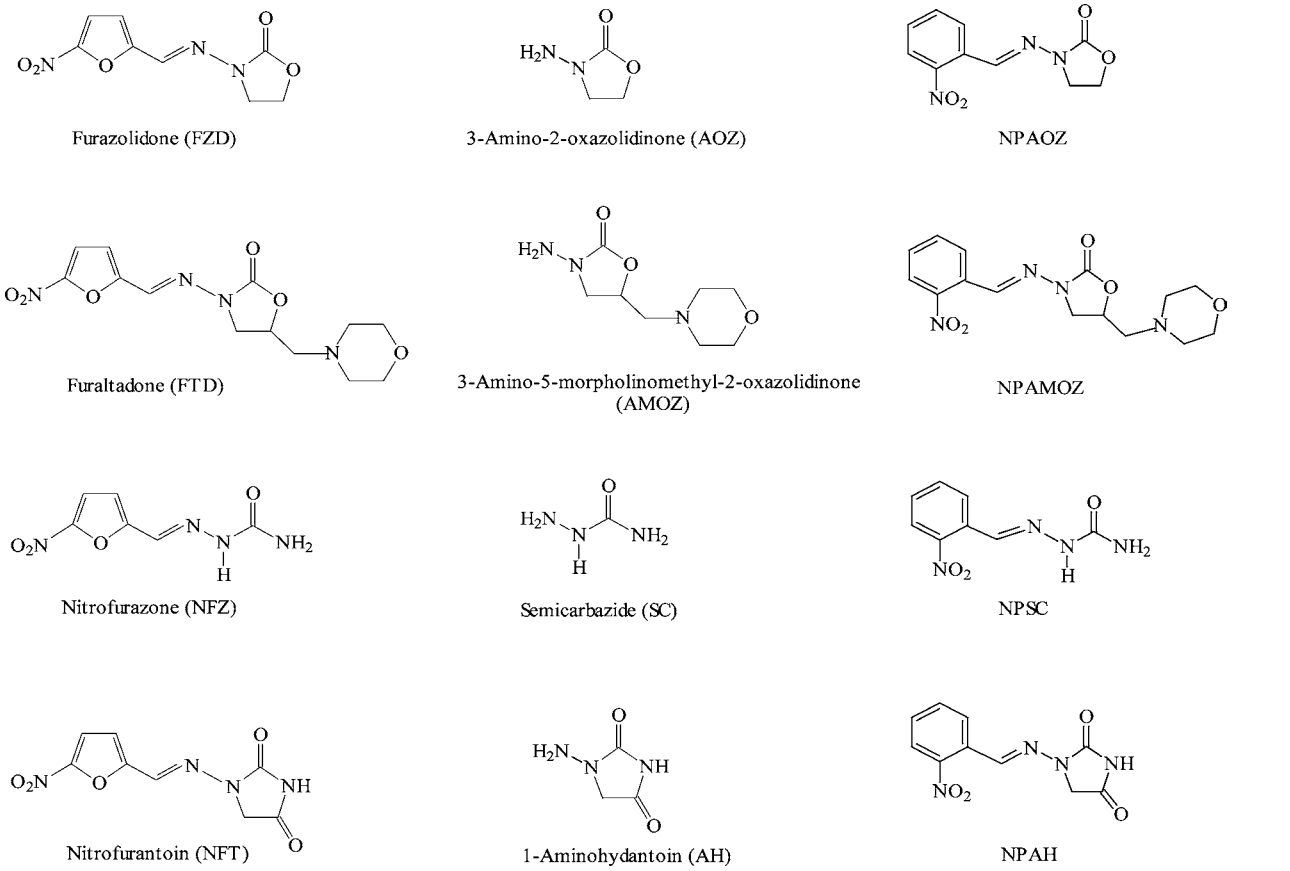

Figure 1. Structures of NFs, their side-chain residues, and nitrophenyl derivatives.

