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RAPID CONFIRMATORY METHOD FOR ANALYSIS OF NITROFURAN METABOLITES IN EGG BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract— **This study has been devoloped and validated a quick method for confirmatory analysis of nitrofuran metabolites according to the European Commission Decision 2002/657/EC requirements. The quantitative and confirmatory determination of nitrofuran metabolites was performed by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS] in the positive ion mode. An in-house method validation was performed and the data reported on specificity, linearity, recovery, CCα and CCβ. The advantage of this method is that avoids the use of clean-up by SPE (Solid-Phase Extraction] and shortens incubation period for derivatisation with nitrobenzaldehyde in overnight or 16 h in the 37 ºC temperature in the dark and then low levels of nitrofuran metabolites are detectable and quantitatively confirmed at a rapid rate in egg samples.**

I. INTRODUCTION

Nitrofurans are broad spectrum antibacterial agents known as Schiff's bases derivates of nitrofuraldehyde [1, 2]. In veterinary medicine, it was used in the treatment of gastrointestinal and dermatological infections in beef, pork, poultry, fish and shrimp, and also applied as a contribution to systemic and feed as growth promoters [3, 4, 5]. In addition, nitrofurans were used in the treatment of bacterial infections in bee colony health [1].

The nitrofurans are known to be unstable and rapidly metabolised, with in vivo half-lives of less than a fewhours. Their metabolites are highly stable [3].

Nitrofurans have been prohibited from use in food-producing animals in the European Union and most countries due to public health and safety concerns, particularly in relation to the carcinogenic potential of either the parent compounds or their metabolites [6]. The use of nitrofurans in food-producing animal was prohibited in Turkey [7].

A minimum required performance limit (MRPL) for nitrofurans is set in European Union for the metabolites in poultry meat and aquaculture products at the level of 1 μg kg^{-1} for all metabolites [8].

Analytically, residues are checked only for marker metabolites of the 4 nitrofuran chemicals, in particular: 3-amino-2-oxazolidinone [AOZ) for furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) for furaltadone, 1-aminohydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone [9].

Several methods have been reported in the analysis of nitrofuran metabolite in food samples. These include thin-layer chromatography (TLC] [10], high performance liquid chromatography diode-array detector (HPLC DAD) [2] and UV detector [11, 12], liquid chromatography-mass spectrometry (LC-MS/MS) [3, 4, 12]. LC-MS/MS analyses were considered very sensitive and commonly used the confirmatory analysis.

In general the study of nitrofuran metabolites in food samples, were used incubation period for derivatisation with nitrobenzaldehyde in overnight or 16 h in the 37 ºC temperature in the dark [4, 12, 13].

In the current study, it has been devoloped and validated a method for quickly confirmatory analysis of nitrofuran metabolites (AOZ, AMOZ, AHD and SEM). Egg samples were achieved acidic by hydrolysis of the protein-bound drug metabolites, followed by derivatisation with nitrobenzaldehyde in ultrasonic bath at a 40 min and liquid-liquid extracted with ethylacetate. The quantitative and confirmatory determination of nitrofuran metabolites was performed by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) in the positive ion mode, according to European Decision 2002/657/EC [14]. The method avoids the use of clean-up by SPE and shorten incubation period for derivatisation with nitrobenzaldehyde in

overnight or 16 h in the 37 ºC temperature in the dark. Therefore, should be performed quickly confirmative analysis in egg samples and used as a routine analysis.

II. MATERIAL AND METHOD

A. Chemicals and reagents

Standards of nitrofuran metabolite; 3-amino-2-oxazolidinone (AOZ) and 1-aminohydantoin (AHD), 3-amino-5-morpholinomethyl-2-oxazolidinon (AMOZ) and Semicarbazide hydrochloride (SEM), internal standards; d5-AMOZ, $(C^{13})_3$-AHD, d4-AOZ and $C^{13}N^{15}N^{15}$-SCA-HCl, standards of derivative; 2NP-AOZ, 2NP-AMOZ, 2NP-AHD and 2NP-SCA, internal standards of derivative; d4-2NP-AOZ, d5-2NP-AMOZ, 2NP-$(C^{13})_3$–AHD and 2NP-$C^{13}N^{15}$-N^{15}-SCA obtained from Witega Laboratorien, Berlin. The purity of all compounds was greater than 99%.

