

New method of analysis of nitrofurans and nitrofuran metabolites in different biological matrices using UHPLC-MS/MS

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Abstract

Introduction: The major difficulty in analysis of nitrofurans in feed, feed water, and food of animal origin is that nitrofurans have low molecular weights and fast metabolism. The principal goal of this study was to prepare a procedure for the determination of nitrofurans and their metabolites by a single method in different types of feed, feed water, and food of animal origin. **Material and Methods:** Two-gram samples were subjected to hydrolysis and derivatisation processes by addition of hydrochloric acid and 2-nitrobenzaldehyde. After incubation the sample was purified by solid phase extraction technique. Nitrofurans were analysed using ultra-high-pressure liquid chromatography-MS/MS (UHPLC-MS/MS). **Results:** The results of validation fulfil the requirement of the confirmatory criteria according to the European Commission Decision 2002/657/EC regarding apparent recoveries (88.9%–107.3%), repeatability (2.9%–9.4%) and within-laboratory reproducibility (4.4%–10.7%). **Conclusion:** The method can be successfully applied to monitor nitrofurans and their metabolites in different matrices.

Keywords: nitrofurans, feed water, feed, food products, UHPLC-MS/MS.

Introduction

The use of nitrofurans has been banned in veterinary medicine within the European Union (Annex IV of Regulation 2377/90/EEC) because of their toxic influence on the health of consumers of animal origin food (4). Formerly, addition of nitrofurans to feeds or water was allowed in order to stimulate growth and in the prophylaxis and treatment of many bacterial and protozoan infections (8, 14).

In 1994, the EU prohibited the use of nitrofurans in veterinary medicine and no maximum residue limit (MRL) was established for these banned compounds due to safety not being possible to claim for any residue levels (4–6). However, the data from the Rapid Alert System for Food and Feed (RASFF) database for the years 2002 to 2017 showed 854 notification events reported for nitrofurans, indicating that contamination in various matrices is still present (12). The notifications referred to a wide range of products such as honey, feed

and feed water, fish and aquaculture products, and meat and meat products (12).

Many methods have been created for analysis of nitrofuran metabolites in biological matrices (8–11, 14), but unfortunately only a few methods have been designed for determination of nitrofurans in feed water (1) and feed (2) using mass spectrometry techniques. Additionally, none of the existing methods could be used for determination of nitrofurans and nitrofuran metabolites in a single analytical procedure, which is why the purpose of this research was to create and validate such a procedure. This method for determination needs to fulfil the requirements of the confirmatory criteria according to European Commission Decision 2002/657/EC (4) and involves the solid-phase extraction (SPE) approach for confirmation and quantitation of residues of these compounds in biological matrices using ultra-high-pressure liquid chromatography-MS/MS (UHPLC-MS/MS). The aim of this research is elaboration of the first method for

determination of nitrofurans and nitrofurans metabolites using UHPLC-MS/MS in a single analytical procedure.

Material and Methods

Materials. All reagents were of minimum analytical grade or higher. Materials were procured as previously described (13) or as follows: ethyl acetate, hexane (95%), acetic acid (99%), ammonium formate, and methanol (HPLC grade) were from J.T. Baker (the Netherlands). Ultrapure water was filtered through a Millipore Milli-Q system (USA). Nanosep MF 0.22 µm filters were supplied by Pall (USA). Ammonia, 35% hydrochloric acid, and anhydrous dipotassium hydrogen orthophosphate were obtained from POCH (Poland). Strata SDB-L SPE cartridges were products of Phenomenex (USA). Two-nitrobenzaldehyde (2-NBA), furazolidone, furaldatone, nitrofurantoin, nitrofurazone and their metabolites, 5-methylamorfolino-3-amino-2-oxazolidone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM), 3-amino-2-oxazolidone (AOZ), and the internal standards 1,2-(15N₂, 13C) SEM, AMOZ-d₅, (3C13) AHD, and AOZ-d₄ were purchased from the Sigma-Aldrich Chemical Company (Germany).

