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# High performance residue analysis: determination of antibacterial agents in foods using liquid chromatography screening and confirmation methods

**Keywords:** determination of veterinary drug residues, screening method, confirmation method, liquid chromatography, sulfonamides, detection capability (CCβ), decision limit (CCα), trimethoprim (diaminopyrimidine), beta-lactams, macrolides, tetracyclines, quinolones, lincomycin (lincosamide) griseofulvin, sulfadimethoxine, sulfadoxine, sulfaquinoxaline, sulfachloropyridazine, sulfamethazine, sulfamethoxazole, sulfadiazine, sulfathiazole and trimethoprim, tylosin, tilmicosin, spiramycin, erythromycin, neomycin, dihydrostreptomycin, streptomycin, apramycin, kanamycin, gentamicin and spectinomycin amoxicillin, ampicillin, penicillin G, penicillin V, oxacillin, nafcillin, cloxacillin, dicloxacillin, new generation cephalosporins (cefquinome, ceftiofur, cefalonium, cefazolin, cefapirin, cefalexin, cefoperazone, chlortetracycline, 4-epi-chlortetracycline, oxytetracycline, 4-epi-oxytetracycline, tetracycline, 4-epi-tetracycline, doxycycline, difloxacin, orbifloxacin, sarafloxacin, ofloxacin, marbofloxacin, enrofloxacin, ciprofloxacin, danofloxacin, norfloxacin, oxolinic acid, nalidixic acid, flumequine, lincomycin.

## 1. Summary

In Hungary, food toxicology monitoring and control analyses, their course and the process of preparation of the monitoring plan for the given year are prescribed and determined by FVM decree 10/2002. (I. 23.). The efficiency of monitoring analyses is increased if the focus is placed primarily on screening methods, and independent confirmation methods are used to test objectionable samples. This allows for the distinguishing between negative samples and those containing drug residues using a simpler, faster and cheaper screening type method, and also for higher certainty in the qualitative and quantitative evaluation of positive samples.

The objective of this paper is to present an analytical concept developed for antibacterial agents, including a multicomponent screening method and independent confirmation measurements for type B1 authorized agents. The screening method allows for the simultaneous identification and semiquantitative evaluation of 54 components with drug residue limit values and griseofulvin in animal tissues (muscle, liver and kidney), milk, eggs and honey, using a liquid chromatography triple quadrupole tandem mass spectrometry method.

Target components detected by the screening method are identified using liquid chromatography confirmation tests and evaluated by optical or tandem mass spectrometric detection. Up until the submission date of this paper, nearly 1,800 samples had been analyzed by the screening method. Some type of drug residue was detected in 24 monitoring samples. The contaminations could also be detected during the confirmation tests. The analytical strategy thus developed has been proven to be effective in multiple international proficiency testing programs.

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**Foreword**

Gábor Domány was the head of laboratory of the NÉBIH ÉTbl Food Toxicology NRL. The analytical concept presented in this article was developed also with his professional support over the last few years. With this paper, we would like to commemorate Gábor.

## 2. Introduction

Over the past decades, developments in analytical chemistry have shifted in the direction to allow for the determination of as many components as possible in the shortest possible time. In the case of liquid chromatography (LC) measurements, these requirements were achieved by ultra high performance liquid chromatography (UHPLC) methods that are able to determine the active ingredients and contaminants of pharmaceutical products in minutes **[1]**. In the case of more complex samples, a large number of target components can be determined simultaneously in a relatively short time using mass spectrometric detectors coupled with the LC, such as a liquid chromatography – tandem mass spectrometry (LC-MS/MS) system **[2]**.

