PhD THESIS

Contributions to the determination of residues of antimicrobial substances in products of animal origin by chromatographic techniques

SUMMARY OF THE PhD THESIS

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INTRODUCTION

In the context of the intensification of livestock production and the globalization of the food chain, the use of antimicrobial agents in veterinary medicine has become indispensable for maintaining the health and productivity of food-producing animals. However, the frequent administration of sulfonamides, trimethoprim, and, to a lesser extent, dapsone, may lead to the persistence of residues in meat, milk, eggs, and honey—products intended for direct human consumption. These residues represent a significant risk to public health, causing allergic reactions, chronic toxicity, and, most importantly, favoring the emergence of antimicrobial resistance, a phenomenon regarded by the World Health Organization as one of the most serious global threats.

Within this framework, the thesis adopts an integrated approach to the issue of antimicrobial residues in food, ranging from the analysis of European and international regulations to the optimization and validation of modern detection methods. Emphasis is placed on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which provides superior sensitivity, specificity, and multi-residue capability compared to classical HPLC methods.

The research pursues four main objectives:

1. A critical analysis of methods used for the detection of sulfonamides, trimethoprim, and dapsone in food of animal origin;
2. The development and validation of a high-performance analytical method, in compliance with European requirements (Commission Decision 2002/657/EC) and international guidelines of good practice;
3. The application of the validated method to real samples of meat, milk, eggs, and honey, to assess the degree of compliance with maximum residue limits (MRLs);
4. The interpretation of results in the context of public health risks and the formulation of recommendations for the efficient monitoring of residues.

By combining the regulatory perspective with advances in analytical techniques, the thesis offers practical solutions for improving food safety measures and reducing the risk of antimicrobial resistance. The results obtained contribute both to the refinement of official control tools and to the protection of consumer health, while also strengthening public confidence in products of animal origin.

THE STRUCTURE OF THE PhD THESIS

The doctoral thesis entitled *"**Contributions to the determination of residues of antimicrobial substances in products of animal origin by chromatographic techniques**"* consists of 151 pages and includes iconography comprising 31 figures and 31 tables. The work is structured into two parts and was prepared in accordance with the writing standards of IOSUD USAMV-Cluj-Napoca.

The first part of the thesis comprises 33 pages and is divided into two chapters. Chapter 1, entitled *"General Introduction"*, outlines the context of antimicrobial use in veterinary medicine, with emphasis on sulfonamides, trimethoprim, and dapsone. It presents their role in the prophylaxis and treatment of diseases in food-producing animals, the risks associated with the persistence of residues in food, and their impact on public health, particularly through the promotion of antimicrobial resistance. This chapter also defines the general aim and objectives of the research, as well as the justification for choosing the topic in relation to European and international regulations on maximum residue limits (MRLs).

Chapter 2 – *"Current State of Knowledge"* – brings together detailed information on the use of antimicrobials in veterinary medicine, their classification according to therapeutic importance and mechanism of action, routes of administration and metabolism in animals, as well as the risks to consumers. European regulations and the current situation of antimicrobial consumption in the EU are analyzed on the basis of EFSA, EMA, and ESVAC reports. A substantial part is dedicated to the pharmacodynamic characterization of sulfonamides, trimethoprim, and dapsone, presenting their chemical structure, mechanisms of action, antibacterial spectrum, and mechanisms of resistance. Finally, the issue of antimicrobial residues in the main food matrices—meat, milk, eggs, and honey—and the mechanisms of their transfer through the food chain are discussed.

The second part of the thesis is structured into six chapters, which include the working hypothesis, specific objectives, materials and methods used, as well as a detailed presentation of the experimental results. Each of the three studies carried out is followed by critical discussions in which the data obtained are interpreted in relation to the scientific literature and current regulations.

The thesis concludes with *"General Conclusions"*, which synthesize the contributions made in the field of detecting and quantifying antimicrobial residues through modern methods of liquid chromatography coupled with mass spectrometry (LC-MS/MS), emphasizing their relevance for food safety and public health. The final chapter highlights the elements of originality and innovative contributions, focusing on the validation of the proposed analytical method and its applicability in official residue monitoring programs.

THE OBJECTIVES OF THE PhD THESIS

The general aim of the research is to evaluate and optimize analytical methods for the detection and quantification of sulfonamide, trimethoprim, and dapsone residues in food of animal origin, with a dual purpose: ensuring compliance with European regulatory requirements and protecting public health. Through this approach, the thesis seeks to strengthen the capacities for official control and monitoring, thereby reducing the risk of consumer exposure to hazardous levels of antimicrobials and limiting the development of bacterial resistance.

