Validation and Application of an Analysis Method of Four Metabolites of Nitrofurans in Honey

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Although nitrofurans are supposed to be absent in foods, they are still used in veterinary medicine for the treatment of infections in animals not bred for consumption. That meant that there are still samples of honey contaminated with residues of nitrofurans because of bees treated with those pharmaceutical substances. Developing accessible methods to detect them is of high interest to food residue monitoring and regulation programs. We propose an immunochemical method as an alternative to detect four toxic metabolites of nitrofurans (1-aminoimidazolidine-2,4-dione, 3-amino-5-morpholinomethyl-2-oxazolidinone, 3-amino-2-oxazolidone and semicarbazide) in honey. The new method has been optimized and validate for the simultaneous determination of the four metabolites of the nitrofurans honey using biochip technology, and it has been used for the quantitative determination of the residues in 16 Romanian honey samples. The evaluated validation parameters included: linearity, sensitivity (IC50≤2.32 μg/kg), specificity and selectivity, precision (intermediate and reproducibility) and accuracy, decision limit (CCα between 0.37 and 1.05 μg/kg), detection capability (CCβ between 0.42 and 1.14 μg/kg), and recovery coefficient (64–192%).

Keywords: nitrofurans, honey, biochip.

Nitrofurans have been used in veterinary practice as antibacterial agents to treat infections caused by bacteria and protozoa. Although their use was banned for the first time in the EU since January 1, 1997 (Annex IV of Regulation 2377/90/EC), they are currently in use for animals not bred for consumption [1].

Nitrofurantoin, furazolidone, nitrofurazone and furaltadone are the most commonly used nitrofurans. Studies have shown that they are rapidly converted to toxic metabolites [2], which bind proteins in high proportions and thus persist for long periods (weeks or even months) in food products. Those metabolites are 1-aminoimidazolidine-2,4-dione (AHD), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidone (AOZ) and semicarbazide (SEM) [3-5].

Analysis of nitrofurans residues is usually done using liquid chromatography tandem mass spectrometry (LC-MS/MS) [1, 6, 7]. Developing accessible methods to detect them is of high interest to food residue monitoring and regulation programs [8-12].

Although honey is considered a very healthy natural product, the incidence of honey samples contaminated with residues of nitrofurans is quite high [13].

We propose an immunochemical method as an alternative to detect residues of nitrofurans in honey. The new method has been validate for the simultaneous determination of four metabolites of the nitrofurans most commonly occurring in honey using biochip technology, and it has been used for the quantitative determination of the residues in honey samples of different origins.

Experimental part

Material and method

All reagents were supplied in a compact kit that included: Antimicrobial Array III - kit (code EV3695, Randox Laboratories, UK) and Antimicrobial Array III Control -kit (code AMC5036, Randox Laboratories, UK).

Required accessories not included in the reagent kit were:

-supplies: pipettes and pipette cones, wash bottle, biopsy bags (EV3664 code), microtubes with sampling and sample lids, sample flasks (50 mL capacity);

-equipment: analytical balance, roller, thermo-agitator;

-reagents: 1M HCl solution, dimethylsulfoxide, Lab-scan (code H34C11X), 10mM 4-nitro-benzaldehyde solution, 0.1M $K_2HPO_4 \times 3H_2O$ solution, Sigma (code P-5504), ethyl acetate, BDH (code 101086J), and bidistilled water.

The validation of the simultaneous quantitative determination of 4 metabolites of nitrofurans in honey using biochip technology followed a protocol that meets the requirements of 2002/657/EC Decision and it may be used in any laboratory that can handle a large number of test samples.

The validation method and the honey analysis procedure were carried out in accordance with the FDA approved validation guidelines, the drug residue validation guides, the guidelines for the implementation of Commission Decision 2002/657/EC and the validation guidelines for screening methods for residues of veterinary drugs. Validation parameters included: linearity, sensitivity (IC50), specificity and selectivity, precision (intermediate and reproducibility), accuracy, detection limit and recovery [14, 15].

The linearity of the method was evaluated by performing a 9-point calibration for each of the 4 nitrofurans using the calibrators included in the Anti Microbial Array III kit. Considering the complex honey composition as a sample matrix, the linearity of the method was also checked by enriching blank honey samples in order to obtain 9 concentration levels 0, 0.001, 0.01, 0.05, 0.1, 1, 4, 10, 50 μg/kg for AHD, AOZ and AMOZ. For SEM, concentrations of 0, 0.001, 0.01, 0.05, 2, 20, 40, 100 and 500 μg/kg were used [16, 17].