In 2007, within the framework of the European Union (EU) Transition Facility project, using the LC-MS/MS instrument obtained by the Food Toxicology National Reference Laboratory of the National Food Chain Safety Office, Directorate of Food and Feed Safety (NÉBIH Étbl), extension of the monitoring analyses of veterinary drugs to previously not investigated target components could begin. Supplementing LC-MS/MS measurements with optical detection methods such as HPLC-DAD (diode array detection) and HPLC-FLD (fluorescent detection), confirmation procedures were developed for seven main groups of authorized drugs and griseofulvin (grisin). The main analytical pathways include the determination of sulfonamides, trimethoprim (diaminopyrimidine), beta-lactams, macrolides, tetracyclines, quinolones, lincomycin (lincosamide) and griseofulvin (***Table 1***). Within the EU, there are group „A" and group „B" components. Group „A" compounds are prohibited substances that do not have maximum residue limit (MRL) values. Group „B" contains authorized components with MRL values, and within this family, there are group „B1" antibacterial agents that are classified into further antibiotics groups **[3]**. The decree on monitoring analyses allows the determination of all components of group B1 from the same sample **[4]**.

By 2016, in addition to confirmation measurements, a multicomponent LC-MS/MS screening method was also developed, allowing the simultaneous determination of the components listed above in foods. The essence of the screening method is the identification of the target components in the samples and their semiquantitative evaluation. In the case of samples in which the presence of a target component can be detected, the confirmation analysis is carried out using a method different from the screening method. Previously, confirmation methods were used in monitoring measurements, however, these procedures had been developed for a group of antibiotics, and so they are not as efficient as a screening method as a multicomponent procedure which ensures the simultaneous analysis of several groups, thus increasing efficiency. Compared to other screening analyses, such as immunoanalytical procedures, the advantage of the multicomponent LC-MS/MS screening method is that the number of components to be measured can be extended, selectivity and identification is ensured by MS/MS detection, and sample preparation is much simpler. At the same time, because of the simultaneous analysis of a large number of molecules with different structures in the LC-MS/MS screening, it is not possible to develop a method which is optimal for each component (which is not necessarily a goal). Thus, neither the efficiency of the extraction, nor the chromatographic resolution will be satisfactory for all components. Sample clean-up (e.g., liquid-liquid extraction or solid phase extraction) is complicated by the different physico-chemical properties of the target components, therefore, often no clean-up is performed during LC-MS/MS screening, but a so-called „dilute-and-shoot" procedure is followed, the sample is only extracted and the extract is injected into the LC-MS/MS after dilution and syringe filtration **[5]**. However, by omitting sample clean-up, the number and concentration of background matrix compounds cannot be reduced prior to the instrumental analysis, which increases the matrix effect (ion suppression/amplification) in the ion source of the MS/MS instrument, thus influencing significantly the quantitative evaluation. This is also the reason why „dilute-and-shoot" procedures can only be used in screening analyses, unless isotope dilution can be used to compensate for the matrix effect **[6]**.

The purpose of our paper is to present the analytical practice for antibacterial agent residues, recently introduced at the NÉBIH ÉTbl Food Toxicology National Reference Laboratory, following the analysis a representative number of samples. Since the beginning of the application of the multicomponent LC-MS/MS screening analysis in monitoring measurements (April 2017), nearly 1,800 samples have been analyzed up until the submission date of this paper. In our manuscript, measurement procedures are only outlined, full descriptions of the methods can be found in the references, our objective was mainly the description of the analytical concept and publication of the results achieved.

## 3. Analytical methods

When developing the multicomponent antibiotics method, the greatest difficulty lies in the different

polarities (hydrophobicity) of the target compounds. While, for example, aminoglycoside type molecules are hydrophilic, water-soluble components, certain antibiotics are hydrophobic, apolar compounds and/or unstable in aqueous media (e.g., macrolides, beta-lactams). Another complication is the sensitivity to the pH of the medium, also because of the different nature of the aminoglycoside and macrolide/beta-lactam groups. The former group can be extracted at strongly acidic pH values (<1), while the latter two are sensitive to low pH media. The composition of the extraction medium therefore has to be developed with a compromise that allows, despite the losses, the reproducible detection of drug residues at half MRL values or below. Additionally, during the liquid chromatography separation that follows sample preparation, a modifier (ion pair reagent) has to be added to the mobile phase which permits the retention of hydrophilic components during reverse phase separation and so the multicomponent screening procedure can be carried out by a single injection **[2]**.