2-nitrobenzaldehyde (2-NBA) (≥98% purity) obtained from (Sigma-Aldrich). Methanol (MeOH) HPLC grade, ethylacetate for analysis, acetic acid (glacial) 100% anhydrous for analysis, hydrochloric acid (HCl), 32% ACS reagent, dimethylsulphoxide (DMSO) for analysis, *n*-hexane for analysis, potassium hydrogen phosphate dibasic (K_2HPO_4) ACS grade obtained from Merck. Reagent water was prepared in-house with a from a Milli-Q purifying system (Elga PureLab Prima).

B. Solutions and Standards

Solution of HCl, 0.1 M; add 9.83 mL concentrated HCl to approxymately 500 mL reagent water and dilute 1.0 L with reagent water.

Solution 2-NBA, 50 mM; accurately weigh 0.76 g 2-NBA into a 100 mL volumetric flask. Add approxymately 50 mL DMSO and dissolve and dilute to the mark with DMSO.

Solution of K_2HPO_4, 1 M; accurately weigh 57.06 g K_2HPO_4 into a 250 mL volumetric flask. Add sufficient reagent water to dissolve and mix well, dilute to mark with reagent water.

Individual stock solution of (1 mg mL^{-1} each); accurately weigh 10 mg of referance compound into a 10 mL class A volumetric flask. Add suffiicient methanol to dissolve and dilute to mark with methanol. But was used DMSO for prepared stock solution of SEM. All stock soutions were stored -20 ºC.

S_2-Working standard solution (10 µg mL^{-1} each); place 0.1 mL of each stock solution into 10 mL volumetric flask and dilute to mark with methanol. All working solution stored in refrigerator (4-6 °C).

S_4-Working standard solution (100 ng mL^{-1} each); place 0.1 mL of S_2-working standard solution into 10 mL volumetric flask and dilute to mark with methanol. All working solution stored in refrigerator (4-6 °C).

Mixed standard spiking solution; prepare spiking solutions at concentrations of 8, 12, 20 and 20 ng mL^{-1} of AMOZ, AOZ, AHD and SEM, respectively. Place 0.8 mL of S_4-AMOZ, 1.2 mL S_4-AOZ, 2.0 mL S_4-AHD and S_4-SEM working solution into 100 mL volumetric flask and dilute to mark with methanol.

Mixed internal standard solution; prepare mixed internal standard solutions at concentrations of 40, 40, 100 and 100 ng $mL^{-1}$of AOZ-d4, AMOZ-d5, AHD-$(C^{13})_3$ and SEM-$C^{13}N^{15}$-N^{15}, respectively. Place 0.4 mL of S_2-AOZ-d4 and S_2-AMOZ-d5, 1 mL of S2-AHD-$(C^{13})_3$ and S2-SEM-$C^{13}N^{15}$-N^{15} working solution into 100 mL volumetric flask and dilute to mark with methanol.

C. Extraction procedure

Collected egg samples were known to be negative in the screning analysis in national program for residue control in Turkey. 30 egg are broken discard shells into wide container and homogenised with manuel blender.

2 g egg sample were weighed into 50 mL polypropylene centrifuge tubes. Mixed standard spiking solution were added 50, 100, 150 and 200 µL and mixed internal standard solution (100 µL) were added to all tubes. To each tube 5 mL of 0.1 M HCl was added. The tubes were capped, mixing by vortex 2 min. 2-NBA (50 mM, 300 µL) was added and the mixture shaken for 2 min by vortex. The tube were capped and were incubated ultrasonic bath at 40 min. After derivatization, the samples were cooled in room temperature and neutralized by addition to this 1 mL of 1 M K_2HPO_4 solution added and by vortexed 2 min. 5 mL ethylacetate is added and mixing by vortex (2 min), and 3 mL n-hexane is added into samples and vortexed (2 min) and centrifuged at 4000 g for 15 min. The 6 mL supernatant were removed using a pipette and transferred into a 15 mL graduated glass tubes. The organic fraction was evaporated to dryness under a stream of nitrogen in a water bath at 42°C.

