VILNIUS UNIVERSITY
INSTITUTE OF CHEMISTRY

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LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF ANTIBIOTICS IN FOOD PRODUCTS

Summary of doctoral dissertation

Physical sciences, chemistry (03 P)

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SKYSČIŲ CHROMATOGRAFIJA–TANDEMINĖ MASIŲ SPEKTROMETRIJA ANTIBIOTIKŲ NUSTATYMUI MAISTO PRODUKTUOSE

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1. INTRODUCTION

The large quantities of antibiotics used in veterinary medicine to prevent and to treat diseases and as supplement to promote growth in food animals have led to their occurrence in the food products. Consumption of harmful or toxic compounds in foods is a major cause for incidents of poisoning and disease pathogenesis, with cancer being the most prevalent. For example, sulfonamide residues in food are of increasing concern due to their carcinogenic potency and their contribution to the increase of antibiotic resistance. In order to protect human health, governments and international organizations are regulating the use of food contaminants such as antibiotics, pesticides, mycotoxins and others, setting maximum residue levels (MRLs) in foods. Some of them (e.g., chloramphenicol, nitroimidazoles etc.) are banned from use in food producing animals within the European Union and other countries. Consequently, effective monitoring of such residues in food of animal origin has become one of the most important duties for public health agencies.

During its 100 year history chromatography together with its various techniques has won a firm place amongst the instrumental methods of analysis. The popularity of this method was determined by the fact that it enables simultaneous separation and quantitative determination of analytes. High-performance liquid chromatography (HPLC) has become the technique of choice for the analysis of antibiotics, which are rather polar, nonvolatile, and in some cases thermally labile, without the need for derivatization. In the past 10 years the on-line combination of HPLC and mass spectrometry (MS) has developed into a widely applied and routinely applicable detection and on-line identification approach for HPLC. More recently, introduction of a second analyzer led to the development of tandem mass spectroscopy (MS/MS), which allows detailed structural information of a fragment ion or the identification of compounds found in complex mixtures such as food. The two mass spectrometers in series are connected by a chamber, the collision cell. Mass analysis is carried out in two stages where the sample is “sorted” and “weighed” in the first mass spectrometer, fragmented in the collision cell, and then a selected fragment or fragments “sorted” and “weighed” in the second mass spectrometer. HPLC coupled with tandem mass spectrometry (MS/MS) offers numerous advantages with respect to selectivity and

sensitivity in low quantities of “dirty” food extracts. The increased selectivity of these techniques reduces the influence of the matrix and also lowers the limits of the detection. However, the chromatographic part of HPLC-MS/MS technique is responsible for some limitations as well. For example, the multi-component analysis of complex samples often requires separation times of 30 min and more.

Recent advances in HPLC instrumentation have enabled the development of a new liquid chromatography technique termed ultra performance liquid chromatography (UPLC). A high speed of analysis, greater resolution, higher peak capacity and sensitivity are obtained due to the novel technology that utilizes a new generation of columns packed with pressure stable 1.7 μm hybrid material particles and novel low dead volume, very high pressure (1000 bar) equipment. As efficiency and speed of analysis has become of a great importance in many routine application of liquid chromatography, UPLC could play a significant role in the future of liquid chromatography.

The aim of this work was systematic investigation and validation of high-performance and/or ultra-performance liquid chromatography coupled with tandem mass spectrometry for the determination of some antibiotics in food products.

The main tasks set to achieve the aim were the following:

- optimization of the HPLC/UPLC and MS/MS conditions for the separation and detection of chloramphenicol, sulfonamides, nitroimidazoles and their metabolites
- development of effective and reliable sample extraction and clean-up procedures for antibiotics studied
- systematic evaluation of the analytical performance of the developed HPLC/UPLC-MS/MS methods in accordance with EU requirements
- application of the methods for the monitoring of antibiotic residues in Lithuanian food products.

2. INSTRUMENTATION

HPLC-MS/MS analyses were performed using a LC Waters Alliance 2695 separations module (Waters, Milford, MA, USA) coupled via an electrospray interface (ESI) to a tandem mass spectrometer Quattro Ultima Pt Micromass (Wythenshawe, UK).

The instrument was operated in multiple reaction-monitoring (MRM) mode. HPLC separations were performed on a C_{18} SymmetryShield column (10 cm × 2.1 mm I.D., 3.5 μm particle size) fitted with a SymmetryShield C_{18} precolumn (1 cm × 2.1 mm I.D., 3.5 μm particle size) (Waters).

UPLC analyses were performed using a Waters Acquity Ultra Performance LC system (Waters). UPLC separations were performed using an Acquity BEH C18 column (100 mm×2.1 mm, i.d., 1.7 μm particle size, Waters). Detection was performed using a Quattro Premier XE tandem quadrupole mass spectrometer (Waters). The instrument was operated using an ESI source in positive mode with MRM.

3. RESULTS AND DISCUSSION

3.1. HPLC-MS/MS determination of chloramphenicol

Chloramphenicol (CAP) is an effective antibiotic that has widely been used since the 1950s to treat food-producing animals (Fig. 1). Because of the well-known risk of aplastic anemia and carcinogenic properties of CAP, its use in human and veterinary medicine is limited by its toxicity. Consequently, since 1994 the European Community has totally banned the use of CAP in food-producing animal. In this part of the present thesis the potential of HPLC-MS/MS technique for the determination of chloramphenicol in milk was systematically investigated.

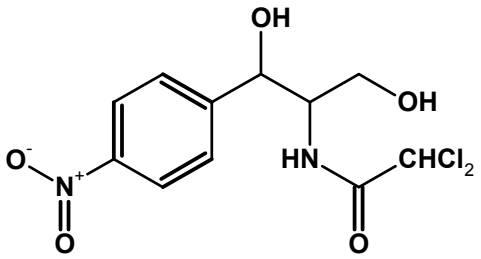

Fig. 1. Structural formula of chloramphenicol.

MS/MS detection. CAP and its deuterated internal standard d_5-CAP were first analyzed in negative ESI-MS mode to select characteristic ions as the precursors (Fig. 2). The full scan mass spectra of CAP and d_5-CAP displayed several intense ions, and the most abundant were *m/z* 321 and *m/z* 326 for CAP and for d_5-CAP, respectively, which correspond to the deprotonated molecular ion $(M–H)^-$. Both CAP and d_5-CAP were then analyzed by HPLC–ESI–MS/MS in negative ionization product ion scan mode by

selecting *m/z* 321 and *m/z* 326 ions as the precursor ion, respectively. The full scan mass spectra of these ions are compared in Fig. 3. As can be seen, two main fragment ions were obtained from the collision induced dissociation experiments of these ions giving rise to respectively *m/z* 257 and *m/z* 152, *m/z* 262 and *m/z* 157. Concentrations were calculated by comparing the ratio of *m/z* 321→152 response CAP with the ratio of *m/z* 326→157 response d_5-CAP.

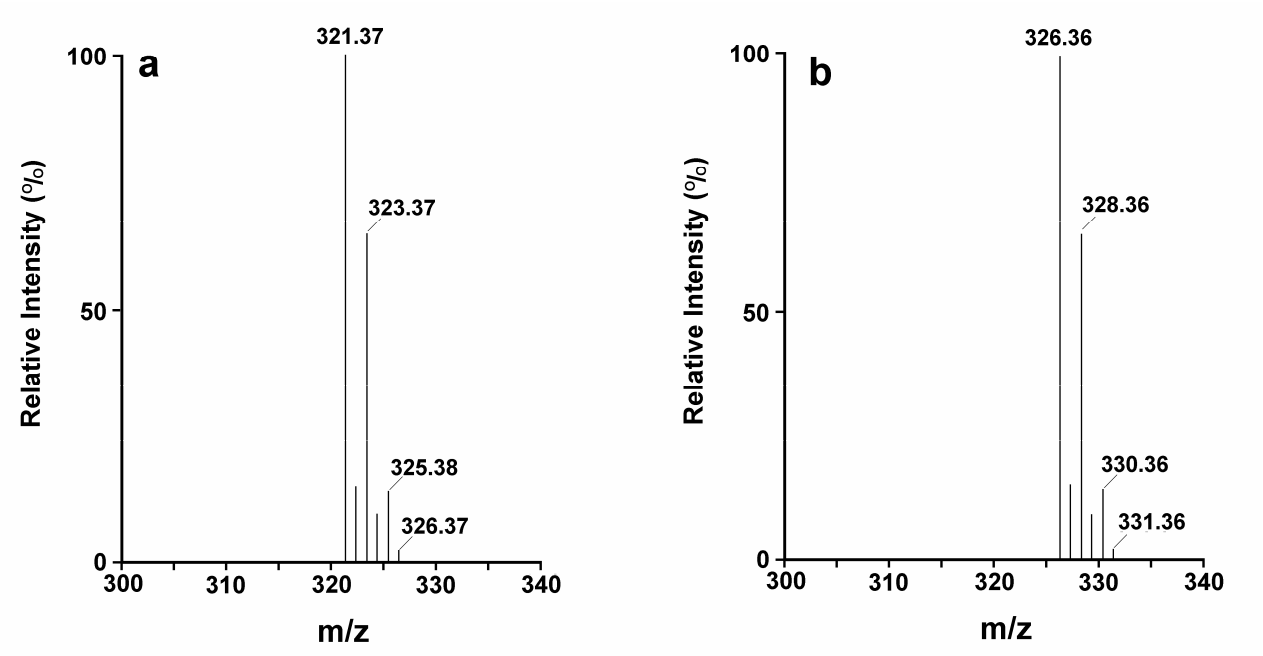

Fig. 2. The full scan mass spectra of (a) CAP and (b) d_5-CAP.