Confirmation methods are already group-specific, and so the extraction of the target components and sample clean-up are „fit for purpose", instrumental analysis can be optimized for the compound group, therefore, analyses will be selective, accurate and reproducible, and will satisfy the requirements of confirmation measurements **[7]**. In the case of confirmation methods, it is worth mentioning that optical detectors (either an UV-VIS spectrophotometric detector or a fluorescence detector (FLD)) may be used in the analysis of authorized drugs **[7]**. More than once, the use of optical detectors proved to be better than mass spectrometric detection, because no isotopically labelled internal standards (ISTD) are required for quantitative evaluation by optical detectors **[8]**. Although isotope dilution mass spectrometry (IDMS) is one of the best ways of quantitative evaluation, stable isotopically labelled analogs that can compensate for the matrix effects on the target component during MS detection are only available in the case of a small number of compounds, thereby improving quantitative determination **[2]**. A specific example is the determination of tetracyclines in foods at the 100 µg/kg (muscle, milk) and 600 µg/kg (kidney) levels as prescribed by the EU. Using a UV detector, tetracyclines can be measured at a higher, more selective wavelength (365 nm), but can also be analyzed by an MS/MS instrument. In the latter case, no isotopically labelled ISTD is commercially available for all components, but their use would be unwarranted from a cost effectiveness point of view. Therefore, when using LC-MS/MS, a matrix-matched calibration is required for quantitative evaluation, in order to compensate for the change in the response signal of the ion source. However, it is not always possible to completely compensate for the matrix effect in the sample with matrix-matched calibration, especially in the case of more complex samples, such as liver, and so optical detection, during which calibration is recorded in a matrix-free solvent, is more appropriate **[8]**.

### 3.1 Multicomponent LC-MS/MS screening method

To check sample preparation and the analysis, the sample is spiked with a five-component surrogate standard mixtures (sulfapyridine, trimethoprim-d9, roxithromycin, penicillin-G-d7 and metacycline) prior to the extraction. The goodness of the measurement is verified by the recovery of the surrogate standards. Tissue and egg samples (2.0 g) were extracted with a mixture of acetonitrile and water containing 0.01 M oxalic acid (25/75, v/v, 10 ml), and the clear supernatant was filtered into an HPLC vial using a syringe filter. Milk samples (5.0 g) were diluted with McIlwain buffer (5.0 mL) and, after centrifugation, were purified and enriched by solid phase extraction (SPE). Honey samples (5.0 g) were dissolved in 10 ml of water containing 0.1% (v/v) heptafluorobutyric acid (HFBA) and hydrolyzed, then cleaned and enriched by SPE. During the SPE, large particle size reverse phase copolymer cartridges (e.g., Strata-XL, 100 µm) were used and 0.1% (v/v) HFBA solution as ion pair reagent. The ion pair reagent is necessary for the retention of polar aminoglycosides.

Target components of the samples thus prepared were separated on a core-shell C18 HPLC column using ion pair chromatography (***Figure 1***). The mobile phase was a mixture of water containing 0.1% (v/v) HFBA and acetonitrile (90/10 v/v) with linear gradient elution. Target compounds were detected using an MS/MS instrument following electrospray ionization (ESI) (***Table 2***) in positive ion mode (ESI+) and MRM (Multiple Reaction Monitoring) scan mode. The ion transitions of ***Table 2*** were also used in the group-specific confirmation methods.

