

Determination of sulfonamides in muscle: a metrological tool for food safety

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ABSTRACT

Sulfonamides represent a wide class of synthetic drugs commonly used in veterinary therapy for the treatment of several bacterial and protozoan infections in cattle, swine and poultry. The use of these drugs in farming can lead to the possibility of having their residues in animal products intended for human consumption. Consequently, to ensure high consumer protection, for sulfonamides European Union (EU) set a Maximum Residue Limit (MRL) equal to 100 µg/kg, either as a single molecule or as a sum of all detected compounds within the class). Official laboratories are directly involved in the execution or residue plans by developing, validating and then applying analytical methods for the measurement of drug residues. Accordingly, official laboratories should update their procedures following the evolution of required drugs and MRLs. A multiclass method previously developed and validated for the determination in animal muscle of ten classes of antibiotics was adjusted to comply with the current European requirements which establish the minimum set of sulfonamides to be determined. Therefore, eight new sulfonamides were added assessing method performance characteristics according to European Regulation (EU) 808/2021.

Section: RESEARCH PAPER

Keywords: sulfonamides; residues; LC-HRMS; multiclass method; food control; measurements

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1. INTRODUCTION

Sulfonamides, N-derivatives of 4-amino-benzensulfonamide, represent a wide range of synthetic antibacterial compounds commonly used in farm and fish aquaculture [1]. This family of drugs was the first effective chemotherapeutic agents to be employed systemically for the prevention and treatment of bacterial infections in humans. In Figure 1, the structures of some of the most used sulfonamides are shown. Sulfonamides, included in Group B of the European Commission (EC) Directive 96/23/EC [2], are comprised among the authorised drugs for which the European Union (EU) set Maximum Residues Limits (MRLs) in foodstuff. The MRL for sulfonamides listed in European Regulation (EU) 2010/37 is equal to 100 µg/kg for individual compounds and their sum [3]. Following that, European Regulation (EU) 2017/625 [4] outlined the requirements for annual, mandatory monitoring plans for residue detection in live animals, their excrements, body fluids, tissues, animal products, animal feed, and drinking water. European

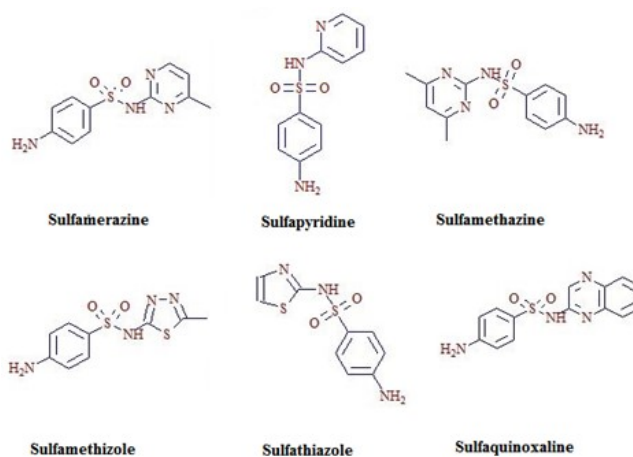


Figure 1. Molecular structure of typical sulphonamides.

Regulation (EU) 2017/625 requires all EU member states to prepare yearly National Residue Plan (NRP). Within Italy, the Health Ministry is in charge of developing NRP in which the type and number of samples to be controlled yearly for single substances or groups of substances. At a national level, the NRP is the main practice for the surveillance of residues of illicit and authorized drugs used in veterinary practice and it represents an essential tool for the management of health risks. The need to measure drug residues in food at a level of a few parts per billion to meet EU requirements is a priority for official laboratories which are faced to develop and validate analytical methods with high sensitivity and selectivity. In this context, method validation based on performance criteria is one of the three pillars of residue control of European Union. The other two cornerstones are the hierarchical organization of official laboratories (European, EURLs, National, NRLs, and Routine laboratories, RLs) and accreditation based on ISO 17025:2017 standard [5]. Within the determination of residues of pharmacologically active substances used in food-producing animals, performance criteria are established by European Regulation (EU) 2021/808 [6] in which a distinction is made between screening and confirmatory procedures. The former are generally cheap, rapid and high-throughput assays used to detect the presence of drugs at the level of interest frequently furnishing binary responses. Their main purpose is to screen a large number of samples for suspect non-compliant results. In contrast, confirmatory methods must provide structural information to unequivocally identify and also quantify analytes and they are based on instrumental techniques, involving liquid chromatographic separation followed by spectrophotometric, fluorescence or mass spectrometric detection. However, it is worth noting that in recent years the progressive availability of liquid-chromatography mass spectrometry (LC-MS) multiclass methods capable of the simultaneous detection and quantification of dozens of veterinary drugs in relatively short times has increased their routine application, both as for screening and confirmatory purposes [7]-[10]. As the number of analytes required continues to increase and gradually lower levels are set, the ability of multiclass methods to bring together the plethora of single-class procedures developed in the past years undoubtedly increases their cost-effectiveness [11], [12]. As a result, single-class protocols for this or that family of drugs are now “dying out” within the official control of the European Union. Changes in specific requirements, such as extension of substances or matrices, are not uncommon and continuous method “maintenance” is fundamental to carry out analysis within NRPs. This paper describes the updating of a multiclass procedure routinely used from about ten years at Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche [13], [14] in order to extend the number of sulfonamides to those currently required by EURLs.