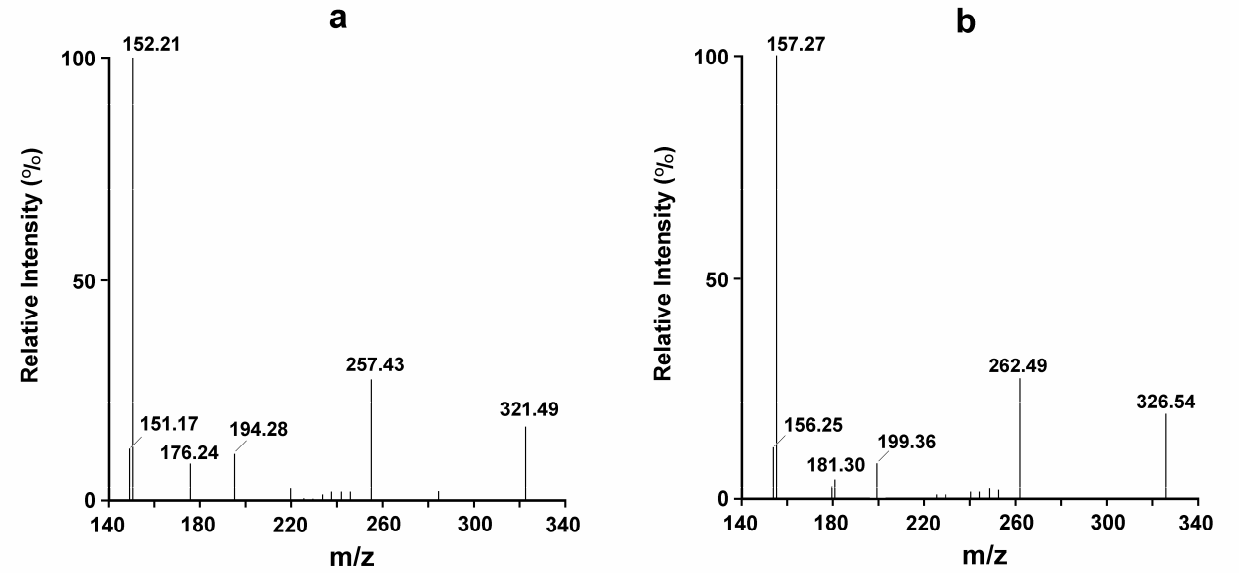

Fig. 3. Collision induced dissociation mass spectra of (a) CAP *m/z* 321 and (b) d_5-CAP *m/z* 326.

Sample extraction and clean-up. The extraction efficiency of CAP from spiked blank samples was studied using two relatively polar extractants: diethyl ether and ethyl acetate. The recoveries were determined by comparing the peak area obtained from spiked blank samples with those obtained from aqueous standard solutions. The obtained results showed that ethyl acetate provided higher recoveries of CAP from spiked blanks. In order to further improve the extraction efficiency, we attempted to increase the extraction time (Fig. 4). This resulted in an increase in the absolute recoveries of CAP up to 65–68%.

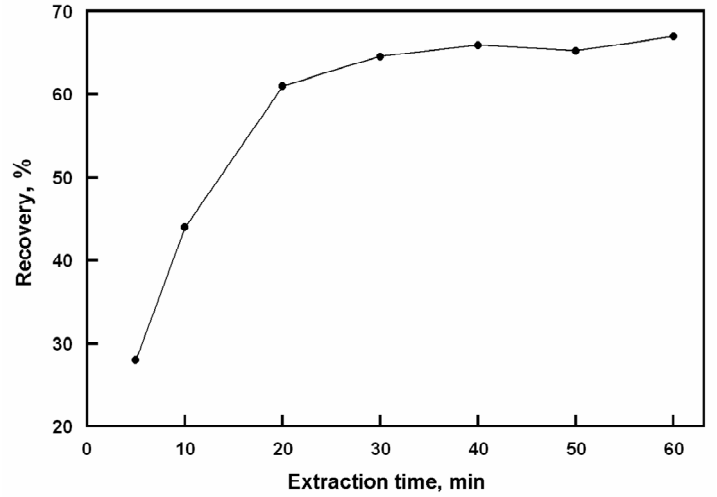

Fig. 4. Effect of extraction time on the absolute recoveries of CAP. 3 mL of defatted milk (dilution 1:1) sample was extracted with 6 mL of ethyl acetate.

The extract from a biological sample contains many diverse compounds in addition to the possible traces of the target analytes. To exclude these interfering substances, an additional clean-up of the extract should be performed. In this study, conventional liquid-liquid extraction was adopted. The following three non-polar extractant systems were studied: carbon tetrachloride, hexane and carbon tetrachloride/hexane (1:1 v/v) mixture. Sample clean-up performance was evaluated by performing a standard addition of CAP to the blank samples just before clean-up procedure in order to avoid the loss of analyte during the first extraction step. The obtained results showed that all three solvents provided adequate recoveries (≥90%), but using carbon tetrachloride and hexane slightly higher amounts of interfering compounds remained in the aqueous phase that did not allow CAP to be screened at low levels.

The complete sample extraction and clean-up procedure optimized for analysis of CAP residue in milk products was as follows. A 10 mL of Milli-Q water was added to 10 g aliquot of milk sample and the sample was defatted by centrifugation for 20 min

(3500×g at -4 ºC). A 3.0 mL of defatted sample was transferred to a glass tube, 6 mL of ethyl acetate and 1 mL of 0.6 ng/mL d_5-CAP standard was added and the mixture was agitated on a minishaker for 45 min. After phase separation, 4 mL of the upper layer (ethyl acetate) was evaporated to dryness at 50 ^{o}C under nitrogen stream. The residue was dissolved in 0.5 mL of the water and then was extracted with 0.5 mL of carbon tetrachloride/hexane (1:1 v/v) for 5 min. After centrifugation for 5 min at 25000 rpm, the aqueous phase was removed and the aliquot of 20 µL was injected on the HPLC column.

Analytical performance. In-house validation was performed applying the alternative validation approach and using specially designed software InterVal. The software is a practical translation of the mathematic-statistical concept, which offers a user-friendly and quick determination of all relevant and requested performance characteristics. The experiment plan in the framework of the matrix-comprehensive in-house validation concept is based on a fractional factorial design. The plan requires the establishment of a so-called leading factor, which can be varied on any number of levels, and of up to seven further factors varying on two levels. The sample matrix (milk, whey, milk powder and water) was defined as leading factor whereas operator, extraction type (static vs. dynamic) and storage of the extracts (0 days vs. 1 day) were used as further factors. Parameters taken into account were: response linearity, decision limit (CC_α), detection capability (CC_β), trueness and precision (repeatability and within-laboratory reproducibility).

The calibration curve showed good linearity in the concentration range from 0.020 to 0.600 µg/kg with correlation coefficient above 0.995. The CC_α and CC_β obtained using the calibration graph approach were 0.050 µg/kg and 0.066 µg/kg, respectively.

Coefficients of variation (CV) of repeatability, within-laboratory reproducibility and apparent recoveries measured at a four spike levels are given in Table 1. Generally, all the charactersitics obtained for CAP in the validated concentration range fulfil the requirements of the EU Commission.

The method was robust against different sample matrix, exchanging the operator, extraction mode and changes in the storage of sample extracts. All these factors showed no significant influence on the determination of CAP. The performance characteristics of the method indicate its fitness for use in food control. As an example, Fig. 5 shows the

HPLC-MS/MS chromatograms of a milk sample and a milk sample spiked with 40 ng/kg CAP.

Table 1. Repeatabilities (CV_r), within-laboratory reproducibilities (CV_{wR}) and apparent recoveries obtained for CAP by HPLC-MS/MS

CAP spike, µg/kg	CV_r, %	CV_{wR}, %	Recovery, %
0.020	30.0	30.3	105.3
0.100	7.9	8.0	100.8
0.300	3.1	4.2	100.0
0.600	1.7	3.5	99.8

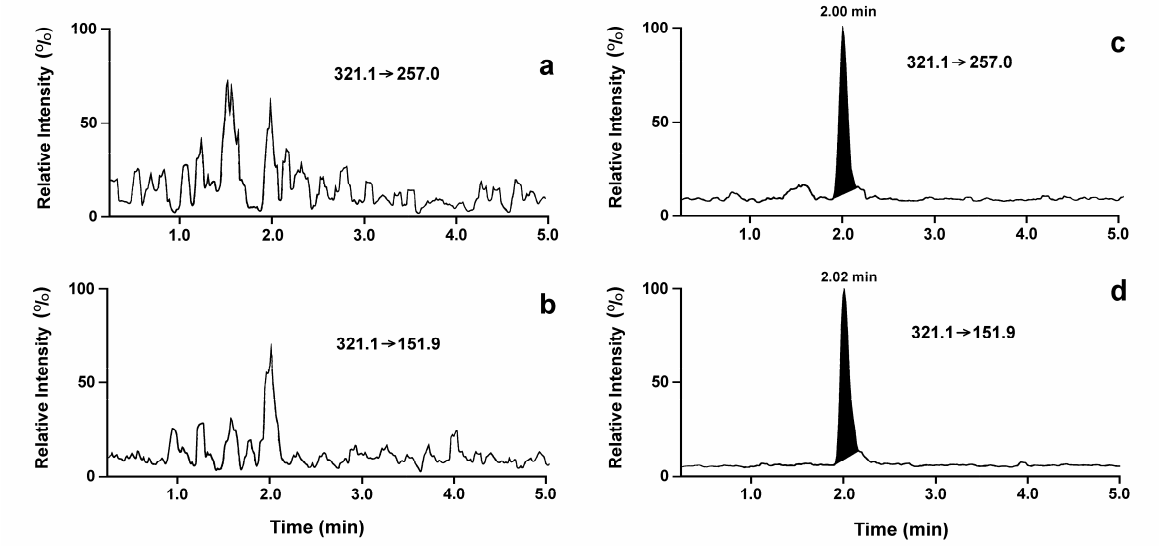

Fig. 5. HPLC-MS/MS chromatograms of a blank milk sample (a, b) and a milk sample spiked with 40 ng/kg CAP (c, d).

