The Pharma Innovation Journal 2020; 9(6): 29-35

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2020; 9(6): 29-35

www.thepharmajournal.com
Received: 16-04-2020
Accepted: 18-05-2020

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Development and validation of analytical methods for monitoring program for control of residues of Pharmacologically active substances in tissues with animal origin

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Abstract
Food safety is a part of the Bulgarian policy, by means of the National monitoring program for control of residues (NMPCR), performed by Central laboratory of veterinary control and ecology (CLVCE) part of Bulgarian Food Safety Agency (BFSA). Herein, two analytical methods are proposed - for simultaneous determination of regulated nitroimidazoles and their by Liquid chromatography with mass spectrometry (LC-MS/MS) and for determination of N-methyl carbamates in liver samples by high-performance liquid chromatography with fluorescence detection (HPLV-FLD). The sample pre-treatment for nitroimidazoles involves one step liquid extraction and purification by solid-phase extraction. The nitroimidazoles were separated on Gemini C18 chromatography column with gradient eluting program. Residues were quantified down to 1.0 µg kg^{-1} with limits of detection and quantification ranging below from determinate limits of 3 ppb. The recoveries at the minimum required performance limits (MRPL) level were between 77 and 120% with RSD values lower than 20%. N-methyl carbamates are part of group B2(c) carbamates and pyrethroids. Four compounds were validated in liver as a target organ - methomyl, aldicarb, carbofuran and propoxur. The separation is performed on a 25cm, C18 column from Pickering and determination by HPLC-FLD with post-column derivatization. The reliability of the method is ensured by validation in accordance with SANTE document. Limits of quantification (LOQs) are below the maximum residue levels (MRLs) with recoveries between 85 and 105% and measurement uncertainty (MU) below 50%, as recommended.

Keywords: Nitroimidazoles, carbamates, pesticides, residue, VMP

Introduction
Bulgarian Food Safety Agency has the responsibility to ensure the food safety throughout the food chain. That includes the control of residues of veterinary medicine products (VMP) and of environmental contaminants in food of animal origin. The function and organization of theses duties are determined in EU Regulation 2017/625 [1] and Directive 96/23/EC [2]. Each EU Member State should ensure the possibility of carrying out official controls and must determine official laboratory for residue control. CLVCE fulfill the criteria to perform the NMPCR. In Bulgaria Central Laboratory of Veterinary Control and Ecology is defined as National Reference Laboratory (NRL)

1. Residues of products with anabolic effect and un authorized substances in biological fluids, raw materials and foodstuffs of animal origin (groups A1, A2, A3, A4, A5 and A6 of Annex I to Directive 96/23/EC).
2. Residues of veterinary medicinal products in biological fluids, raw materials and food of animal origin (groups B1, B2a, B2b, B2d, B2e and B3e of Annex I to Directive 96/23/ EC).
3. Residues of environmental pollutants (contaminants) in raw materials and food of animal origin (groups B2c, B3a, B3b, B3c and B3d of Annex I to Directive 96/23/ EC).
4. Residues of gamma emitting radionuclides in waters, beverages, feed and food (Group B3f of Annex I to Directive 96/23/ EC).
5. Safety and quality of honey and bee products.

The extensive use of veterinary drugs in husbandry can result in residues of (VMP) in food with animal origin as eggs, milk, honey etc. To ensure the health safety of the consumers the health authorities have determinate strict tolerance levels for these compounds. There are