2. EXPERIMENTAL

2.1. Chemical and reagents

Ammonium acetate analytical grade was obtained from VWR chemicals (Milan, Italy) and deionized water for LC was generated by a MilliQ purification system. EDTA sodium salt dehydrate and acetonitrile (ACN) ultra-gradient grade for LC-MS and methanol ultra-gradient grade for LC-MS were from Sigma-Aldrich (St. Louis, MO, USA). Sulfaclozine, sulfadoxine, sulfisoxazole, sulfamethizole, sulfamethoxypridazine, sulfamer, sulfamoxole, sulfachloropyridazine and

Table 1. Chromatographic run conditions.

Total flow (μL/min)	Time (min)	Methanol (%)	Formic acid 0.1 % (%)
250	0	5	95
	1	5	95
	20	95	5
	25	95	5
	26	5.0	95
	30	5.0	95

sulfametazine 13C6, analytical standards (reference materials) were purchased from Sigma-Aldrich.

2.2. Standard and working solutions

For the eight sulfonamides and isotopically labelled internal standard individual stock solutions at 100 μg/mL were prepared in MeOH and stored at -20°C for 24 months [15], [16]. The intermediate working solutions (1 μg/mL) were prepared in MeOH, too.

2.3. Chromatographic and MS conditions

Chromatographic analysis was performed by means of a Thermo Ultimate 3000 HPLC System (San Jose, CA, USA). Analyte separation was achieved using an Agilent Poroshell 120 EC-C18 column as described in Moretti et al. [14]. The mobile phases were methanol and 0.1% formic acid in water. The gradient is shown in Table 1. The autosampler was kept at 16°C and the column at 30 °C. The gradient flow was 250 μL/min and sample injection volume 5 μL.

Experiments were carried out with high resolution mass spectrometry (HRMS) detection (Q Exactive™, Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI-II) source. The mass spectrometer was controlled by the Xcalibur 4.4 software (Thermo Fisher Scientific). The exact mass of compounds was calculated using Qualbrowser in Xcalibur 4.4. Retention times, m/z of precursor ion and m/z of two fragment ions for analyte identification are reported in Table 2.

2.4. Sample preparation

One-half grams of swine muscle were weighed in a 15 mL Falcon tube. The sample was spiked with 15 μL of the IS solution at 1 μg/mL corresponding at 10 μg/kg. 100 μL of an EDTA solution 0.1 M was added and muscle was extracted with 3 mL of a mixture ACN/H₂O 4:1 v/v acidified with 0.05 % formic acid. After vortexing, shaking and centrifugation, a second ultrasonic-assisted extraction with 3 mL of acetonitrile was carried out. The reunited extracts were evaporated and then dissolved in 1.5 mL of 0.2 M ammonium acetate solution. After centrifugation, the sample was injected.

2.5. Method validation

Validation is the demonstration by examination and the provision of effective evidence that the particular requirements of a specific intended use are fulfilled. Method performance characteristics estimated in this study were selectivity, linearity, accuracy (trueness and precision), decision limit (CC_α) and matrix effect. These parameters are required by Regulation 2021/808. In addition, detection and quantification limits along with measurement uncertainty were assessed, too. Selectivity was tested by analysing 20 blank muscle samples from different animal species. Linearity was evaluated in solvent and matrix in the range 5–150 ng/mL, (5, 10, 33, 100 and 150 ng/mL). Solvent

Table 2. Retention times and mass spectrometric parameters used for analyte identification.