### 3.2 Confirmation measurements

#### 3.2.1 Determination of sulfonamides and trimethoprim

Components analyzed: sulfadimethoxine, sulfadoxine, sulfaquinoxaline, sulfachloropyridazine, sulfamethazine, sulfamethoxazole, sulfadiazine, sulfathiazole and trimethoprim. Since sulfapyridine is not used in veterinary medicine, it can be used as a surrogate standard, because this component is unlikely to contaminate foods of animal origin. With the exception of honey, samples (5.0 g) were extracted with dichloromethane and purified using normal phase, silica gel SPE. The aqueous eluate was extracted by ethyl acetate in a liquid-liquid extraction step, and the organic phase was collected. Following solvent exchange, sulfonamides were determined by HPLC-DAD ($\lambda$ = 267 nm). Honey samples (5.0 g) were dissolved in water containing formic acid or acetic acid (5%, v/v) and hydrolyzed, then purified by copolymer SPE. For the analyses, HPLC-FLD ($\lambda_{ex}$ = 420 nm, $\lambda_{em}$ = 480 nm) or LC-ESI(+)-MS/MS

was used. LC-MS/MS allows for the simultaneous determination of sulfonamides and trimethoprim. If the test sample is not honey, it is sufficient to extract the sample (2.0 g) with acetonitrile when using an LC-MS/MS system. Following solvent exchange, the extract can be injected into the LC-MS/MS system. For the determination of trimethoprim, isotope dilution mass spectrometry (IDMS) was used, and samples were spiked with trimethoprim-d9 ISTD **[9]**.

### 3.2.2 Determination of macrolides

Components analyzed: tylosin, tilmicosin, spiramycin and erithromycin. Samples were extracted with aqueous TRIS buffer (0.1 M, pH = 10) and the extract was purified by copolymer SPE. Samples were analyzed using LC-ESI(+)-MS/MS with an acidic eluent on a C18 stationary phase **[10]**.

### 3.2.3 Determination of aminoglycosides

Components analyzed: neomycin, dihydro-streptomycin, streptomycin, apramycin, kanamycin, gentamicin and spectinomycin. Samples (2.0 g) were extracted with aqueous trichloroacetic acid (5%, m/m, pH=1), and were injected directly into the LC-ESI(+)-MS/MS instrument. In the case of sample clean-up, a copolymer SPE column can be used and HFBA as an ion pair reagent. HPLC separation is also carried out by ion pair chromatography, the eluent is water containing 0.1% (v/v) HFBA and acetonitrile (90/10 v/v) with linear gradient elution, the stationary phase being reverse phase C18 **[11]**.

### 3.2.4 Determination of beta-lactams

The analysis of beta-lactams includes the combined measurement of classical penicillins (amoxicillin, ampicillin, penicillin G, penicillin V, oxacillin, nafcillin, cloxacillin and dicloxacillin) and new generation cephalosporins (cefquinome, ceftiofur, cefalonium, cefazolin, cefapirin, cefalexin and cefoperazone) in foods. With the exception of milk, samples (2.0 g) were extracted with phosphate buffer (pH=6 or 8) or a mixture of acetonitrile and water (50/50 v/v). Cephalosporins can be better extracted from the sample using the latter one. Samples can be purified by SPE or injected directly into the LC-ESI(+)-MS/MS system. Milk samples (5.0 g) were diluted with water (1:1 v/v), centrifuged, purified by copolymer SPE and concentrated. Penicillin G as determined by IDMS, following the dilution of the sample with penicillin G-d7 ISTD **[12]**. Separation was carried out on a C18 stationary phase with an acidic eluent.

### 3.2.5 Determination of tetracyclines

Components analyzed: chlortetracycline, 4-epi-chlor-tetracycline, oxytetracycline, 4-epi-oxytetracycline, tetracycline, 4-epi-tetracycline and doxycycline. Samples (5.0 g) were extracted with McIlwain buffer (pH = 4) and the extract was purified by reverse phase (C-18 or copolymer) SPE. The samples thus prepared were analyzed by HPLC-DAD ($\lambda$ = 365 nm) or LC-ESI(+)-MS/MS using an acidic eluent and a core-shell C-18 HPLC column **[8]**. In the case of UV detection, application of a ternary mobile phase (methanol/acetonitrile/aqueous oxalic acid) is necessary to achieve the required chromatographic resolution.