UHPLC-MS/MS. The UHPLC-MS/MS system consisted of an AB Sciex ExionLC UHPLC system connected to an AB Sciex API 5500 Qtrap mass spectrometer (AB Sciex, Canada). Analyst 1.6.3 software (AB Sciex) controlled the UHPLC-MS/MS system and Multiquant 3.2 (AB Sciex) was used to process the data. The MS/MS was operated as previously described with some modifications (10). Briefly, desolvation temperature was set at 500°C, gas 1 (air) at 40 psi; gas 2 (air) at 40 psi; collision gas (N₂) to high; nebuliser gas (N₂) at 40 psi; and curtain gas (N₂) at 40 psi. The voltage of electron multiplier and the electrospray capillary were set at 2,100 V and 5,500 V, respectively. The chromatography was performed in a C8(2) column 3 µm × 2 mm, 75 mm (Phenomenex, USA), connected to a C8 precolumn 3 µm × 2 mm × 4 mm (Phenomenex). The mobile phase was composed of two solutions: A (ammonium formate – 5 mmol/L) and B (methanol) in a gradient which started with 80% of A and 20% of B. From 0 to 2.5 min, the concentration of B was raised to 95% and left for 2.5 min. Finally, after

0.2 min, the B concentration was decreased to 20% and left for 9 min. The flow rate was 0.4 mL/min, the column operated at 40°C, and the ions were monitored in MRM mode (Table 1).

Hydrolysis. Nitrofurans and their metabolites were prepared using the same method as previously described, with some modifications (13). A 2.0 ± 0.05 g portion of each sample was transferred to centrifuge tubes. The samples were submitted to hydrolysis and derivatisation processes by adding internal standard mixture (1,2-(15N₂, 13C) SEM, AMOZ-d₅, (3C13) AHD, and AOZ-d₄), 10 mL of the 0.2 mol/L hydrochloric acid, and 240 µL of 2-NBA (10 mg/mL in methanol). The samples were capped securely in the centrifuge tubes and vortexed for 30 s, then placed in a water bath at 40 ± 3°C and incubated overnight. After the incubation process, the samples were removed from the water bath and cooled down to room temperature. The pH-value of about 7.0 was adjusted by the addition of 10 mL of 0.2 mol/L di-potassium hydrogen orthophosphate solution and 800 µL of 1 mol/L ammonia solution. The samples were centrifuged at 4,500 × g for 15 min and transferred to the SDB-L cartridges.

Cleanup. SPE cartridges were placed on a vacuum manifold and the cartridges were sequentially conditioned with 3 mL of ethyl acetate, 3 mL of methanol, and 5 mL of water. A small portion (0.5 mL) of water was left on the cartridges until the application of the sample extracts. The latter were passed through the SDB-L cartridges, washed with 5 mL of water and 5 mL of hexane, and eluted with ethyl acetate. The eluates were vortexed for 10 s and then evaporated to dryness on a heating block at 40°C under a gentle stream of nitrogen. The residues were dissolved in 200 µL of 60% methanol solution in water and filtered through Nanosep MF 0.22 µm filters before being injected into the LC column.

Results

The procedure was validated according to the Commission Decision of 2002/657/EC (4). The analysis of 20 blank samples for each of the matrices did not reveal any interference. The criteria concerning relative retention time of the analytes corresponded to those of the calibration solution at a tolerance of ±2.5%.

Table 1. Precursor ions and product ion transitions of nitrofurans metabolites and internal standards – mass spectrometry parameters

Analyte	Precursor ion (m/z)	Ion transition	Declustering potential	Collision energy (eV)
AOZ	235.95	134.0	116	15
		104.0		29
AMOZ	335.00	291.0	71	16
		262.1		26
SEM	209.00	166.0	96	13
		192.0		15
AHD	249.00	134.0	126	15
		104.0		27
AOZ-D4 (IS)	239.95	134.0	136	17
AMOZ-D5 (IS)	340.00	296.1	106	15
SEM-C ₁₃ , 2N ₁₅ (IS)	212.00	167.9	86	13