Ammonium acetate, anhydrous potassium phosphate dibasic, and sodium hydroxide were of ACS reagent grade (Sigma-Aldrich, St. Louis, MO). Methanol (MeOH), hexane, and ethyl acetate (EtOAc) were high-purity solvents obtained from Burdick & Jackson (Muskegon, MI). Dimethyl sulfoxide (DMSO) was obtained from EM Science (Gibbstown, NJ). 1-Aminohydantoin hydrochloride (AH·HCl), semicarbazide hydrochloride (SC·HCl), and 2-nitrobenzaldehyde (2-NBA) were purchased from Sigma-Aldrich. 3-Amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 2-nitro-benzaldehyde-semicarbazone (NPSC), 5-morpholin-4-ylmethyl-3-[(2-nitro-benzylidene)amino]oxazolidin-2-one (NPAMOZ), 3-[(2-nitro-benzylidene)amino]oxazolidin-2-one (NPAOZ), and 1-[(2-nitro-benzylidene)-amino]imidazolidine-2,4-dione (NPAH) were obtained from Witega (Berlin, Germany). The internal standards semicarbazide hydrochloride-$^{13}C,^{15}N_2$ (SC+3·HCl), and 3-amino-2-oxazolidinone-d4 (AOZ-d4) were obtained from Sigma-Aldrich while 3-amino-5-morpholino-methyl-2-oxazolidinone-d5 (AMOZ-d5) was obtained from Witega. In this paper, the letters NP are added to the abbreviated notation for the side chains when referring to the nitrophenyl derivatives (e.g., SC vs NPSC).

Oasis HLB SPE columns (30 mg, 3 mL) were obtained from Waters Corp. (Milford, MA), and the 20 mL reservoirs were obtained from Varian Inc. (Palo Alto, CA). Nylon syringe filters (13 mm, 0.2 μm) were obtained from Gelman Sciences (Ann Arbor, MI). A TurboVap LV evaporator (Zymark, Hopkinton, MA) was used for sample concentration. A Jouan model CR 4.22 centrifuge (Winchester, VA) equipped with a swing-out rotor with 15 and 50 mL centrifuge tube holders was used for centrifugation. Wide mouth polypropylene bottles (1 L, Nalge 2110 series) with polypropylene screw closures used for milk collection and polypropylene centrifuge tubes (50 mL, Corning brand) for analysis were obtained from Thomas Scientific (Swedesboro, NJ). Containers closed with lids sealed with plastic gaskets must not be used for milk sampling, because these gaskets may leak out SC.

Standard Solutions. *Concentrated Stock Solutions (100 μg/mL).* On the basis of purity and composition, the amount of AOZ, SC, AH, and AMOZ needed to prepare 100 mL of the individual 100 μg/mL standard solution was calculated. All standards are expressed as the unionized moiety equivalent. Each standard was weighed to the nearest ±0.1 mg into a respective 100 mL volumetric flask, brought to the mark with MeOH, and sonicated for 5 min. These solutions were used immediately for the preparation of the solutions below.

Intermediate Mixed Stock Solution (2 μg/mL). Two milliliters of each of the concentrated stock solution (100 μg/mL) was pipetted into a 100 mL volumetric flask and brought to the mark with MeOH. The volume pipetted was adjusted for the concentration difference of the primary stock solution from the nominal 100 μg/mL. This solution was stored at −20 °C.

Table 1. SRM Transitions and Ion Ratios Used for Analyte Confirmation

derivative	precursor ion (*m/z*)	product ion (*m/z*)	collision energy (eV)	ion ratios used for confirmation
NPAH	249	104	31	134/104
		134	19	178/104
		178	21	
NPSC	209	134	17	192/166
		166	15	134/166
		192	17	
NPAOZ	236	104	29	104/134
		134	17	149/134
		149	19	
NPAMOZ	335	128	39	262/128
		262	19	291/128
		291	21	
int. standard NPSC+3	212	168	17	
int. standard NPAOZ-d4	240	134	17	
int. standard NPAMOZ-d5	340	296	17	