### 3.2.6 Determination of quinolones

Components analyzed: difloxacin, orbifloxacin, sarafloxacin, ofloxacin, marbofloxacin, enrofloxacin, ciprofloxacin, danofloxacin, norfloxacin, oxolinic acid, nalidixic acid and flumequine. Samples (5.0 g) were extracted with phosphate buffer and the extract was purified on a C18 SPE column, then the target components were determined by HPLC-FLD ($\lambda_{ex}$ = 260/280 nm, $\lambda_{em}$ = 366/450 nm) on a C18 reverse phase **[13]**. Although quinolones can be measured with high selectivity and sensitivity on an LC-MS/MS system, this separation technique still cannot be considered the most suitable, because quantitative evaluation is greatly influenced by the matrix effect when IDMS (isotope dilution mass spectrometry) is not used.

### 3.2.7 Determination of lincomycin

Lincomycin belongs to the group of lincosamides. It is well extractable from the test sample with pure acetonitrile and then, following a solvent exchange, it can be purified on a cation exchange SPE column in aqueous acetic acid solution (pH = 4.7). Lincomycin can be separated isocratically by LC-ESI(+)-MS/MS on a core-shell C18 HPLC column using an acidic eluent **[14]**.

### 3.2.8 Determination of griseofulvin

Grisin can also be extracted with acetonitrile and then directly purified on a C18 SPE column. Its quantitative analysis is carried out by HPLC-DAD ($\lambda$ = 290 nm) on a C18 HPLC column using an acidic eluent.

## 4. Results and evaluation

### 4.1 Method validation

For the validation of screening methods for veterinary drugs, a uniform procedure was issued by the European Union Reference Laboratory (EU-RL) in 2010 **[15]**. According to Annex I of this, analytical methods of drug residues can also be validated as follows: select twenty different blank samples (e.g., twenty different honey samples) and analyze them in parallel, with and without spiking. The level of spiking should be half the MRL value or less. If the range of the detected signals in the twenty spiked samples (for each component) does not overlap with the range of detected signals in the 20 blank samples within the retention time window of the given target compound, then the method can be considered

validated for the given component. Cut-off values can be given for each component as the smallest response of the spiked samples. The dimension of the analytical response is usually absorbance or count. In this case, the detection capability (CCβ) is equal to the spike level set during the validation (≤0.5 x MRL). CCβ has several definitions; in the case of a screening method, the CCβ is the concentration of the target component in the sample which can be detected by the given analytical method with β error. For group B compounds the permissible error in the case of screening procedures is 5%, i.e. β=5. Calculation of the detection capability is also prescribed for confirmation methods by Commission Decision 2002/657/EC **[7]**. At the same time, the CCβ calculated with the formula for confirmation methods is always higher than the decision limit (Ccα) of the confirmation method, which is erroneous in principle, since the CCβ should be lower than the MRL value. For components with limit values, the CCα is always higher than the MRL: $CC\alpha = MRL + 1.64 \times S_{MRL}$, where $S_{MRL}$ is the standard deviation of the 20 samples spiked to the MRL value during the validation. During the review of the regulation, evaluation of the CCβ is suspended in the case of confirmation methods, and only giving of the CCα value will be mandatory for confirmation procedures. ***Table 1*** shows the detection capability of the multicomponent LC-MS/MS screening method in various matrices for different components. Validation was achieved for each component, but the recovery of neomycin and gentamicin proved to be low, the reason for which was the weakly acidic extraction medium.