The analyzer software used for calibration a specific equation for the competitive immune-enzymatic detection method [4]: $y = D + [(A-D)/1+(x/C)^B]$ where: x = analyte concentration (μg/kg), y = the intensity of the chemi-luminescent signal expressed as relative light units (RLU), A, B, C, D = parameters of the competitive method,

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predefined in the analyzer software as A = the intensity of the chemiluminescent signal when the concentration of the analyte is 0, B = slope factor, C = the inflection point of the calibration curve and D = the intensity of the luminous response signal at an infinite theoretical concentration of the analyte [18, 19].

An initial estimate was made for each parameter, then it was optimized by minimizing the sum of the squares of the residuals using the Microsoft Excel Solver.

For the calibration curves, the following steps were taken:

- extracting the support with 9 biochips from the protective packaging;
- adding 50 µL of each calibrator to the surface of each biochip followed by the addition of 150 µL of reaction buffer (AM III DIL ASY);
- incubating the biochips at 25°C while stirring at 370 rpm for 30 min;
- adding 100 µL of enzyme conjugate solution to each biochip;
- incubating the biochips at 25°C while stirring at 370 rpm for 30 min;
- removing the reaction mixture by washing the reaction surface; that operation was repeated 6 times, thus removing the components that did not bind to the polyclonal antibodies present on the solid substrate represented by the surface of the biochip;
- after complete removal of the reaction mixture, adding 250 µL of working signal reagent (obtained by separate mixing luminol and peroxide in equal volumes);
- placing the biochips support in the dark for the development of the reaction and exactly 2 min after placing it in the image capture chamber for the processing and interpretation of the signal;
- placing the biochips support in the image capture chamber for signal processing and interpretation.

In order to evaluate the sensitivity of the method the half maximal *inhibitory concentration* (IC50) for each assayed analyte was calculated using 50% of the RLU signal value generated by the zero concentration calibrator and extrapolating the RLU value thus obtained on the x-axis of the calibration curve on which the concentration units were expressed as ìg/kg. The concentration thus obtained corresponded to the inhibitory concentration producing 50% inhibition.

The specificity and selectivity of the method was analyzed by adding each analyte separately in known concentrations of (10 and 100 µg/kg) to the zero concentration calibrator in serial dilutions. To assign the cross reactivity percentage, three replicates were assessed for each analyte level in the serial dilution

According to the validation guides, the cross-reactivity percentage (% CR) for the determination of drug residues in honey must not exceed 25% for the analyte concentration at the minimum limit of quantification. Cross-reactivity was calculated as % CR = [IC50 (analyte)/IC50 (cross-reactant)] × 100.

Antimicrobial Array III Control - code AMC5036, manufactured by Randox Laboratories, UK, was used to evaluate the accuracy and precision. Also mean concentration, standard deviation, and coefficient of variation (% CV) were calculated.

Because the residues of nitrofurans have a 1µg/kg reference point for action (RAP), that was the reason the target concentration for screening was set at 0.5 µg/kg (50% of RAP). (http://eur-lex.europa.eu).

According to current legislation and validation guidelines for methods of drug determination in honey, precision and accuracy must be assessed for the minimum required performance limit (MRPL) which was 1 µg/kg, and 50 and 150% MRPL [20, 21].

The precision in the same analytical series was determined by analyzing 20 replicates of negative honey samples, enriched at 3 different concentration levels: 0.5, 1.0 and 1.5 µg/kg.

The accuracy in different analytical series was determined by analyzing 2 replicates of blank honey samples enriched with nitrofurans at three different concentration levels (0.5, 1.0 and 1.5 µg/kg) in 10 different rounds of work. Accuracy and accuracy are acceptable if the coefficient of variation in the concentration of the control samples measured does not exceed ± 15% for determinations on the same day or on different days or analytical series.

In order to determine the decision limit (CCα) and the detection capability (CCâ) 20 blank honey samples were selected and spiked at the target concentration for screening - 0.5 µg/kg. CCα was calculated as the arithmetic mean of the concentration in 20 spiked samples. The concentration level of each analyte was MRPL plus 1.64 × standard deviation of repeatability at α = 5%. CCβ was calculated as the arithmetic mean of the concentration analyte at CCα plus 1.64 × standard deviation of repeatability at α = 5%.

The validated method was used for the determination of 11 samples of honey purchased from supermarkets and 5 samples purchased from private producers from different geographic regions of Romania. The samples had been stored at room temperature and in the dark.