Table 2. Validation report for nitrofurans metabolites

Matrix	Repeatability (RSD _r ,%)	Within-lab reproducibility (RSD _{wR} ,%)	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	Apparent recovery (%)	Matrix effect (%)	Expanded uncertainty ($\mu\text{g kg}^{-1}$)
AOZ							
feed water	4.4 \pm 3.4	5.6 \pm 4.1	0.50	0.52	104.5 \pm 2.8	4.0 \pm 1.9%	1.0 \pm 0.14
feed	4.9 \pm 4.4	6.6 \pm 4.3	0.50	0.54	103.5 \pm 6.8	6.1 \pm 2.9%	1.0 \pm 0.24
urine	5.0 \pm 3.7	6.3 \pm 4.4	0.50	0.56	104.0 \pm 4.2	6.0 \pm 2.7%	1.0 \pm 0.2
plasma	3.1 \pm 3.6	5.7 \pm 3.2	0.50	0.52	98.0 \pm 3.8	4.5 \pm 3.5%	1.0 \pm 0.23
muscle	2.9 \pm 4.5	4.5 \pm 3.6	0.50	0.54	103.1 \pm 4.2	5.4 \pm 5.5%	1.0 \pm 0.18
eggs	7.4 \pm 6.1	10.7 \pm 6.5	0.50	0.53	97.0 \pm 0.3	7.0 \pm 2.5%	1.0 \pm 0.25
milk	3.2 \pm 3.8	4.8 \pm 4.4	0.50	0.54	96.0 \pm 4.9	6.4 \pm 4.3%	1.0 \pm 0.18
honey	6.1 \pm 3.9	8.7 \pm 5.7	0.50	0.53	98.7 \pm 6.3	7.3 \pm 5.5%	1.0 \pm 0.28
AMOZ							
feed water	4.1 \pm 3.4	6.6 \pm 3.1	0.50	0.53	89.5 \pm 3.8	4.0 \pm 2.0%	1.0 \pm 0.18
feed	5.1 \pm 5.1	5.3 \pm 3.4	0.50	0.54	96.5 \pm 6.8	6.3 \pm 4.0%	1.0 \pm 0.26
urine	6.2 \pm 3.9	7.3 \pm 4.1	0.50	0.53	103.0 \pm 4.1	6.3 \pm 5.2%	1.0 \pm 0.15
plasma	3.6 \pm 3.9	4.4 \pm 4.2	0.50	0.53	95.0 \pm 4.5	4.5 \pm 5.5%	1.0 \pm 0.23
muscle	5.9 \pm 3.5	6.4 \pm 3.6	0.50	0.52	106.2 \pm 4.1	5.3 \pm 3.6%	1.0 \pm 0.16
eggs	5.4 \pm 4.2	5.7 \pm 6.5	0.50	0.53	97.2 \pm 5.3	5.0 \pm 4.5%	1.0 \pm 0.25
milk	4.2 \pm 3.9	5.8 \pm 4.4	0.50	0.52	105.2 \pm 3.9	4.0 \pm 4.3%	1.0 \pm 0.16
honey	5.9 \pm 4.8	7.7 \pm 4.7	0.50	0.53	95.7 \pm 5.3	8.0 \pm 4.5%	1.0 \pm 0.28
SEM							
feed water	4.2 \pm 2.4	8.4 \pm 4.1	0.50	0.54	88.9 \pm 3.8	3.0 \pm 1.0%	1.0 \pm 0.19
feed	9.4 \pm 5.4	8.4 \pm 5.2	0.50	0.55	93.3 \pm 5.6	6.3 \pm 5.5%	1.0 \pm 0.35
urine	8.6 \pm 4.6	8.4 \pm 4.3	0.50	0.53	102.0 \pm 6.3	6.3 \pm 5.1%	1.0 \pm 0.25
plasma	4.1 \pm 3.9	5.7 \pm 4.6	0.50	0.53	92.0 \pm 4.3	6.5 \pm 4.5%	1.0 \pm 0.24
muscle	3.9 \pm 3.5	5.4 \pm 4.6	0.50	0.54	107.3 \pm 5.1	5.3 \pm 5.2%	1.0 \pm 0.26
eggs	7.4 \pm 6.1	10.6 \pm 6.8	0.50	0.55	97.0 \pm 5.3	9.0 \pm 4.5%	1.0 \pm 0.25
milk	5.1 \pm 3.2	6.8 \pm 5.4	0.50	0.54	95.0 \pm 3.9	7.0 \pm 3.5%	1.0 \pm 0.26
honey	7.0 \pm 5.8	8.4 \pm 5.7	0.50	0.55	95.7 \pm 6.3	10.0 \pm 4.6%	1.0 \pm 0.31
AHD							
feed water	4.9 \pm 3.4	7.4 \pm 4.1	0.50	0.54	100.5 \pm 2.8	5.2 \pm 2.0%	1.0 \pm 0.23
feed	5.2 \pm 3.7	8.4 \pm 6.4	0.50	0.54	94.9 \pm 6.8	5.0 \pm 4.3%	1.0 \pm 0.32
urine	8.2 \pm 5.7	7.5 \pm 5.5	0.50	0.52	106.2 \pm 5.3	6.3 \pm 4.6%	1.0 \pm 0.25
plasma	4.3 \pm 3.4	4.3 \pm 3.9	0.50	0.53	95.0 \pm 5.8	6.7 \pm 4.7%	1.0 \pm 0.28
muscle	3.9 \pm 3.2	4.4 \pm 3.9	0.50	0.53	102.1 \pm 4.3	5.3 \pm 5.5%	1.0 \pm 0.23
eggs	6.3 \pm 5.2	8.7 \pm 4.5	0.50	0.54	94.3 \pm 5.3	4.0 \pm 5.5%	1.0 \pm 0.25
milk	5.1 \pm 3.3	6.8 \pm 5.4	0.50	0.53	95.0 \pm 6.9	5.2 \pm 4.3%	1.0 \pm 0.28
honey	7.3 \pm 4.3	8.1 \pm 5.1	0.50	0.55	93.4 \pm 7.3	10.2 \pm 5.5%	1.0 \pm 0.32