specific rules for samples and group of substances which have to be look for. In regard of effective, strong and responsible control Directive 96/23/EC [2] obligate the EU member states to do annually monitoring plan for substances and their residues, used legally or illegally during the process of animal growth and the food with animal origin. The substances according Directive are divided into two groups. One of the groups includes unauthorized substances. As unauthorized substances, one of the major classes is nitroimidazoles group. Because of high misuse potential, nitroimidazoles are included in the monitoring programs for residues of veterinary drugs (Council Regulation 2377/90/EC [3], Directive 96/23/EC [2], Commission Regulation (EU) No 37/2010) [4]. In order to do the official determination and confirmation, exist specific requirements for analytical methods by which analysis and control of official samples has to be done. These rules were determinate in Commission Decision 2002/657/EC [5]. To guide the member states and to harmonize the work of national reference laboratories (NRLs), there is a Guide Paper 2007 (CRL Guide 2007) [6], with technical recommendations for the analytical methods, so called "recommended concentration", determinates as 3µg.kg^{-1} for nitroimidazoles. Nitroimidazoles are broad-spectrum of antimicrobial agents used for prophylactic and therapy of different disease of livestock and aquaculture, applied against anaerobic and bacterial, and as parasitic agents. Nitroimidazoles have potential mutagenic and carcinogenic effect, that's why their use in husbandry for food producing animal is banned. The nitroimidazoles have not been licensed for use neither in EU member states, nor in the USA, Canada, China, and Japan. In some countries their use is not prohibited. That means potential presence of these compounds in food with origin. Because of high misuse potential, nitroimidazoles are included in the monitoring programs for VMPs residues of EU countries. According scientific studies, the metabolites contain the imidazole ring and these nitroimidazloe-products can provoke the same mutagenic and carcinogenic symptoms as the main nitroimidazoles. As a consequence no MRLs could be determinate neither for main nitroimidazoles, nor for their metabolites. It is known that the European human health authorities have imposed criteria for food quality, as in Council Regulation 2377/90/EC. The need of monitoring of a number of drug residues in a broad spectrum of matrices provokes intense research on developing and validation of analytical methods for residues determination at trace levels in ecologically and user friendly manner. The presence of them in food of animal origin requires detection of the main compounds and their hydroxyl metabolites at trace levels [7]. Even cooking the main part of nitroimidazoles molecule and their hydroxyl metabolites were not destroyed [8, 9]. In literature different analytical methods in different type of matrices are described. Thorough reviews on the recent advances in nitroimidazoles determination in food samples have been published [11, 27]. In the recent years, since 2010 up to now, there are some studies representing analysis of nitroimidazoles in milk [10, 14], eggs [12, 13, 15, 17], milk and eggs [12, 13]. Relative rare are the articles for honey [11, 18, 19], cosmetics [8], management of the control of the selected VMPs [9, 20] and proficiency testing of food control [21].

The Directive 96/23/EC, 1996 [2] defines the pesticides as environmental contaminants (group B), and their use is not prohibited. Therefore, some traces of them can be found in foods, but maximum residue levels (MRLs) are set in Regulation 396/2005 for foods of animal and plant origin (REGULATION EC No396/2005) [22]. The aim of the European commission is to lower as much as possible the legal MRLs. This depends on the capability of the laboratories and the techniques they perform at the lab. The better the methods, the lower the limits of quantifications (LOQs). Specific requirements on analytical quality control and validation procedures for pesticide residues analysis in food and feed are written in SANTE/12682/2019 [23]. These documents require each member of the EU to establish annual monitoring program for the control of residues in foods and feed (NMPCR).

Traditionally, the sample preparation of complex samples, such as products of animal origin, is carried out in a sequence of extraction and clean-up steps. LeDoux summarized that the most widely used pesticide extraction technique from foods of animal origin are: solid–liquid extraction, the traditional Soxhlet extraction method, supercritical fluid extraction, accelerated solvent extraction (ASE), microwave-assisted extraction, matrix solid-phase dispersion and the clean-up steps are mainly performed by: freezing centrifugation, liquid–liquid partitioning, gel permeation chromatography (GPC), solid-phase extraction and solid-phase micro-extraction [30]. Nowadays, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method is widely used for extracting a wide range of pesticides in fruit and vegetables and become very popular since it was introduced in 2003 by Anastassiades and coworkers [24]. The method is characterized by using the polar solvent acetonitrile for extraction of water containing matrices with addition of salts in order to get phase separation. Since its introduction, this method has been readily accepted by many pesticide residue analysts because of its low cost, fast, the accurate procedures are no time consuming. The QuEChERS approach is very flexible and it serves as a template for modification depending on the analyte properties, matrix composition, etc. The ruggedness characteristics of the QuEChERS approach have been thoroughly evaluated in the original [24] and subsequent publications [25, 29]. The disadvantage of that methodology is the possibility of lower recoveries and respectively higher matrix effect if the final extract is not clean enough. After the extraction and purification procedures, pesticides need to be separated and further determined. N-methyl carbamates are LC-amendable pesticides. Normally they are analyzed by LC-MS/MS or HPLC-Fld with post-column derivatization [30].

The aim of this work is to present a development and validation of an analytical method for the simultaneous determination of banned nitroimidazoles as metronidazole, dimetridazole, ipronidazole, ronidazole and their hydroxyl metabolites in milk, as well as new methodology for determination of 4 N-methyl carbamates in liver samples since new, lower, MRLs are set in the legislation.