3.2. Comparison of HPLC and UPLC coupled to MS/MS for the determination of sulfonamides in egg and honey

Sulfonamides (SAs) are a class of antimicrobial drugs that are widely used in food producing animals as growth promoters as well as for therapeutic and prophylactic purposes (Fig. 6). Residues of SAs in the food chain are of increasing concern due to their carcinogenic potency and their contribution to the increase of antibiotic resistance. The maximum residue limit in the European Union countries for SAs in food of animal origin was established at the total level of 100 µg/kg. In this part of the thesis the performance of HPLC-MS/MS and UPLC-MS/MS for quantification of 10 sulfonamide residues in egg and honey was studied and compared.

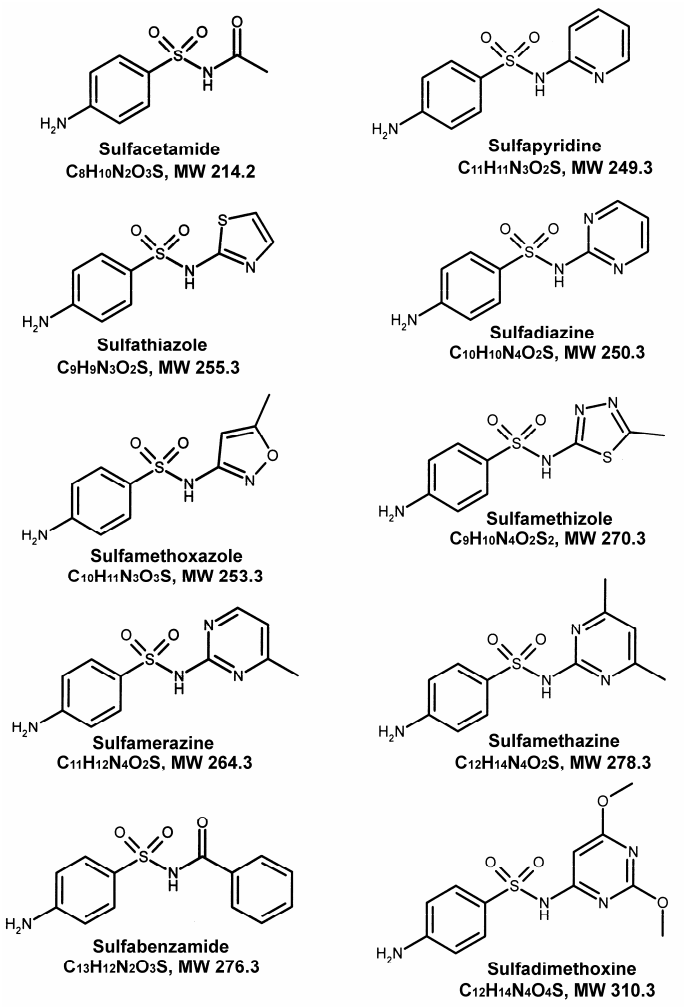

Fig. 6. Chemical structures, raw formulas and molecular weights of sulfonamides studied.

Chromatographic separation. In order to optimize the HPLC separation of SAs a serial of preliminary experiments was performed testing different mobile phases consisting of acetonitrile, methanol or mixture of acetonitrile and methanol as an organic phase and water containing formic or acetic acid at various (0.01–0.20%) concentrations. Both acids were found to provide acceptable separation and detection performance. The optimal HPLC separation of SAs was achieved using a gradient elution with 0.05% aqueous formic acid and acetonitrile/methanol (90:10 *v/v*). The chromatogram showing the HPLC separation of 10 sulfonamides is given in Fig. 7a. Then, the HPLC method was transferred to UPLC technique. Elution with the same gradient conditions at different mobile phase flow rates showed that the optimal performance, which is based on a compromise between separation time, resolution, and column backpressure is obtained using 0.5 mL/min (Fig. 7b). Using UPLC the separation time was shortened in about 30% reducing the run time by 8 min and a better resolution was achieved compared to HPLC. In general, the height equivalent to theoretical plate values were about two times lower for UPLC system. Finally, due to the narrower peaks obtained with UPLC, similar signal-to noise ratios were obtained by both methods even using 10 μL of the same standard as injection volume in UPLC comparing to 20 μL injected in HPLC analysis.

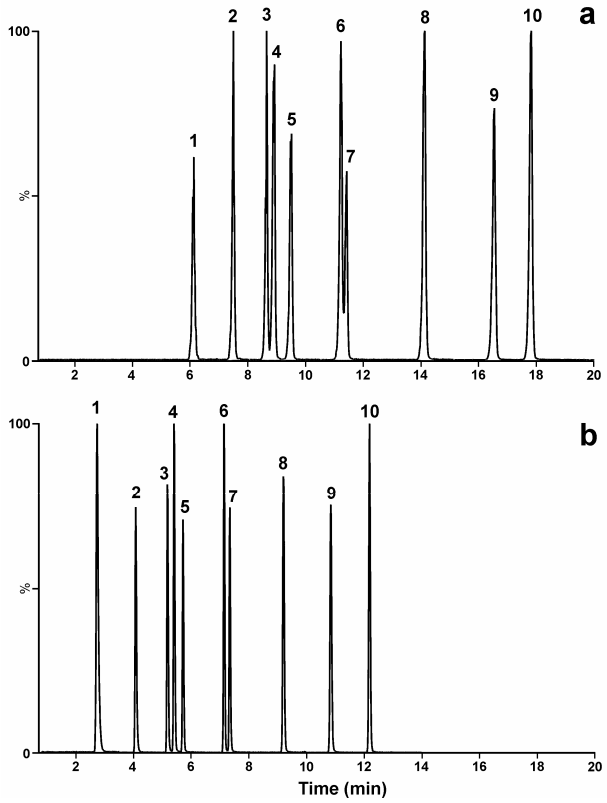

Fig. 7. Comparison of HPLC (a) and UPLC (b) separation of 10 sulfonamides (15 μg/L standard solution). 1-sulfacetamide; 2-sulfadiazine; 3-sulfathiazole; 4-sulfapyridine; 5-sulfamerazine; 6-sulfamethazine; 7-sulfamethizole; 8-sulfamethoxazole; 9-sulfabenzamide; 10-sulfadimethoxine.

MS/MS detection. Mass spectra of all analytes were acquired in the full scan mode with cone voltage of 25 V, using positive and negative electrospray ionization. All compounds produced higher signal-to-noise ratios in the positive ion mode. As expected, sulfonamides show relatively simple mass spectra. For all SAs except two internal standards most intense fragments in the positive ion mode were detected at *m/z* 156 and 108, although the abundance was different depending on the variable compounds. Cone voltages were optimized for maximum signal intensity of typical ions during injection of single compounds into the mass spectrometer. The selected MRM transitions as well as the individual cone and collision energy voltages applied for the analytes and internal standards are summarized in Table 2.

Extraction and sample clean-up. Preliminary studies were conducted to optimize solvents and extraction conditions for both egg and honey matrices. The extraction efficiency together with sample defatting procedure of SAs from spiked (at a 100 μg/kg fortification level) blank samples was studied using two solvents (10 mL): acetonitrile and ethyl acetate with subsequent defatting with 5 mL of pure hexane or hexane/CCl_4 (1:1, v/v) mixture. The obtained results (Fig. 8) showed that acetonitrile provides higher

absolute recoveries of SAs from spiked blanks. In the next step, the extraction of the SAs from the spiked blank samples was optimized by varying the volume of acetonitrile from 5 to 15 mL. However, acetonitrile volumes greater than 10 mL did neither provide cleaner extracts nor higher analyte recoveries. Furthermore, the influence of the number of repeated extractions was tested by performing one, two and three consecutive extractions. The results obtained indicated that a volume of 10 mL of solvent and two consecutive extractions with subsequent defatting provided absolute recoveries between 60% and 80% of all the analytes from both matrices, except sulfathiazole and sulfamethazine, for that obtained recoveries from egg matrix were about 26%. After extraction, egg samples were additionally defatted with hexane.

