



An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

13F484 - Detection of Cephalosporins and quaternary ammonium compounds in food

Final Report

This project was funded under the Department of
Agriculture, Food and the Marine Competitive Funding
Programme.

SUMMARY

The objectives of the project were as follows:

- To improve and validate methods for cephalosporin residues in food.
- Apply and validate a method for QAC measurement in milk.
- To identify the source of QACs in milk (if detected).
- Transfer the methodology for cephalosporins to the VPHRL so they can be used for monitoring purposes.
- Investigate the impact of cephalosporins on native starter cultures, stability during product manufacture and distribution into different dairy products.
- To disseminate research to key stakeholders

Upon completion of this project, two new sensitive and accurate tests were developed, which allow the combined analysis of 30 or more cephalosporins, penicillin's and carbapenem residues in meat and milk. The research carried out during the development of this method has produced many key findings that improve the analysis of these difficult to analyse residues including stability improvements, sample preparation and improved chromatographic separation. This method is very important for monitoring compliance with EU regulatory limits and providing new knowledge on the occurrence of these residues in food. The methodology can also be potentially applied to monitor residues in the environment to combat antimicrobial resistance. The residues of beta-lactams are unlikely to impact on starter cultures used in dairy manufacturing processes, but residues have been shown to survive processing and can transfer into products. Thus, the application of the newly developed tests is important to ensure milk is free of residues to prevent product contamination and protect the reputation of Irish dairy products.

A sensitive method was developed to analyse two different groups of QAC residues in milk. QACs are an emerging group of residues and research has shown that the frequency of occurrence in Irish farm milk is low. However, further research is required to investigate the fate of their occurrence in different processed dairy products.

KEYWORDS

Antibiotic, Biocides; Residues;

ACRONYM

DuCATti

PROJECT COORDINATOR, INSTITUTION

Dr Martin Danaher, Teagasc.

EMAIL

martin.danaher@teagasc.ie

COLLABORATORS, INSTITUTION

Dr. Kieran Jordan, Ms. Róna Sayers, Dr David Gleeson & Dr Mary Moloney, Teagasc.

Dr Celine Mannion & Dr. Montserrat Gutierrez,
Department of Agriculture, Food and the Marine.

Dr Ambrose Furey, Cork Institute of Technology.

PUBLICATION DATE

May 2020.

Section 1 - Research Approach & Results

Start Date

01 April 2014

End Date

31 March 2018

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 4: Technology validated in
lab

NRPE Priority area

Sustainable Food Production and Processing

Total DAFM Award

€389,199.20

Total Project Expenditure

€298,593.45

Rationale for undertaking the Research

Preliminary research carried out by Teagasc, identified the presence of quaternary ammonium compound (QAC) residues in milk and dairy products. QACs are used in many detergent and disinfectant products to improve the antimicrobial effectiveness of the cleaning agent. Some products may be used inadvertently at farm or processing level to maintain a high standard of plant hygiene. This project was undertaken to assess the frequency of occurrence and the potential source of QACs in dairy products.

The project also carried out research on cephalosporin residues in meat, milk, and dairy products.

Cephalosporins belong to the Betalactam group and are important antibiotics used in human and veterinary medicine. The European Medicinal Agency (EMA) advise that 3rd and 4th generation cephalosporins should only be administered systemically to animals in the event that clinical conditions respond poorly to narrow spectrum antibiotics. This is because resistant bacteria might transfer resistance genes to human pathogens. At the start of the project, no chemical methods were available nationally to measure the presence of cephalosporin residues in food. Rapid low-cost inhibition tests are used to screen food samples for antibiotics but these tests cannot tell what drug is present or quantity it. In the event

that a positive sample is found, residues should be confirmed by LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry). This project aimed to address this deficiency by establishing LC-MS/MS tests for cephalosporin residues in meat and milk.