Materials and methods

1. Nitroimidazoles

An instrumental method for the chromatographic analysis of these substances in muscle, applied in CLVCE, was a starting point for the present study. The nitroimidazole determination was carried out in a LC–MS/MS System TSQ Quantum Discovery MAX (Thermo Electron Corporation). The electrospray ionization–tandem mass spectrometry (ESI–MS/MS) detection of the nitroimidazoles was achieved using a triple stage quadrupole instrument. The negative ionization mode was used, and the ions were monitored in the multiple-reaction monitoring (MRM) mode. The ESI MS/MS conditions were the following: spray voltage (-4) kV; sheath gas (N_2, >95%) 50 (arbitrary units); auxiliary gas (N_2, >95%) 0 (arbitrary units); capillary offset/voltage (-5) V, capillary temperature 300° C). Instrumental control and data analysis

were performed by Qualbrauser application software from Thermo Electron Corporation. The chromatographic column was Phenomenex Gemini C18, 5µm, 110 Å, 150 x 2mm ID equipped with the same pre-column.

The presented method was developed, optimized and validated according to Commission Decision 2002/657/EC [1] in one of the food matrices, recommended by European health authorities: milk. The method can be used as screening and confirmatory method according to the recommendation of health agencies.

The nitroimidazoles, their hydroxyl metabolites and deuterated analogs, used as internal standards, were extracted, cleaned and analyzed by LC-MS/MS, using gradient eluation programme, shown in table 1.

Table 1. Chromatographic conditions Nitroimidazoles

Time, min	Mobile phase A, %	Mobile phase B %	Flow rate, ul/min	Detected compounds
0.0	95	5	250.0	MNZ, DMZ, MNZ-OH, DMZ-OH, RNZ, IPZ, IPZ-OH
0.25	95	5		
12.25	20	80		
12.5	95	5		
19.0	95	5		

It is in compliance with the EU Regulations and Directives regarding the official control, screening and confirmation of banned substances, for substances whose use is not authorized or are specifically prohibited in the Community, in order to ensure harmonized implementation of Directive 96/23/EC [5].

2. N-methyl carbamates

2.1. Reagents and chemicals

Dichloromethane, cyclohexane and methanol (for HPLC gradient grade) were obtained from VWR, water was purified through a Milli-Q system from Millipore (USA). Anhydrous sodium sulphate was supplied by Supelco (USA) and was heated at least 6 hours at 600°C. Strata NH_2 (55um, 70A) 500mg/6mL cartridges.

2.2. Standard solutions

Five certified standards solutions were used: Aldicarb, 99,9% purity, Propoxur, 99,8% purity, Carbofuran, 99,0% purity, Methomyl, 100% purity and 4-bromo-3,5-dimethylphenyl-N-methylcarbamate (BDMC), 99,5% purity. All of them were purchased from Dr. Ehrenstorfer (Germany). The working standard solutions were prepared at 3 levels-50%, 100% and 150% from the maximum residue level (MRL), for each compound according to its MRL, published in Regulation 396/2005 [22] (Table 3). Standard solution for the internal standard (BDMC) was prepared only in concentration 1µg/ml. All standards were diluted in acetonitrile.

2.3. Sample preparation

The liver was homogenized with mixer to generate representative sample of the product. 10 g of the homogenized product were extracted with 10 mL of 50:50 dichloromethane and cyclohexane (v/v) mixture. 100µl of the internal standard solution (1µg/mL) BDMC were added as well. The solvent was collected in a flask and rotary-evaporated till dryness and dissolved with 1mL dichloromethane.

The SPE-NH_2 cartridge is conditioned by 1mL dichloromethane. The sample is eluted by 8mL of dichloromethane: methanol mixture 99/1 (v/v). The eluate is evaporated by rotary evaporator and the dry extract is re-solved with 1mL 85/15 water/methanol (v/v) and put into vial for further HPLC determination.

2.4. HPLC-Fluorescence analysis

Residue levels in liver samples were determined by reversed-phase high-performance liquid chromatography with fluorescence detection after post column derivatization. The separation of the 4 aim carbamates is achieved with the Pickering 5µm, C_{18} column, maintained at constant temperature and with water-methanol gradient as mobile phase (Tab. 2).

Table 2: Gradient of mobile phase

Time	Flow Rate (mL/min)	% A Water	% B Methanol
Initial	0.80	85	15
2.00	0.80	85	15
20.00	0.80	30	70
23.00	0.80	30	70
35.00	0.80	0	100

The derivatization is performed by a specialized module from Pickering with two consecutive reactors. The reagents are ready-to-use mixtures- reagent 1 is 0.05M NaOH and reagent 2 is *o*-phtaldehide (OPA) and thyofluor stabilized in 0.05 M borate buffer.

The separated carbamates are first saponified by treatment with sodium hydroxide at 100 °C. Further, the released methylamine reacts in post-column reaction according to Figure 1, to form a highly fluorescent 1-methyl-2-dimethyl-ethylamine thioisoindole derivate.