Peak	Analyte	RT (min)	Molecular formula	Precursor ion	Exact mass (m/z)	Fragment 1 (m/z)	Fragment 2 (m/z)
1	Sulfamoxole	8.70	C ₁₁ H ₁₃ N ₃ O ₃ S	[M+H] ⁺	268.0750	156.0	108.1
2	Sulfameter	8.82	C ₁₁ H ₁₂ N ₄ O ₃ S	[M+H] ⁺	281.0703	156.0	108.1
3	Sulfamethizole	9.00	C ₉ H ₁₀ N ₄ O ₂ S ₂	[M+H] ⁺	271.0318	156.0	108.1
4	Sulfamethoxy pyridazine	9.40	C ₁₁ H ₁₂ N ₄ O ₃ S	[M+H] ⁺	281.0703	108.1	156.0
5	Sulfachloropyridazine	9.96	C ₁₀ H ₉ ClN ₄ O ₂ S	[M+H] ⁺	285.0208	156.0	108.1
6	Sulfadoxine	10.51	C ₁₂ H ₁₄ N ₄ O ₄ S	[M+H] ⁺	311.0809	156.0	108.1
7	Sulfisoxazole	10.60	C ₁₁ H ₁₃ N ₃ O ₃ S	[M+H] ⁺	268.0750	156.0	108.1
8	Sulfaclozine	12.02	C ₁₀ H ₈ ClN ₄ NaO ₂ S	[M+H] ⁺	307.0027	156.0	108.1

calibration curves were prepared dissolving standards in 0.2 M ammonium acetate, whereas the matrix-matched ones were prepared processing a swine blank muscle and then adding the analytes immediately prior to LC injection. Calibration curves were built applying linear regression analysis. To perform spiking experiments a nested design was carried out [17], [18]. Blank muscle was spiked before extraction with an appropriate volume of standard solutions. The spiking levels were five: 3.3, 10, 33, 100, and 150 µg/kg. Four replicates ($n = 4$) were analysed during the same day along with a matrix-matched calibration standard. Each series was repeated on three different days at each of the five concentrations varying time, muscle, operator and calibration status of liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) system. Precision evaluated in repeatability and within-laboratory reproducibility conditions, recovery (trueness), LOD and LOQ were estimated for each analyte. Decision limit ($CC\alpha$) was calculated adding to MRL, 1.64 times the standard deviation observed at MRL in intralaboratory reproducibility conditions (swR). Matrix effect (ME) was assessed by comparing the slope of a five-point standard curve prepared in ammonium acetate with the slope of the same five-point curve prepared adding antibiotic standards to a blank muscle extract immediately prior to LC injection. Finally, measurement uncertainty was estimated, too.

3. RESULTS AND DISCUSSION

The choice of analytes included in the original method was based on EU Regulation 37/2010, manuals of veterinary drugs for food-producing animals and consultation of experts in the field of livestock production. In this update, eight additional sulfonamides have to be added to comply with the current required compounds [11]. The chromatographic conditions and sample preparation have been maintained as in the original method since they were satisfactory also for the new analytes. As matter of fact, the chromatographic conditions allowed a good separation of new and already included sulfonamides, although some of them are isomers such as sulfamethoxy pyridazine, sulfameter and sulfamonomethoxine (C₁₁H₁₂N₄O₃S). In Figure 2, the chromatograms of a blank swine muscle (a) and of a spiked one at 10 µg/kg (b) are shown, demonstrating an excellent separation of eight sulfonamides.

3.1. Selectivity

Following IUPAC definition “selectivity refers to the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour” [19]. Interference of homologs, isomers, degradation products, endogenous substances, analogs, metabolic products of the residue of

interest, matrix compounds, or any other possibly interfering substance shall be determined and, if needed, the method shall be amended to avoid the identified interferences. In addition, for detection by mass spectrometry, a minimum of four identification points (IPs) must be achieved for permitted substances (Section 1.2.4.2 of Regulation 2021/808). With the applied method, one IP was from chromatography separation and six and a half IPs were obtained from the acquisition of one full scan ion (1.5) and two HRMS product ions (2.5+2.5) for a total of seven and a half IPs.

