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Analysis of Matrix-Bound Nitrofuran Residues in Worldwide-Originated Honeys by Isotope Dilution High-Performance Liquid Chromatography−Tandem Mass Spectrometry

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A sensitive and selective isotope dilution liquid chromatography−electrospray ionization tandem mass spectrometry (LC-ESIMS/MS) method is presented for the simultaneous analysis of the metabolites of four nitrofuran veterinary drugs, that is, furazolidone, furaltadone, nitrofurantoin, and nitrofurazone, in honey samples. The method entails a combined hydrolysis of protein-bound drug metabolites and derivatization of the resulting metabolites with 2-nitrobenzaldehyde (NBA) during an overnight incubation, followed by a liquid−liquid extraction and a cleanup on a polymeric solid-phase extraction cartridge. Mass spectral acquisition is carried out in the positive ion mode by applying multiple reaction monitoring (MRM) of three diagnostic transition reactions for each analyte under survey. A reliable quantification is obtained by the use of one deuterated analogue per analyte (NBA-d_4 derivative). The method has been validated in honey according to the European Union criteria for the analysis of veterinary drug residues in food. Expressed in underivatized nitrofuran metabolite concentrations, the decision limits (CCα) ranged within 0.07−0.46 μg/kg, and the detection capabilities (CCβ) were within 0.12−0.56 μg/kg. The method has been successfully applied in a survey of honeys of various geographical origins, showing that furazolidone is the main nitrofuran antibiotic administered to treat bacterial diseases of bees.

KEYWORDS: Nitrofuran metabolites; honey; mass spectrometry; 1-aminohydantoin (AH); 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ); 3-amino-2-oxazolidinone (AOZ); semicarbazide (SC); furazolidone; furaltadone; nitrofurantoin; nitrofurazone; veterinary drugs

INTRODUCTION

Nitrofurans belong to a group of broad-spectrum antibiotics used against both Gram-positive and Gram-negative bacteria. These drugs are used for the treatment of gastrointestinal and dermatological infections including salmonellosis in cattle, swine, poultry, and fish. These antibiotics are also employed to treat bees infected with bacterial diseases, and consequently residues of these compounds may be found in honey (*1*). Among the hundred listed nitrofurans, four of them, that is, furazolidone, furaltadone, nitrofurantoin, and nitrofurazone, have been banned from use in food-producing animals within the European Union (EU) [Commission Regulation 1442/95 (1995) for furazolidone and Regulation 2901/93 (1993) for other nitrofurans], Australia (1993), The Philippines (2001), Brazil (2002), Thailand (2002), and the United States (2002) because of significant concerns about their carcinogenicity and mutagenicity and those of their respective metabolites (*2*). Several studies have shown that animals rapidly metabolize nitrofurans and that their in vivo stability is not longer than a few hours (*3*, *4*). Consequently, the detection of parent drugs in animal tissues is impractical. However, it was also demonstrated that the decrease of the parent compounds in plasma levels led to a concomitant accumulation of some metabolites in proteins (*5*, *6*). Unlike the parent molecules, protein-bound metabolites are stable and persistent in the body and can be analyzed even several weeks following administration. These metabolites can be released from proteins under mildly acidic conditions (*7*) and derivatized in situ with 2-nitrobenzaldehyde (NBA). The NBA adduct can then be detected by LC-UV (*8*) and also improves selectivity if measured by mass spectrometry. So far, no study has been conducted on the metabolism in and depletion of nitrofurans from bees, and no maximum residue limits (MRLs) have been set for nitrofuran antibiotics in honey. However, a minimum required performance limit (MRPL) for each method dealing with the analysis of these drugs in poultry meat and aquaculture products has been proposed by the EU at 1 μg/kg for each nitrofuran metabolite (*9*). To increase the specificity of the detection of these metabolites, the use of tandem mass spectrometry (MS/MS) is required to obtain the lowest limit of

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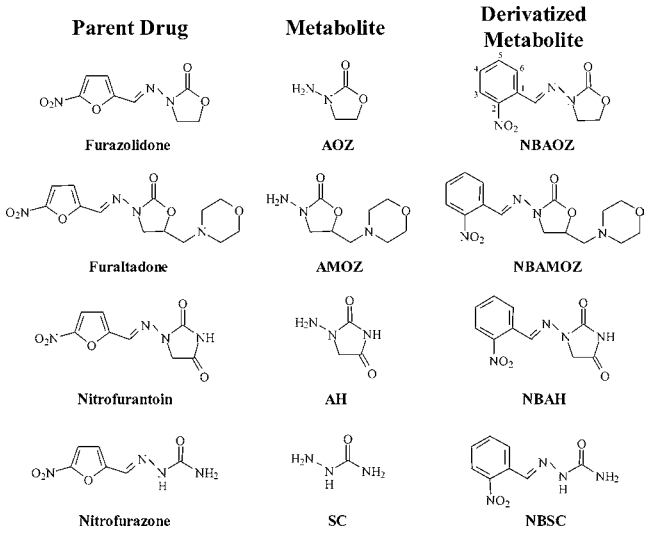