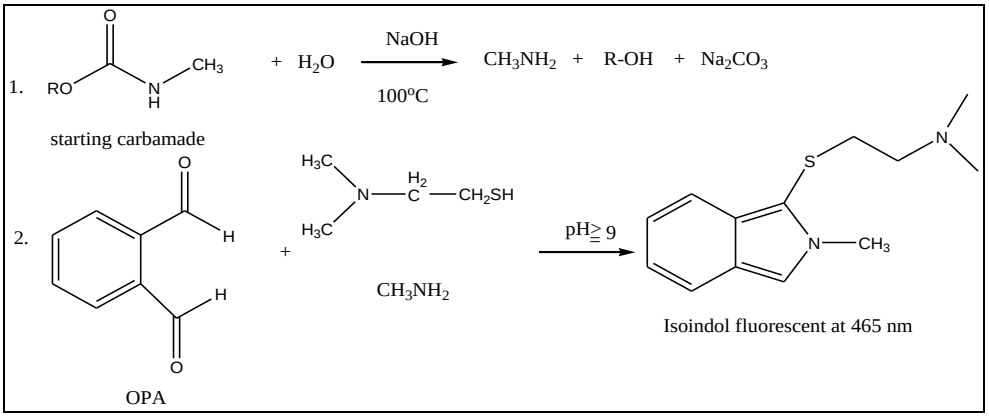

Fig 1: The principle of post-column derivatization reaction.

2.5 Validation

The calibration curve was used for quantitative analysis. For the evaluation of recovery and precision (repeatability and within-laboratory reproducibility), blank liver samples spiked with mixed standard solution at three concentration levels of 50%, 100% and 150% of the MRL values for each compound were used. Spiked samples at each concentration level were analyzed in three series, each on the different day, and each in six replicates. The recoveries were calculated by comparing the determined concentrations of spiked samples to their target level. The precision was determined by calculating the relative standard deviation (RSD). The working range was determined in relation to procedural calibration curve (from spiked samples) of mixed standard solutions prepared on five levels. The linearity was checked by calculation of regression curve and correlation coefficients for each compound of mixed standard solution. The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated on the basis of blank sample analyses (Tab. 3).

Table 3: Validation parameters of investigated 4 N-methyl carbamates

Pesticide	MRL (R. 396/2005) [mg/kg]	Linearity	LOQ [mg/kg]	Spike level [mg/kg]	Recovery [%], (n=18)	Repeatability RSDr [%], (n=6)	Within-laboratory reproducibility RSD_{wR} [%], (n=18)	Uncertainty [%]
Methomyl	0.01	R^2 = 0.9917	0.002	0.005	94.5	10.8	16.7	14.6
				0.010	96.0	6.2	8.6	
				0.015	98.9	10.3	12.1	
Aldicarb	0.01	R^2 = 0.9863	0.003	0.005	101.1	11.4	12.3	17.8
				0.010	104.7	10.6	14.7	
				0.015	111.2	9.4	9.6	
Carbofuran	0.01	R^2 = 0.982	0.007	0.005	92.7	8.4	16.4	16.8
				0.010	93.1	9.5	9.0	
				0.015	94.4	5.1	6.3	
Propoxur	0.05	R^2 = 0.994	0.03	0.025	100.9	5.5	11.3	17.9
				0.050	103.6	7.9	7.6	
				0.075	106.0	9.3	7.7	