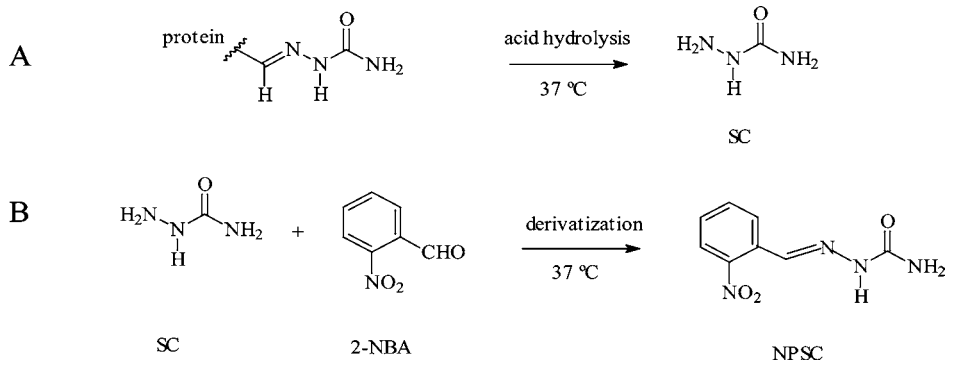

Figure 2. Reaction scheme for NFZ showing (**A**) the release of the side chain through hydrolysis of protein-bound residues and (**B**) simultaneous derivatization with 2-nitrobenzaldehyde. Adapted from ref *17*.

Table 2. Method Validation of NF Residues in Milk—Fortified Samples ($N = 6$)

		internal standard		external standard		derivative standard	
analyte	fortification level (ng/g)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)
AH	1	85	11	85	14	92	3
	2	83	8	82	9	84	9
	4	90	9	86	10	84	8
SC	1	101	3	100	9	86	8
	2	100	4	99	7	81	9
	4	100	6	96	11	76	11
AOZ	1	100	4	100	6	97	7
	2	101	4	96	6	90	8
	4	104	3	98	4	90	4
AMOZ	1	102	5	117	5	87	10
	2	98	3	113	5	82	8
	4	100	5	113	7	82	6

Table 3. Method Validation of NF Residues in Milk—Incurred Samples ($N = 6$)

		internal standard		external standard		derivative standard	
analyte	incursion	ng/g found	CV (%)	ng/g found	CV (%)	ng/g found	CV (%)
AH	level 1	1.4	13	1.4	12	1.4	7
	level 2	3.0	7	2.8	4	2.8	6
SC	level 1	1.8	4	1.8	6	1.5	6
	level 2	3.7	3	3.5	5	2.8	6
AOZ	level 1	0.76	6	0.74	6	0.74	10
	level 2	1.5	2	1.5	3	1.4	6
AMOZ	level 1	1.1	5	1.3	6	1.0	7
	level 2	2.3	5	2.6	4	1.9	4

Stock Solution (0.2 μg/mL). One milliliter of the intermediate mixed stock solution (2 μg/mL) was pipetted into a 10 mL volumetric flask and brought to the mark with 50% aqueous MeOH. This solution was stored at −20 °C.

Fortification Standard (0.04 μg/mL). The stock solution (0.2 μg/mL) was diluted with 50% aqueous MeOH to prepare a 0.04 μg/mL fortification solution, which was used for sample fortification and for preparing four calibration standards at the following concentrations: 0.5, 1, 2, and 5 ng/g (ppb). The fortification standard was stored at −20 °C.

Solutions for the internal standards (AOZ-d4, AMOZ-d5, and SC+3) and the derivative standards (NPAH, NPSC, NPAOZ, and NPAMOZ) were prepared in a similar manner, except that smaller quantities were weighed out. The concentrations of the derivative standards were expressed as the underivatized side chain equivalents based on the molar mass difference between the side chains and their nitrophenyl derivatives.

Liquid Chromatograph. The liquid chromatography system consisted of two Perkin-Elmer (Norwalk, CT) Series 200 micropumps and a Perkin-Elmer Series 200 autosampler equipped with a 100 μL loop. The LC column was an Inertsil ODS-3 5 μm, 150 mm × 2.1 mm with a guard column of the same packing (Ansys Technologies, Inc., Lake Forest, CA). An optional precolumn filter (Upchurch, Oak Habour, WA) was installed between the autosampler and the guard column.

The HPLC mobile phase consisted of MeOH−20 mM NH_4Ac (55 + 45, v/v). The 20 mM NH_4Ac solution was prepared by dissolving 1.54 g of NH_4Ac in 1 L of water. The HPLC mobile phase was prepared by measuring 550 mL of MeOH and 450 mL of the 20 mM NH_4Ac solution followed by mixing and filtering through a 0.45 μm filter.