The specific objectives can be summarized in five major directions:

1. A critical analysis of the literature regarding existing methods for the determination of antimicrobial residues in meat, milk, eggs, and honey;
2. The development of a modern analytical method, focusing on LC-MS/MS techniques, capable of accurately identifying and quantifying the compounds under study;
3. The validation of the proposed method in accordance with European legislation and international guidelines, by assessing key performance parameters such as sensitivity, specificity, precision, accuracy, stability, and robustness;
4. The application of the validated method to real samples collected from the distribution chain, in order to determine the current levels of residues and compliance with maximum residue limits (MRLs);
5. The interpretation of the results obtained in relation to public health risks, and the formulation of practical recommendations for the effective monitoring and control of antimicrobial residues in food.

Study 1. Determination of sulfonamide residues in poultry using HPLC-FLD technique

Introduction

Intensive animal farming has led to the widespread use of antimicrobials in veterinary medicine, with sulfonamides being among the most frequently administered substances due to their broad spectrum of activity against both Gram-positive and Gram-negative bacteria. However, the persistence of residues in food of animal origin

may cause allergic reactions and, more importantly, may contribute to the development of antimicrobial resistance. The monitoring of antibiotic residues, in accordance with European legislation, is a priority for food safety and public health.

Aim of the study

The objective of this work was the development, validation, and application of a selective and sensitive method for the identification and quantification of 12 sulfonamides in poultry meat, using high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The study aimed to evaluate the level of residues in real samples and to compare the results with the maximum residue limits (MRLs) established by European legislation.

Materials and Methods

A total of 24 poultry muscle tissue samples were analyzed, collected monthly over the course of one year from a defined area. The method involved the extraction of sulfonamides with acetonitrile, purification using SPE C18 Strata-X cartridges, pre-column derivatization with fluorescamine, and analysis by HPLC-FLD. Method validation was performed in accordance with Commission Decision 2002/657/EC, by determining performance parameters such as linearity, selectivity, specificity, sensitivity, limits of detection and quantification, precision, reproducibility, robustness, and measurement uncertainty.

Results and discussions

The developed method showed recoveries ranging between 87–118%, good repeatability (RSDr <6%) and reproducibility (RSDR <15%), as well as detection limits between 3.3–5.2 µg/kg, values compliant with legislative requirements. When applied to real samples, residues of sulfaquinoxaline were detected in two samples (8.33% of the total), at levels of 31.98 µg/kg and 23.70 µg/kg, both below the maximum permitted level of 100 µg/kg. The positive concentrations originated from the same batch, suggesting a possible failure to observe the required withdrawal period after treatment. These results confirm the relevance of the method for monitoring residues and for preventing risks associated with consumer exposure.

Conclusions

This study demonstrates, for the first time in Romania, the applicability of an HPLC-FLD method for the detection and simultaneous quantification of 12 sulfonamides in poultry meat. The validated method meets European standards and can be used in official control programs. The results highlight the importance of continuous monitoring of antimicrobial residues, both for ensuring food safety and for reducing the risk of antimicrobial resistance.

Study 2 – Development and validation of an LC-MS/MS method for the simultaneous detection of sulfonamides, trimethoprim and dapsone in honey

Introduction

Honey, valued for its nutritional and therapeutic properties, is also vulnerable to contamination with antimicrobial residues used in apiculture for the treatment of bacterial and parasitic diseases. Sulfonamides, trimethoprim, and dapsone are compounds of major concern, due to their frequent use and the risks associated with their residues: induction of bacterial resistance, allergic reactions, and possible genotoxic effects. In the European Union, legislation prohibits the presence of sulfonamides and dapsone in honey, enforcing a zero-tolerance policy, which makes it necessary to develop highly sensitive and specific analytical methods.

Aim of the study

The aim of this work was to develop and validate a robust ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous detection of 22 sulfonamides, trimethoprim, and dapsone in honey samples. The main objective was to provide a high-precision analytical tool applicable in official monitoring programs for veterinary drug residues in Romania.

Materials and Methods

The method involved: sample preparation through acid hydrolysis and solid-phase extraction (SPE) to separate antimicrobials from the complex honey matrix; chromatographic analysis carried out on a submicron C18 column, with detection by triple quadrupole mass spectrometry in positive ESI mode; calibration on a polyfloral honey matrix at concentrations between 5–150 µg/kg for sulfonamides and trimethoprim and 0.5–15 µg/kg for dapsone; and validation in accordance with Commission Decision 2002/657/EC and SANCO guidelines, by testing performance parameters including linearity, sensitivity, specificity, recovery, precision (RSDr, RSDR), detection and quantification limits, CCα, CCβ, stability, and measurement uncertainty.

Results and discussions

The developed method demonstrated excellent sensitivity, with detection limits below the minimum recommended performance values (LOD <1 µg/kg for most sulfonamides and trimethoprim, and <0.5 µg/kg for dapsone). Recoveries ranged between 80–120%, and correlation coefficients exceeded 0.99. Precision, both in terms of repeatability and reproducibility, was within the required limits (RSD <20%). In the real honey samples analyzed, no residues above decision limits (CCα) were identified, confirming the efficiency of the method in detecting prohibited compounds at very low levels.