Methodology

The project was coordinated by the Teagasc Food Research Centre (TFRCA), Ashtown, Dublin. TFRCA were responsible for the development and validation of analytical methods for the measurement of beta-lactam antibiotics and QAC residues using LC-MS/MS in collaboration with the VPHRL (Veterinary and Public Health Laboratory, Backweston, Celbridge, Co. Kildare) (Tasks 1 to 3). Researchers in the Teagasc

Animal & Grassland Research and Innovation Centre (AGRIC), Moorepark, Co Cork were responsible investigation of QAC contamination on Irish farms through sampling of bulk milk tanks (Task 4). The milk samples collected for QAC analysis were tested using the method developed at TFRCA. The new analytical methods for the determination of Beta-lactam antibiotics were assessed through inter-laboratory studies by exchanging samples between VPHRL and TFRCA (Task 5). In addition, external proficiency test samples were provided by RIKILT based in the Netherlands. The final standards operating procedures (SOPs) for meat and milk methods were transferred to the VPHRL by Teagasc. In addition, one month was spent by Teagasc staff at Backweston to transfer the methodology. Researchers based in the Teagasc Food Research Centre (TFRCM), Moorepark were responsible for assessing the impact of the nine cephalosporin antibiotic residues on 7 *L. lactis* starter cultures and 8 *S. thermophilus* strains (Task 6). As part of this task 6, the stability of a cephalosporin, namely, cefquinome, was assessed in the manufacturing of different dairy products (milk, skim milk, cream, cheese, curd, whey, buttermilk and butter). TFRCA developed and validated a bespoke to test residues in the samples collected on Task 6. The research activities on the project was disseminated on Task 7 through e-Newsletters, workshops, seminars, national conferences, international conferences and peer reviewed publications.

Project Results

Results

Tests were developed and validated to measure ≥ 30 beta-lactam residues in meat and milk. The developed methods were fully validated according to EU Commission Decision 2002/657/EC. The methods are far superior to existing published methods because of the range of analytes covered and the simplicity of the sample preparation procedures used. Methodology was evaluated through inter-laboratory studies and SOPs were transferred to the VPHRL, who were trained on the methods.

An UPLC-MS/MS method was established for the simultaneous determination of 15 QACs in milk samples based on a method developed by the EURL. The method was applied to analyse bulk milk tank samples collected from Irish farms showing that only 8 out of 316 samples contained measurable QAC residues. The concentration of QACs in milk samples were ≤ 0.012 mg/kg.

The impact of nine cephalosporin antibiotics on 7 *L. lactis* starter cultures and 8 *S. thermophilus* strains was studied. The results showed that the minimum inhibitory concentration (MIC) varied between the antibiotics tested, did not vary much between the *L. lactis* starter cultures tested, but did vary between the *S. thermophilus* strains tested. For cefadroxil and cephalexin the MIC was 50-100 mg/L for *L. lactis* starters, 20 mg/L for cefoperazone and 1.5-5 mg/L for the remaining six antibiotics. For the *S. thermophilus* cultures the MIC was generally about 5 mg/L, apart from one strain that had an MIC of 8 mg/L, the others were < 1 mg/L.

The fate of the cephalosporin, cefquinome, was investigated in milk samples showing that cefquinome residues were stable and could be detected in all of the dairy products analysed following the production of dairy products from contaminated milk. Cefquinome residues were found to mainly concentrate in the polar dairy fractions including skim, whey and buttermilk.

Conclusion

This project significantly advanced beta-lactam residue analysis in food by developing two comprehensive, accurate and robust LC-MS/MS methods. These methods can confirm samples that have been found positive by low-cost screening tests or can be used as standalone tests in official food inspection laboratories to detect residues at the Maximum Residue Limits (MRLs) and below.

The impact of different cephalosporin antibiotics were assessed on starter cultures showing that the MICs are very significantly higher than the MRLs. Therefore, it is extremely unlikely that milk for cheesemaking would contain cephalosporin residue levels that would inhibit starter cultures.

The fate of the cephalosporin, cefquinome was investigated in different dairy processes showing that residues did not degrade significantly during processing and persisted in dairy products. Also, the partitioning of cefquinome between the different dairy products depends on the physico-chemical properties of the drug. Research on QAC residues showed that these compounds were detected in a low number of samples. Further research is needed to investigate the fate of QAC residues during dairy processing and transfer into different dairy products.

Ongoing monitoring of residues needs to be carried out to ensure milk is not contaminated prior to processing because many residues survive dairy processing and transfer into dairy products.

