TERMS OF REFERENCE FOR R&D PROJECT

Food and Agriculture Department

Food and Agriculture Sectional Committee, FAD 28

1 TITLE OF THE PROJECT

Development and Validation of method for determination of nitrofuran metabolites and chloramphenicol in aquaculture and the applicability of the method in milk, honey, poultry and egg.

2 BACKGROUND

2.1 Antibiotics play a crucial role in veterinary medicine, particularly in animal feed and food animal production. The human health risks associated with antibiotic residues are significant. These residues contribute to the development and acceleration of antibiotic resistance, facilitate the transfer of antibiotic-resistant bacteria to humans, and may trigger allergies, especially in the case of penicillin. Additionally, certain antibiotics like sulfamethazine, oxytetracycline, and furazolidone have been linked to severe health issues such as cancer. Other adverse effects include anaphylactic shock, nephropathy associated with gentamicin, bone marrow toxicity, mutagenic effects, and reproductive disorders related to chloramphenicol.

2.2 The national focus on imported or exported consignments of animal-origin food primarily revolves around fish, milk, honey, poultry, and eggs. International and national regulatory agencies, such as Food and Agriculture Organization/WHO, Food and Drug Administration, Canadian Food Inspection Agency, the Australian Pesticides and Veterinary Medicines Authority, European Commission, European Food Safety Authority, Food Safety and Standards Authority of India (FSSAI) and the Ministry of Health of each country, continuously attempt to regulate antibiotic use with international standards, considering the specific realities of each country (Krishnan et.al 2022, Okocha et al. 2018).

2.3 Nitrofurans are antibacterial, antiprotozoan and growth promoters. In animal studies the parent drugs and their metabolites showed carcinogenic and mutagenic characteristics. For that reason, nitrofuran use in the treatment of animals used for food production is prohibited. Despite being prohibited, residues continue to appear in the food supply (Krishnan et. al 2022). In particular, metabolites of nitrofurans can be tissue or protein bound resulting in residue remaining long after administration of the parent drug (Nouws and Laurensen 1990; McCracken et al. 1995). Common parent nitrofuran drugs, and their respective side chains (the R groups), include: furazolidone (side chain: 3-amino-2-oxazolidinone = AOZ), furaltadone (side chain: 3-amino-5-morpholinomethyl-2-oxazolidinone = AMOZ), nitrofurantoin (side chain: 1- aminohydantoin = AHD) and nitrofurazone (side chain: semicarbazide = SEM) with a new addition nifursol (side chain: 3, 5-dinitrosalicylic acid hydrazide =DNSH). Initially, testing for residues of nitrofurans in animal tissues was conducted using methods directed at the parent compounds, using high-performance liquid chromatography–ultraviolet (HPLC-UV) and later using liquid chromatography–mass spectrometry (LC-MS) techniques. A method based on similar acidic hydrolysis and NBA derivatisation was developed for tissue-bound metabolites of five nitrofurans, using AOZ, AMOZ, AHD, DNSH and SEM as the marker metabolites, in food of animal origin determination (Finzi et al 2005) of residues by liquid chromatography–tandem mass spectrometry (LC-MS/MS). This method uses a 37°C incubation for 16 hr which is time consuming. There are presently method using lesser time of incubation with accelerated temperature condition, however these methods are not always reproducible. The method used for chloramphenicol is independent and extraction of chloramphenicol from

sample matrices is most often carried out using solvent extraction, commonly with ethyl acetate but also using aqueous/solvent mixtures such as dilute salt solution and acetonitrile which is fast and rapid. There is a need for multiclass rapid method for food testing laboratories globally and accordingly a method based on microwave assisted derivatization followed by clean up would reduce considerable time in analysis for nitrofuran metabolites including chloramphenicol in food of animal origin.

3 OBJECTIVE

To develop and validate the method for determination of nitrofuran metabolites and chloramphenicol in aquaculture and the applicability of the method in milk, honey, poultry and egg for various performance characteristics *i.e* applicability, specificity, identification, confirmation, calibration curve, trueness, precision, (repeatability and within-laboratory reproducibility), LOQ, ruggedness and relative matrix effect as detailed under method validation protocol.

4 SCOPE

4.1 Method development and validation for determination of nitrofuran metabolites and chloramphenicol in aquaculture using LC-MS/MS, covering performance characteristics given in the objectives through a designed multi lab validation by at least eight (8) participating laboratories involved in analysis of these analytes as per the method and criteria mentioned in ToR.