Figure 1. Chemical structures of nitrofuran parent drug compounds along with their metabolites and NBA derivatives. The deuterium labeling of the corresponding internal standards were located in positions 3, 4, 5, and 6.

detection with a high degree of confidence regarding the analyte identity. Recent LC-MS/MS methods have been reported to determine the nitrofuran metabolites 1-aminohydantoin (AH), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ), and semicarbazide (SC) in foods of animal origin, using the acid-mediated release of protein-bound metabolite/derivatization procedure. These methods employ either an analogue compound (4-nitrobenzaldehyde derivative of SC) as internal standard (IS) to improve the quantitative aspect of the method (*10*), no IS (*11*), or two ISs (*12*). Signal suppression is a commonly observed effect in electrospray ionization (*13*), and thus the best approach for a reliable quantification is clearly the use of a stable isotopically labeled IS for each analyte under study.

In the present work, we describe a confirmatory and quantitative LC-ESIMS/MS method for the determination of four metabolites of nitrofuran antibiotics (**Figure 1**) in honey. To our knowledge, no such mass spectrometry based method has been previously reported. In-house synthesized NBA-d_4 derivatives of these drug residues were employed as ISs, and the method was validated following the EU criteria for the analysis of veterinary drug residues in food (*14*). Ionization, confirmation criteria, calibration considerations, honey matrix effects, and performance data of the method are discussed. The study also focuses on the sensitivity of the method reflected by the calculated CCα and CCβ values. Finally, the applicability of this analytical method is demonstrated in a survey of honeys of different geographical origins.

MATERIALS AND METHODS

Chemicals and Reagents. AOZ, SC (hydrochloride form), NBA, and AH (hydrochloride form) were supplied by Aldrich (Buchs, Switzerland). AMOZ and NBA derivatives of nitrofuran metabolites (i.e., NBAH, NBAMOZ, NBAOZ, and NBSC) were obtained from Witega (Berlin, Germany). NBA-*3,4,5,6*-d_4 (chemical purity > 99%, isotopic purity > 99%) was supplied by Toronto Research Chemicals Inc. (North York, ON, Canada). Acetonitrile (ultra gradient HPLC grade) was purchased from J. T. Baker (Deventer, The Netherlands) and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Steinheim, Switzerland). All other solvents and reagents, as well as Lichrolut EN (200 mg, 3 mL) solid-phase extraction (SPE) cartridges were purchased from Merck (Darmstadt, Germany). Deionized and distilled water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA). Individual NBA-d_4 derivatives of nitrofuran metabolites were obtained by condensation of NBA-*3,4,5,6*-d_4 with an excess of AOZ, AMOZ, AH, and SC, respectively, and were further purified by preparative HPLC according to a procedure described elsewhere (*15*).

Standard Solutions. Individual stock standard solutions of NBAH, NBAMOZ and NBAOZ (2 mg/mL) were prepared by dissolution in methanol and that of NBSC (0.2 mg/mL) in acetonitrile/distilled water (50:50 v/v). The corresponding deuterated NBX-d_4 solutions were prepared in a similar way. Stock standard solutions were stored at −20 °C. Individual concentrations of derivatized nitrofuran metabolite solutions were regularly checked by UV spectrophotometry using their molar extinction coefficients [($L \cdot mol^{-1} \cdot cm^{-1}$) NBAH (264 nm), 18396; NBAMOZ (256 nm), 17723; NBAOZ (259 nm), 16305; and NBSC (263 nm), 15477] after adequate dilution in water to reach a concentration of ∼10 μg/mL (*15*). Multicomponent solutions, either unlabeled or labeled, were prepared in water and aliquots stored at − 20 °C.

Honey Samples. Honeys of different flowers and geographical origins were collected from various honey suppliers or purchased from retail outlets in Switzerland. Samples were collected during the 2002/2003 period and were stored under dry conditions in the dark at 4 °C. For method validation, honey obtained under strictly controlled conditions ("bio" label) was used.