Section 2 - Research Outputs

Summary of Project Findings

Two new comprehensive and sensitive methods were developed for the measurement of Beta-lactam residues in meat and milk samples. These methods are advantageous over existing published methods because they include the widest number of beta-lactam residues and allow detection of residues to 0.5 µg kg⁻¹ for unapproved substances and a quarter of the MRL for licensed medicines. Research on the project reported a number of solutions to improve the accuracy and precision of beta-lactam residues analysis. The new tests can be used to monitor the compliance with EU MRLs and can also measure lower concentrations of residues, which is important for monitoring the usage of products. The methods reduce the risk of false positives and more importantly false negative results because of the superior accuracy of the methods and a complete range of beta-lactam residues covered. The safety of meat and milk products produced and consumed on the island will be underpinned through improved residue surveillance methods, which will protect the pure image of Irish food. The impact assessment of cephalosporin antibiotics on cheese starter cultures showing that the MICs are very significantly higher than the MRLs. Therefore, it is extremely unlikely that contaminated milk would affect cheese manufacturing. Although, it was shown the cephalosporin drug cefquinome was found to be stable did not degrade significantly during processing and persisted in dairy products. Thus, ongoing of milk is required to protect public health.

A new method was developed to detect different classes of QAC disinfectant residues in milk and dairy products. The application of this new test showed that these compounds were detected in a low number of samples. Further research is needed to investigate the fate of QAC residues during dairy processing and transfer into different dairy products.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
Post Doctorates	0	2	2
PhD Students	0	1	1

Summary of Academic Outputs

Research Outputs	Total Number	Details
Publications in Peer Reviewed Scientific Journals	2	<ol style="list-style-type: none">1. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2017) Development and validation of a quantitative confirmatory method for 30 β-lactam antibiotics in bovine muscle using liquid chromatography coupled to tandem mass spectrometry. J. Chrom. A. 1500, 121-135.2. Rocco, M., Moloney, M., Haren, D., Gutierrez, M., S. Earley, S., Berendsen, B., Furey, A., Danaher, M. (2020) Improving the chromatographic selectivity of βlactam residues analysis in milk using phenyl-column chemistry prior to detection by tandem mass spectrometry. Analytical and Bioanalytical Chemistry (accepted in press).

1. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2016) Development of a multi-residue method for β -lactam antibiotics in bovine muscle using UHPLC-MS/MS. In the proceedings of EuroResidue VII conference. Egmond aan Zee, The Netherlands. 23 – 25th May 2016.
2. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2017) Development and validation of a multi-residue method for B-lactam antibiotics in bovine muscle using ultra-high performance liquid chromatography coupled to tandem mass spectrometry. In the proceedings of IUFOST 2016 conference, Dublin, 21st-25th August 2016.
3. Ramkumar, Abilasha & Danaher, Martin. (2015). Evaluation of various QuEChERS sample preparation methods for the determination of Quaternary ammonium compounds in milk (Poster). In the proceedings of HPLC 2015: 42nd International Symposium on High Performance Liquid Phase Separations and Related Techniques. Geneva, Switzerland, June 21–25th 2015.

1. Di Rocco, M., Furey, A., Danaher. Development of a multi-residue method for β lactam antibiotics in food of animal origin by UHPLC-MS/MS. 43rd Annual Food Research Conference. University College Dublin, 10-11th December 2014.
 2. Danaher, M. Residues in milk and dairy products. Milk and Product Quality Forum meeting. The Heritage Hotel, Portlaoise, May 14th, 2015.
 3. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Development of a multi-residue UHPLC-MS/MS method for 33 β lactam antibiotics in meat (poster). 44th Annual Food Research Conference, Teagasc Moorepark, 14th December 2015.
 4. Danaher, M. New test methods and their role in identifying emerging residue threats in food. Chemical Residues Knowledge Network Conference. Maldron Hotel, Dublin, 26th February 2016.
 5. Danaher, M. Development and application of modern methods for the detection and quantification of residues and contaminants in milk. IDF Scientific Symposium, Dublin, 31st March 2018.
 6. Danaher, M. Recent developments in the analysis of residues in milk and dairy products. Teagasc Gateways - Advanced Analytical Methodologies for the Food Industry. Teagasc Moorepark, 16th November 2017.
 7. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Detection of β -lactam compounds in meat and milk by UHPLCMS/MS. DAFM Workshop. Backweston, 31st March 2018.
-

8. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Detection of β -lactam compounds in meat and milk by UHPLCMS/MS. Food Safety Department Seminar. Teagasc, Ashtown, Dublin, 30th June 2018.
9. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Development of a multi-residue method for β -lactam antibiotics in bovine muscle using UHPLC-MS/MS. 46th Annual Food Science and Technology Conference. Teagasc, Ashtown, Dublin, 6-7th December 2017.