Results and Discussion

Milk was chosen as a high protein liquid matrix. Despite scarcity of scientific data for nitroimidazles hydrophilicity (acidic, basic or amphoteric ones) or their lipophilicity, number of papers implies references for extraction by polar organic or hydro-organic solvents [7, 36]. Sample extraction protocol was optimized in terms of number of the extractions, type and volume of the extraction solvent, solvent evaporation technique. The method was validated according to Commission Decision 2002/657/EC [5]. The validation parameters evaluated were specificity, linear range, recovery, precision (repeatability and intra-laboratory reproducibility), and decision limit and detection capability. The method clean-up procedure was optimized. In this work the clean-up procedure based on N-vinylpyrolidone-DVB copolymer cartridge was proposed for the determination of nitroimidazoles in matrices of animal origin milk and is the same as procedure for other class VMP [10, 20]. The sorbent was conditioned with methanol and equilibrated using ammonium acetate buffer. The sample dry extract was dissolved priory to Solid Phase Extraction SPE in ammonium acetate buffer to convert the analytes in the ionic form more firmly trapped in the copolymer sorbent of the column. Matrix components were washed using pure water. A volume of basic methanol (5% NH_3) was used as an elution solution, followed by a volume of pure methanol. The obtained elute was evaporated under N_2 stream at 60 ˚C and the dry residue was dissolved in 1 mL mobile phase prior to the chromatographic analysis. Sample preparations with liquid-liquid extraction and solid phase extraction, developing, validation were described in previous work of Stoilova *et al.* l [31, 32]. It was a starting point for the present investigation. The unification of sample preparation stage for different matrices, numerous compounds and different classes of target analytes, is very helpful for daily and routine laboratory praxis. The obtained recoveries of all of nitroimidazoles (each lower than determinate MRPL of 3.0 μg kg^{-1}) were around 100% with good signal resolution. The combination of the proposed extraction and HLB-type cartridge based clean-up procedure using acetonitrile as a solvent improves the target analytes isolation and concentration. No matrix interference was noticed in studied tissue milk. The method was validated as per Decision 2002/657/EC [39]. According Wang *et al.* [33] this all overlaps the requirements of Codex Alimentarius Commission, which includes mandatory determining of analyte stability, ruggedness/robustness, calibration curve, analytical range, linearity, sensitivity, selectivity/specificity, accuracy, recovery, precision/repeatability and reproducibility, measurements uncertainty, sample stability, method comparisons, and an extra establishing of limit of detection and limit of quantification. Under Commission Decision 2002/657/EC [39] for analytes with established maximum required performance limit (MRPL), the validation parameters were estimated at concentration levels consistent with these MRPL-values: 0.5, 1, 1.5 times MRPL. All samples were submitted to liquid extraction, SPE and LC-MS/MS procedure by triplicate. Finally, CC_α and CC_β values were determined (Table 4). The CC_α, defined as the concentration above which it can be concluded that a sample is non-compliant with an error probability α, was calculated using the approach of calibration curves procedure. The blank samples were fortified at three concentration levels of mixture of analytes each consistent with MRPL concentration. CC_α was calculated as a sum of 0.5MRPL and 2.33 times standard deviation (α=5%). Detection capability CC_β is the minimum concentration at which the method can detect contaminated samples with an error probability β. It was calculated using calibration curve approach.

Table 4: Decisions limit (CCα) and detection capability (CCβ) for all the nitroimidazole residues in matrix milk:

Milk	DMZ	DMZ-OH	MNZ	MNZ-OH	RNZ	IPZ	IPZ-OH
CCα/CCβ, [µg/kg]	1.07/1.38	1.52/1.95	1.49/1.92	1.28/1.64	1.27/1.63	0.69/0.89	1.01/1.29

The blank matrix samples were fortified at and below MRPL. The CC_{β} was calculated as the decision limit plus 1.64 times the corresponding standard deviations when analyzing 20 blank samples spiked at lower than MRPL level. Tipical LC-MS/MS chromatograms of a blank solvent, a blank milk sample, and a milk sample spiked with nitroimidazoles mixture at MRPL before sample preparation is shown at Figure 2. The results are summarized in Tables 5 and 6.