Sample Preparation. An aliquot of honey (2 g) was homogenized and weighed into a 50-mL Falcon polypropylene tube (Becton Dickinson, Le Pont de Claix, France). A 25-mL portion of 0.125 M hydrochloric acid and the NBA derivatization agent (250 μL of a 50 mM solution in DMSO) were added, and the mixture was vigorously shaken. The slurry was then incubated for 16 h in a water bath at 37 °C. After cooling at room temperature, the mixture was neutralized by first adding 2.5 mL of sodium hydroxide (1 M) and 1 mL of hydrogen dipotassium phosphate (1 M), and the final pH was adjusted at 7.0 ± 0.2 with a few drops of NaOH (1 M). An aliquot of 500 μL containing the four d_4-derivatized nitrofuran metabolites (50 ng/mL stock solution, each) was added, the mixture was well mixed and allowed to stand for 15 min. Then, 15 mL of ethyl acetate was added to the slurry before being thoroughly hand-shaken for 2 min and centrifuged at 3600*g* for 10 min (centrifuge Mistral 2000, MSE Scientific Instrument, Leicestershire, U.K.). The organic phase was collected into a 15-mL Falcon tube and evaporated to dryness under a stream of nitrogen at 40 °C; the dry residue was reconstituted with distilled water (3 mL). A Lichrolut EN SPE cartridge was conditioned successively with 9 mL of ethyl acetate, 3 mL of methanol, and 9 mL of distilled water. The reconstituted water extract was loaded onto the cartridge and, after penetration, was successively washed with distilled water (3 mL) and hexane (3 mL) and dried by sucking air through after each solvent addition (Visiprep vacuum manifold, Supelco, Buchs, Switzerland). The derivatized nitrofuran metabolites were finally eluted with 9 mL of ethyl acetate and collected in a 15-mL Falcon tube. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C and subsequently reconstituted with 250 μL of acetonitrile/distilled water (30:70 v/v). The resulting solution was filtered through a 0.2-μm nylon filter (Nalgene, Rochester, NY) directly into an HPLC vial.

LC-ESIMS/MS. Analyses were performed on a Perkin-Elmer 200 Micro Pump series system (Perkin-Elmer, Uberlingen, Germany) coupled to an Applied Biosystem API 3000 triple-stage quadrupole mass spectrometer equipped with a TurboIonSpray ionization source (Applied Biosystem, Foster City, CA). The HPLC column was a SymmetryShield C_{18} reversed-phase (15 cm × 2.1 mm, 3.5 μm) connected to a SymmetryShield RP_{18} precolumn (1 cm × 2.1 mm, 3.5 μm) (Waters, Milford, MA). The mobile phase was constituted by solvent A [water containing concentrated acetic acid 0.025% (v/v) (pH ∼3.6)] and solvent B (acetonitrile). A linear gradient program was realized with 0−0.5 min 10% B, 0.5−3 min 35% B, then hold at 35% B for 5 min, 8−9 min 100% B, and hold at 100% B for 3 min before coming back to 10% B in 1 min (the HPLC column was reconditioned at 10% B for a 9-min additional time). The flow rate was 0.3 mL/min, and 50 μL of the extract was injected onto the column. The HPLC flow was directed into the MS detector between 0.5 and 9 min using a VICI diverter (Valco Instrument Co. Inc., Houston, TX). Analytes were detected using electrospray ionization in the positive mode. Nitrogen was used both as TurboIonSpray and curtain gas at flow rates

Table 1. Transition Reactions and Their Corresponding Collision Energies Monitored by LC-ESIMS/MS in Positive Mode

analyte	transition reactions (*m*/*z*) (used for quantification)	collision energy (eV)	transition reactions (*m*/*z*) (used for confirmation)	collision energy (eV)
NBAH	249 → 134	19	249 → 104	29
			249 → 178	20
NBAH-d_4	253 → 138	19	253 → 108	29
NBAMOZ	335 → 291	18	335 → 128	30
			335 → 262	24
NBAMOZ-d_4	339 → 295	18	339 → 266	24
NBAOZ	236 → 134	18	236 → 101	18
			236 → 149	20
NBAOZ-d_4	240 → 138	18	240 → 153	20
NBSC	209 → 166	15	209 → 134	17
			209 → 192	17
NBSC-d_4	213 → 170	15	213 → 196	17

of 7.5 L/min and 10 mL/min, respectively. The nebulizer and collision gas (both nitrogen) was used under a pressure of 776 Torr (15 psi) and 5 mTorr, respectively. The block source temperature was maintained at 350 °C, and the electrospray capillary voltage was set at 5.5 kV. The quantitative analysis was performed using tandem MS in MRM alternating three transition reactions for each nitrofuran derivatized metabolite and two transition reactions for their corresponding IS with a constant dwell time of 25 ms (**Table 1**). Data processing was done using Analyst software (version 1.1).

Calibration. The derivatized nitrofuran metabolites were quantified with six concentration calibrants (external calibration curves in water) ranging from 0 to 4 ng (5 ng of each deuterated IS) injected on-column, thus covering the 0−10 μg/kg range. The area ratio of the analyte versus that of the deuterated IS was plotted against the analyte/IS concentration ratio. The linearity of the MS response was checked by calculating the standard deviation of the average of response factors (RFs, ratio of the peak area of the analyte and that of its IS divided by the corresponding ratio of the concentration of the analyte and that of its IS), which should be below 15% (*16*). Calibration curves were also constructed in various honeys to check whether any potential matrix effects could be identified. All final results were expressed as the free nitrofuran metabolite, that is, AH, AMOZ, AOZ, and SC, by multiplying the concentrations of derivatized nitrofuran metabolites obtained from the calibration curves by a factor of 0.464, 0.602, 0.434, and 0.361, respectively (obtained from the molecular weight ratio).