Validation of honey samples was carried out for the veterinary drugs suitable for the treatment of bees (tetracyclines, sulfonamides, and among aminoglycosides, mainly streptomycin), complemented by the determination of lincomycin and griseofulvin. For the first three groups, recommended values between 20 µg/kg and 50 µg/kg in honey were determined by the EU-RL (ANSES, Fougeres, France) **[16]**. Antibiotics may be effective against the American or European foulbrood of honey bees, however, for the time being, their use in the European Union has not been allowed **[17]**, and so no regulatory limit values exist for them in honey. Regarding the regulation of the veterinary drug content of honeys, a step forward could be the 2013 Codex recommendation for the establishment of an MRL in honey **[18]**. The 20 µg/kg value recommended by the EU-RL applies to the group of macrolides, including erythromycin and tylosin. At the same time, according to recent studies, these components rapidly metabolize in honey, so only their degradation products (anhydroerythromycin, erythromycin enol ether and desmycosin) can be detected **[19]**, **[20]**, for which, for the time being, there is no recommended value. So far, our measurements have not been extended to include the analysis of these metabolites.

Validation of the confirmation methods is carried out according to the above-mentioned Commission Decision 2002/657/EC **[6]**. In this case, a considerably larger number of analytical performance characteristics have to be determined, such as selectivity, identification, accuracy, reproducibility, linearity, CCα, etc. As a new parameter, the evaluation of the matrix effect will be included after the review of the guidance, which will be required in the case of mass spectrometric detection. In our paper, the decision limits (CCα) of confirmation methods are given for each component, depending on the matrix. For new components that are yet to be validated, the limit of quantification (LOQ) is reported (***Table 1***).

### 4.2 International proficiency testing

The EU-RL role of food antibiotics testing is performed by the laboratory of EU-RL (Fougeres, France). In the proficiency tests (PT) organized by them, in which participation is mandatory for the national reference laboratories (NRL) of member states, the screening and confirmation measurements of the samples have to be evaluated and reported separately. Previously, authority laboratories had to participate in group-specific proficiency tests (e.g., the detection of tetracyclines in pig muscle, or the determination of beta-lactams in milk), but today it is necessary to analyze samples that are contaminated with antibacterial agents that belong to different antibiotics groups (e.g., the analysis of type B1 antibiotics in eggs or in muscle). In the 2016 and 2017 proficiency tests, the multicomponent LC-MS/MS method was already used by us for the screening of the samples.

In the 2016 proficiency tests, the antibiotics content of four honey samples had to be determined, one of which was negative. In the other three samples, various veterinary drug residues were detected. In the first sample sulfathiazole, in the second tetracycline and 4-epi-teracycline, and in the third sample streptomycin was detected. Identification of the components was adequate using the screening method, the same components could be detected by the confirmation measurements based on LC-MS/MS, while the blank sample did not contain antibiotics according to the confirmation measurements (**Table 3**). Concentrations detected by the confirmation measurements were adequate. In the case of sulfathiazole, a value of 4.76 µg/kg was obtained by the LC-MS/MS measurement, the 6.0 µg/kg value obtained by HPLC-FLD detection could not be reported. However, the higher value obtained using the optical detector proved to be better, based on the report of the organizers of the proficiency test. In the case of the LC-MS/MS measurement, the evaluation of sulfathiazole was performed without IDMS, using the matrix-matched calibration method. The smaller detected value can be explained by the different matrix effects in the matrix-matched calibration samples and the proficiency test sample: if the ion suppression (matrix effect) is greater in the

test sample than in the calibration sample, then it can lead to the underevaluation of the contaminants of the test sample. In the absence of an IDMS, the matrix effect, which occurs in the ion source of the instrument due to background components and influences quantitative evaluation decisively, could not be compensated by the calibration. This hypothesis is supported by the fact that, in the case of optical detection (FLD), a value within the acceptability range (5.7 µg/kg – 14.7 µg/kg) could be obtained.

This is so because optical detection is not influenced by background matrices, as long as they do not generate a fluorescent signal at the wavelengths where the target components are detected.