A linearity (R^2) for all concentration levels (0.50–10.0 $\mu\text{g kg}^{-1}$) was obtained, within the range of 0.990–0.998. The apparent recoveries for all concentration levels (0.50–10.0 $\mu\text{g kg}^{-1}$) were in the range of 88.9% to 107.3% with a repeatability of less than 9.4% (2.9%–9.4%), and within-laboratory reproducibility below 10.7% (4.4%–10.7%). The expanded uncertainty was calculated at the 1.0 $\mu\text{g kg}^{-1}$ concentration level applying a coverage factor of 2, which provided a level of confidence of approximately 95%. The calculated ion suppression of the matrix effects for nitrofurans metabolites in all matrices did not exceed 15%. The decision limit (CC α) and the detection capability (CC β) values were also determined and are presented in Table 2. The individual stock solutions stored at $<-18^\circ\text{C}$ were stable for six months. The stability of the stock solutions stored at 6°C was six months, and working solutions were stable for three months. The stability of extracts for each of the analysed matrices has been estimated at least one week at $<-18^\circ\text{C}$.

Discussion

To the best of our knowledge, the current method is the first published method for determination of nitrofurans and nitrofurans metabolites using UHPL-MS/MS in a single analytical procedure. Based on our earlier experience (13) we intended to use positive ionisation with the ions presented in Table 1, and the following parameters were optimised by direct flow infusion: source temperature, desolvation gas temperature, desolvation gas, capillary voltage, cone voltage, and collision energy. For all studied analytes one precursor ion and two product ions were selected. Separation of nitrofurans metabolites is generally performed by LC using reversed-phase C18 columns with a mixture of water and acetonitrile, water buffer, or methanol as the mobile phase (8, 9, 13). Since in our previously described method the time of analysis was about 20 min (13), we decided to use a shorter column with C8 reversed phase. The composition of the mobile

phase in the previously described method gave a symmetrical peak shape and minimised any matrix effect (13), and this decided us on using this mobile phase (5 mM ammonium formate, pH 4.5; methanol), but with gradient modification. Example chromatograms of honey (blank and sample fortified with $1.0 \mu\text{g kg}^{-1}$) are shown in Fig. 1.

The main problem in the determination of nitrofurans in feed and feed water and nitrofuran metabolites in food of animal origin is that nitrofurans have low molecular weights and fast metabolism. Another difficulty in determining nitrofurans is their instability, because these compounds are extremely sensitive to daylight (8, 10, 11, 14). To solve these problems we added hydrolysis and derivatisation steps. The latter step increases the molecular weight, thus leads to a significantly increased sensitivity of the method. Thus we decided to build on the method which had previously been developed for the determination of metabolites of nitrofurans in eggs (3, 7, 10, 13). In order to prove the conversion of nitrofurans to nitrofuran

metabolites, we developed a method to verify the presence of the parent compounds after the hydrolysis step. The tests were performed at a concentration of $10 \mu\text{g kg}^{-1}$ to completely exclude the presence of parent compounds. The experiment demonstrated that furaltadone, nitrofurantoin and nitrofurazone were completely converted into their metabolites, while furazolidone was an exception, being 96% converted into its metabolite (Fig. 2). Our results clearly prove that this method can be used to determine the residues of both nitrofurans and their metabolites.