Sample processing included derivatization and extraction of nitrofurans from honey samples and it was carried out as follows:

-1g of honey sample was mixed with 4 mL distilled water, incubated at 37°C for 30 min, and stirred 10 min until dissolved;

-0.5 mL of 1M HCl and 100 µL of 10 mM 4-nitrobenzaldehyde solution were added to the sample solution; the mixture was stirred for 10 min, and then incubated for 16-24 h at 37°C;

-after incubation, 5 mL dipotassium phosphate 0.1M solution was added to each sample, and the *p*H was adjusted to 7.4 with 1M NaOH solution;

-15 mL ethyl acetate were added to 5 mL of derivatized honey sample, which was stirred on a Vortex shaker for 2 min and then homogenized for another 10 min; the sample was centrifuged for 10 min at 4500 relative centrifugal force;

-6 mL of supernatant was transferred from each sample into microtubes, which were then evaporated for 30 minutes at 60°C and 15 psi;

-the residue was mixed with 375 µL of sample diluent provided in the kit (AM III DIL SPE) and stirred for 2 min.

Confirmation of the results obtained using the biochip method for the analyzed honey samples was performed by using a LC-MS/MS method [21] using an Agilent 1100 LC (Agilent Technologies, USA) coupled with a 4000 Q TRAP mass spectrometer (Applied Biosystems, USA). Performance parameters of the method are shown in table 1. To confirm the results, the following parameters were followed: signal/noise ratio > 3, ± 2.5% differentiation of analyte retention time and corresponding standard, and ± 20% deviation of the relative abundance of the analyte and ± 50% deviation of the corresponding standard.

Analyte	Mean concentration ± DS (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)	Precision (%)	Accuracy (%)
AOZ	0.4 ± 0.17	0.68	0.96	6.26	11.70
AMOZ	0.6 ± 0.20	0.93	1.26	5.11	9.73
AHD	0.5 ± 0.14	0.73	0.96	4.41	16.23
SEM	0.5 ± 0.28	0.96	1.42	4.90	12.60