3.2. Linearity

Although Regulation 2021/808 does not give a precise criterion to assess linearity, the use of coefficient of determination (R^2) is suggested (Section 2.8). However, it is well recognised that R^2 is not a suitable parameter to judge linearity. Therefore, as criterion, the deviation of back-calculated concentration from true concentration was applied and values of deviation lower than $\pm 20\%$ were considered acceptable [20]. For

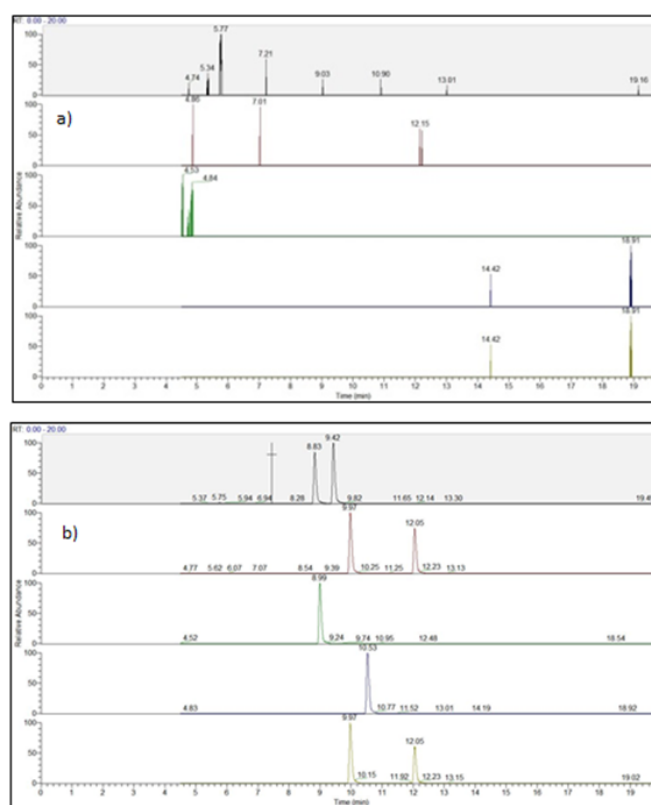


Figure 2. LC-HRMS chromatograms of a) a blank swine muscle and b) a swine muscle spiked at 10 µg/kg.

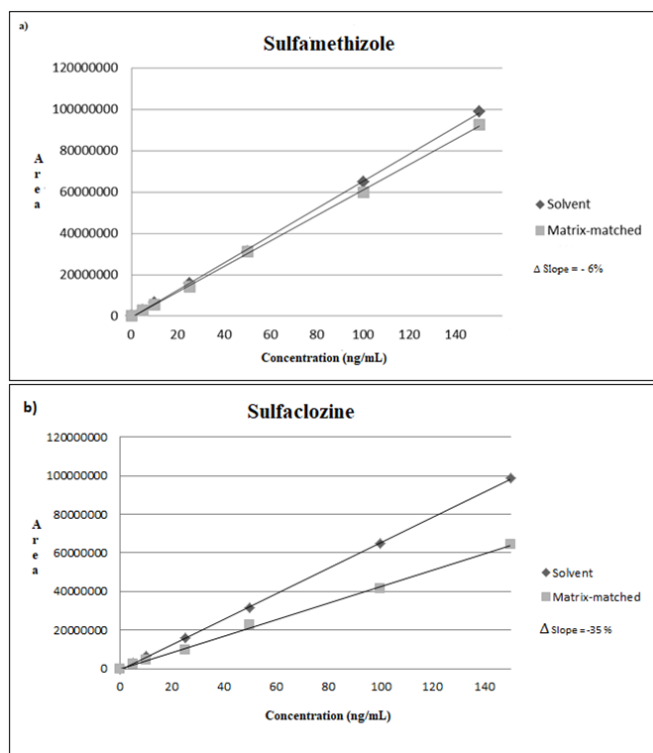


Figure 3. Evaluation of matrix effect of a) sulfamethizole and b) sulfaclozine .

solvent curves, the linearity was ascertained in the interval 10 ng/mL-150 ng/mL, whereas for the matrix-matched ones was from 5 ng/mL - 150 µg/kg. Therefore, when suspect muscle samples with concentration higher than 150 µg/kg (150 ng/mL) have to be confirmed, the final extract is diluted 10 times so as to be in the linearity interval.