The dry residue was redissolved 2 mL n-hexane and vortexed 2 min. The samples were added 0.75 mL MeOH/Water (5/95) and vortexed 2 min and allowed 15 min in room temperature. 0.5 mL samples were taken from the lower phase with the help of syringe or automatic pipette and filtered using a 0.2 µm syringe filter into an autosampler vial.

D. Equipment

Chromatographic analyses were performed on a LC-MS/MS equipmentconsisted of a Thermo Electron TSQ Quantum Access Max, mass spectrometercontrolled by the Xcalibur (2.2 SP1) software.

Chromatographic separations were achieved on Phenomenex Synergy Hydro RP (150x2,00 mm 80A 4µ) and protected with a C18 guard column. The mobile phase was deionised water/methanol (80/20) (A) and methanol acidified 0.1% acetic acid and flow rate of 0.25 mL/min. The linear gradient was: 0-2 min 100% A, 2-9 min 10% A and 9-15 min 100% A. Injection volume of 50 µL. The column was thermostated at 40 ◦C. The analysis of samples were carried in the positive ESI-MS-MS ion mode.

E. MS/MS Condition

The mass spectrometer was operated in the positive electrospray ionisation (ESI) mode. The collision gas was argon at 0.22 mL/min, spray voltage was 3.0 kV, vaporiser temperature 300 °C, capillary temperature was 300°C, sheath gas was 35 psi, ion sweep gas was 0.2 psi and aux gas 10 psi.

MS/MS parameters and precursor-product ions of each compound were tuned by direct infusion in the SRM mode and 0.25 mL/min flow rate mobile phase 50:50 (A:B). Table 1 summarizes the cone and collision parameters and the selected reaction monitoring (SRM) transitions used for this study.

F. Calculations

Limit of detection (LOD); determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Limit of quantification (LOQ); determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Decision limit (CC_α) and detection capability (CC_β); the CC_α and CC_β for banned substances were calculated with the application of the following formula;

$CC\alpha = C_1 + 2.33 \times SD_{wIR}$

where in C_1 is lowest concentration level of the validation study (MRPL) and SD_{wIR} is the standard deviation from within-laboratory reproducibility.

$CC\beta = CC\alpha + 1.64 \times SD_{wIR,CC\alpha}$

where in $SD_{wIR,CC\alpha}$ is standard deviation at CC_α concentration.

TABLE I: LC–MS/MS CONFIRMATION PARAMETERS FOR THE ANALYTES

Analyte	MS MH+ (m/z)	MS-MS (m/z)	Collision Energy	Width	Tube Lens	Dwell Time
2-NP-SEM	209.0	166.0*	11	0,05	98	0,1
		192.0	13	0,05	98	0,1
2-NP-SEM- $C^{13}N^{15}N^{15}$	212.0	168.0	10	0,05	115	0,1
2-NP-AHD	249.0	134.0	12	0,05	71	0,1
		104.1*	22	0,05	71	0,1
2-NP-AHD- $(C^{13})_3$	252.0	134.0	12	0,05	71	0,1
2-NP-AOZ	236.0	134.0	13	0,05	64	0,1
		104.0*	22	0,05	64	0,1
2-NP-AOZ-D4	240.0	133.9	12	0,05	65	0,1
2-NP-AMOZ	335.0	291.0	12	0,05	70	0,1
		261.9*	17	0,05	70	0,1
2-NP-AMOZ-D5	340.0	296.0	12	0,05	71	0,1

*Confirmative ion

III. RESULTS

A. Method validation

Method validation was performed with consideration of the criteria and recommendations of the European Commission Decision 2002/657/EC implementing the Council Directive 96/23/EC.