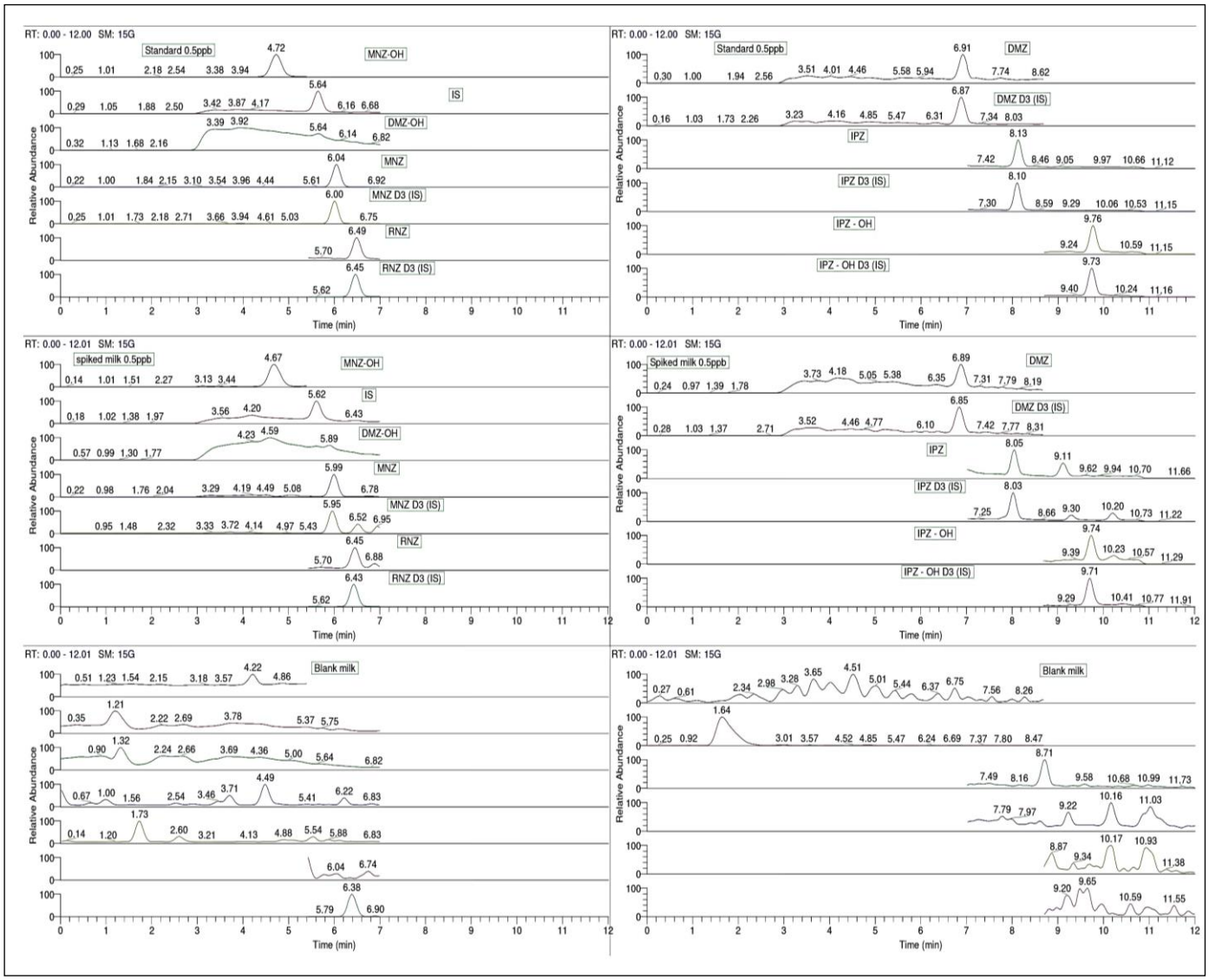

Fig 2: LC-MS/MS chromatograms of a blank solvent, a blank milk sample, and a milk sample spiked with nitroimidazoles mixture at MRPL before sample preparation: (1) MNZ-OH; (2) DMZ-OH; (3) MNZ; (4) DMZ; (5) RNZ; (6) IPZ; (7) IPZ-OH

Table 5: Transitions followed for identification; only one is used for quantitation (in bold)

Analyte	Parent ion, m/z	1 Product ion m/z	2 Product ion m/z
MNZ	172.1	142.6	128.1
MNZ-OH	188.07	126.03	123.03
MNZ-D3			
DMZ	142.06	113.03	96.06
DMZ-OH	158.06	140.05	111.06
DMZ-D3			
RNZ	201.06	140.05	126.03
IPZ	170.09	140.11	124.1
IPZ-OH	186.09	168.08	128.05
IPZ D3			

Table 6: Validation results for milk

	MRPL, µg kg^{-1}	Recovery, %	RSD, %	Range, µg kg^{-1}	Slope	Intercept	r
MNZ	3.0	93	32	0-4.5	30.586	8.8038	0.9441
MNZ-OH	3.0	116	32	0-4.5	0.2233	0.0418	0.9988
DMZ	3.0	75	32	0-4.5	2.7521	0.4819	0.9754
DMZ-OH	3.0	101	33	0-4.5	0.8784	0.1125	0.9913
RNZ	3.0	65	29	0-4.5	0.3309	0.1798	0.9916
IPZ	3.0	110	22	0-4.5	0.426	0.0117	0.9776
IPZ-OH	3.0	94	36	0-4.5	1.9278	0.0187	0.9906

Recovery and RSD were determinate at concentration level of 1.0 ppb; n=54

The method for determination of N-methyl carbamates was validated using liver samples, spiked at three concentration levels, according to the MRL values for each compound in mg/kg.

Performance characteristics, such as linearity, LOQ, precision and recovery were studied. All validation parameters obtained for N-methyl carbamates determination in spiked samples are listed in Table 3. Estimated validation parameters of the method were satisfactory. According to the legislation (SANTE/12682/2019 Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed) [23], LOQ must be ≤ MRL, R^2 must be ≥0.95, for recovery: 70-120% is allowed, for precision (repeatability and reproducibility): RSD value must be ≤ 20% and for measurement uncertainty: ≤ 50%. The accuracy of the method was expressed as mean recoveries, and they were all higher than 92% for all spiked levels and all compounds. The method shows good linearity and LOQs for the studied pesticides in the ranged from 0.002 to 0.03 mg/kg.

The newly developed analytical method for determination of N-methyl carbamates residues, allows very low concentration levels to be determined. A presented sample preparation is specific for this compound group and performed good clean-up and respectively lowers matrix effect. The validation is performed in accordance with the recommendations in the Document SANTE/12682/2019 [22] and it meets the acceptability criteria for precision, mean recovery and limits of quantification.