Table 2. MRM transitions and MS operating parameters selected for analysis of sulfonamides and internal standards (in bold)

Compound	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)
Sulfacetamide	215>156	17	8
	215>108		17
Sulfapyridine	250>156	26	17
	250>108		25
Sulfadiazine	251>156	24	14
	251>108		24
Sulfathiazole	256>156	25	16
	256>108		24
Sulfamerazine	265>156	28	16
	265>108		28
Sulfamethoxazole	254>156	25	16
	254>108		23
Sulfamethoxazole-$^{13}C_6$	260>162	25	18
Sulfamethizole	271>156	20	14
	271>108		18
Sulfamethazine	279>186	31	18
	279>108		26
Sulfamethazine-$^{13}C_6$	285>186	27	15
Sulfabenzamide	277>156	16	13
	277>108		22
Sulfadimethoxine	311>156	31	20
	311>108		30

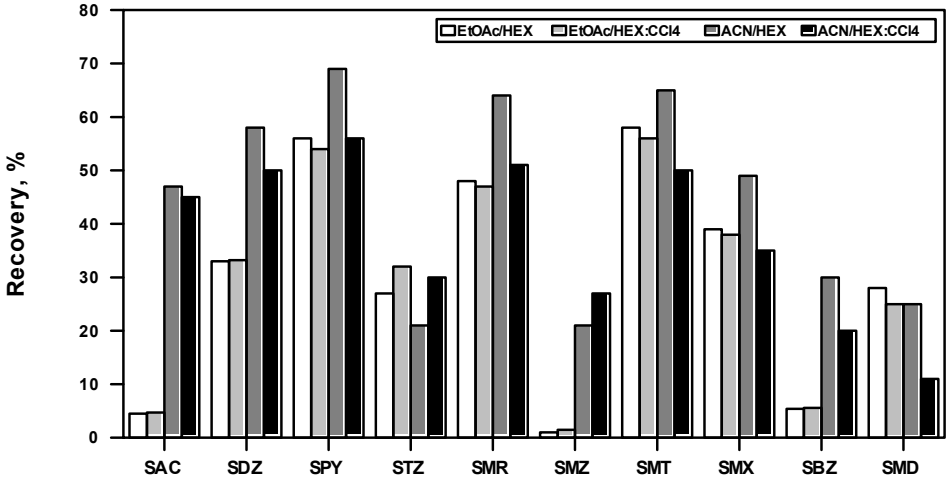

Fig. 8. Effect of extraction (10 mL) and defatting (5 mL) solvents on the absolute recoveries of SAs from egg sample spiked at 100 μg/kg.

Strata-X cartridges (200 mg/6 mL) from Phenomenex were tested for the solid-phase extraction (SPE) clean-up of both egg and honey samples. According to the manufacturer, the unique surface properties of this surface modified styrene-divinylbenzene polymeric stationary phase offer numerous retention mechanisms, including hydrophobic, hydrogen bonding and π-π interactions. Aliquots (20 mL) of blank egg and honey extracts spiked at 100 μg/kg were processed through all steps of the SPE to monitor the recoveries. We investigated the volume of acetonitrile to completely elute the SAs from the cartridge. In this experiment, the adsorbed analytes (2 μg each) were eluted from the cartridge as 1 mL fractions for the determination of SAs in each fraction. The obtained results showed, that complete desorption of the analytes requires at least 7 mL CH_3CN. Higher acetonitrile volume did not result in higher SAs recoveries. This optimized SPE procedure yielded satisfactory high (≥80%) absolute recoveries for all SAs from spiked blank extracts.

The complete sample extraction, lipid removal and clean-up procedure optimized for analysis of SAs residues was as follows. A 2 g of egg sample or 2 g of honey sample dissolved with 2 mL water was placed into a beaker, a volume of 100 μL of internal standard solution (0.1 μg/mL) and/or an appropriate volume of working standard solution (1 μg/mL) was added to the sample and the sample was vortexed and left to equilibrate for 10 min. After this, 10 mL of acetonitrile was added, and the mixture was agitated on a minishaker for 10 min. The mixture was then centrifuged for 10 min at 3512 *g* and the supernatant was decanted. This extraction procedure with acetonitrile was

repeated once more and the combined extracts were evaporated to dryness at 50 °C under a nitrogen stream. The residue was dissolved in 20 mL of 0.2 mol/L ammonium acetate buffer (pH 5.3) and defatted with 5 mL of *n*-hexane followed by centrifugation for 10 min at 3512 *g*.

For solid-phase extraction, the Strata-X cartridge was preconditioned with 5 mL methanol followed by 5 mL 0.2 mol/L ammonium acetate buffer (pH 5.3). After the sample loading washing was done with 5 mL of water and then the cartridge was dried under vacuum. The analytes were eluted with 7 mL of acetonitrile, which was then evaporated to dryness at 50°C under a nitrogen stream. The residue was reconstituted in 200 µL 0.05% formic acid and filtered into an autosampler vial.

Analytical performance. The main validation parameters obtained by InterVal at 10 µg/kg concentration level with all factors, without HPLC and without UPLC factor level are compared in Table 3. Due to higher peak efficiency achieved with UPLC, the decision limit values obtained by both techniques are almost equal despite the fact that in UPLC twice lower sample volumes were injected. Satisfactory and comparable recoveries (80–110%) were obtained by UPLC and HPLC for all of the sulfonamides, except sulfacetamide by HPLC and sulfabenzamide by both techniques. Although recoveries obtained by UPLC and HPLC were similar, for a majority of the spiked compounds, UPLC gave significantly better precision. This might in part be due to better peak shapes obtained with UPLC and the fact that faster separation displayed higher reproducibility. Furthermore, narrower peaks will result in better separation and therefore higher spectral purity of the analytes. At all concentration levels investigated by both techniques sulfabenzamide gave unaceptable CV_r values (30-70%) and therefore this compound can not be quantified.

InterVal shows the overall calibration curves with the prediction intervals as well as the calibration curves for a chosen factor. For the majority of SAs the factor *Method* showed no significant difference between the slopes and the correlation coefficients of the calibration curves measured by UPLC and by HPLC. The deviations of the slopes, however, were always smaller for UPLC than for HPLC. For most analytes the deviations of the slopes obtained by UPLC were below 10.3%. Only in the case of sulfadimethoxine, the deviation of the slope was 14.6%. Generally, for HPLC the deviations of the slopes were about 1.3-2 times higher.

Table 3. Comparison of validation parameters (at 10 µg/kg concentration level) obtained with all factors, without HPLC and without UPLC factor level

Analyte	CC_α, µg/kg			Recovery, %			CV_r, %		
	All factors	UPLC	HPLC	All factors	UPLC	HPLC	All factors	UPLC	HPLC
Sulfacetamide	8.96	7.10	10.9	118.2	110.0	125.9	23.2	8.5	29.5
Sulfadiazine	9.07	9.43	9.90	104.2	106.6	101.7	14.2	12.1	16.3
Sulfathiazole	7.75	7.61	8.91	104.5	103.1	105.9	13.4	9.8	16.2
Sulfapyridine	7.55	7.40	8.36	106.5	103.8	109.1	12.1	8.8	14.4
Sulfamerazine	7.41	7.25	8.54	100.4	103.2	97.8	11.7	9.3	13.7
Sulfamethazine	7.28	7.40	7.89	103.7	104.2	103.2	9.5	8.5	10.6
Sulfamethizole	9.07	7.87	11.4	100.9	101.3	100.8	14.0	9.1	17.9
Sulfamethoxazole	6.66	6.61	7.25	99.6	99.8	99.4	6.0	5.5	6.1
Sulfabenzamide	19.5	25.5	18.8	97.6	79.7	116.4	52.7	66.9	41.3
Sulfadimethoxine	9.78	9.23	11.9	104.8	106.4	103.9	18.5	15.6	21.2

In principle, all selected factors may be presented individually as boxplots. As an example, a boxplot of measured concentrations for sulfacetamide for the factor *Method* is given in Fig. 9. As regards the range of the „box“ (25-75%-quantile) and the range covered by the minimum and maximum (whiskers) in most cases they are bigger for the factor level HPLC. This indicates that measured results show a greater scatter for HPLC than for UPLC. The boxplots of all the other analytes showed similar tendencies.

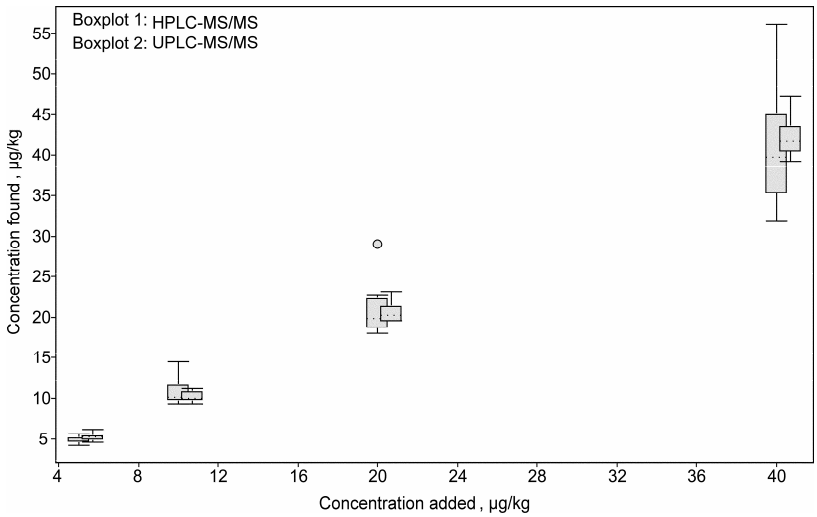

Fig. 9. Boxplot of measured concentrations for sulfacetamide for the factor *Method*.

The method was robust against different sample matrix, exchanging the operator, changes in the storage and in the evaporation temperature of sample extracts. These factors showed no significant influence on any of the analytes. As an example, Fig. 10 shows the UPLC–MS/MS chromatograms for (a) egg and (b) honey samples fortified with 5 µg/kg of ten sulfonamides and (c) blank honey sample plus the two internal standards (5 µg/kg of each).

In conclusion, on the basis of the obtained results it can be concluded that both UPLC and HPLC methods provide acceptable analytical performance but UPLC displayed shorter run-time, higher efficiency with better resolution and better precision, making this technique more suitable for confirmatory assay of sulfonamide residues in egg and honey.