The proposed method was used to analyse the nitrofuran metabolites and nitrofurans in water, feed water, milk, honey, urine, muscle, and egg samples within the official control programme in Poland. Over the last three years, nine honey samples containing furazolidone (AOZ) have been found: the lowest concentration was $0.8 \mu\text{g kg}^{-1}$ and the highest $12.36 \mu\text{g kg}^{-1}$. These results showed that this method is important and useful in qualitative and quantitative routine analysis for nitrofurans and nitrofuran metabolites.

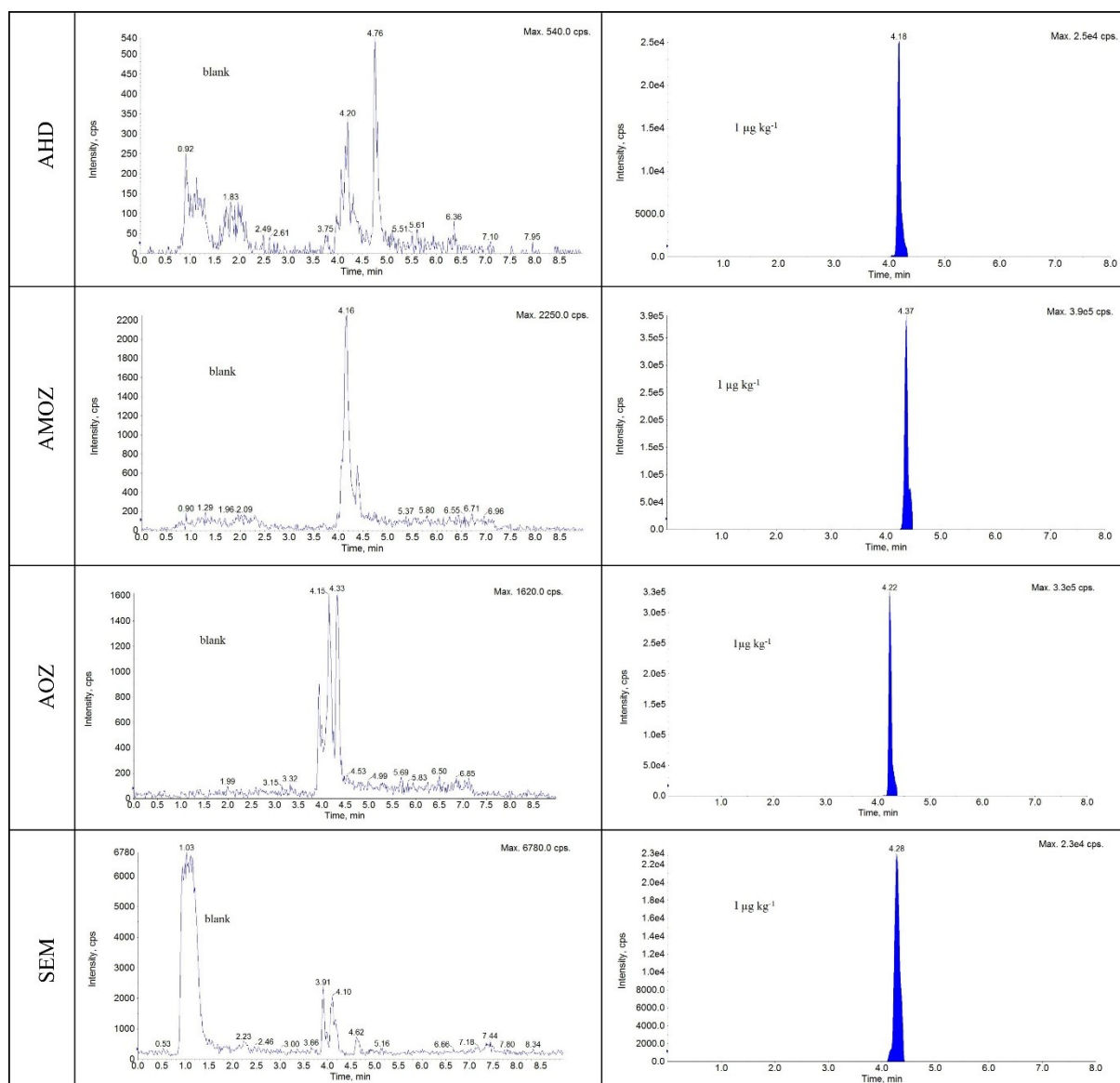


Fig. 1. Chromatograms of blank and spiked honey matrices with nitrofurans ($1.0 \mu\text{g kg}^{-1}$)