In the 2017 proficiency test, the task was to investigate antibacterial agents in freshwater fish for type B1 components. During the LC-MS/MS screening analysis, of the four samples received no target compound was detected in sample no. 1. However, the other samples were contaminated: oxytetracycline and 4-epi-oxytetracycline were detected in the second sample, sulfadiazine and trimethoprim were detected in the third sample, and the last sample contained oxolinic acid. For the confirmation of oxytetracycline, a HPLC-DAD method was used, while for the detection of oxolinic acid the HPLC-FLD technique. For the confirmation analysis of sulfadiazine and trimethoprim, LC-MS/MS was used (***Table 3***). Evaluation of the proficiency test is still ongoing.

During the measurement of tetracyclines and quinolones, optical detection was preferred **[7]**, because this way no IDMS was required for accurate concentration determination. After the results had been submitted, the samples contaminated with oxytetracycline and containing oxolinic acid were analyzed with LC-MS/MS as well. When analyzing oxytetracycline, the sample preparation steps of the HPLC-DAD and LC-MS/MS measurements were identical, and the samples were evaluated using matrix-matched calibration.

Calibration was recorded with a matrix from carp, the type of the proficiency test sample was unknown. The values detected using the HPLC-DAD technique were 33.6 µg/kg 4-epi-oxytetracycline and 81.8 µg/kg oxytetracycline. 4-Epi-oxytetracycline concentrations were 30.6 µg/kg and 36.9 µg/kg, while oxytetracycline concentrations were 132 µg/kg and 232 µg/kg for the samples prepared for LC-MS/MS analysis in duplicate. The difference between the retention times of 4-epi-oxytetracycline and oxytetracycline (5.8 min and 6.5 min) is 0.7 min (***Figure 2***). The quantitative determination of the components was not negatively affected by the background in the elution time window of the epimer, however, the matrices entering the ion source of the instrument together with the parent compound (oxytetracycline) had a significant and irreproducible effect on the signal of oxytetracycline. The matrix effect in the test sample, exhibiting lower ion suppression compared to the matrix effect in the calibration samples, could not be compensated by matrix-matched calibration, and so the concentration detected in the proficiency test sample found to be higher. Additionally, the matrix effect of the test sample could not be repeated in the parallel samples, so in the future further sample clean-up steps or the use of an IDMS might be necessary when using the LC-MS/MS technique. The Z-score value calculated for the value detected by HLC-DAD was -1.3, therefore, it was in the appropriate range.

When analyzing the sample containing oxolinic acid using an LC-MS/MS system, the concentration was found to be 2.5 times higher than the value obtained by FLD detection. The 105 µg/kg value measured by the fluorescent detector is more likely, because optical detection was not influenced by interference, and so the accuracy of the quantitative evaluation was considered satisfactory. The calculated Z-score value for the concentration of 105 µg/kg was -0.9, so the evaluation of the analytical result of the sample contaminated with oxolinic acid was also considered satisfactory. In the absence of an IDMS, detection of oxolinic acid by MS/MS showed a great ion amplification, as a result of which the measured concentration appeared to be several times of the assigned value.

### 4.3 Monitoring analysis

The applicability of analytical methods can always be verified by the examination of real samples, since during validations, mostly spiked samples are analyzed and samples coming from untreated animals. In the case of real samples, target components are located in the pores between cells or in the intracellular space, whereas spiked samples carry the target compounds on their surface. This may result in significant differences in the recovery of the components. In the course of monitoring studies, samples containing real contamination or blank samples are analyzed. Monitoring samples are predominantly animal tissues (muscle, liver and kidney), or milk, eggs and honey. Commercially available control samples usually also come from treated animals, of these, a honey sample contaminated with sulfaquinoxaline and sulfathiazole was analyzed using the multicomponent LC-MS/MS system (***Table 3***). Target components could be detected and their confirmation analysis proved to be satisfactory as well **[9]**.