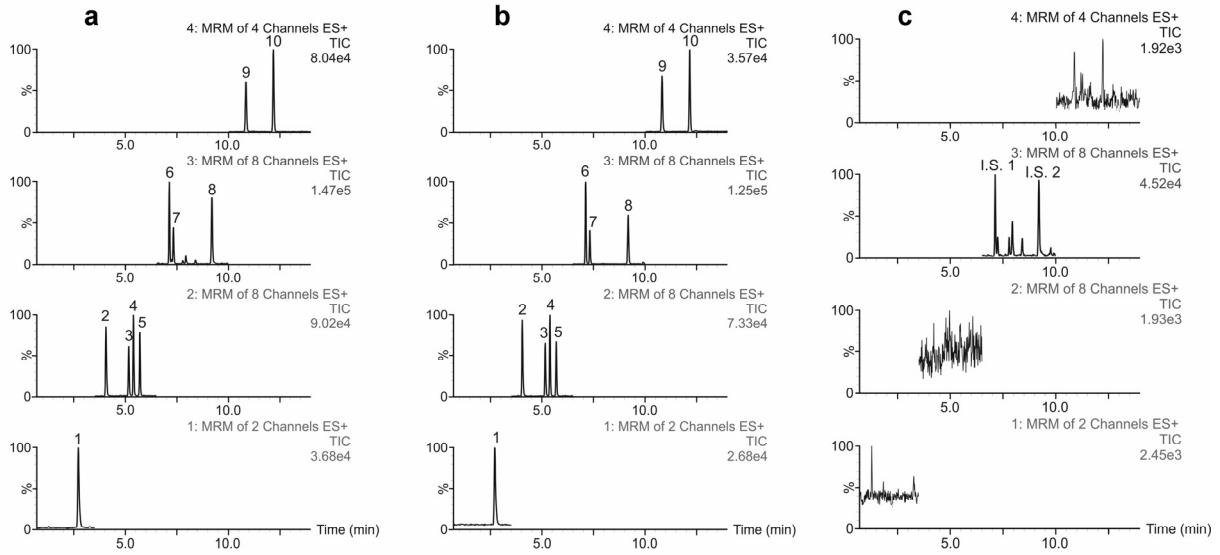

Fig. 10. UPLC–MS/MS chromatograms for (a) egg and (b) honey samples fortified with 5 µg/kg of each ten sulfonamides and (c) blank honey sample plus the two internal standards (5 µg/kg of each). 1-sulfacetamide; 2-sulfadiazine; 3- sulfathiazole; 4-sulfapyridine; 5-sulfamerazine; 6-sulfamethazine; 7-sulfamethizole; 8-sulfamethoxazole; 9-sulfabenzamide; 10-sulfadimethoxine; I.S.1-sulfamethoxazole-$^{13}C_6$; I.S.2-sulfamethazine-$^{13}C_6$.

3.3. UPLC-MS/MS for the determination of 5-nitroimidazoles and their metabolites

Dimetridazole (DMZ, 1,2-dimethyl-5-nitroimidazole), ipronidazole (IPZ, 1-methyl-2-isopropyl-5-nitroimidazole), metronidazole (MNZ, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), ronidazole (RNZ, 1-methyl-2-(carbamoyloxymethyl)-5-nitroimidazole) and ternidazole (TNZ, 1-(3-hydroxyisopropyl)-2-methyl-5-nitroimidazole) are 5-nitroimidazole-based veterinary drugs with antibiotic and anticoccidial activities. The 5-nitroimidazoles are known to be rapidly metabolised. The main metabolites result from oxidation of the side-chain in the C2 position of the imidazole ring. For DMZ and RNZ, the major metabolite is 1-methyl-2-hydroxymethyl-5-nitroimidazole (MHMNI), MNZ gives 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (MNZOH) and IPZ gives 1-methyl-2-(2‘-hydroxyisopropyl)-5-nitroimidazole (IPZOH). For their potentially harmful effects on human health DMZ, MNZ and RNZ are banned from use in food producing animals within the European Union and other countries. IPZ and TNZ have never been autorized as veterinary drugs and, analytically, are therefore to be treated like a banned substances. The present part describes a method for the determination of five 5-nitroimidazoles and three of their metabolites (Fig. 11) in egg matrices employing UPLC-MS/MS technique.

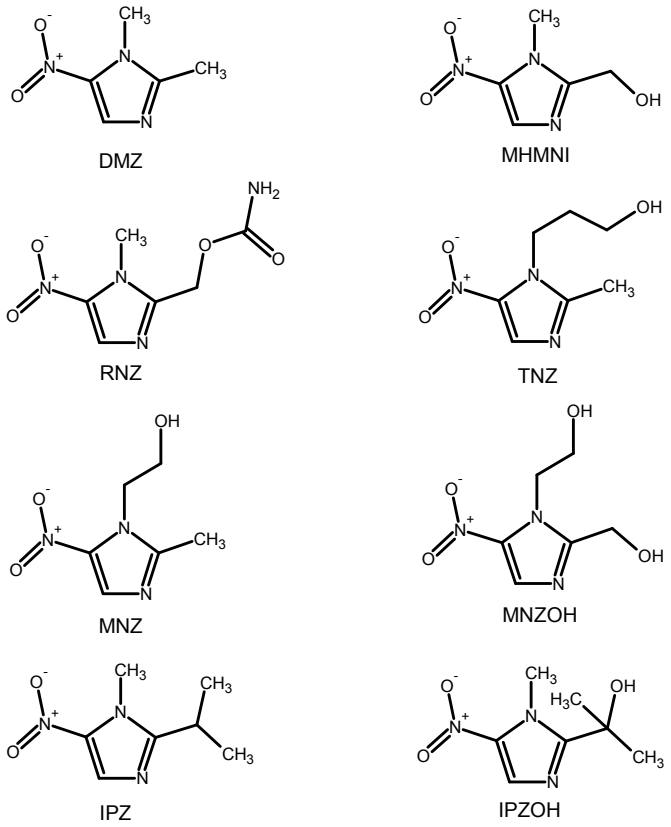

Fig. 11. Chemical structures of 5-nitroimidazoles and their metabolites studied.

UPLC separation. Usually, separation of nitroimidazoles by HPLC technique is performed on conventional reversed-phase columns using volatile mobile phases consisting of acidified aqueous-acetonitrile solution. Acidic additives are known to promote protonation of basics compounds and as a result an increase of signal in ESI+ source takes place. Initial transfer of the HPLC assay to UPLC was accomplished by simply applying a mobile phase similar to that used in previous papers. The optimal UPLC separation of five nitroimidazoles and three hydroxylated metabolites was achieved using a gradient elution with aqueous-acetonitrile mobile phase containing 0.05% formic acid. Elution with the same gradient conditions at different mobile phase flow rates showed that the optimal performance, which is based on a compromise between separation time, resolution, and column backpressure is obtained using 0.6 mL/min. Through UPLC separation the signals of the eight analytes were well-resolved in about 6 min with a total run time of 12 min (including the reequilibration time).

MS/MS detection. Mass spectra of all analytes were acquired in the full scan mode with cone voltage of 25 V, using positive and negative electrospray ionization. All compounds produced higher signal-to-noise ratios in the positive ion mode. Protonated molecules $(M + H)^+$ were obtained for the 5-nitroimidazoles and their hydroxylated metabolites. Cone voltages were optimized for maximum signal intensity of typical ions during injection of single compounds into the mass spectrometer. The selected MRM transitions as well as the individual cone and collision energy voltages applied for the analytes and internal standards are summarized in Table 4. As an example, Fig. 12

demonstrates the full scan mass spectra for DMZ, RNZ and RNZ-d3 and collision induced dissociation mass spectra of the most intensive molecular ion for each compound. The most intensive fragment ion from each precursor ion was selected for quantification and a less sensitive secondary transition was used for confirmation purposes. Three acquisition periods were set during the analysis, one (1.2–2.9 min retention window) for MNZOH, second (2.9–4.6 min) for DMZ, MHMNI, MNZ, TNZ and RNZ and the third (4.6–7.0 min) for IPZ and IPZOH.