4.2 The applicability of the method in milk, honey, poultry and egg through validation of the test method including various performance criteria, i.e., specificity, identification, confirmation, calibration curve, trueness, precision, (repeatability and within-laboratory reproducibility), LOQ, ruggedness and relative matrix effect as detailed under method validation protocol.

5 RESEARCH METHODOLOGY

5.1 Detailed review of existing literature and national, regional and International Standards, research publications for the determination of nitrofuran metabolites and chloramphenicol in aquaculture using microwave digestion and LC-MS/MS.

5.2 The proposer/project leader should have the experience and competence in the field of analytical chemistry including antibiotic residue analysis.

5.3 The proposer lab shall take consent of the participating labs before initiating study regarding facility available for carrying out Multi Lab Validation (MLV).

5.4 Laboratories engaged in the project shall have experience and infrastructure in antibiotic residue analysis (LC-MS/MS, Microwave Digestion System etc.), and method validation as per IS/ISO 11843 (Part 1 to Part 8) and requirements of IS/ISO/IEC 17025: 2017.

5.5 Develop a method using microwave assisted derivatization following by analysis using LCMSMS for simultaneous determination of nitrofuran metabolites (AOZ, AMOZ, AHD, SEM and DNSH) and Chloramphenicol in aquaculture shrimp and applicability of the method is checked in poultry, milk, honey and egg.

5.6 The validation protocol of proposed method shall be planned following an approach of two main phases: Phase 1- Single Laboratory Validation and Phase 2- Multi Laboratory Validation.

5.6.1 Phase 1: Single Laboratory Validation

The proposed method has to be developed and validated for performance characteristics as per IS/ISO 11843 (Part 1 to Part 8). The parameter for validation shall include applicability, specificity, identification, confirmation, calibration curve, trueness, precision, (repeatability and within-laboratory reproducibility), LOQ, ruggedness and relative matrix effect. Naturally contaminated in house quality control sample can be used as part of single laboratory validation. The proposer shall develop and undertake single laboratory validation of the proposed method in their laboratory covering all performance characteristics as per the table below for aquaculture shrimp samples. The applicability of the method would be checked in milk, honey, poultry and egg during which the method would be validated at three level covering performance characteristics like specificity, LOQ, trueness, precision (repeatability), relative matrix effect.

The proposed experiment plan for SLV is given in the table below; however, minor modifications may be made by the proposer as deemed necessary for practical implementation:

Validation	**Sample type**	**Levels**	**Number of extractions**	**Concentration (µg/kg-1)**	**Performance characteristics**
Series 1	Calibration samples	1	7	0 (fortified matrix)	calibration
		2		0.25 (fortified matrix)	
		3		0.5 (fortified matrix)	
		4		0.75 (fortified matrix)	
		5		1.0 (fortified matrix)	
		6		1.5 (fortified matrix)	
		7		2.0 (fortified matrix)	
	Blank samples	1	7	0 (fortified matrix)	specificity LOQ, trueness, precision (repeatability) within lab reproducibility)
	Validation samples	2	7	0.25 (fortified matrix)	
		3	7	0.5 (fortified matrix)	
		4	7	0.75 (fortified matrix)	
Series 2	Calibration samples	1	7	0 (fortified matrix)	calibration
		2		0.25 (fortified matrix)	
		3		0.5 (fortified matrix)	
		4		0.75 (fortified matrix)	
		5		1.0 (fortified matrix)	
		6		1.5 (fortified matrix)	
		7		2.0 (fortified matrix)	
	Blank samples	1	7	0 (fortified matrix)	specificity LOQ, trueness, precision (repeatability within lab reproducibility)
	Validation samples	2	7	0.25 (fortified matrix)	
		3	7	0.5 (fortified matrix)	
		4	7	0.75 (fortified matrix)	
Series 3	Calibration samples	1	7	0 (fortified matrix)	calibration
		2		0.25 (fortified matrix)	
		3		0.5 (fortified matrix)	
		4		0.75 (fortified matrix)	
		5		1.0 (fortified matrix)	
		6		1.5 (fortified matrix)	
		7		2.0 (fortified matrix)	
	Blank samples	1	7	0 (fortified matrix)	specificity LOQ, trueness, precision (repeatability within lab reproducibility)
	Validation samples	2	7	0.25 (fortified matrix)	
		3	7	0.5 (fortified matrix)	
		4	7	0.75 (fortified matrix)	
Series 4	Blank samples (3 changes in a method in duplicate)	2	6	0.25 (fortified matrix)	ruggedness
	Blank samples	2	20	0.25 (fortified matrix)	relative matrix effect
	Standard solution	2		0.25	

5.6.2 Phase 2: Multi laboratory Validation (MLV)

5.6.2.1 Number of Participating Laboratories (Min.): Eight labs which are proficient in Antibiotic Residue Analysis as required at **5.4** above. The multi laboratory validation would be coordinated as a nodal lab, by the proposer lab using the same method for which SLV has been successfully carried out.