B. Specificity/Selectivity

Specificity/selectivity were evaluated via analysis of blank matrix samples fortified mixed benzimidazole and nitroimidazole standards (concentration of 1 µg kg^{-1} each) with standards of nitrofuran metabolites. According to analysis no significant peaks with an S/N ratios of 3 or more and chromatographic interference were being observed at the retention times of the targeted nitrofuran metabolites.

C. Linearity

The matrix calibration curves at four levels 0.2, 0.4, 0.6 and 0.8 µg kg^{-1} for AMOZ, 0.3, 0.6, 0.9 and 1.2 µg kg^{-1} for AOZ, 0.5, 1.0, 1.5 and 2.0 µg kg^{-1} for AHD and SEM which is in accordance with the MRPL levels. For each compound were made three matrix calibration curves for three days. The obtained linearity was satisfactory for all the tested concentrations. No significant differences were found between the different matrix curves (r^2>0.991) show in Table 2.

D. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD were calculated between 0.1-0.22 µg kg^{-1} and LOQ were calculated 0.2-0.54 µg kg^{-1} presented in Table 2.

E. Decision limit (CCα) and detection capability (CCβ)

For each compound of CC_α and CC_β were calculated from linearity study. The mean value CC_α between 0.22-0.57 µg kg^{-1} and the mean value CC_β between 0.23-0.62 µg kg^{-1} in samples are presented in Table 2.

TABLE II: SUMMARY OF CC_α, CC_β AND LINEARITY

Analyte	Calibration Range (ppb)	Linearity r^2	CC_α (ppb)	CC_β (ppb)	LOD (ppb)	LOQ (ppb)
AOZ	0.3-1.2	0.997	0,32	0,34	0,10	0,31
AMOZ	0.2-0.8	0.999	0,22	0,23	0,07	0,20
AHD	0.5-2	0.996	0,55	0,59	0,18	0,50
SEM	0.5-2	0.991	0,57	0,62	0,22	0,54

F. Accuracy

The accuracy was obtained by analysing blank egg samples fortified with at 0.3, 0.6 and 0.9 µg kg^{-1} for AOZ, 0.2, 0.4 and 0.6 µg kg^{-1} for AMOZ, 0.5, 1.0 and 1.5 µg kg^{-1} for AHD and SEM according to MRPL levels. According to results, accuracy was observed 96.2-101.5% and presented in Table 3.

G. Reproducibility

The within-laboratory reproducibility were calculated in blank egg samples fortified at 0.3, 0.6 and 0.9 µg k^{g-1} for AOZ, 0.2, 0.4 and 0.6 µg k^{g-1} for AMOZ, 0.5, 1.0 and 1.5 µg k^{g-1} for AHD and SEM. They were analysed for three different days, with the different instrument and different operator. The within-laboratory reproducibility were found satisfactory for the four compounds. CV (%) values are less than 9.3% for all samples and presented Table 3.

H. Stability

Stability tests of nitrofuran metabolites AOZ, AMOZ, AHD and SEM in fresh egg, stored at−20 ◦C, are stable at least for 6 weeks. In standard solution the analytes AOZ, AMOZ,

AHD, and SEM are stable for at least 1 year when stored at +4 ◦C in the dark [15].

I. Evaluation

In order to evaluate this method, it eventually participating in the Progetto Trieste (Laboratory Proficiency Testing for Food Safety Analysis), the test of "Lyophilised egg, confirmatory" (2014, 4th round. Test Material E1472, Lab No:92). AOZ total assigned value is 3.35 µg kg^{-1}. The result of our laboratory was obtained 3.4 µg kg^{-1}. Z-score were 0.07, as well as good and satisfactory.

TABLE III: REPEATABILITY AND ACCURACY IN SPIKED EGG SAMPLE.