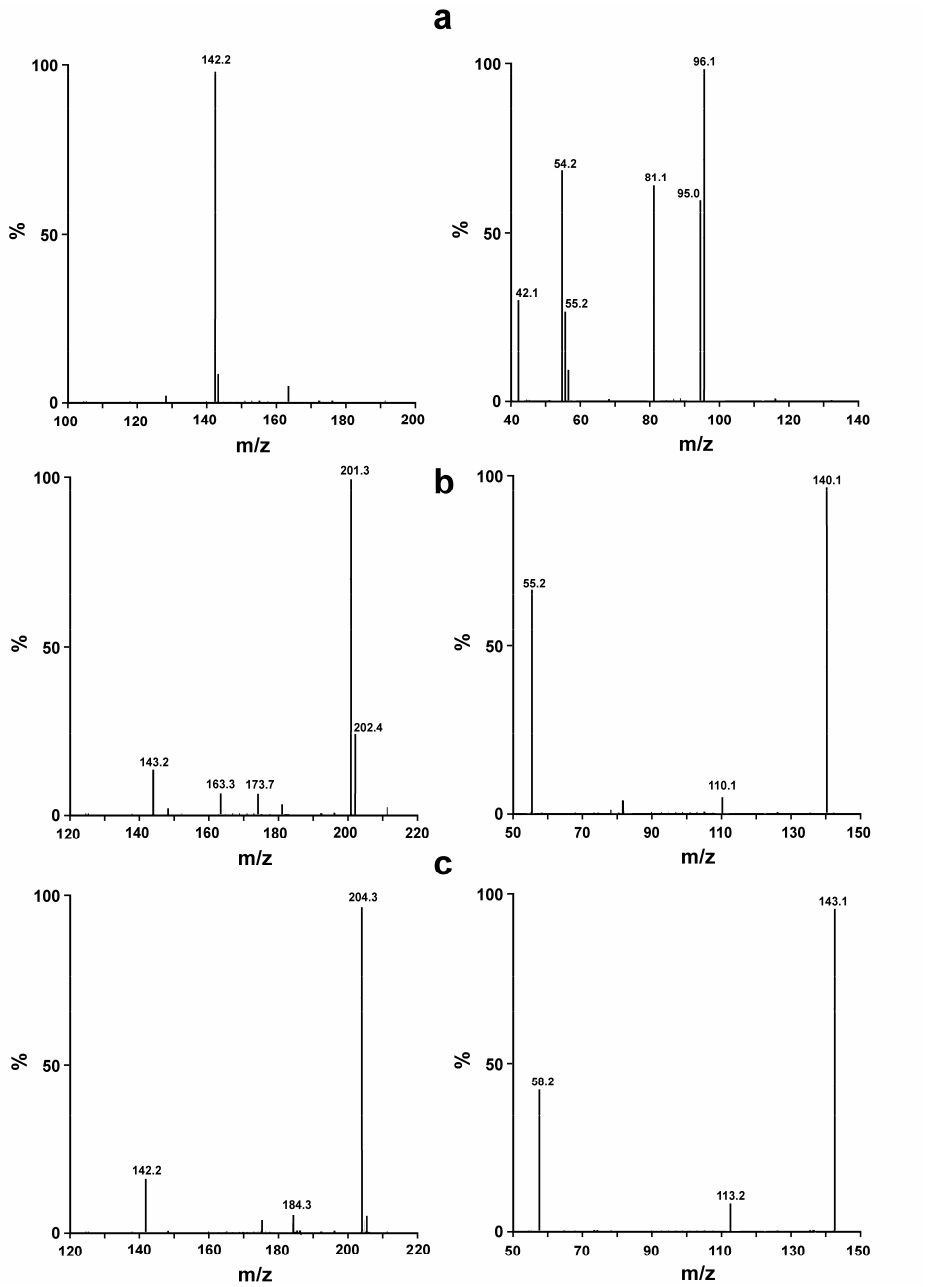

Fig. 12. The full scan mass spectra of DMZ (a), RNZ (b) and RNZ-d3 (c) (left) and collision induced dissociation mass spectra of the most intensive molecular ion for each compound (right).

Table 4. Monitored MRM transitions and MS operating parameters of 5-nitroimidazoles, their metabolites and deuterated internal standards (in bold)

Analyte	Precursor ion (*m/z*)	Daughter ions (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
MNZOH	188	68 144	0.45	22	21 13
MHMNI	158	55 140	0.20	23	17 12
TNZ	186	82 128	0.02	26	26 15
MNZ	172	82 128	0.02	24	24 14
DMZ	142	54 96	0.02	28	26 15
RNZ	201	55 140	0.02	16	21 12
IPZOH	186	122 168	0.08	24	25 14
IPZ	170	109 124	0.08	31	24 17
MHMNI-d3	161	143	0.02	23	13
MNZ-d3	175	131	0.02	24	14
DMZ-d3	145	99	0.02	28	16
RNZ-d3	204	143	0.02	18	12
IPZOH-d3	189	171	0.08	22	13
IPZ-d3	173	112	0.08	32	25

Sample preparation. The 5-nitroimidazole residues are usually extracted from biological matrices with an organic solvent like acetonitrile, ethyl acetate, toluene and further clean-up is made by solid-phase extraction. In some cases, the extract is additionally washed with hexane to remove the lipids before clean-up. However, this defatting step was shown to reduce the recovery of the less polar 5-nitroimidazoles, *e.g.*, IPZ and IPZOH. In this study, aqueous trichloroacetic acid (TCA) solution (20 mL) was chosen as extraction solvent because 5-nitroimidazoles partitioned well into the aqueous phase under acidic conditions. The final percentage of TCA was evaluated by testing several concentrations from 0 to 5% with a 1% step. These experiments showed that above 1% TCA no significant improvement in the extraction efficiency was obtained. In the next step, the influence of the number of repeated extractions was tested by performing second and third consecutive extractions with 5 mL of 5% TCA. Two

consecutive extractions with 20 and 5 mL of acidified water provided satisfactory extraction yields for all analytes.

For the clean-up of egg extracts, a mixed-mode polymeric sorbent with reversed-phase and cation-exchange functionalities, Strata-X-C (Phenomenex), was tested. Aliquots of spiked blank egg extracts were processed through all steps of the SPE to monitor the recoveries. The influence of the pH (from 1 to 6) of the aqueous extract and various compositions of the eluting solvent were tested to isolate the analytes. However, at lower pH values significantly higher background noise and poorer precision were observed. Loading of the neutralized to about pH 5 samples produced the cleanest extracts. During the subsequent washes with acidified water and methanol, no loss was recorded for all the studied analytes.

Elution of the basics compounds from cation-exchange sorbent materials usually is achieved by alkaline solution, which deprotonates the analytes and breaks the ionic interaction. In this study, the eluting properties of water/triethylamine/acetonitrile and water/triethylamine/ethylacetate (2.5:2.5:95, v/v) mixtures were examined. It was found that the higher extraction yields were obtained when water/triethylamine/ethylacetate mixture was used as the eluting solvent. In the next step, the concentration of triethylamine was optimized. These experiments showed (Fig. 13) that above 2.0 % triethylamine no significant improvement in the extraction efficiency was obtained.

Finally, to establish the volume of elution solvent required, blank extracts spiked at two concentration levels (2 and 10 μg/kg) were investigated. During SPE, 1 mL fractions were collected, evaporated to dryness, re-dissolved with 150 μL of the mobile phase A, filtered and analyzed. At both concentration levels, the total solvent volume required to extract ≥80% of the analytes was about 6 mL. To ensure robustness of the method, we used 7 mL in further method development.

The complete sample extraction and lipid removal procedure optimized for analysis of nitroimidazole residues was as follows. A well-homogenized portion (5 g) of egg was weighed into a 50 mL polypropylene tube, fortified with an aliquot of the ISs, mixed and allowed to stand for 5 min in darkness. After this, 10 mL 0.2 mol/L sodium acetate buffer (pH 5) and 10 mL of 5% trichloroacetic acid (TCA) were added, the mixture was shaken for 10 min and then centrifuged for 10 min at 3500 *g*. The supernatant was collected in a second 50 mL polypropylene tube and the residual was additionally

extracted with 5 mL of 5% TCA. The supernatants were combined, then adjusted to pH 5 with 0.5 mL of 5 mol/L NaOH and this solution was loaded onto a Strata-X-C cartridge (200 mg/6 mL, Phenomenex), preconditioned sequentially with methanol (5 mL), water (5 mL) and 0.2 mol/L acetate buffer (5 mL). The cartridge was further rinsed with 5 mL of 5% TCA, 3 mL of methanol and then dried under vacuum. The analytes were finally eluted with water/triethylamine/ethylacetate (2.5:2.5:95, v/v) mixture (7 mL), which was evaporated under a stream of nitrogen at 35 °C, reconstituted in 150 µL of the mobile phase A and filtered into an autosampler vial.

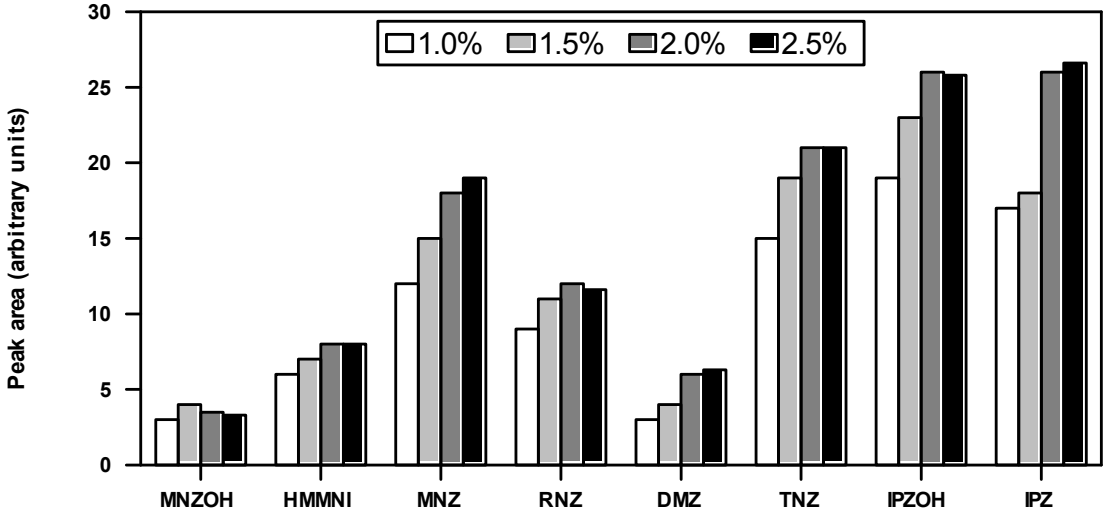

Fig. 13. Effect of triethylamine concentration on the desorption efficiency of nitroimidazoles (10 µg/kg) and their metabolites. Desorption mixture water/triethylamine/ethylacetate (4.0-2.5/1.0-2.5/95, v/v).

Method validation. The sample matrix was defined as leading factor in the validation study. As further factors which might influence the analysis, the operator, the SPE cartridge, the storage of extracts and the evaporation temperature of extracts were chosen. The decision limits and detection capabilities are summarized in Table 5. Coefficients of variation of repeatability, within-laboratory reproducibility and apparent recoveries measured at a second spike level are also given in Table 5. These data were also evaluated for other spike levels (results not shown). Generally, the apparent recoveries obtained for all analytes in the validated concentration range were within the range of 76–109% and thus fulfill the requirements of the EU Commission. For most analytes the recovery rate was almost constant over the whole concentration range, whereas the CV_r and the CV_{wR} values were lower for higher concentrations than for

lower ones. The linearity of the overall calibration curves was good for all compounds with correlation coefficients always higher than 0.99.