5.6.2.2 Protocol to be Followed

This MLV for determination of nitrofuran metabolites and chloramphenicol in aquaculture using LC-MS/MS with applicability in milk, honey, poultry and egg by microwave assisted digestion and extraction method would be used for MLV covering performance characteristics like recovery, repeatability and reproducibility covering all matrices and all laboratories. Initially trial samples along with the developed method would be sent by the proposer laboratory which have given consent to participate in the MLV.

6 EXPECTED DELIVERABLES

Detailed project report of the work done, in hard copy and digital formats, as per the scope specified under **4**, with the following as appendices:

a) Report on Single laboratory Validation (SLV) for the method proposed.
b) Report of analysis of all the experiments conducted by the participant laboratories along with the data obtained and statistical analysis of the data collected during the Multi laboratory Validation (MLV).
c) Working Draft of the method as per IS format.

7 TIMELINE AND METHOD OF PROGRESS REVIEW

7.1 Timeline for the project is 6 months from the date of award of the project.

7.2 Stages for Progress Review

Stage	Timeline
Stage I Review of existing literature and national, regional and International Standards, research publications.	1 month
Stage II Development and single lab validation of method in shrimp aquaculture by the proposer lab and checking the applicability of the method in milk, honey, poultry and egg.	1st to 3rd month
Submission of mid-term report	End of 3rd month
Stage III Multi lab validation for determination of nitrofuran metabolites and chloramphenicol in aquaculture using LC-MS/MS with applicability in milk, honey, poultry and egg.	3rd to 5th month

Stage IV Draft report submission – Sectional Committee will evaluate the draft report and provide feedback/recommend changes, if required.	6th month

At the end of 6th month, project allottee to submit final project report incorporating recommendations/feedback of the Committee, if any.

Note: The timelines given above are indicative and calculation of time will start from the date of award of sanction letter for the project to the Project leader.

8 SUPPORT FROM BIS

8.1 Access to Indian and International Standards.

8.2 Letters from BIS to concerned stakeholders, wherever required for support in research project.

9 NODAL OFFICER

Ms. Disha Zanwar, Sc. C/Deputy Director, FAD, BIS, may be contacted at fad28@bis.gov.in for any queries on the research project.

10 REFERENCES

a) Krishnan, Anoop A., Venkateswarlu Thelukutla, Anoop S. Sengar, Archa Vijayan, Sisira Raveendran, Praveen Malekadi, Saskia S. Sterk, Ravi Shanker, and J. S. Reddy. 2022. "Simultaneous Determination of Five Metabolites of Nitrofurans Including the Metabolite Nifursol in Shrimp and Fish by UPLC- MS/MS: In-House Method Validation According to Commission Implementing Regulation (EU) 2021/808." Food Additives & Contaminants: Part A 40 (2): 222–34. doi:10.1080/19440049.2022.2154855.

b) Okocha, R.C., Olatoye, I.O. & Adedeji, O.B. Food safety impacts of antimicrobial use and their residues in aquaculture. Public Health Rev 39, 21 (2018).

c) McCracken RJ, Blanchflower WJ, Rowan C, Mccoy MA, Kennedy DG. 1995. Determination of furazolidone in porcine tissue using thermospray liquid chromatographymass spectrometry and a study of the pharmacokinetics and stability of its residues. Analyst. 120(9):2347–2351. doi:10.1039/an9952002347

d) Nouws JFM, Laurensen J. 1990. Postmortal degradation of furazolidone and furaltadone in edible tissues of calves. Vet Q. 12(1):56–59. doi:10.1080/01652176.1990.9694243

e) Finzi JK, Donato JL, Sucupira M, De Nucci G. 2005. Determination of nitrofuran metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 824(1–2):30–35. doi:10.1016/j.jchromb.2005.05. 012

f) ISO 11843-1:1997 Capability of detection – Part 1: Terms and definitions.