Mass Spectrometer. An Applied Biosystems (Foster City, CA) Sciex API 2000 triple quadrupole mass spectrometer with atmospheric pressure chemical ionization (APCI) source in the positive ion mode was used. The protonated molecules, $[M + H]^+$ at *m*/*z* 249 (NPAH), 209 (NPSC), 236 (NPAOZ), and 335 (NPAMOZ), were selected as the precursor ions for collision-induced dissociation (CID). The product ions were identified, and the collision energies for selected reaction monitoring (SRM) for LC-MS/MS analysis are shown in **Table 1**. The dwell time for each monitored transition was 150 ms, which generated 24−28 data points for each chromatographic peak. Both Q1 and Q3 were set at unit resolution. The source temperature and ion spray voltage were set at 350 °C and 5500 V, respectively. Peak areas for quantitation were computed by summing the areas of the product ions of the respective analytes upon integration using PE-Sciex Analyst version 1.3.2 software. In other words, the area counts were obtained by integration of the analyte peaks in the reconstructed total ion chromatograms of the product ion scan of each analyte.

Animal Dosing. A capsule, which contained 0.88 mg/kg body weight each of FZD, NFZ, and FTD and 4.4 mg/kg body weight of NFT was administered orally to one 736 kg cow. Milk samples were collected at 12 h intervals for 2 weeks, a collection period sufficient to allow milk residues to fall below the detection limit. The milk samples were stored at −80 °C until analysis. Control raw milk used for method development was obtained from cows that had not been treated with NFs.

Incurred Milk for Method Validation. Two levels of incurred milk at approximately the target concentration were prepared for method validation as follows. The incurred level 1 milk was prepared by mixing 40 mL of the 36 h dosed milk with 40 mL of control milk; the incurred level 2 milk was the 36 h dosed milk.

Extraction Procedure. Milk (2.0 ± 0.2 g) was weighed into a 50 mL polypropylene centrifuge tube. After the samples had been weighed, fortification of milk samples and calibration standards was performed at this step. Four calibration standards were prepared at 0.5, 1, 2, and 5 ng/g by adding 25, 50, 100, and 250 μL of the 0.04 ng/μL fortification solution to blank 50 mL centrifuge tubes. These standards were processed along with the milk samples. Milk samples for method accuracy determination were fortified at 1, 2, and 4 ng/g by adding 50, 100, and 200 μL of the 0.04 ng/μL fortification solution to 2 g of milk, respectively. To all samples and standards was then added 100 μL of the working internal standard solution (0.04 ng/g).

Five milliliters of 0.125 M HCl and 400 μL of freshly prepared 2-NBA solution (50 mM in DMSO) were added to each sample. The