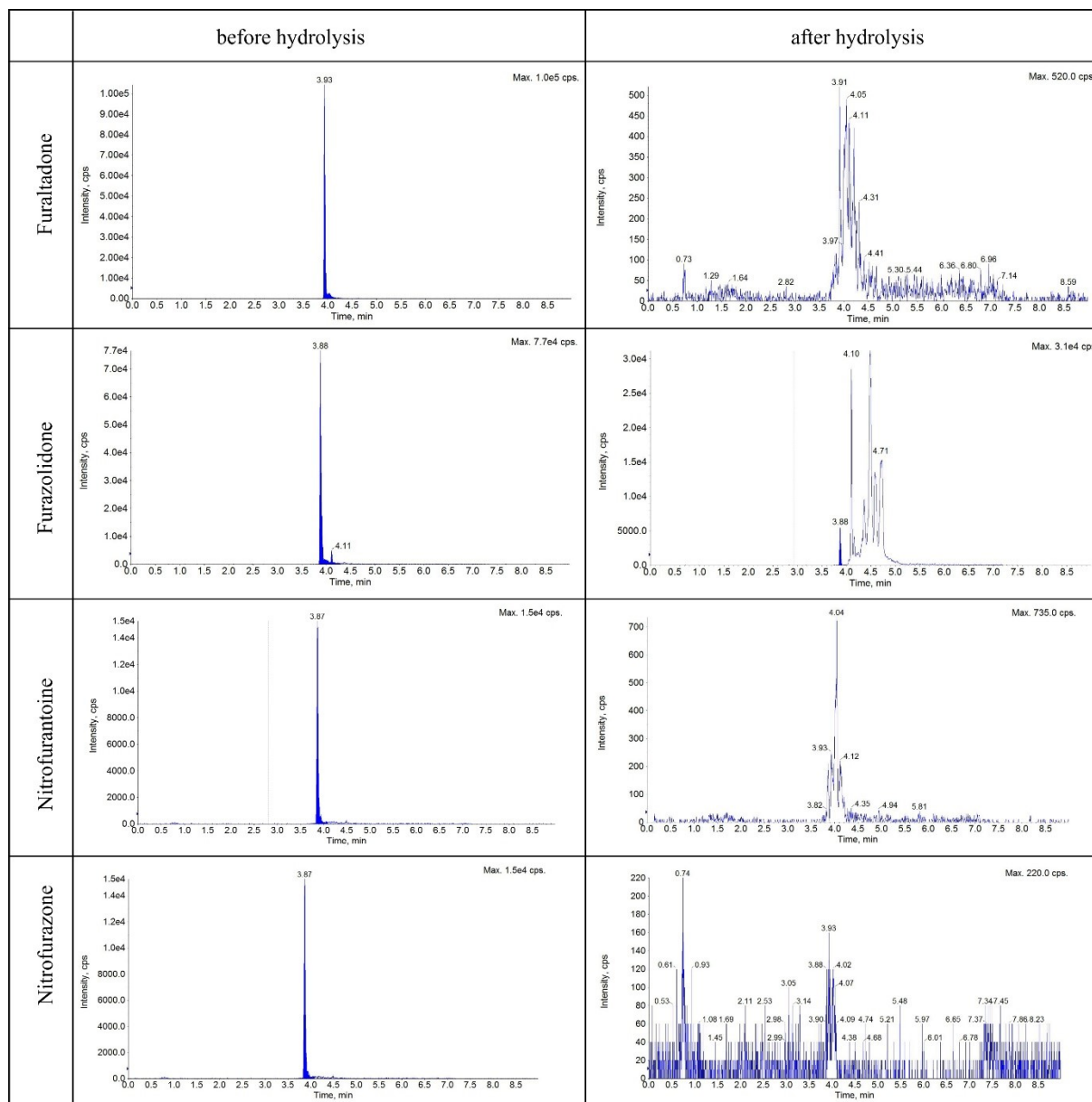


Fig. 2. Chromatograms of nitrofurans, before and after hydrolysis ($10.0 \mu\text{g kg}^{-1}$)

The procedure presented in the current article is able to detect nitrofurans and nitrofurans metabolites in various matrices like feed, feed water, urine, plasma, muscle, eggs, milk, and honey in one method, and its validation parameters show that it meets the European Union criteria. The method is now used for the effective routine analysis of nitrofurans and their metabolites in the National Monitoring Plan in Poland.

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