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S Kalpana
Chemical Residues Laboratory, ICAR-National Research Centre on Meat, Chengicherla, Hyderabad, Telangana, India

CH Umamaheswara Rao
Chemical Residues Laboratory, ICAR-National Research Centre on Meat, Chengicherla, Hyderabad, Telangana, India

M Muthukumar
Chemical Residues Laboratory, ICAR-National Research Centre on Meat, Chengicherla, Hyderabad, Telangana, India

Corresponding Author:
S Kalpana
Chemical Residues Laboratory, ICAR-National Research Centre on Meat, Chengicherla, Hyderabad, Telangana, India

Analysis of residual furaltadone metabolite AMOZ in dried meat powder using liquid chromatography-tandem mass spectrometry

S Kalpana, CH Umamaheswara Rao and M Muthukumar

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Abstract
A sensitive liquid chromatography–tandem mass spectrometric method has been optimized for determination of residual 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) in dried meat powder. Dried meat powder were extracted by acid hydrolysis followed by derivatization with 2-nitrobenzaldehyde, aliquot was evaporated to dryness and reconstituted in mobile phase.LC separation was carried out on a BEH C_{18} column (100 mm × 2.1 mm, 1.7 µm) column with gradient elution mode using mobile phase consisting of methanol and 2 mM ammonium format. LC-MS/MS analysis was done in multiple reaction monitoring (MRM) mode through an ESI interface operated in positive ionisation mode, with deuterated AMOZ-D_5 as internal standard. Four identification points were obtained for AMOZ with one precursor ion and two product ions. Identification and quantification of the AMOZ were performed based upon the intensities of mass fragments from the precursor ions, MRM of NP-AMOZ: 335 > 291, 335 > 127; MRM of NP-AMOZ-D5 – 340.1 > 101.9, respectively. Linear matrix-matched calibration curves were obtained (regression coefficient ≥ 0.99) over the quantitation range of 0.1-5 µg/Kg with the limit of quantitation (LOQ) being 0.13 µg/Kg. Mean recoveries ranged from 81% to 108%, with the corresponding intra-day variation ranging from 2.7% to 6.6%, based on the spike concentration around MRPL. The proposed tandem mass spectrometric method is quite adequate for analysis of residual AMOZ in dried meat powder at and below EU MRPL (0.5 µg/Kg).

Keywords: AMOZ, antimicrobial residues, meat powder, LC-MS/MS

Introduction
Furaltadone is a synthetic antimicrobial agent from nitrofuran group that has a broad spectrum of activity. This nitrofuran is commonly used in veterinary medicine owing to their low cost and high efficiency. Furaltadone have low *in vivo* stability and half-life, and mostly metabolize quickly into secondary metabolites i.e. 3-amino 5-morpholino ethyl-2-oxazolidinone (AMOZ) (EunChae *et al*. 2021) [7]. In any case, reports indicate these metabolites are not destroyed during grilling, cooking and baking (Cooper and Kennedy, 2007) [5] and can be detected in food for over a month as stable protein-bound adducts causing side effects like carcinogenicity and mutagenicity, in addition to allergic reactions and antimicrobial resistance. In lieu of these side effects reported nitrofurans were banned and a zero-tolerance policy was adopted by many countries (Barbosa *et al*. 2011) [1]. The EU has currently set a minimum required performance limit (MRPL) of 0.5 µg/Kg for analytical testing of AMOZ in food commodities (EC/1871/2019) to ensure food safety.

Many physiochemical and immunological methods have been devised to detect nitrofuran residues in food matrices are documented like Chemiluminescence (Liu *et al*. 2012) [11], electrochemical method (Cai, *et al*. 2021) [3], biosensor assay (Su *et al*. 2021) and ELISA method (Gaudin *et al*. 2020; Yan *et al*. 2020) [8, 18]. Also, reports are available on chromatographic methods for quantification of AMOZ using HPLC-FLD (Sheng *et al*. 2013; Luo *et al*. 2019) [13, 12], and HPLC-PDA (Wang *et al*. 2020) [17]. Presently, the gold standard method which is widely opted for this banned antimicrobial is LC-ESI-MS/MS for its confirmatory ability in different food matrices *viz*. meat (Verdon *et al*. 2007; Leitner *et al*. 2008) [16, 10], aquaculture products (Guichard *et al*. 2020) [9], and animal feed (Barbasa *et al*. 2007).