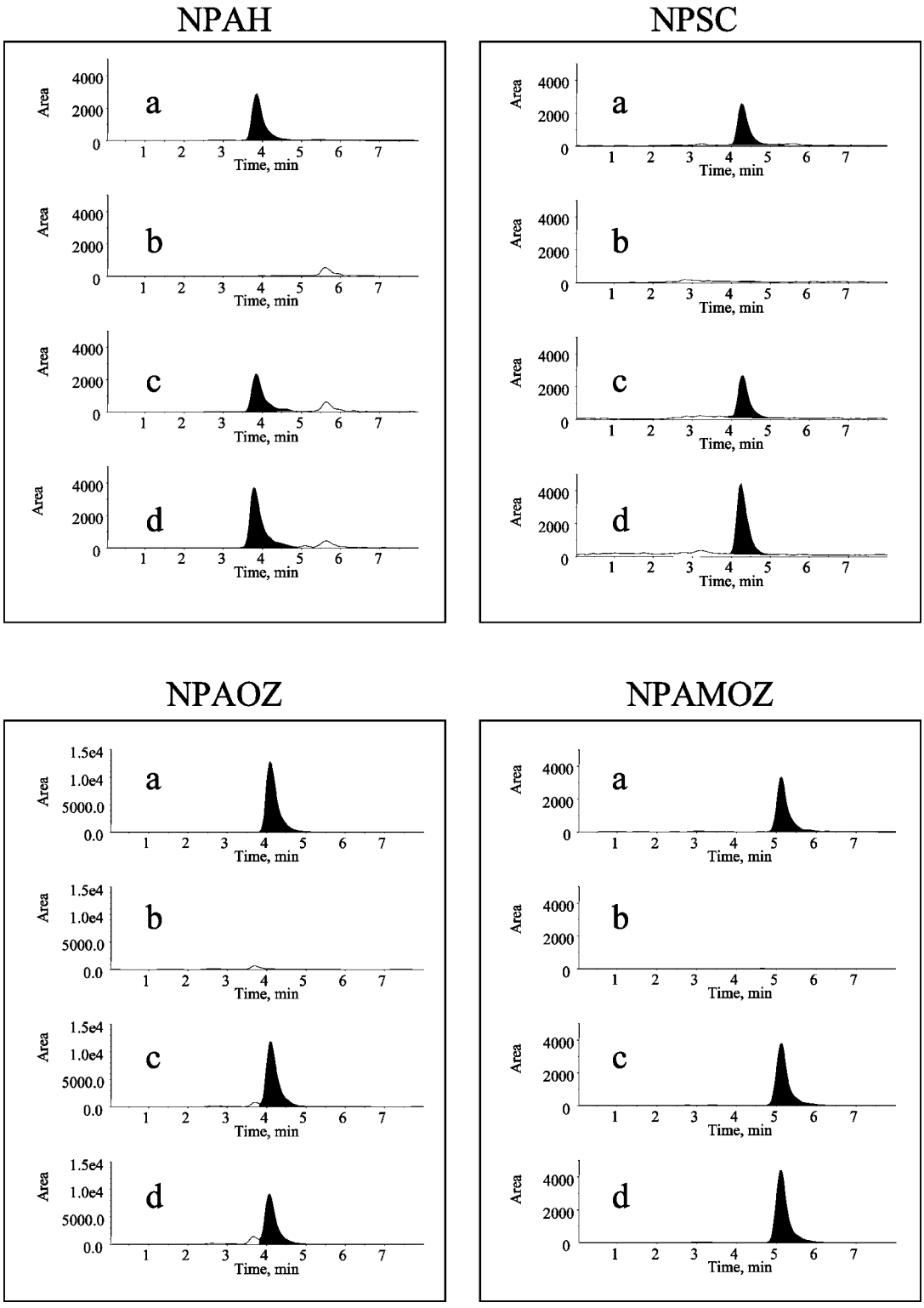

Figure 3. Reconstructed ion chromatograms of four derivatized NF residues extracted from milk: (**a**) 2 ng/g mixed standard, (**b**) control, (**c**) control fortified at 2 ng/g, and (**d**) incurred.

sample was vortex mixed for 15 s and then placed in a 37 °C water bath overnight (~14−16 h) with gentle shaking. After incubation and cooling, 3 mL of 0.1 M K_2HPO_4 was added to each sample and the pH was adjusted to 7.0 ± 0.25 by adding 0.8 M aqueous NaOH. Hexane (10 mL) was added to each sample. The tube was gently hand-mixed and then centrifuged at 4 °C for 10 min at 4000 rpm (3400 g) to effect phase separation. After centrifugation, three layers were formed as follows: a bottom solid layer, a middle aqueous layer, and a top hexane layer.

SPE Cleanup. The middle aqueous layer was pipetted into an SPE column (Waters Oasis, HLB, 30 mg, 3 cm^3) equipped with a 20 mL reservoir, which had been conditioned sequentially with 3 mL of ethyl acetate, 3 mL of MeOH, and 3 mL of water. The sample was allowed to drip at 1−2 drops/s. Vacuum was applied as needed. After the sample had been eluted, 3 mL of water was added to the reservoir. Both eluents and washes were discarded. The reservoir was then removed. The SPE column was washed with 1 mL of water. The column was vacuum-dried for 2 min. The analytes were eluted off of the column with 3 mL