Confirmation Criteria. Nitrofuran metabolites were considered as positively identified in honey when the following criteria were met: (a) the ratio of the chromatographic retention time of the analyte to that of the corresponding IS, that is, the relative retention time of the analyte, corresponded to that of the calibration solution within a ±2.5% tolerance; (b) the presence of a signal was determined at each of the three diagnostic transition reactions for the endogenous metabolite and at each of the two transition reactions for the corresponding IS; (c) the peak area ratio from the different transition reactions recorded was within the tolerances as fixed by the EU criteria (*14*).

RESULTS AND DISCUSSION

Sample Preparation and IS Supplementation. The sample treatment procedure was essentially adapted from that described by Leitner et al. (*10*) with the following modifications: (a) a liquid−liquid extraction step using ethyl acetate was performed before the SPE cleanup, as omitting this step led to a poor recovery of the analytes under survey (i.e., the introduction of the liquid extraction step increased the overall recoveries by 100, 25, 70, and 5%, for NBAH, NBAMOZ, NBAOZ, and NBSC, respectively); (b) a further washing step of the SPE cartridge with hexane eliminated excess water trapped by the polymer-based phase of the cartridge before the elution of the compounds; and (c) one deuterated analogue per analyte was used as IS to improve the quantification. Our preliminary studies revealed a degradation of the underivatized metabolites spiked in a blank honey during the hydrochloride acid-mediated hydrolysis. Leitner et al. reported similar observations with losses of metabolites of ∼26−34% during the acidic treatment of animal muscle tissues (*10*). We observed that these losses were even more important when the addition of the derivatization agent was delayed (e.g., from 1 to 4 h) after the underivatized metabolites' spiking (data not shown). This suggests that the supplementation of underivatized isotope-labeled compound prior to the acidic treatment could lead to an overestimation of the level of matrix-bound metabolite, as a different behavior of such an IS compared to that of the endogenous analyte may be expected. Consequently, our ISs were NBA-d_4-derivatized metabolites, and their posthydrolysis addition allowed an accurate and precise quantification of the released nitrofuran metabolites in the extract. This posthydrolysis addition also avoided a possible clip-off of IS molecules, which would have led to false positive results (considering that the deuterium species are located on the NBA part of the compound). Initially, we used ^{13}C-based ISs (*15*), but due to the cost of the starting material ([$^{13}C_6$]toluene) and the considerable efforts to produce sufficient quantities of these isotopically labeled materials, we chose deuterated nitrofuran metabolites synthesized from commercially available NBA-d_4.

The extraction procedure described here provides absolute analyte yields of ca. 30% for NBAH, 70% for NBAMOZ and NBAOZ, and 40% for NBSC. These yields were calculated from the ratio of the peak area of the internal standard in the sample to the mean peak area of the internal standard of the corresponding standard calibration and thus included both the loss of analyte during the extraction/purification steps and potential matrix effects during ionization.

LC-ESIMS/MS. In positive ionization mode, protonated molecules $(M + H)^+$ were obtained for NBAH, NBAMOZ, NBAOZ, and NBSC at *m*/*z* 249, 335, 236, and 209, respectively. Collision-induced dissociation (CID) mass spectra were then recorded for each analyte and the corresponding IS at various collision energies before the optimal MS/MS transition reactions and electronic parameters were selected (**Figure 2**). The CID fragmentation pathways of the derivatized nitrofuran metabolites have been described previously by Delatour et al. (*15*) and will not be elaborated further. The MS/MS experiments revealed a common characteristic fragment ion observed at *m*/*z* 134, which corresponds to the NBA part of the molecule. Considering that hundreds of nitrofurans have been synthesized, drugs other than the ones described here may be used to treat animals and consequently found as residues in foods. Similarly, they will be released during the acidic treatment and derivatized with NBA. Thus, a potential uses of mass spectrometry to identify the presence of other nitrofuran-related chemicals would be as follows: (a) to perform a first survey experiment in precursor ion mode by fixing *m*/*z* 134 in the third quadrupole and scanning the first quadrupole to identify potential protonated molecules; (b) once a predefined threshold is reached, a second MS/MS experiment would be realized in CID mode by automatically selecting the protonated molecule in the first quadrupole and scanning the third one at several collision energies; (c) from the fragmentation pathway obtained at these different collision energies, unknown nitrofuran molecule could be identified.