Table 1
PERFORMANCE PARAMETERS OF THE LC-MS/MS METHOD

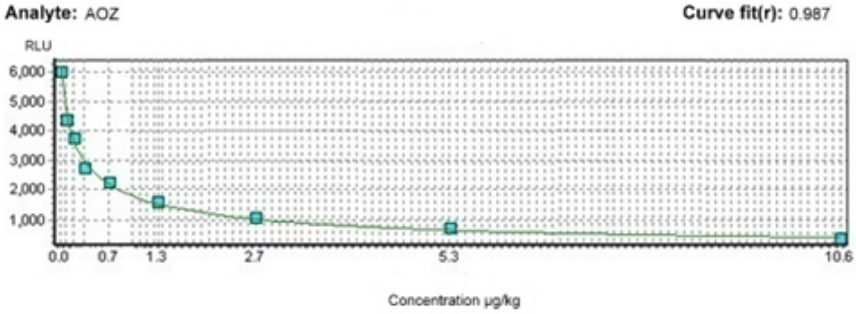

Fig. 1. Calibration curve for AOZ

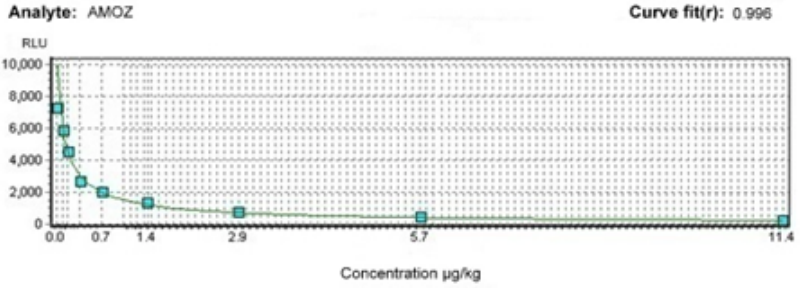

Fig. 2. Calibration curve for AMOZ

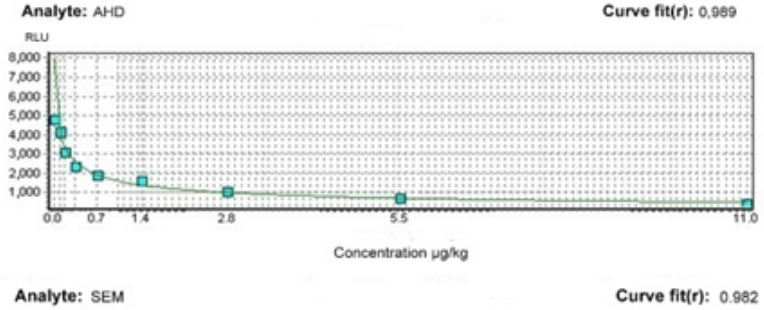

Fig. 3. Calibration curve for AHD

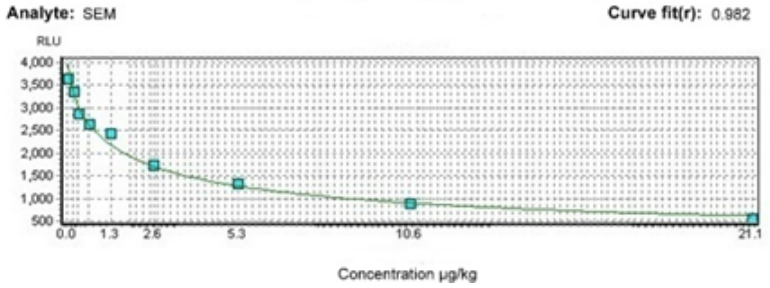

Fig. 4. Calibration curve for SEM

Results and discussions

The calibration curves for all 4 metabolites of nitrofurans are presented in figures 1-4. Following the determinations, correlation coefficients greater than 0.98 were obtained. The lowest value for the correlation coefficient was obtained for SEM (r = 0.982). The obtained results prove that the admittance criteria for the calibration curve has been met as the correlation coefficients must be higher than 0.949.

The sensitivity of the method expressed as IC50 for the simultaneous quantitative determination of the 4 nitrofurans had values equal or even lower than 2.32 µg/kg (table 2).

The values obtained for the cross-reactivity of the analytes analyzed are shown in table 3.

Parameter	Analyte			
	AOZ	AMOZ	AHD	SEM
Calibration range (µg/kg)	0-10	0-20	0-10	0-10
Correlation coefficient r	0.987	0.996	0.989	0.982
IC 50 (µg/kg)	0.09	0.44	0.40	2.32

Table 2
LINEARITY AND SENSITIVITY

Table 3
SPECIFICITY AND SELECTIVITY

Cross-reactivity (%)								
Nitrofuran metabolite	4-NP-AOZ	100	4-NP-AMOZ	100	4-NP-AHD	100	4-NP-SEM	100
Cross- reactant	furazolidone	9	furaltadone	18	nitrofurantoin	16	5-nitro-2-furaldehyde semicarbazone	24

Parameter		Analyte				
		AOZ	AMOZ	AHD	SEM	CAP
1	Concentration (µg/kg)	0.62	0.62	0.68	0.96	0.32
	Recovery (%)	1.24	124	136	192	64
2	Concentration (µg/kg)	1.06	1.11	1.19	1.25	0.75
	Recovery (%)	106	111	119	125	75
3	Concentration (µg/kg)	1.84	1.73	1.81	2.25	1.06
	Recovery (%)	123	115	121	150	71
Mean concentration (µg/kg)		0.62	0.62	0.68	0.96	0.32
Standard deviation		0.05	0.05	0.05	0.06	0.03
1.64 × Standard deviation		0.08	0.08	0.08	0.09	0.05
CCα (µg/kg)		0.70	0.70	0.76	1.05	0.37
CCβ (µg/kg)		0.78	0.78	0.84	1.14	0.42
n = 20						

Table 4
RECOVERY, DECISION LIMIT AND DETECTION CAPABILITY

Concentration level		AOZ	AMOZ	AHD	SEM
Same series					
1	Concentration (µg/kg)	0.62	0.62	0.68	0.96
	CV (%)	8.05	7.87	7.32	5.86
2	Concentration (µg/kg)	1.06	1.11	1.19	1.25
	CV (%)	7.32	8.10	5.72	5.59
3	Concentration (µg/kg)	1.64	1.82	1.81	2.08
	CV (%)	7.36	6.88	6.89	3.93
Different series					
1	Concentration (µg/kg)	0.68	0.64	0.61	0.96
	CV (%)	10.15	7.87	8.32	5.86
2	Concentration (µg/kg)	1.16	1.14	1.07	1.25
	CV (%)	8.02	8.10	5.72	7.46
3	Concentration (µg/kg)	1.84	1.73	1.81	2.25
	CV (%)	8.33	11.78	8.71	12.93
n = 20					

Table 5
PRECISION AND ACCURACY

The recovery percentage was calculated for 3 concentration levels representing 50, 100 and 150% of the minimum required performance limit for nitrofurans residues in honey (MRPL = 1 µg/kg). Honey samples were spiked with nitrofurans at the following concentrations levels 0.5, 1, 1.5 µg/kg. Recovery percentages were calculated by plotting the ratio of the analyte concentration in the sample against the theoretical concentration of the analyte in the standard solution.