Conclusions

A multispecies method for nitroimidazoles determination based on liquid chromatography with masspectrometry detection has been introduced, as well as method for determination of four N-methyl carbamates - aldicarb, methomyl, carbofuran and propoxur. An acetonitrile based extraction protocol and clean-up procedure were proposed for nitroimidazoles. The sample preparation was proved to be efficient enough for simultaneous extraction from every of the studied matrices. The method was validated for determination of metronidazole, dimetridazole, ipronidazole, ronidazole and their metabolites in samples of animal origin: milk. For N-methyl carbamates the used extraction includes dichloromethane/cyclohexane mixture, followed by SPE clean-up. The instrumental analysis was done by HPLC-Fld with post-column derivatization. By the validation procedures was proved that the both methods are reliable and can ensure the food safety. Both methods are approved as a within-laboratory reference method at the Bulgarian Reference Laboratory.

Acknowledgements

The work is part of annual plan for developing of new analytical methods in Central Laboratory of Veterinary Control and Ecology, supported by Bulgarian Food Safety Agency.

References

1. Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/ EC and Council Decision 92/438/EEC (Official Controls Regulation); SANCO/2004/2726-rev 4-December 20081 Guidelines for the implementation of decision 2002/657/EC
2. Council Directive 96/23/EC
3. EEC 1990. Council regulation (EEC) no 2377/90 of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Commun. L 224 (1e8), 18.8.90
4. EU 2010. Commission regulation (EU) no 37/2010 of 22 December 2009 on pharmacologically
5. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. Eur. Communities, L221/8/17.8.2002.
6. CRL Guidance paper 2007.
7. Mitrowska K, Posyniak A, Zmudzki J. Selective determination of fourteen nitroimidazoles in honey by high-performance liquid chromatography–tandem mass spectrometry. Analytical Letters 2014; 47(10):1634-1649.
8. Rose D, Bygrave J, Sharman M. Effect of cooking on veterinary drug residues in food, Part 9 Nitroimidazoles, Analyst, 1999, 12-289.
9. Baynes RE, Dedonder K, Kissell L, Mzyk D, Marmulak T, Smith G *et al*. Health Concerns and Management of Select Veterinary Drug Residues Food and Chemical Toxicology. 2016; 88:112e122.
10. Blazevic N, Kayfez F, Suvijic V. σ -Values of some nitroimidazoles. J Heterocy Chem. 1970; 7:227.
11. Constantinou S, Kanari P, Papachrysostomou C, Hadjigeorgiou M. Determination of nitroimidazoles and their metabolites in milk using GC//MS-NC II Symposium 6-7 November 2006 Limassol Cyprus, Veterinary Drug Residues Laboratory, State General Laboratory. Kimonos, Nicosia, Cyprus.
12. Piatkowska M, Jedziniak P, Zmudzki J. Comparison of