3.3. Matrix effect

Matrix effects were evaluated by comparing the slope of solvent calibration curve (without matrix) versus that of matrix-matched calibration one. A slight ion suppression (20 %- 40 %) was measured for all sulfonamides, except sulfamethizole. In Figure 3 solvent and matrix-matched calibration curves of sulfamethizole and sulfaclozine are shown [21] [22].

3.4. Accuracy and Decision Limit (CC α)

A strategy following “other validation approaches” (Section 2.2.3 of Regulation 202/808) was adopted to assess accuracy (trueness and precision) and Decision Limits by means of a nested design at five concentrations encompassing sulfonamide MRL [14], [18]. Since the same MRL (100 µg/kg) is fixed for each individual sulfonamide as well as for their sum, it is important to evaluate method accuracy also at low

Table 3. Decision limits (CC α) of the eight sulfonamides.

Analyte	CC α (µg/kg)
Sulfamoxole	112
Sulfameter	111
Sulfamethizole	112
Sulfamethoxyipyridazine	113
Sulfachloropyridazine	113
Sulfadoxine	113
Sulfisoxazole	120
Sulfaclozine	117

concentrations since, when suspect samples containing more than one sulfonamide have to be confirmed, accurate measurements until one tenth of MRL are needed. The mean recoveries were in the range 75 % – 81 %, comparable to those measured for the sulfonamides already included in the original procedure [14]. Coefficients of Variation (CVwR) in intra-lab reproducibility conditions were satisfactory and lower than 20 %. Decision limits (CC α), estimated according “method 2” described for authorized substances in section 2.6 of EU Reg. 808/2021, are listed in Table 3.

3.5. Limit of detection (LOD), limit of quantification (LOQ) and measurement uncertainty

In 2002, updating the performance criteria of analytical methods for residues of pharmacologically active substances used in food-producing animals, Commission Decision 2002/657/EC [23] (now repealed by Regulation 2021/808) introduced, for the first time in the context of official controls, the parameter called Decision Limit (CC α) which must be evaluated in order to verify the sample conformity. In contrast, during 2000s, in documents establishing method performance criteria for other groups of residues such as heavy metals, mycotoxins, dioxins or pesticides, the concept of measurement uncertainty prevailed. In analytical chemistry, limit of detection (LOD) and limit of quantification (LOQ) are used to define lower limits at which an analyte can be detected and quantified, irrespective to its “legal status” (authorized substance or not). However, Regulation 2021/808 which is mainly aimed at verifying the conformity of sample does not consider neither LOD or LOQ. All that premised, although LOD, LOQ and measurement uncertainty are not mentioned in Regulation 2021/808, in certain circumstances as in external audits according to ISO 17025 Standard, their evaluation can be required. At the first validation level (3.3 µg/kg) some analytes demonstrated unsatisfactory CVwR and, therefore, LOD and LOQ were fixed both at 10 µg/kg at which also quantitative performance were satisfactory. It is worth noting that it is not necessary to set the exact LOD or LOQ of each analyte, but to set so-called operational limits. In other words, the “true” LOD and LOQ might be lower than those stated, but they are guaranteed not to be higher. Also the values of relative expanded uncertainties (U_{rel}) were satisfactory being lower than 40 % (Table 4). The estimation of U_{rel} was achieved considering precision and bias contributions and applying a coverage factor k equal to 2.

3.6. Ruggedness

Analytical methods shall be tested for their performance characteristics under different experimental conditions, which include, for example, different animal species and minor changes of operational parameters. The scope is to verify method ruggedness, i.e. if these changes can influence or not results. In Table 5 recoveries and standard deviations obtained analyzing three animal species different than swine used for the validation study are reported. Four replicates were performed for each species spiking blank muscles at 33 µg/kg. Analysis of Variance (ANOVA) demonstrated that performance characteristics did not significantly vary as a function of animal species.

4. CONCLUSION

As well known, one of the main advantages of multiclass procedures is their flexibility, since they are already designed to analyse compounds with very different physicochemical properties. This is an important feature because, in the context

Table 4. Relative expanded uncertainty at the four validation levels.