Analyte	Fortified Level (µg kg^{-1})	Mean (µg kg^{-1})	RSD %	Accuracy %	Ion Ratio %	Retention Time (min)
AOZ	0.3	0.30	2.4	101.5	47.30	8.4
	0.6	0.58	4.5	97.0	48.08	
	0.9	0.89	1.7	98.4	45.15	
AMOZ	0.2	0.20	5.1	97.8	29.84	8.0
	0.4	0.39	2.2	98.6	30.56	
	0.6	0.61	1.4	101.2	31.92	
AHD	0.5	0.48	5.5	96.2	47.30	8.3
	1.0	0.98	6.3	97.8	44.82	
	1.5	1.48	2.1	98.8	42.53	
SEM	0.5	0.49	9.3	97.6	63.76	8.4
	1.0	0.96	4.2	96.5	62.46	
	1.5	1.47	2.6	98.1	59.04	

IV. DISCUSSION

To measure nitrofuran metabolites using the selective reaction-monitoring (SRM) mode, full scan and product ion spectra of the analytes were investigated under the LC conditions described in mass spectrometry. Nitrofuran metabolites could be detected under the positive ionization mode ESI-MS conditions. Figure 1 and 2 show LC/MS/MS extracted-ion chromatograms obtained from analysis of a blank and spiked egg sample.

The majority of the method for nitrofuran metabolites then employ a solid phase extraction (SPE) step in order clean-up and incubation period for derivatisation with nitrobenzaldehyde in overnight or 16 h in the 37 ºC temperature in the dark.

The study by P. Mottier et al. [11] quantitative determination of four nitrofuran metabolites in meat samples. In this study, used of extraction method clean-up (Solid Phase Extraction) and incubation period for derivatisation in overnight 16 h. The decision limits (CC_α) were 0.11–0.21 µg kg^{-1}, and the detection capabilities (CC_β) 0.19–0.36 µg kg^{-1}, thus below the minimum required performance limit (MRPL) set at 1 µg kg^{-1} by the EU.

In other study by S. Szilagyi and B. de la Calle [12], determination of semicarbazide in fresh egg and in egg powder based on the use of liquid chromatography tandem mass spectrometry. In this study, only semicarbazide was determinated and used method of extraction clean-up and incubation period for derivatisation in overnight 16 h. The CC_α values obtained were 0.41, 0.89 and 0.91 µg $kg^{-1}$for fresh whole egg, industrial whole egg and egg white powders, respectively. The CC_β values were 0.46, 0.97 and 0.95 µg kg^{-1} for fresh whole egg, industrial whole egg and egg white powders, respectively.

Another study, conducted by Back C. et al. [15] a method for the detection and determination of nitrofuran derivatives in egg by liquid chromatography–tandem mass spectrometry (LC–MS/MS) was validated with the software InterVal and can be applied for the confirmation of nitrofuran metabolites in fresh or lyophilised eggs. Method of extraction clean-up and incubation period for derivatisation in overnight 16 h was used in this study.