The four analytes were well separated and eluted successively at retention times of 3.4, 5.3, 5.6, and 6.5 min for NBAMOZ, NBSC, NBAH, and NBAOZ, respectively. The total run time per sample was 22 min, enabling >30 samples to be analyzed

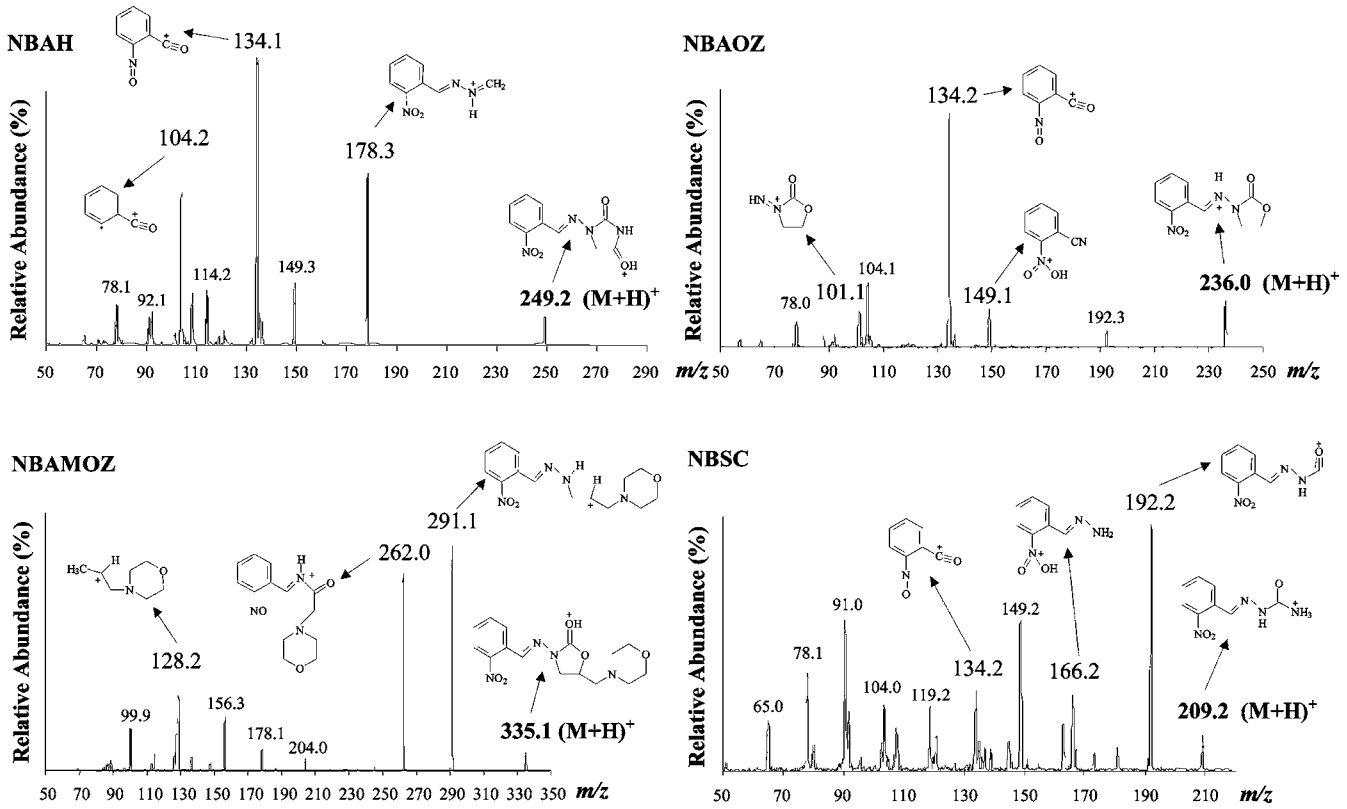

Figure 2. CID mass spectra of a standard solution of the four nitrofuran derivatized metabolites obtained at a collision energy of 25 eV under positive electrospray ionization.