Concentration (µg/kg)	U_{rel} (%)							
	Sulfameter	Sulfamoxole	Sulfisoxazole	Sulfamethizole	Sulfadoxine	Sulfamethoxyppyridazine	Sulfachloropyridazine	Sulfaclozine
10	18.55	19.7	39.40	24.30	23.54	23.05	22.75	26.8
33	16.09	17.1	24.09	16.83	18.64	18.15	18.57	22.4
100	15.85	16.9	22.20	15.98	18.13	17.64	18.15	22.0
150	15.83	16.9	22.06	15.93	18.10	17.60	18.12	21.9

Table 5. Result of ruggedness experiment.

Analyte	Swine		Bovine		Poultry		Trout	
	Recovery (%)	s (%)	Recovery (%)	s (%)	Recovery (%)	s (%)	Recovery (%)	s (%)
Sulfameter	87	4.8	89	5.3	85	4.6	82	3.1
Sulfamoxole	73	8.2	82	4.4	82	3.3	74	5.0
Sulfisoxazole	73	4.6	76	7.6	77	5.5	70	6.8
Sulfamethizole	82	5.0	79	5.1	76	3.8	80	2.9
Sulfadoxine	78	7.2	82	2.4	90	3.5	81	4.1
Sulfamethoxyppyridazine	82	3.8	85	4.4	82	4.0	80	3.8
Sulfachloropyridazine	82	5.7	83	5.1	80	2.4	79	4.0
Sulfaclozine	73	8.2	82	7.4	80	4.1	75	6.6

of official food control, changes of specific requirements such as extensions of substances or matrices, are not uncommon. This work precisely confirms that the introduction of eight new sulfonamide drugs can be achieved with little effort and without any modification of the original method (chromatographic conditions and sample preparation). Thus, the updated method can be applied within the EU official control. Experiments are in progress to extend the procedure also to other food such as milk and eggs.

REFERENCES

- [1] W. M. A. Niessen, Analysis of antibiotics by liquid chromatography-mass spectrometry, *J. Chromatography A* 812 (1998), pp. 53-75. DOI: [10.1016/S0021-9673\(98\)00281-7](https://doi.org/10.1016/S0021-9673(98)00281-7)
- [2] European Commission (1996). Council Directive (EC) n° 96/23 of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Communities, L125, 10-32. Online [Accessed 09 March 2024] <https://eurlex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31996L0023>
- [3] European Commission (2010). Commission Regulation (EU) n° 2010/37 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, Official Journal of the European Communities, L15, 1-72. Online [Accessed 09 March 2024] <https://eurlex.europa.eu/legalcontent/EN/TXT/?uri=CELEX%3A32010R0037>
- [4] The European Parliament and The Council of European Union (2017). 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, Official Journal of the European Communities, L 95: 1-142. Online [Accessed 09 March 2024] <https://eur-lex.europa.eu/eli/reg/2017/625/oj>
- [5] International Standard Organization ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories, Int. Stand. 2005. Online [Accessed 09 March 2024] <https://www.iso.org/standard/39883.html>
- [6] Commission Implementing Regulation (EU) 2021/808, of 22 march 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. Official Journal of the European Communities, L180, 84. Online [Accessed 09 March 2024] <https://op.europa.eu/en/publicationdetail/publication/3dc2b06b-b9cf-11eb-8aca-01aa75ed71a1/language-en>
- [7] S. C. Barros, A. S. Silva, D. Torres, Multiresidues multiclass analytical methods for determination of antibiotics in animal origin food: A critical analysis, *Antibiotics* 12(2) (2023), art. no. 202. DOI: [10.3390/antibiotics12020202](https://doi.org/10.3390/antibiotics12020202)
- [8] A. Masiá, M. M. Suarez-Varela, A. Llopis-Gonzalez, Y. Picó, Determination of pesticides and veterinary drug residues in food by liquid chromatography-mass spectrometry: A review, *Anal. Chim. Acta* 936 (2016), pp. 40-61. DOI: [10.1016/j.aca.2016.07.023](https://doi.org/10.1016/j.aca.2016.07.023)
- [9] L. Mainero Rocca, A. Gentili, V. Pérez-Fernández, P. Tomai, Veterinary drugs residues: a review of the latest analytical research on sample preparation and LC-MS based methods, *Food Additives & Contaminants: Part A* 34(5) (2017), pp. 766-784. DOI: [10.1080/19440049.2017.1298846](https://doi.org/10.1080/19440049.2017.1298846)
- [10] R. Rossi, G. Saluti, S. Moretti, D. Giusepponi, I. Diamanti, R. Galarini, Multiclass methods for the analysis of antibiotic residues in milk by liquid chromatography coupled to mass spectrometry: A review, *Food Additives & Contaminants: Part A* 35(2) (2018), pp. 241-257. DOI: [10.1080/19440049.2017.1393107](https://doi.org/10.1080/19440049.2017.1393107)
- [11] Introduction to evaluation of EU-MS RMP 2020'' – EURL – ANSES - FOUGERES (A6-B1-B2f-B3e), Laboratory of Fougères. Online [Accessed 09 March 2024] https://www.anses.fr/en/content/reference_activitesfoug%C3%A8reslaboratory
- [12] EURL Guidance on Minimum Method Performances Requirements (MMPEs) for Specific Pharmacologically Active Substances in Specific Animal Matrices, September 2020. Online [Accessed 09 March 2024] https://sitesv2.anses.fr/en/minisite/eurl_fougères/uidance_minimum_method_performance_requirements
- [13] S. Moretti, G. Dusi, D. Giusepponi, S. Pellicciotti, R. Rossi, G. Saluti, G. Cruciani, R. Galarini, Screening and confirmatory method for multiclass determination of 62 antibiotics in meat, *J. Chromatography A* 1429 (2016), pp. 175-188.