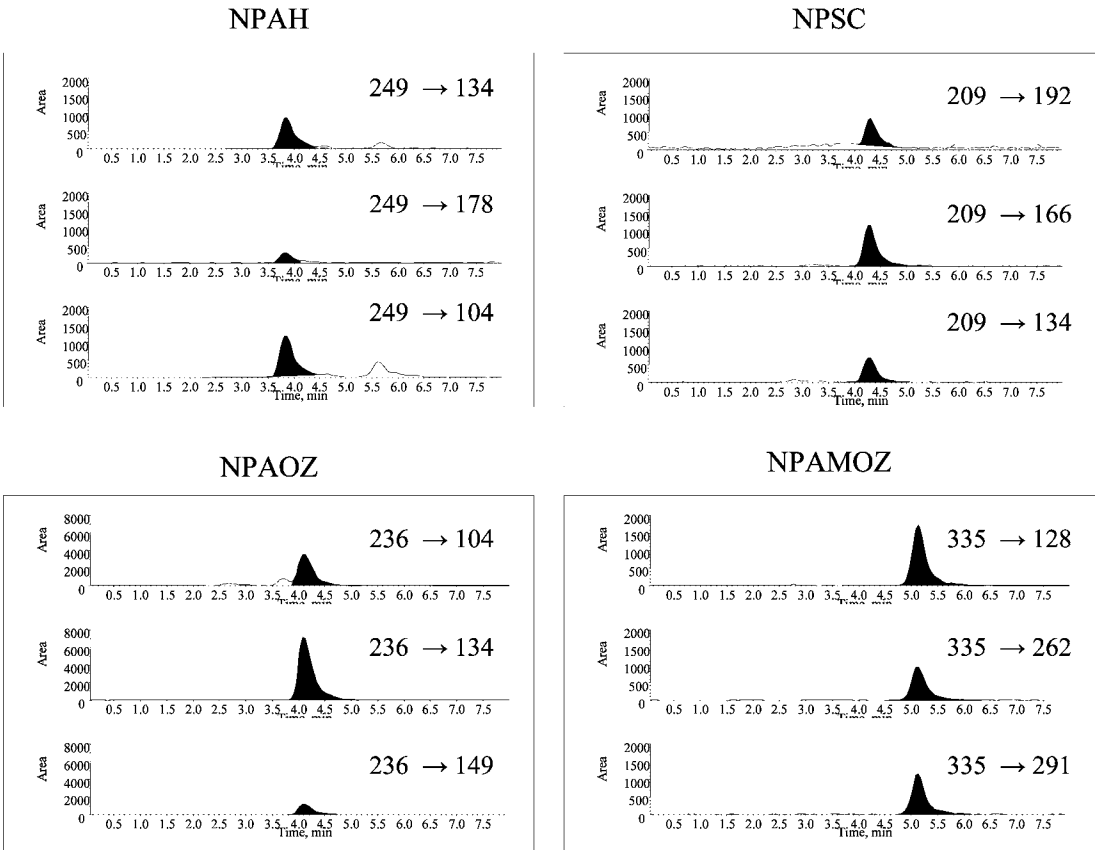

Figure 4. Reconstructed ion chromatograms of a fortified milk sample at 2 ng/g showing the individual SRM transitions.

of ethyl acetate into a 12 mm × 75 mm glass tube. The column was vacuum-dried for 2 min. To the ethyl acetate eluate was added Milli-Q water (1 mL). After vortexing and phase separation, the bottom aqueous layer was pipetted out and discarded. The remaining EtOAc layer was evaporated to dryness using a Zymark evaporator at 50 °C. The residue was dissolved in 200 μL of the HPLC mobile phase followed by vortexing, sonication for 2 min, and vortexing again. The final extract was filtered through a 0.2 μm Gelman nylon filter into an autosampler vial with a 300 μL insert.

Chromatographic Conditions. Milk extracts were analyzed for total bound and extractable residues of NFs using the following isocratic LC conditions: mobile phase, MeOH−20 mM NH_4Ac (55 + 45, v/v); and flow rate, 200 μL/min. The injection volume was 40 μL. The typical injection sequence was as follows: a solvent blank to equilibrate the LC system, a standard set, a solvent blank, a sample set, a solvent blank, and the standards again. At the end of each day's analyses, the analytical and the guard column were flushed with water−MeOH (10 + 90, v/v) for at least 30 min to remove retained materials.

Quantitation Using Internal Standard Ratio Curves. Internal standard ratio curves were constructed by plotting the ratio of each analyte area/internal standard area vs the ratio of each analyte concentration/internal standard concentration. The following internal standards were used for each analyte: AOZ-d4 for AOZ, SC+3 for SC, and AMOZ-d5 for AMOZ. Because an isotopically labeled analogue was not available for AH at the time this study was conducted, SC+3 was used as the internal standard instead. The sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y = (analyte peak area)/(IS peak area) and X = (concentration of standard in ng/g)/(concentration of IS in ng/g). Correlation coefficients for each of the standard curves were routinely >0.99. For method validation, a four-point standard curve (ranged from 0.5 to 5 ng/g) was plotted for each analyte, and curve weighting was not used. For the incursion study, which covered a wider range from 0.2 to 100 ng/g, a weighting of $1/x$ was needed to accomplish a good linear fit. When unknown or incurred samples were assayed, a negative and a fortified control at the target level were always processed along with each set of samples to assess method performance.