Analytical results of the nearly 1,800 samples tested since the introduction of the LC-MS/MS technique as a screening method into the monitoring analyses (April 2017) show that the most common veterinary drug residues belong to the group of tetracyclines. Of the four tetracycline derivatives having legal limit values, all of them could be detected in the different samples (***Table 3***). Contaminated samples included

pig and bovine tissues (muscle, liver and kidney), and a muscle coming from goose. In the urine of a cow treated with tetracycline, a corticosteroid type compound, dexamethasone could be detected using another LC-MS/MS technique **[21]**. It is likely that the animal was treated with a combined drug containing several active ingredient. The other contaminated samples contained different antibacterial agents. Enrofloxacin and ciprofloxacin (quinolone type antibiotics) were detected in chicken muscle. In honey, sulfadimethoxine (sulfonamide) and trimethoprim (diaminopyrimidine) were detected simultaneously. The role of trimethoprim in the product is to enhance the effect of the sulfonamide, so in addition to sulfonamides, the analysis of trimethoprim residues is definitely necessary.

This is also illustrated by the analysis of a bovine kidney that contained sulfadiazine and trimethoprim as well (***Table 3***). From among beta-lactams, penicillin G (benzylpenicillin) was detected in bovine kidney. In animal tissue samples, penicillins are unstable even at -20 °C **[22]**. During the repeat analysis of the sample ten days later, only half the amount of the target component could be detected, despite storage at -18 °C. It is advisable to store samples contaminated with beta-lactam type veterinary drugs in a pH=6 medium at -70 °C for longer shelf life **[21]**.

In cow's milk samples, cefalonium could be detected, which is a cephalosporin, i.e., belongs to the beta-lactam family. Besides this, no other antibacterial agent was found in milk. Of the monitoring samples analyzed, no contamination was detected in eggs so far, and of the target compounds, macrolides, lincomycin and griseofulvin has not yet been identified in any of the samples. One year after the introduction of the multicomponent screening method it can be said that the number of samples containing detectable amounts of contamination has increased considerably over recent years.

The main reason for this is that the measurements are not specific for certain antibiotics groups (e.g., beta-lactams or quinolones, etc.), but the majority of agents belonging to type B1 are covered by a multicomponent screening analysis. Another advantage of the method is that the material cost is minimal, since samples are only extracted during sample preparation and they are analyzed without a purification step so, for example, no solid phase extraction is necessary, which would significantly increase the time and cost of sample preparation. In addition to reducing costs, the efficiency of the analyses was increased successfully with the multicomponent screening method.

## 5. Method adaptation

Within the framework of the cooperation between the National Food Chain Safety Office and Shahid Behesti University of Medical Sciences (Tehran, Iran) five Iranian experts arrived at the NÉBIH Étbl in May 2017, to learn the multicomponent screening methods as part of an LC-MS/MS training. During the one-week course, the procedure was fully demonstrated and it was tried and learned by the colleagues from Iran. The second half of the training will take place in Tehran, and the objective is to fully implement the method in the Iranian NRL. The training will then continue with the description of confirmation measurements, two of which (tetracyclines and sulfonamides) have already been tried during the first training in Budapest.

Adaptation of the multicomponent method was also successfully tried at the National Reference Laboratory (NRL) in Turkey as part of a one-week training. Within the framework of a twinning project, another training is planned for May 2018, and as a continuation of this, validation of the method is planned by the Turkish colleagues in the near future.

## 6. Conclusions

The efficiency of the veterinary drug residue analytical concept presented in this paper has already been proven with the analysis of 1,800 samples. One year after the introduction of the multicomponent screening method, the efficiency of monitoring analyses has increased considerably, a larger number of contaminations could be detected, compared to previous years. Confirmation of the target components detected by the screening method is carried out by an independent confirmation procedure. Confirmation analyses of the samples containing antibiotic residues confirmed in all cases the presence of the target compound detected by the screening method in the sample.

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