PhD Theses	1	Di Rocco, M. PhD Thesis. Research on Cephalosporin Residues in Milk and Meat. Cork Institute of Technology (expected submission August 2020).
------------	---	---

Intellectual Property

A total of three analytical methods were developed on the project to detect residues of beta-lactam antibiotics and Quaternary ammonium compounds in food. These methods have been written up in ISO17025 format and validation reports have been prepared, which are all stored within the Teagasc quality system. Two of the methods will be published in peer reviewed literature and can be reproduced in expert laboratories if they have the equipment to do so.

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Technology	<ol style="list-style-type: none"> 1. A method was developed to analyse antibiotic residues in meat and was published in Journal of chromatography A. 2. A method was developed to analyse antibiotic residues in milk has been accepted for publication in Analytical and Bioanalytical Chemistry April 2020. 	2
Other	A method was established for the analysis of QAC residues on milk, but more extensive validation work is required before this method will be published.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Socio-Economic	Methods developed for the measurement of residues in food can be used to comply with international regulatory guidelines and support access to export markets.
Other	The safety of milk and meat produced on the island can be improved through application of improved residue surveillance technologies to improve consumer protection.

Dissemination Activities

Activity	Details
Workshops at which results were presented	Research from the project was disseminated at Teagasc Gateway Events and IDF symposia to the dairy industry. In addition, the project research was disseminated at Milk Quality workshops organised by the Teagasc Moorepark, DAFM-Backweston and the SafeFood chemical residues research network.
Other	Project research was disseminated at a number of International conferences including EuroResidue, HPLC 2015 and IUFOST.

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.	<p>The project produced new improved approaches for the detection of beta-lactam antibiotic residues that provide accurate and sensitive detection for a wide range of residues.</p> <p>Research on the project identified and remedied problems around the issues affecting the stability of beta-lactam residues in laboratory analysis. In addition, the project developed a chromatographic method that provides improved selectivity in betalactam residues in a short injection cycle. These findings will have a widespread impact in the field of veterinary drug residue analysis. The application of this methodology demonstrated that the cephalosporin drug cefquinome did not degrade significantly during the manufacture of different dairy products. Therefore, it is important to continue monitoring of antibiotic residues at farm and factory level to prevent residue contamination.</p> <p>A sensitive method was developed to analyse QAC residues ion milk use a novel chromatographic separation process. This method allows sensitive analysis of two different QAC residues in milk in the low part per billion range. The application of this method demonstrated that the incidence of QAC residues in milk is low but further research is required for high fat dairy products.</p>
Identify any knowledge transfer activities executed within the project.	<p>Methods for the measurement of beta-lactam antibiotics were transferred to the VPHRL, DAFM, Backweston by Teagasc. This included establishment of method LC-MS/MS detection method in the DAFM laboratory and training on the sample preparation procedure. Two standard operating procedures for the analysis of beta-lactam methods in meat and milk was wrote up in ISO17025 format and transferred to DAFM. Research from the project was presented at a workshop organised at Backweston. Methodology was additionally transferred to the ISO17025 laboratory at Teagasc.</p>
List any impacts resulting from the knowledge transferred during the project.	<p>The chromatographic separation technologies used on this project have been applied on a number of EU and SFI funded projects at Teagasc.</p> <p>The sample preparation methodology developed on the project has been adapted to the analysis of high polar residues, namely, chlorates and antiviral drugs in different foods. Methodology for QAC residues is now routinely used by industry milk analysis.</p>

Section 3 – Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
EU R&I programmes	€342,125.00	EU-CHINA-SAFE was funded through the European Union's Horizon 2020 research and innovation programme under grant agreement No. 727864 and from the Chinese Ministry of Science and Technology (MOST).