- DOI: [10.1016/j.chroma.2015.12.021](https://doi.org/10.1016/j.chroma.2015.12.021).
- [14] S. Moretti, G. Cruciani, S. Romanelli, R. Rossi, G. Saluti, R. Galarini, Multiclass method for the determination of 62 antibiotics in milk, *J. Mass. Spectrom.* (2016), pp. 792–804. DOI: [10.1002/jms.3834](https://doi.org/10.1002/jms.3834)
- [15] S. Pellicciotti, S. Moretti, G. Saluti, R. Galarini, G. Dusi, Stability of antibiotics in solution: a critical issue during the development of a multi-class method by LC-HRMS (Orbitrap), in 7th International Symposium On Hormone and Veterinary Drug Residues Analysis, Ghent (Belgium), 2-5 June, 2014.
- [16] B. J. Berendsen, I. J. Elbers, A. A. Stolker, Determination of the stability of antibiotics in matrix and reference solutions using a straightforward procedure applying mass spectrometric detection, *Food Additives & Contaminants: Part A* 28 (2011), pp. 1657-1666. DOI: [10.1080/19440049.2011.604045](https://doi.org/10.1080/19440049.2011.604045)
- [17] EURL Guidance Document on the extension of a quantitative confirmation methods, Version 1.0, July 2021. Online [Accessed 09 March 2024]. <https://sitesv2.anses.fr/en/minisite/eurl-fougeres/eurlguidance>
- [18] R. Galarini, S. Moretti, G. Saluti, Quality assurance and validation general considerations and trends, *Chromatographic Analysis of the Environment: Mass Spectrometry Based Approaches*, 4th ed.; CRC Press: Boca Raton, FL, USA, 2017, p. 45.
- [19] J. Vessman, R. Stefan, J. V. van Staden, K. Danzer, W. Lindner, D. Burns, A. Fajgelj, H. Müller, Selectivity in analytical chemistry (IUPAC Recommendations 2001), *Pure Appl. Chem.*, 73(8) (2001), pp.1381–1386.
- [20] SANTE/11312/2021, Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed, 2021. Online [Accessed 09 March 2024] https://www.accredia.it/en/documento/guidance_sante_11312_2021
- [21] B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, *Anal. Chem.* 1 75(13) (2003), pp. 3019-3030. DOI: [10.1021/ac020361s](https://doi.org/10.1021/ac020361s)
- [22] M. Cortese, M. R. Gigliobianco, F. Magnoni, R. Censi, P. Di Martino, Compensate for or minimize matrix effects? Strategies for overcoming matrix effects in liquid chromatography-mass spectrometry technique: A Tutorial Review, *Molecules* 25(13) (2020), art. no. 3047. DOI: [10.3390/molecules25133047](https://doi.org/10.3390/molecules25133047)
- [23] European Commission (2002), Commission Decision n° 2002/657/EC of 12 August 2002 concerning the performance of analytical methods and the interpretation of results, *Official Journal of the European Communities*, L22, 8. Online [Accessed 09 March 2024] <https://eurlex.europa.eu/legalcontent/EN/ALL/?uri=CELEX%3A32002D0657>