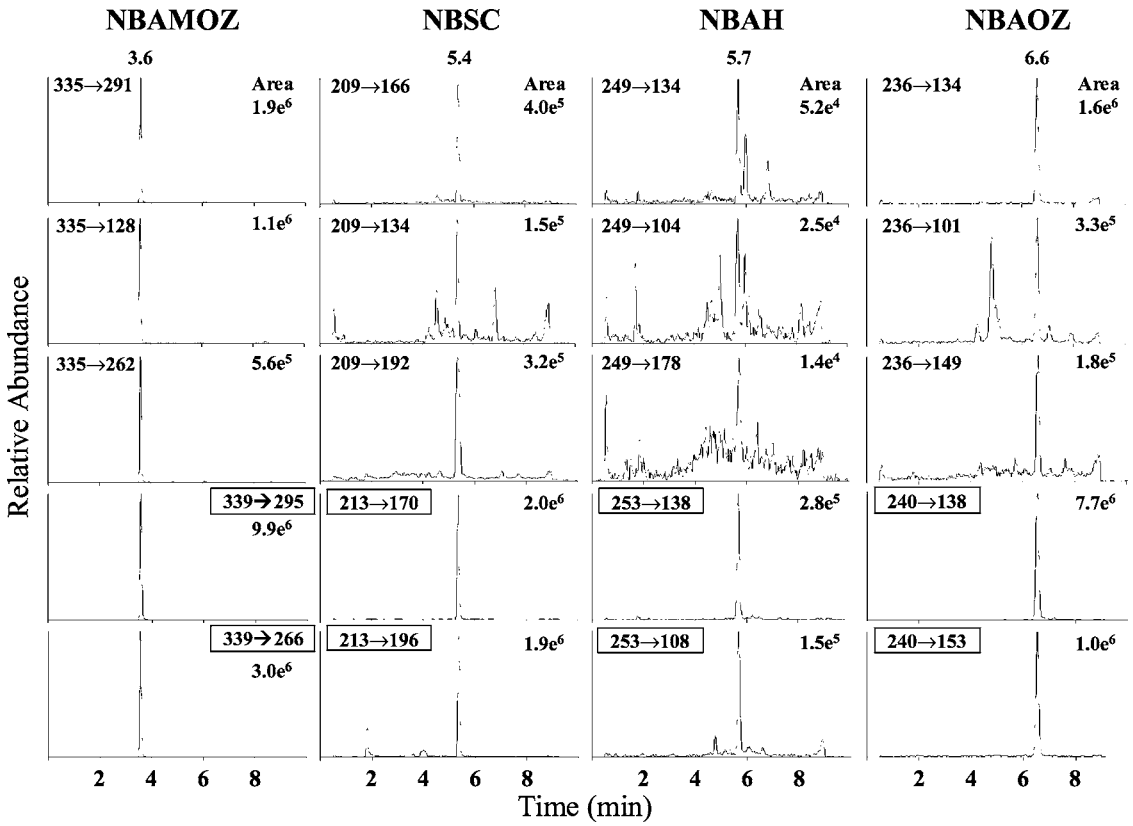

Figure 3. MRM chromatograms obtained by LC-ESIMS/MS in positive mode of a fortified honey extract at a 2 μg/kg level of each derivatized nitrofuran metabolite (corresponding to 1.20, 0.72, 0.93, and 0.87 μg/kg of AMOZ, SC, AH, and AOZ, respectively). The different transition reactions of the internal standards are boxed.

in an overnight sequence**.** No interferences at the expected retention times were noticed in blank matrices. **Figure 3** shows chromatograms of a spiked honey extract (at the 2 μg/kg level of derivatized nitrofuran metabolites). Addition of ammonium

Table 2. Water and Matrix-Matched Calibration Curves (Established in Different Honey Extracts)

	NBAH		NBAMOZ		NBAOZ		NBSC	
matrix	slope	r^2 [a]	slope	r^2	slope	r^2	slope	r^2
water	1.068	0.999	1.022	0.989	1.186	0.998	1.187	0.998
sunflower, Switzerland	1.215	0.976	1.131	0.999	1.131	0.999	1.151	0.996
acacia, Switzerland	1.041	0.992	1.092	0.996	1.109	1.000	1.243	0.976
multiflower, Switzerland	1.075	0.997	1.114	0.997	1.249	0.999	1.340	0.997
multiflower, Argentina	1.300	0.999	0.932	0.995	1.143	1.000	1.285	0.999
multiflower, Spain	1.180	0.987	0.992	0.999	1.030	0.998	1.334	0.998

[a] r^2 = coefficient of determination.

acetate to the mobile phase resulted in a decrease in signal intensities, contrary to previous observations (*10*), whereas the addition of acetic acid in the mobile phase proved to be worthwhile and resulted in both signal enhancement and a better repeatability of the retention times.

Matrix Effects. In food analysis by mass spectrometry, signal suppressions may occur during the ionization process of the extracts due to the composition (salts, proteins, lipids, carbohydrates, ..., etc.) of the matrix under survey. This matrix effect can be minimized by an efficient cleanup of the extract, but usually cannot be totally avoided. Verzegnassi et al. highlighted a strong matrix effect when analyzing 10 sulfonamide-related antimicrobial agents in honey by LC-ESIMS/MS, with slopes of matrix-matched calibration curves differing not only from a solvent-based curve but also among honey extracts of different origin. Only one isotopically labeled IS was used during this study (*17*). In this context, we checked the matrix effects of different honeys on the analysis of nitrofuran metabolites by constructing matrix-matched calibration curves and comparing their slope with those of calibration curves constructed in water. **Table 2** summarizes these data and shows that the responses were linear over the range of concentrations considered (i.e., from 0 to 10 μg/kg NBX), with small differences between the slope values whatever the type of honey used. These results also show the importance of using isotopically labeled compound as IS to efficiently compensate the matrix effect. Consequently, only water-based calibration curves were considered during this validation.