different sample preparation procedures for multiclass determination of selected veterinary drug, coccidiostat and insecticide residues in eggs by liquid chromatography-tandem mass spectrometry. Anal. Methods. 2014; 6:30-34.
13. Granja R, Nino A, Reche K, Giannotti F, Lima A, Wanschel A *et al*. Determination and confirmation of metronidazole, dimetridazole, ronidazole and their metabolites in bovine muscle by LC-MS/MS. Food Additives and Contaminants: Part A, 2013; 30(6):970-976.
14. Shimelis O, Wihlborg A, Rudolfsson M, Boyd B, Trinh A. Extraction of Nitroimidazoles from milk and egg samples using Supel MIP SPE Nitroimidazoles and LC-MS/MS, Sigma-Aldrich Technical Report.
15. Mitrowska K, Posyniak A, Zmudzki J. Selective determination of fourteen nitroimidazoles in honey by high-performance liquid chromatography–tandem mass spectrometry. Analytical Letters. 2014; 47(10):1634-1649.
16. Mitrowska K, Antcsak M, Posyniak A. Confirmatory method for the determination of nitroimidazoles in milk by high-performance liquid chromatography–tandem mass spectrometry. Bulletin Veterinary Institute in Pulawy. 2014; 58:581-587.
17. Stoyke M, Polzer J, Radeck W, Gowik P. Proficiency testing in food control: an evaluation of more than 13 years. Journal für verbraucherschutz und Lebensmittelsicherheit, 2015. DOI: 10.1007/s00003-015-0955-7.
18. Cronly M, Behan P, Foley B, Malone E, Martin S, Doyle M *et al*. Rapid multi-class multi-residue method for the confirmation of chloramphenicol and eleven nitroimidazoles in milk and honey by liquid chromatography tandem mass spectrometry. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 2010. DOI: 10.1080/19440049.2010.489579.
19. Feketeova L, Postler J, Zavras A, Scheier P, Denifld S, Richard A *et al*. Decomposition of nitroimidazole ions: Experiment and theory. Phys. Chem. Chem. Phys. 2015; 17:125-98.
20. Cho MJ, Kurtz RR, Lewis C, Machkovech SM, Houser DJ. Metronidazole Phosphate-A Water-Soluble Prodrug for Parenteral Solutions of Metronidazole. Pharmaceutical Research and Development Division. 1982; 71(4):410-414.
21. Piatkowska M, Jedziniak P, Zmudzki J. Multiresidue method for the simultaneous determination of veterinary medicinal products, feed additives and illegal dyes in eggs using liquid chromatography–tandem mass spectrometry. Food Chemistry. 2016; 197:571-580.
22. Regulation 396/2005.
23. SANTE/12682/2019 Main changes introduced in Document № SANTE/12682/2019 with respect to the previous version (Document № SANTE/11813/2017) Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed.
24. Anastassiades M, Lehotay J, Stajnbaher D, Schenck J. Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. J AOAC Int. 2003; 86:412-431.
25. Lehotay J, Son A, Kwon H, Koesukwiwat U, Fu W, Mastovska K *et al*. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. J. Chromatogr. 2010; A, 1217: 2548-2560.
26. Aguilera-Luiz M, Vidal M, Romero-González R, Frenich G. Multi-residue determination of veterinary drugs in milk by ultra high pressure liquid chromatograph- tandem mass spectrometry. J. Chromatogr. 2008; A, 1205:10-16.
27. Cunha C, Lehotay J, Mastovska K, Fernandes O, Beatriz M, Oliveira P *et al*. Evaluation of the QuECHERS sample preparation approach for the analysis of pesticide residues in olives. J Sep. Sci. 2007; 30(4):620 -632.
28. Lehotay J, Mastvoska K, Yun J. Evaluation of Two Fast and Easy Methods for Pesticide Residue Analysis in Fatty Food Matrixes. J AOAC Int. 2005a; 88:630 -638.
29. Stoykova I, Yankovska-Stefanova T, Yotova L, Danalev D. Modified QuEChERS method for determination of methomyl, aldicarb, carbofuran and propoxur in liver. Chemistry: Bulgarian Journal of Science Education 2015; 24:390-396.
30. LeDoux M. Analytical methods applied to the determination of pesticide residues in foods of animal origin. A review of the past two decades, Journal of Chromatography A. 2011; (1218):1021-1036.
31. Stoilova N, Surleva A, Stoev G. Determination of Quinolononones in Food of Animal Origin by Liquid Chromatography Coupled with Fluorescence and Mass Spectrometric Detection. Acta Chromatographica. 2014; 26(4):599-614.
32. Stoilova N, Surleva A, Stoev G. Simultaneous Determination of Nine Quinolones in Food by Liquid Chromatography with Fluorescence Detection, Food Anal. Methods. 2013; 6:803-813.
33. Wang J, MacNail SD, Kaj JF. Chemical analysis of antibiotic residues in food. John Wiley & Sons, Inc. Publication. PDF ISBN: 978-1-118-06718-5.
34. Baynes E, Dedonder K, Kissell L, Mzyk D, Marmulak T, Smith G *et al*. Health Concerns and Management of Select Veterinary Drug Residues Food and Chemical Toxicology. 2016; 88:112e122.
35. Blazevic N, Kayfez F, Suvijic V. σ -Values of some nitroimidazoles. J. Heterocy. Chem, 1970.
36. Shimelis O, Wihlborg A, Rudolfsson M, Boyd B, Trinh A. Extraction of Nitroimidazoles from milk and egg samples using Supel MIP SPE Nitroimidazoles and LC-MS/MS, Sigma-Aldrich Technical Report.
37. Xianshuang M, Qiang M. Simultaneous determination of 15 nitroimidazoles in cosmetics by HPLC coupled with electrospray ionization-tandem mass spectrometry. Journal of AOAC International. 2014; 97(6):13-35.
38. Yuanyuan W, Xiaowei L, Zhiwen Z, Shuangyang D, Haiyang J, Jiancheng L *et al*. Simultaneous determination of nitroimidazoles, benzimidazoles, and chloramphenicol components in bovine milk by ultra-high performance liquid chromatography–tandem mass spectrometry. Food Chemistry. 2016; 192:280-287.
39. Commission Decision 2002/657/EC establish criteria and procedures for the quality and comparability of analytical results obtained by analysis in different laboratories in matrices with animal origin