Quantitation Using External Standard Curve. The sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y = peak area and X = concentration of standard in ng/g. Correlation coefficients for each of the calibration curves were routinely >0.99.

Quantitation Using External Derivative Standard Curve. Calculation was performed similar to that of the external standard curves described above, except that commercially available synthetic nitrophenyl derivatives of the four side chains were used in the construction of standard curves. A four-point derivative standard curve at 0.5, 1, 2, and 5 ng/g was prepared by diluting 25, 50, 100, and 250 μL of a 0.08 ng/μL solution to 400 μL using the mobile phase. These were solvent-based standards without going through the extraction and cleanup procedures.

RESULTS AND DISCUSSION

The method described in this paper is based on the SPE approach described by Leitner et al. for the determination of NF residues in animal tissues (*17*). A hydrolysis and derivatization scheme for NFZ as adapted from Leitner et al. is shown in **Figure 2**. During hydrolysis, the side chains are released from the protein-bound residues (and from any compounds having the side chains intact). Simultaneously, the released side chains are derivatized with 2-nitrobenzaldehyde to their nitrophenyl derivatives. As discussed in Leitner's article, the derivatization serves several purposes: (i) to increase the molecular masses of the side chains, (ii) to improve the MS detection sensitivity by shifting the monitored ions away from a region of high background noise, and (iii) to increase the specificity of the fragment ions.

In the early stage of method development, we employed liquid−liquid extraction (LLE) for sample cleanup, an approach we had successfully used for shrimp (*20*). However, for milk, we had a problem with emulsion formation during liquid−liquid

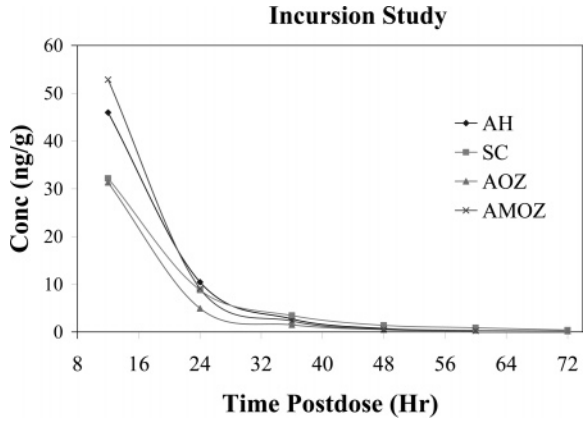

Figure 5. Levels of NF residue in milk of a dairy cow administered a single oral dose containing 0.88 mg/kg body weight each of FZD, NFZ, and FTD and 4.4 mg/kg body weight of NFT.

partitioning between the ethyl acetate and the hydrolyzed milk. Complete separation and removal of ethyl acetate from the aqueous phases were difficult. Hence, the SPE approach was used instead. At the outset, occasional clogging of SPE and slow SPE flow rates were observed in some samples. To reduce the problem, we added a hexane partitioning step to remove the fat and lipid materials prior to the SPE steps. In our experience, it was critical to hand-shake the samples and not to vortex; otherwise, an emulsion could form. With the hexane step, samples exhibited a faster SPE flow rate and no clogging. For elution, MeOH, ACN, and EtOAc are the most commonly used solvents for reversed-phase SPE columns. EtOAc was selected because the eluate could be partitioned with water without the need of first evaporating off the solvent. Such water wash provided further sample cleanup, which was much needed to minimize matrix effects during ionization in the mass spectrometer.

Initially, DMSO was used as the dissolving solvent for 2-NBA. Subsequently, it was replaced with MeOH, because it is more commonly used and readily available in most laboratories. Little difference in method performance was found between these two solvents; therefore, MeOH can be used in lieu of DMSO as the dissolving solvent.

Recent studies have shown that certain plastic gaskets used in containers can leak out SC, causing food products to be found positive for NFZ (*22*, *23*). These investigators traced the origin of SC to the use of azodicarbonamide, a blowing agent often used in the manufacture of plastic gaskets. In light of these findings, we avoided the use of such types of containers for milk sampling and handling. With such precaution, SC contamination did not present a problem to us.