Method Performance Characteristics. According to the EU criteria for the analysis of veterinary drug residues in live animals and animal products (*14*), a system of identification points (IPs) is used to define the number of ions and their corresponding ratios that should be measured when using confirmatory MS techniques. For the analysis of nitrofurans, which are listed in Annex IV (compounds with no MRL) of Council Regulation 2377/90, a minimum of 4 IPs is required. In our case, the consideration of three transition reactions for each analyte fulfills this requirement with a total of 5.5 IPs. Validation was conducted following the EU criteria: for performance data under repeatability conditions, six blank honeys were spiked at each of the three fortification levels, that is, 1.0, 1.5, and 2.0 μg/kg of derivatized nitrofuran metabolites after the hydrolysis step, and then analyzed by the same operator on three separate occasions. The same protocol was applied for performance data under intermediate reproducibility conditions but analyses (i.e., six blank honeys spiked at each of the three fortification levels) were conducted by two analysts on one occasion over a 1-month period. **Table 3** summarizes these data (after conversion of values into nitrofuran metabolites concentrations). Within- and between-day, as well as within-laboratory, precisions were below 18%, whereas recoveries ranged between 82 and 112%. Precisions at fortification levels close to the MRPL set by the EU (i.e., 1 μg/kg) were even better and below 12%. A potential drawback of our validation scheme is that it allows only the liquid−liquid extraction and the SPE cleanup recoveries to be verified and not the extraction recovery of the bound metabolites from the matrix. However, due to the losses observed when spiking underivatized nitrofuran metabolites in blank honey and the nonavailability of isotope-enriched metabolites bound to matrix components to be employed as ISs, the posthydrolysis addition of labeled derivatized nitrofuran metabolites remains the best approach to provide an accurate and precise quantification of the endogenous analytes effectively released in the extract.

Determination of Decision Limits (CCα) and Detection Capabilities (CCβ). The EU criteria propose two options for the calculation of CCα: either by the calibration curve procedure (plot of the experimental versus the nominal spiked concentration values obtained by two operators, determination of the calibration line *y*-intercept, the corresponding concentration at the *y*-intercept plus 2.33 times the standard error of the intercept is CCα) or by analyzing 20 blank materials and calculating 3 times the signal-to-noise (S/N) ratio at the retention time at which the analyte is expected. Both ways of calculating these limits present drawbacks; for example, the calibration curve procedure gives a decision limit, which is extrapolated from the intercept of the calibration curve and not experimentally found, whereas the S/N ratio method does not take into consideration that MS signals are time-related due to either instrument instability or instrument cleanliness. Nevertheless, our determination of CCα limits was based on the calibration curve procedure as values obtained by this method are more representative than those obtained from the S/N ratio method (e.g., two operators involved and analyses performed over a long interval of time). Calculated CCα values, expressed in underivatized nitrofuran metabolites (**Table 4**), were found to be very low and far from the lowest fortification level considered during the validation of the method (i.e., 0.46, 0.60, 0.43, and 0.36 μg/kg for AH, AMOZ, AOZ, and SC, respectively). Consequently, further spiking experiments were performed at these calculated limits. Results showed that only the CCα value for AMOZ was valid, whereas that of AOZ was found to be 3 times higher than the calculated one (at a S/N of 3). For AH and SC, a worse case scenario based on the considerations by Jülicher et al. (*18*) was followed, with CCα similar to the lowest fortification level used during the validation of the method (i.e., 0.46 and 0.36 μg/kg for AH and SC, respectively). CCβ values were derived from the CCα limits and were calculated as CCα + 1.64 × within-laboratory precision at the lowest fortification level, as described by Polzer et al. (*19*).

Analysis of Incurred Honeys. The method was applied to >120 honeys of various geographical origins, mainly from South and Central America and from the Pacific Zone, but also from Europe, Africa, and the Middle East. It is worth noting that some of these samples were blended honeys, and therefore their precise origin is tentative. AH was not detected in these samples,

Table 3. Performance Data of the LC-ESIMS/MS Method for the Analysis of the Four Derivatized Nitrofuran Metabolites in Honey (Values Are Expressed in Micrograms per Kilogram of Nitrofuran Metabolites)

	AH, at fortification level of			AMOZ, at fortification level of			AOZ, at fortification level of			SC, at fortification level of		
	0.46 μg/kg	0.70 μg/kg	0.93 μg/kg	0.60 μg/kg	0.90 μg/kg	1.20 μg/kg	0.43 μg/kg	0.65 μg/kg	0.87 μg/kg	0.36 μg/kg	0.54 μg/kg	0.72 μg/kg
under repeatability conditions[a]												
overall mean ± SD (n = 18)	0.45 ± 0.07	0.65 ± 0.08	0.89 ± 0.09	0.50 ± 0.04	0.84 ± 0.05	1.14 ± 0.09	0.38 ± 0.02	0.62 ± 0.02	0.84 ± 0.03	0.37 ± 0.03	0.58 ± 0.06	0.81 ± 0.06
overall recovery ± SD (%)	97 ± 16	94 ± 12	96 ± 9	83 ± 7	93 ± 6	94 ± 8	88 ± 4	95 ± 3	97 ± 3	102 ± 9	108 ± 12	112 ± 9
within-day precision (%)	18	13	9	7	6	8	4	3	3	7	7	7
between-day precision (%)	16	12	10	9	7	8	4	3	3	10	12	9
under intermediate reproducibility conditions[b]												
overall mean ± SD (n = 12)	0.46 ± 0.06	0.66 ± 0.06	0.98 ± 0.11	0.49 ± 0.04	0.80 ± 0.06	1.08 ± 0.05	0.38 ± 0.02	0.59 ± 0.03	0.82 ± 0.02	0.36 ± 0.04	0.54 ± 0.03	0.73 ± 0.06
overall recovery ± SD (%)	100 ± 12	95 ± 9	106 ± 11	82 ± 6	88 ± 6	90 ± 4	86 ± 5	91 ± 5	95 ± 3	98 ± 11	99 ± 7	101 ± 8
within-laboratory precision (%)	12	11	11	9	8	4	7	6	3	11	7	9