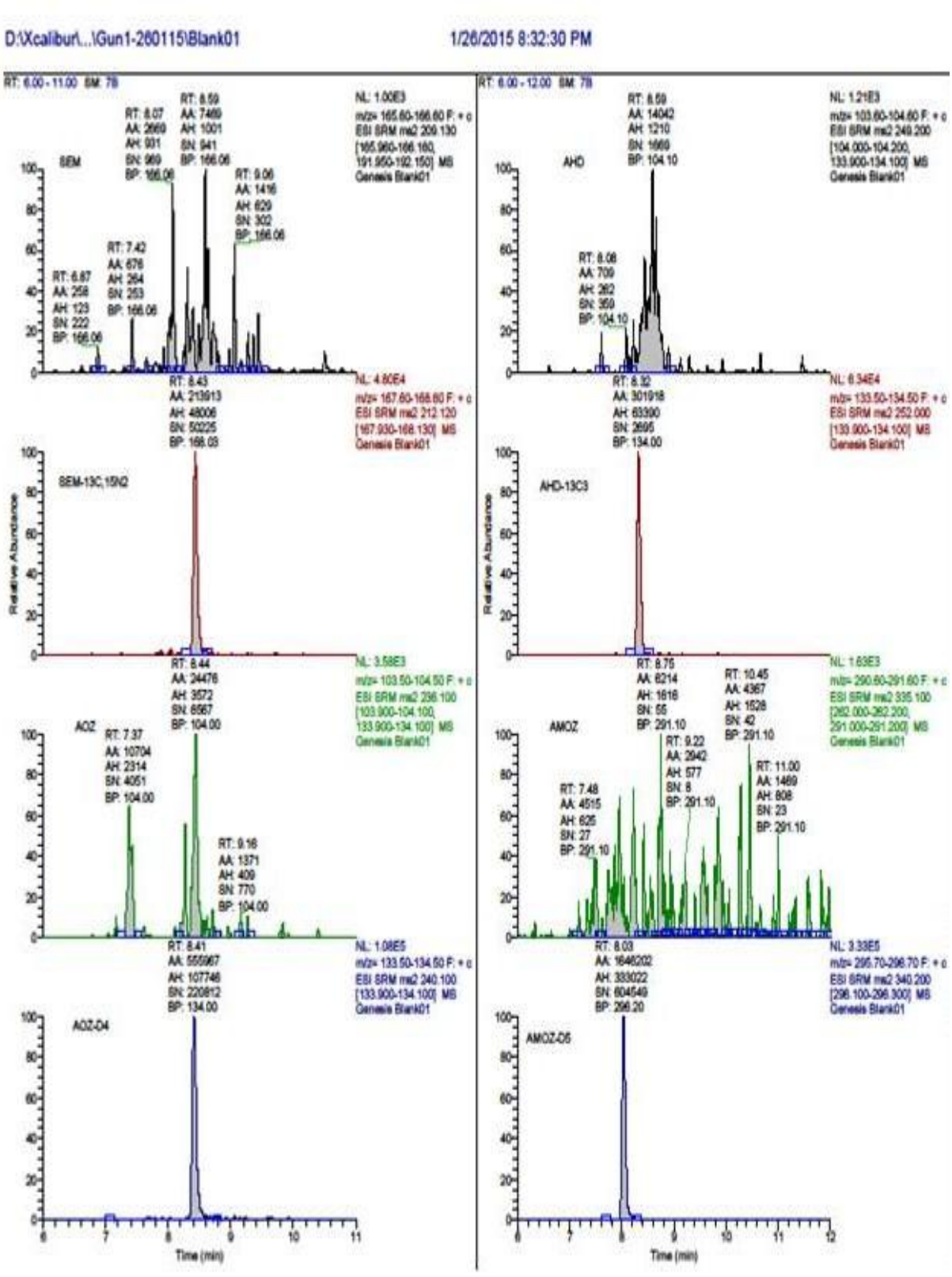

Figure 1: Chromatograms of blank egg sample

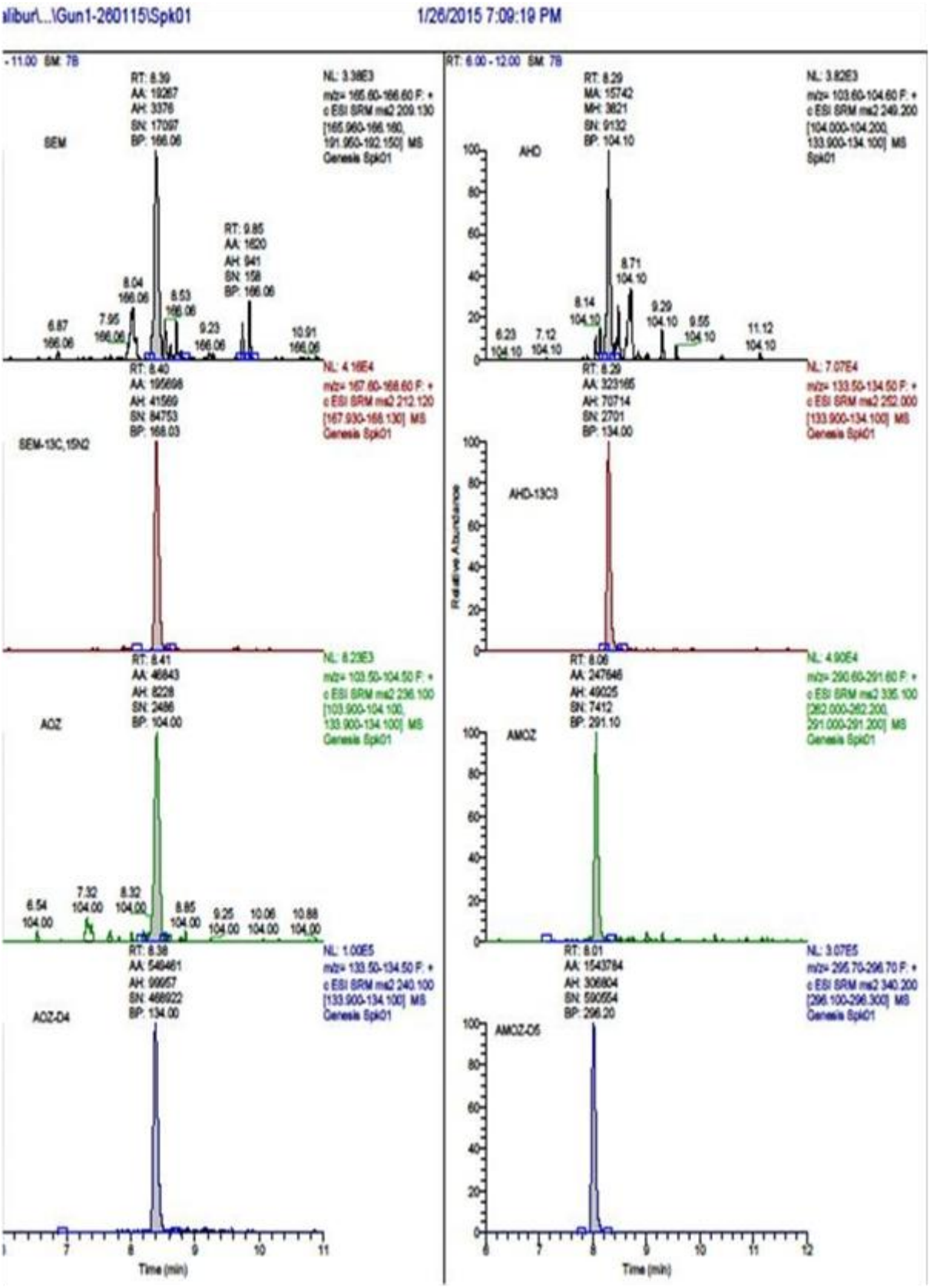

Figure 2: Chromatograms of blank egg sample fortified at 0.2 µg kg^{-1} for AMOZ, 0.3 µg kg^{-1} for AOZ, 0.5 µg kg^{-1} for AHD and SEM.

The obtained validation results indicate the accordance of the method with Decision 2002/657/EC. The repeatability and

within-laboratory reproducibility (precision) of the method are less than 9.86 % for all analytes. The $CC_{\alpha}$and CC_{β} are below the MRPL of 1 µg kg^{-1}. This new analysis method has been used for routine confirmatory analysis of nitrofuran metabolites in egg samples.

V. CONCLUSION

A rapid and sensitive method described in this paper provides reliable, simultaneous quantitative analysis of nitrofuran metabolites residues in egg samples. The optimized procedure provides significant advantages including simplicity, employment of usual laboratory equipment, avoids the use of clean-up by SPE and short extraction time.

Thus should be performed quickly confirmative analysis in egg samples and used as a routine analysis.

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