Future Strategies

The project has comprehensively addressed current gaps in the multi-residue analysis of beta-lactam residues in milk and meat, extending research beyond the state-of-the-art. Further research through is required to develop methods of cefapirin and ceftiofur residues, which undergo complex metabolism in food producing animals. Now that methodology has been established it should be applied for a more comprehensive and cost-effective assessment of beta-lactam residues in different dairy products. Similarly, methodology for the QAC residues should undergo a more rigorous validation and be adapted to the analysis of QAC residues in different dairy products.

Project Publications

Peer review papers

1. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2017) Development and validation of a quantitative confirmatory method for 30 β -lactam antibiotics in bovine muscle using liquid chromatography coupled to tandem mass spectrometry. *J. Chrom. A.* 1500, 121-135.
2. Di Rocco, M., Moloney, M., Haren, D., Gutierrez, M., S. Earley, S., Berendsen, B., Furey, A., Danaher, M. (2020) Improving the chromatographic selectivity of β -lactam residues analysis in milk using phenyl-column chemistry prior to detection by tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* (accepted in press).
3. Scollard, J., Di Rocco, M., Sayers, R., Furey, A., Danaher, M., Jordan, K. Migration of cefquinome antibiotic residues from milk to dairy products (submitted to the *International Dairy Journal*).

International Conference Proceedings

4. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2016) Development of a multi-residue method for β -lactam antibiotics in bovine muscle using UHPLC-MS/MS. In the proceedings of EuroResidue VII conference. Egmond aan Zee, The Netherlands. 23 – 25th May 2016.
5. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. (2017) Development and validation of a multiresidue method for B-lactam antibiotics in bovine muscle using ultra-high performance liquid chromatography coupled to tandem mass spectrometry. In the proceedings of IUFoST 2016 conference, Dublin, 21st-25th August 2016.
6. Ramkumar, Abilasha & Danaher, Martin. (2015). Evaluation of various QuEChERS sample preparation methods for the determination of Quaternary ammonium compounds in milk (Poster). In the proceedings of HPLC 2015: 42nd International Symposium on High Performance Liquid Phase Separations and Related Techniques. Geneva, Switzerland, June 21–25th 2015.

National Conferences

7. Di Rocco, M., Furey, A., Danaher. Development of a multi-residue method for β -lactam antibiotics in food of animal origin by UHPLCMS/MS. 43rd Annual Food Research Conference. University College Dublin, 10-11th December 2014.
8. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Development of a multi-residue UHPLC-MS/MS method for 33 β -lactam antibiotics in meat (poster). 44th Annual Food Research Conference, Teagasc Moorepark, 14th December 2015.
9. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Development of a multi-residue method for β lactam antibiotics in bovine muscle using UHPLC-MS/MS. 46th Annual Food Science and Technology Conference. Teagasc, Ashtown, Dublin, 6-7th December 2017.
10. Danaher, M. Development and application of modern methods for the detection and quantification of residues and contaminants in milk. IDF Scientific Symposium, Dublin, 31st March 2018.

Workshops

11. Danaher, M. Residues in milk and dairy products. Milk and Product Quality Forum meeting. The Heritage Hotel, Portlaoise, May 14th2015.
12. Danaher, M. New test methods and their role in identifying emerging residue threats in food. Chemical Residues Knowledge Network Conference. Maldron Hotel, Dublin, 26th February 2016.
13. Danaher, M. Recent developments in the analysis of residues in milk and dairy products. Teagasc Gateways - Advanced Analytical Methodologies for the Food Industry. Teagasc Moorepark, 16th November 2017.
14. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Detection of β -lactam compounds in meat and milk by UHPLC-MS/MS. DAFM Workshop. Backweston, 31st March 2018.

Seminars

15. Di Rocco, M., Moloney, M., O'Beirne, T., Earley, S., Berendsen, B., Furey, A., Danaher, M. Detection of β -lactam compounds in meat and milk by UHPLC-MS/MS. Food Safety Department Seminar. Teagasc, Ashtown, Dublin, 30th June 2018.

PhD Thesis

16. Di Rocco, M. PhD Thesis. Research on Cephalosporin Residues in Milk and Meat. Cork Institute of Technology (expected submission August 2020).