Conclusions

This study reports, for the first time in Romania, the development and validation of a UHPLC-MS/MS method for the simultaneous detection of sulfonamides, trimethoprim, and dapsone in honey. The method complies with European requirements, is applicable in official laboratories, and represents a strategic tool for monitoring the safety of apicultural products. Through its high sensitivity and specificity, the method contributes to the implementation of the European zero-tolerance policy on antimicrobial residues in honey, ensuring the protection of public health and strengthening consumer confidence in local apicultural products.

Study 3. Identification and quantification of residues of trimethoprim and dapsone sulfonamides in table eggs

Introduction

Sulfonamides represent, after tetracyclines, one of the most widely used classes of veterinary antibiotics in the European Union, due to their broad spectrum of activity and low cost. Their administration, as well as that of adjuvant compounds such as trimethoprim and dapsone, may lead to the persistence of residues in food, including eggs, with possible adverse effects on consumer health: allergic reactions, chronic toxicity, and the promotion of antimicrobial resistance. While maximum residue limits (MRLs) have been established for animal tissues under European regulations, no MRLs have been defined for eggs, which enforces a zero-tolerance policy. This situation requires extremely sensitive and selective analytical methods, capable of detecting and quantifying residues at very low levels.

Aim of the study

The study aimed to develop, optimize, and validate an ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous identification and quantification of 22 sulfonamides, trimethoprim, and dapsone in table eggs.

Materials and Methods

Twenty egg samples from conventional and organic farms in Cluj County were analyzed. The samples were homogenized, subjected to successive extraction with acetonitrile, defatted with hexane, and purified using Strata-X SPE cartridges. Chromatographic analysis was carried out on a Pursuit XRs Ultra C18 column, with an acidified binary mobile phase, under gradient conditions. Detection was performed by LC-MS/MS in positive electrospray mode, monitoring multiple reaction transitions specific to each analyte. Validation was conducted in accordance with Commission Decision 2002/657/EC and SANCO guidelines, testing parameters such as linearity, selectivity, specificity, sensitivity, precision, recovery, decision limits (CCα), detection capability (CCβ), robustness, and measurement uncertainty.

Results and discussions

The developed method demonstrated excellent sensitivity, with detection limits below 1 µg/kg for most sulfonamides and trimethoprim, and below 0.5 µg/kg for dapsone. Recoveries were within 80–120%, and calibration curve correlation coefficients exceeded 0.99. Method precision was confirmed by repeatability and reproducibility values below 20% for all analytes. Analysis of real egg samples revealed no exceedances of decision limits, confirming the compliance of the tested samples. However, the presence of traces below the quantification limits highlights the utility of the method for early detection of contamination.

Conclusions

This study validates, for the first time in Romania, the application of a UHPLC-MS/MS method for the simultaneous detection of a wide spectrum of sulfonamides, along with trimethoprim and dapsone, in table eggs. The method complies with European standards, shows high sensitivity and robust precision, and is suitable for use in official monitoring programs. The implementation of this technique contributes to ensuring food safety and reducing the risk of consumer exposure to antimicrobial residues, while supporting the European strategy to combat bacterial resistance.

GENERAL CONCLUSIONS

The doctoral thesis aimed to develop, validate, and apply modern methods for the detection and quantification of antimicrobial residues in food of animal origin, focusing on three major matrices: poultry meat, honey, and eggs.

The results obtained demonstrate that the proposed methods—HPLC-FLD for poultry meat and LC-MS/MS for honey and eggs—comply with European and international validation requirements, being characterized by high specificity, excellent sensitivity, appropriate recoveries, and solid reproducibility. Application to real samples confirmed both the robustness of the methods and their practical relevance for food safety monitoring. The detection of sulfaquinoxaline residues in two poultry meat samples, at levels below the MRL, illustrates the importance of respecting the withdrawal period after treatment. In the case of honey, the validated method successfully passed an international proficiency test (Fapas), confirming its applicability in the global monitoring network. For eggs, the developed method proved extremely sensitive and robust, with potential for extension to other complex products.

The work underscores the need to strengthen national monitoring plans, to improve the legislative framework (particularly for honey and eggs, where clearly defined MRLs are lacking), and to implement a One Health approach, linking animal health, food safety, and public health.

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

The originality of the thesis lies in the integration of modern, reliable, and immediately applicable methods into veterinary sanitary control and residue monitoring. The major contributions include:

- the first study in Romania on the development of a multiresidue HPLC-FLD method for poultry meat;
- the validation and application of an LC-MS/MS method for honey, confirmed by international proficiency testing schemes;
- the extension of the LC-MS/MS method to table eggs, a complex matrix scarcely addressed at national level;
- the creation of a unitary methodological platform, adaptable to different food products;
- the provision of primary data on food contamination with antimicrobials in Romania;
- the support offered to control authorities and official laboratories through analytical tools with immediate practical applicability.

Overall, the research makes a significant scientific and practical contribution to the field of food safety, by validating high-performance methods comparable to those used internationally and by promoting an integrated approach aimed at protecting public health and limiting the impact of antimicrobial resistance.