The method was validated using a single source of control milk fortified with AH, AOZ, SC, and AMOZ at 1, 2, and 4 ng/g for each analyte. A fixed level of mixed internal standards (2 ng/g) was added to each sample at the beginning of the procedure. The results were calculated using three different methods: (i) external standard curves, (ii) external derivative standard curves, and (iii) internal standard ratio curves. The results are presented in **Tables 2** and **3**. With external standard curves, method accuracies ranged from 82 to 100% for NPAH, NPSC, and NPAOZ and ranged from 113 to 117% for NPAMOZ. The method accuracies for NPAMOZ were slightly higher than the acceptable value of 110% as set forth by our internal method validation guideline. Such positive bias was likely caused by matrix effects, which is a common problem for LC-MS.

Results quantitated using external derivative standard curves are shown in **Tables 2** and **3**. In a previous paper, we described our efforts of replacing the in situ derivatized side chain standards with the commercially available synthetic NPAH, NPAOZ, NPSC, and NPAMOZ derivative standards for quantitating NF residues in shrimp, so as to maximize the number of samples assayed per batch (*20*). Such effort, however, was not successful, as method accuracies were higher than 110% for AH. We postulated that the problem might be due to (i) degradation of the derivative standards when stored at 4 °C and (ii) matrix effects upon MS ionization. In this project, we repeated the experiments of using external derivative standards for quantitation on milk. Particular measures were taken to prevent degradation of derivative standards upon storage. As such, all derivative standards were stored at −20 °C instead of at 4 °C. With derivative standard curves, method accuracies were between 76 and 97% and CVs within 11% for all four analytes. These results suggest that derivative standards can be used in lieu of the in situ derivatized side chain standards when assaying milk samples.

For comparison, results were also calculated using an internal standard ratio curve. This quantitation method corrects for procedural recovery loss and compensated for mass spectrometer ionization differences due to matrix effects. As **Tables 2** and **3** show, the method accuracies are between 83 and 104% with CVs of <20% for all analytes. The use of internal standards markedly improves the AMOZ accuracies to values close to 100%. Therefore, it is the method of choice for quantitation. Typical reconstructed ion chromatograms of control and fortified control milk are shown in **Figure 3**. No major interference peaks were detected in the retention times of the nitrophenyl derivatives of AH, SC, AOZ, and AMOZ. There was a small peak eluted before NPAOZ, which was originated due to the *m*/*z* 104 ion in milk matrix. The origin of this peak is clearly shown in **Figure 4** where the individual SRM chromatograms of a fortified milk samples at 2 ng/g are displayed. The limits of detection were calculated as the concentration corresponding to a signal three times the noise level of the background and were estimated to be 0.1, 0.2, 0.2, and 0.1 ng/g for AOZ, SC, AH, and AMOZ, respectively. The lowest calibration standard (0.2 ng/g) used in this study was defined as the limit of quantitation.

The next stage of our method validation involved testing the method using milk derived from a single treated cow. A preliminary incursion study was initiated solely for the purpose of finding the right concentration range for method validation. The levels of NF residues in milk are shown in **Figure 5**. Although milk samples were collected beyond 72 h postdose, their concentrations were below 0.2 ng/g and therefore were not reported in **Figure 5**. The dose of NFT used was five times higher than the other three drugs. The rationale was based on our previous experience with NFs in other species, including shrimp (*20*) and catfish (unpublished results), in which incurred AH levels were always several times lower than the other three analytes. Consistent with our findings for shrimp and catfish, the AH levels found in incurred milk are much lower than the other three side-chain residues.

The method can also be used to confirm the identity of analytes in milk samples. Confirmation of structure is accomplished by comparing the retention times of the analytes and their ion ratios against known standards. In determining the ion ratios, the most intense ion is used as the denominator while the other two less intense ions are used as the nominator. The ion ratios used in our laboratory for confirmation are

provided in **Table 1**. A sample is confirmed positive if its retention time matches within 5% and the two ion ratios match arithmetically within 20% of those of standards. In addition, each confirmation ion must have a signal-to-noise ratio of at least 3 to 1. Using these criteria (*24*), analytes in all fortified and incurred samples were confirmed, whereas false positives were never detected in negative controls.

In this article, we describe a method suitable for determination and confirmation of NF residues in milk. The method is simple, involving only one SPE cleanup. Eight samples and four standards can easily be processed as a batch, and the entire procedure takes 2 days for completion.

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