Table 5. Decision limits (CC_{α}), detection capabilities (CC_{β}), repeatabilities (CV_r), within-laboratory reproducibilities (CV_{wR}) and apparent recoveries of the analytes (n = 24)

Analyte	CC_{α}, µg/kg	CC_{β}, µg/kg	CV_r, %[a]	CV_{wR}, %[a]	Recovery, %[a]
MNZOH	2.62	4.29	15.3	20.9	100.6
MHMNI	1.25	1.77	11.5	11.8	95.2
TNZ	0.22	0.32	11.6	13.2	94.6
MNZ	0.30	0.44	16.3	17.3	98.7
DMZ	2.37	3.39	11.8	11.9	100.6
RNZ	1.03	1.42	9.1	11.2	96.9
IPZOH	0.21	0.30	10.3	11.1	93.0
IPZ	0.19	0.26	8.4	9.0	96.2

[a] Measured at a second spike level.

None of the factors examined in this validation showed any significant effect on the measurement results. As an example, Fig. 14 shows the UPLC–MS/MS chromatograms for (a) blank egg sample and (b) egg sample spiked at the concentration level close to CC_{α}.

3.4. Interlaboratory comparative study and monitoring of Lithuanian food products

The performance of the developed methods was additionally evaluated by interlaboratory comparative analysis of certified reference materials for milk and egg matrices. The performance statistical test to assess the result in term of accuracy was the *z*-score:

$$z = \frac{x - \mu}{\sigma}$$

where x is the obtained result, μ - the assigned value and σ – the standard deviation for assigned value. The *z*-scores are classified as follows:

$|z| \leq 2$ – good result.

$2 > |z| \geq 3$ – satisfactory result.

$|z| > 3$ – unsatisfactory result.

The results obtained showed that developed methods are capable of determining CAP, SA and NIZ residues in food matrices with good accuracy. Only two results obtained for sulfonamides gave satisfactory accuracy.

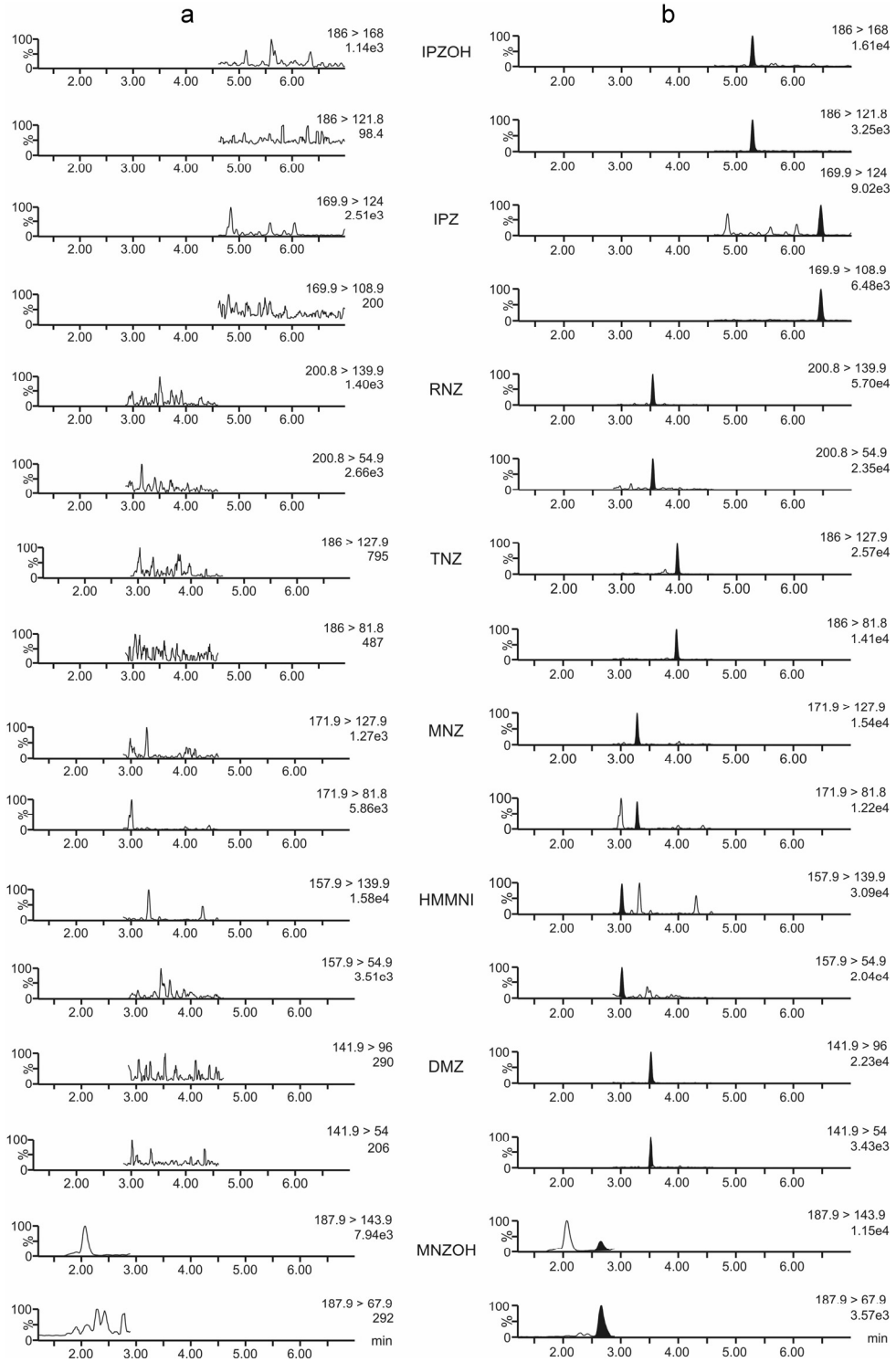

Fig. 14. UPLC–MS/MS analysis of (a) blank egg sample and (b) egg sample spiked at the concentration level close to CC_{α}. Spike levels: 0.22 μg/kg for IPZ, IPZOH, MNZ and TNZ; 1.1 μg/kg for RNZ and MHMNI; 2.2 μg/kg for DMZ and MNZOH.

Finally, the developed techniques were applied for the monitoring of antibiotic residues in Lithuanian food products. As an example, Fig. 15 shows the results obtained for CAP in Lithuanian milk products during the 2005-2008 period. As can be observed, the number of incurred samples gradually decreases during this period indicating the positive impact of strict control. In addition, 17 egg/3 honey and 11 egg samples were analysed during 2008 for sulfonamides and nitroimidazoles, respectively, and none were found contaminated by these drugs.

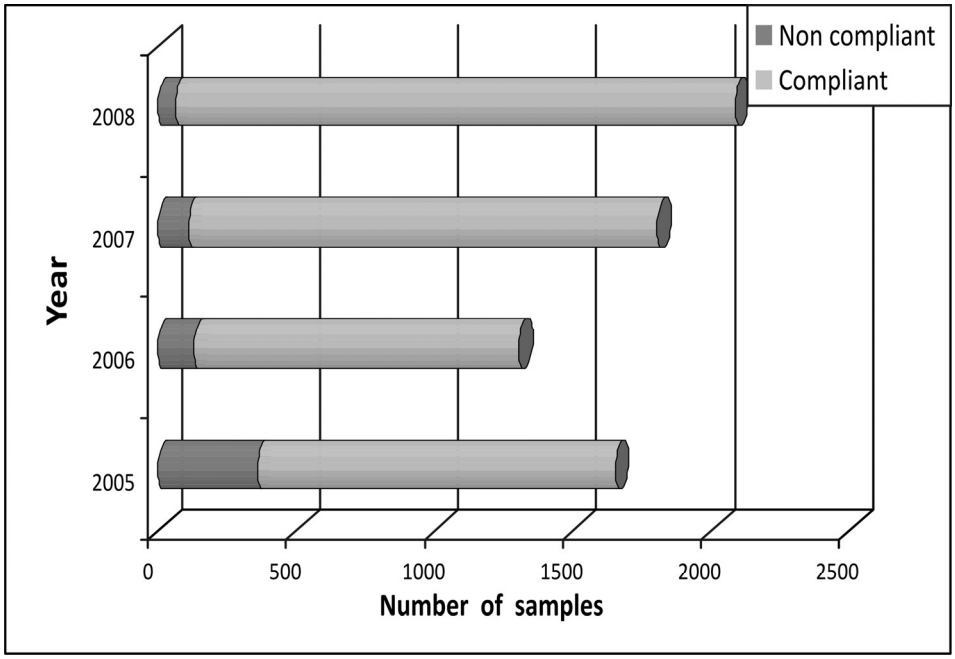

Fig. 15. Results obtained for CAP in Lithuanian milk products during the 2005-2008 period.