[a] Six negative honeys spiked at each of the three fortification levels and analyzed on three separate occasions by the same operator using the same equipment over a 2-week period. [b] Six negative honeys spiked at each of the three fortification levels and analyzed by two operators using the same equipment over a 1-month period.

Table 4. Decision Limit (CCα) and Detection Capability (CCβ) Values for the Four Nitrofuran Metabolites

metabolite	CCα[a] (μg/kg)	CCα[b] (μg/kg)	CCβ (μg/kg)
AH	0.11	0.46	0.56[d]
AMOZ	0.07	0.07	0.12[c]
AOZ	0.04	0.12	0.18[d]
SC	0.04	0.36	0.43[d]

[a] From the calibration curve procedure according to the EU criteria, CCα = corresponding concentration at the y-intercept + 2.33 × standard error of the intercept. [b] Final corrected CCα (found experimentally). [c] From the calibration curve procedure according to the EU criteria, CCβ = corresponding concentration at the y-intercept of CCα + 1.64 × standard error of the intercept. [d] CCβ calculated as CCα + 1.64 × standard deviation of the within-laboratory reproducibility of the lowest fortification level (*19*).

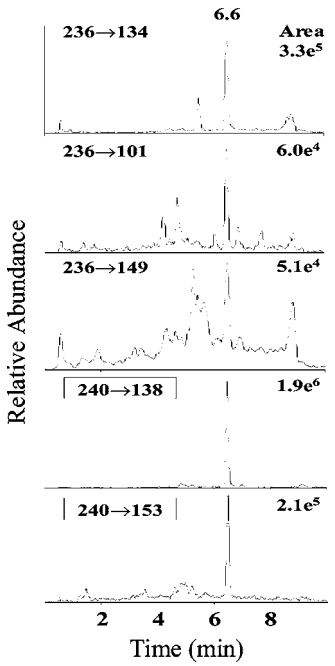

Figure 4. MRM chromatograms of an incurred honey containing 0.6 μg/kg AOZ. The different transition reactions of the internal standard are boxed.

thus confirming previous findings that the parent drug nitrofurantoin is less frequently used in veterinary medicine (*11*). Similarly, AMOZ was not detected in any of the samples analyzed, although this metabolite is frequently found in meat-based products (data not shown). AOZ (metabolite of furazolidone) and SC (metabolite of nitrofurazone) were detected in 14% (maximum content = 5.1 μg/kg) and 21% (maximum content = 24.5 μg/kg) of analyzed honeys, respectively, without any obvious geographical preference. **Figure 4** shows the chromatogram of an incurred honey contaminated with AOZ (at the 0.6 μg/kg level). However, SC in food products may originate from "non antibiotic" sources. Indeed, we recently demonstrated that SC is a thermal decomposition product of azodicarbonamide (ADC), used as a blowing agent in the gasket of lids of certain food jars (*20*). Thus, honeys packaged and stored in such containers may be potentially contaminated with SC. Furthermore, SC has also been found in foods after treatment with sodium hypochlorite, related to certain food-processing methods used for disinfection or bleaching (*21*). These recent findings indicate that SC is not an established marker for nitrofuran administration in animal food production.

Conclusion. We describe a quantitative LC-ESIMS/MS method for the determination of four metabolites of nitrofuran

veterinary drugs in honey. Tandem mass spectrometry using MRM transitions together with the use of one isotopically labeled IS for each analyte under survey enables a selective and confirmatory detection at the sub parts-per-billion level, below the MRPL set at 1 μg/kg. The calculation of CCα and CCβ following the EU criteria for the analysis of veterinary drug residues in food highlighted some limitations in regard to the experimental feasibility of these limits. As already mentioned (*19*), an improved standardized procedure to calculate these values is highly desirable. Our limited survey on different honeys shows that furazolidone is the main nitrofuran antibiotic used to treat bacterial diseases of bees, whereas the presence of SC in honey could have origins other than the illegal use of nitrofurazone.

SAFETY

NBA is a possible mutagen. Avoid inhalation and use only in a chemical fume hood.

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