According to the validation guidelines, the requirement for the recovery percentage for the determination of drug residues in honey must be higher than 70%. The recovery rate obtained was in the range of 64-192% relative to the initial concentration, as shown in table 4.

The decision limit (CCα) for the determined nitrofurans ranged between 0.70 µg/kg and 1.05 µg/kg. The detection capability (CCβ) obtained for nitrofurans ranged between 0.78 µg/kg and 1.14 µg/kg (table 5).

The precision of the method (table 5) was very good, both within the same analytical series and in different analytical series, with typical values lower than 15% for concentrations of 0.5, 1 and 1.50 µg/kg. Within the different analytical series, the coefficients of variation reached higher values than those for samples from the same analytical series, but those values were within the acceptability limit.

The validated biochip method was applied to the analysis of 16 samples of honey from various geographic regions in Romania. One of the analyzed samples was found positive with values higher than 1 µg/kg for AOZ and SEM.

All results were confirmed by the LC-MS/MS method. The performance of the biochip method was very good, the values obtained were comparable to the results obtained for both positive samples and negative samples (table 6).

Table 6
COMPARISON OF RESULTS DETERMINED THROUGH BIOCHIP AND LC-MS/MS METHODS

Sample N°	Method	Analyte (µg/kg)			
		AOZ	AMOZ	AHD	SEM
1	biochip	0.50	0.74	0.46	0.75
	LC-MS/MS	0.17	0.12	0.32	0.19
2	biochip	0.76	0.75	0.78	0.66
	LC-MS/MS	0.41	0.42	0.13	0.28
3	biochip	1.24	2.88	4.16	7.21
	LC-MS/MS	1.11	1.46	2.34	6.54
4	biochip	0.68	0.53	0.55	0.54
	LC-MS/MS	0.08	0.34	0.11	0.38
5	biochip	0.35	0.65	0.73	0.44
	LC-MS/MS	0.12	0.18	0.65	0.22

6	biochip	0.44	0.88	0.67	0.57
	LC-MS/MS	0.43	0.65	0.60	0.52
7	biochip	0.41	0.54	0.65	0.72
	LC-MS/MS	0.09	0.16	0.45	0.43
8	biochip	0.28	0.78	0.89	0.34
	LC-MS/MS	0.22	0.98	0.99	0.21
9	biochip	0.27	0.99	0.54	0.88
	LC-MS/MS	0.07	0.76	0.76	0.72
10	biochip	1.07	0.99	1.14	0.88
	LC-MS/MS	0.72	0.77	0.76	0.72
11	biochip	0.43	0.43	0.54	0.88
	LC-MS/MS	0.44	0.76	0.76	0.72
12	biochip	0.76	0.65	0.42	0.63
	LC-MS/MS	0.42	0.48	0.53	0.26
13	biochip	0.43	0.74	0.89	0.34
	LC-MS/MS	0.87	0.81	0.19	0.21
14	biochip	0.89	0.89	0.54	0.88
	LC-MS/MS	0.80	0.16	0.26	0.42
15	biochip	0.89	0.79	0.54	0.88
	LC-MS/MS	0.80	0.76	0.46	0.62
16	biochip	0.89	0.89	0.54	0.88
	LC-MS/MS	0.48	0.32	0.32	0.21

Table 6
CONTINUATED

Conclusions

The biochip technology allowed the simultaneous and selective measurement of residues of nitrofurans in honey at levels much lower than minimum required performance limits.

The simultaneous quantitative determination of 4 nitrofurans had specificity for each target analyte, and the sensitivity of the method expressed as all IC50s were lower than 2.32 μg/kg.

The method presented very good accuracy both within the same analytical series and in different analytical series with typical values lower than 15% for concentration levels of 0.5, 1 and 1.5μg/kg. The decision limit obtained for the determination of metabolites of 4 nitrofurans ranged between 0.37μg/kg and 1.05 μg/kg. The detection capability had values ranging from 0.42-1.14 μg/kg. The recovery coefficient obtained was in the 64-192% range of the initial concentration. The validated biochip method was applied to the analysis of 16 samples of honey from various geographic regions in Romania, and the results were confirmed by a LC-MS/MS method.

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