CONCLUSIONS

1. A high–performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed for the determination of chloramphenicol (CAP) in milk products. Two solvents (diethyl ether and ethyl acetate) were compared for the extraction of CAP and significantly higher (53% vs. 38%) absolute recoveries were obtained with ethyl acetate. Carbon tetrachloride/hexane (1:1 v/v) mixture was optimal solvent for clean-up of the extracts by means of liquid-liquid extraction. The method was validated according to EU criteria applying the alternative validation approach, and the results showed that the method is selective, accurate, reproducible and sensitive enough.
2. Separation of 10 sulfonamides was optimized and compared using HPLC and ultra-performance liquid chromatography (UPLC). It was shown that by acidifying of the mobile phase with formic acid higher MS/MS detection sensitivity was obtained compared to acetic acid. Peak shapes were improved by using acetonitrile with small amounts (5-10% v/v) of methanol as a tertiary mobile phase component. Using UPLC the separation time was significantly shortened and about twice higher efficiency with better resolution was achieved compared to HPLC.
3. By liquid-liquid extraction of sulfonamides from eggs with ethyl acetate the emulsion formed causes decrease in detection sensitivity and the extractability for some analytes was relatively low due to their protonation. In contrast, acetonitrile showed good sample deproteinisation and extraction properties. Strata-X cartridges were investigated for the clean-up of both egg and honey extracts. It was found that the cleanest extracts were obtained by loading slightly acidic (pH<6) samples and complete desorption of the analytes requires at least 7 mL CH_3CN.
4. The analytical performance of the both methods was compared applying the alternative validation approach using specially designed software InterVal. Satisfactory and comparable recoveries (80–110%) were obtained by UPLC and HPLC for all of the sulfonamides, except sulfacetamide by HPLC and sulfabenzamide by both methods. For a majority of the compounds, UPLC gave significantly better precision due to the higher efficiency and faster separation.

5. UPLC-MS/MS method was developed for the determination of five 5-nitroimidazoles and three of their metabolites in egg matrices. A liquid-liquid extraction with aqueous trichloroacetic acid (≥1%) solution provided satisfactory recoveries for all analytes. Using extraction with water the defatting and extract evaporation/reconstitution steps are not necessary which saves time and prevents sample losses.
6. A Strata-X-C cartridges with reversed-phase and cation-exchange functionalities were investigated for clean-up of the extracts. It was found that loading of the neutralised to about pH 5 samples produced the cleanest extracts. The eluting properties of water/triethylamine/acetonitrile and water/triethylamine/ethylacetate (2.5:2.5:95, v/v) mixtures were examined and higher recoveries were obtained when water/triethylamine/ethylacetate mixture was used as the eluting solvent.
7. The UPLC–MS/MS method was robust against different sample matrix and soli-phase extraction cartridges, exchanging the operator and changes in the storage of sample extracts. The apparent recoveries obtained for all analytes in the validated concentration range were within the range of 93.0–100.6% with acceptable repeatability ($CV_r \leq 15.3\%$) and within-laboratory reproducibility($CV_{wR} \leq 20,9\%$).
8. The results obtained from the analysis of certified reference materials showed that proposed techniques completely fulfill the requirements for the methods designed to control the veterinary drug residues. Developed techniques were applied for the monitoring of antibiotic residues in Lithuanian food products.

The List of Original Publications by the Author

Articles in journals

1. **V. Tamošiūnas**, J. Petraitis, A. Padarauskas. Chloramphenicol determination in milk by liquid chromatography-tandem mass spectrometry. *Chemija*, Vol. 17 (2006) 25-29.
2. **V. Tamošiūnas**, A. Padarauskas, D. Babičienė, T. Petrėnas. High-performance liquid chromatography-tandem mass spectrometry for the determination of sulfonamides in eggs. *Chemija*, Vol. 18, (2007) 20-24.
3. **V. Tamošiūnas**, A. Padarauskas. Comparison of LC and UPLC coupled to MS-MS for the determination of sulfonamides in egg and honey. *Chromatographia*, 67 (2008) 783-788.
4. **V. Tamošiūnas**, A. Padarauskas. Ultra performance liquid chromatography–tandem mass spectrometry for the determination of 5-nitroimidazoles and their metabolites in egg. *Central European Journal of Chemistry*, (2009) in press. DOI: 10.2478/s11532-009-0008-0.

Published contributions to academic conferences

1. V. Tamošiūnas, A. Padarauskas, A. Tamošiūnienė, A. Kaminskas, J. Petraitis. Determination of chloramphenicol in animal muscle by LC-MS/MS. 1-st Baltic conference on food science and technology FOODBALT - 2006, Kaunas, 2006, 51.
2. **V. Tamošiūnas**, A. Padarauskas, E. Naujalis. Determination of Residual Sulphonamides in Egg and Honey by Ultra-Perfomance Liquid Chromatography. Thesis of the 4th Nordic Separation Science Society International Conference, Kaunas 26-29 August 2007.
3. **V. Tamošiūnas**, T. Petrėnas, I. Jarmalaitė, J. Petraitis, A. Padarauskas. Determination of nitroimidazole residues in eggs by ultra performance liquid chromatography-tandem mass spectrometry. Proceedings of the 6th International conference "EuroResidue VI", The Netherlands, 2008, p. 979-980.

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SKYSČIŲ CHROMATOGRAFIJA–TANDEMINĖ MASIŲ SPEKTROMETRIJA ANTIBIOTIKŲ NUSTATYMUI MAISTO PRODUKTUOSE

SANTRAUKA

Šioje daktaro disertacijoje apibendrintų mokslinių tyrimų tikslas – nuodugniai ištirti ir pritaikyti efektyviosios ir/arba ultraefektyviosios skysčių chromatografijos-tandeminės masių spektrometrijos metodus kai kurių antibiotikų nustatymui maisto produktuose. Ištirtas ir optimizuotas efektyviosios skysčių chromatografijos-tandeminės masių spektrometrijos metodas chloramfenikolio nustatymui pieno produktuose. Chloramfenikolio ekstrakcijai iš pieno mėginių palyginus du tirpiklius (dietileterį ir etilacetatą) nustatyta, kad ekstrahuojant etilacetatu, chloramfenikolis išekstrahuojamas efektyviau: absoliučios išgavos siekia 38% ekstrahuojant dietilo eteriu ir 53% ekstrahuojant etilacetatu. Optimalus tirpiklis mėginių valymui – anglies tetrachlorido/heksano mišinys (1:1 v/v). Įvertinus metodo atrankumą kitiems veterinariniams vaistams, kalibracinės kreivės tiesiškumą, sprendimo ribą, aptikimo gebą, teisingumą ir glaudumą nustatyta, kad metodas gerai tenkina ES reikalavimus, keliamus veterinarinių vaistų likučių nustatymui maisto produktuose.

Optimizuotas ir palygintas sulfonamidų atskyrimas efektyviosios (ESCh) ir ultraefektyviosios skysčių chromatografijos (UESCh) metodais. Nustatyta, kad UESCh metodas yra dvigubai efektyvesnis, atrankesnis ir greitesnis. Ekstrahuojant sulfonamidus etilacetatu iš kiaušinio mėginių susidaro emulsija, o dalies analičių tirpumas dėl protonizacijos sumažėja. Acetonitrilas puikiai nusodina baltymus bei gerai tirpina ir protonizuotas analites. Ekstraktų valymui kietafazės ekstrakcijos metodu ištirtas naujas sorbentas Strata-X, pasižymintis mišria (hidrofobine , π-π ir vandenilinio ryšio) sąveika su analitėmis. Atrankiausiai sulfonamidai adsorbuojami iš truputį parūgštintų (pH<6) tirpalų. Desorbcijai palyginus metanolį ir acetonitrilą nustatyta, kad efektyviau, t.y. mažesniu tirpiklio tūriu, analites desorbuoja acetonitrilas. Įvertinus metodų analizines charakteristikas nustatyta, kad visoms analitėms, išskyrus sulfacetamido nustatymą ESCh metodu ir sulfabenzamido nustatymą abiem metodais, gaunamos ES reikalavimus tenkinančios išgavos (80–110%). Beveik visoms analitėms UESCh rezultatų santykiniai standartiniai nuokrypiai buvo žymiai mažesni. Geresnį UESCh metodo glaudumą sąlygoja geresnė smailių forma bei greitesnis atskyrimas.

Nitroimidazolų ir jų metabolitų nustatymui kiaušiniuose optimizuotas UESCh-MS/MS metodas. Nustatyta, kad nitroimidazolai puikiai ekstrahuojasi iš kiaušinio matricos vandeniu su trichloracto rūgšties priedu (≥1%). Ekstrahuojant vandeniniu tirpalu visiškai nereikalingas papildomas ekstraktų nuriebalinimas (lipidai netirpsta vandenyje) bei ekstraktų nugarinimas ir pertirpinimas vandeninėje judrioje fazėje. Tai ženkliai pagreitina ir supaprastina mėginio paruošimo analizei procedūrą. Nitroimidazolų ekstraktų valymui kietafazės ekstrakcijos būdu ištirtas mišrus polimerinis sorbentas Strata-X-C, pasižymintis ir atvirkščių fazių adsorbcinėmis ir katijonų mainų savybėmis. Maksimalios analičių išgavos bei gryniausi mėginiai gauti valant mažiau parūgštintus (pH 5) ekstraktus. Nitroimidazolų desorbcijai palyginus du mišinius (trietilaminas/vanduo/acetonitrilas ir trietilaminas/vanduo/etilacetatas (2,0:3,0:95, v/v)) nustatyta, kad trietilamino/vandens/etilacetato mišiniu analitės desorbuojamos efektyviau. Įvertinus UESCh-MS/MS metodo analizines charakteristikas nustatyta, kad visoms analitėms gaunami ES reikalavimus tenkinantys išgavų (93,0–100,6%), pakartojamumo (SSN_r≤15,3%) ir atkuriamumo (SSN_{wR} ≤20,9%) rezultatai. Pagrindinis metodo privalumas lyginant su iki šiol publikuotais metodais – žymiai mažesnė (2-3 kartus) analizės trukmė ir paprastesnis mėginio paruošimas. Nauji metodai įteisinti Nacionalinėje veterinarijos laboratorijoje ir sėkmingai naudojami veterinarinių vaistų likučių monitoringui maisto produktuose.