Considering the recent state of food safety and quality assurance issues there is still a great need for sensitive tandem mass spectrometric methods to determine AMOZ residue with confirmation and to comply the current regulation.

Therefore, the objective of the study is to optimize a sensitive liquid chromatography-tandem mass spectrometric method for analysis of trace level AMOZ in dried meat powder below its set MRPL.

Materials and Methods

Chemicals and reagents

AMOZ, analytical standard purity > 99% (HPC standard GmbH), analytical standard of AMOZ-D5 (HPC standard GmbH) used as an internal standard (IS). Methanol LCMS grade (J.T. Baker), Water LCMS grade (J.T. Baker), ethyl acetate LC grade (Merck) and 2 Nitro-benzaldehyde LC grade (Sigma Aldrich) were used.

Preparation of Stock and working standard solutions

Analytical grade individual standards of AMOZ and its internal standard AMOZ-D5, with 99.5% purity were used for this study. Stock solution of 1mg/ml concentration of AMOZ were prepared in methanol. From this stock solution, working standards solutions of the following concentrations *viz*. 0.1, 0.2, 0.5, 1.5, 1,2 and 5 ng/ml were prepared by diluting with the methanol. All stock and working standard solutions were stored at 4 ºC in the refrigerator until use.

Instrumentation and conditions

Liquid chromatography

Chromatography was performed using UPLC (Acquity; Waters, USA) with a vacuum degasser and autosampler. The chromatographic separation was achieved on C_{18} column, 100mm X 2.1 mm; 1.7µm (Acquity UPLC, BEH). Injection volume was set at 10 µL and the analysis was carried out in a gradient elution mode using methanol and 2 mM ammonium format as the mobile phase at a flow rate of 0.30 mL/min.

Mass spectrometry

LC–MS/MS system, Xevo TQ-S micro mass spectrometer (Waters, USA) that was connected to LC system via an electrospray ionisation (ESI) interface was employed. Analysis of AMOZ was performed in positive ionisation mode. For quantitation purposes, samples were analysed in a multiple reaction monitoring (MRM) mode. MRM parameters for the optimal yield of product ions were defined in individual time windows for AMOZ. Data acquisition was done using Mass Lynx (version 4.2) software. MRM transitions, along with their collision energies and dwell times were monitored. Quantification and a confirmation transition for AMOZ and a transition for the internal standard D5-AMOZ were obtained.

Sample preparation

For extraction purpose, 0.2 g meat powder weighed in a polypropylene centrifuge tube and a known amount of working standard solutions along with its internal standards were spiked. To this spiked sample 10ml of 0.125M hydrochloric acid and 400µl of 50 mM 2-nitrobenzaldehyde (2-NBA) in methanol were added and vortexed for 1 minute. Following that the mixture was incubated for 16 hours at 37 °C for derivatization. After cooling the 2-NBA derivatized sample to room temperature, it was then neutralized with 0.1M K_2HPO_4 and 1ml 0.8M sodium hydroxide. The resultant sample was extracted with 15 ml of ethyl acetate, vortexed and centrifuged for 10 minutes at 6000 rpm in a refrigerated centrifuge. The supernatant extract were collected into a polypropylene tube, evaporated to dryness using nitrogen gas at 40 °C and reconstituted with 1ml n-hexane and 0.5ml methanol: water (50:50; v/v). Final aliquot was then filtered using 0.2 µ PVDF syringe filter and transferred into an autosampler vial for LC-MS/MS analysis.

Calibration

Matrix-matched calibration curves were constructed using AMOZ at different spiking levels ranging from 0.1 to 5.0 µg/Kg. The internal standard, D_5-AMOZ, was spiked at 10pg/µL in all samples.Spiked samples were extracted in triplicates at each spiking level and analysed by LC–MS/MS. The lower limit of quantification (LLOQ) was estimated from the lowest concentration of matrix-matched standard.

Recovery

Recovery was calculated using AMOZ spiked at three levels around MRPL *viz*. 0.25, 0.5 and 0.75 µg/Kg which corresponds to 0.5, 1 and 1.5 times MRPL, respectively and were analyzed in six replicates at each level.

% Recovery = 100 X measured content/fortification level

Sensitivity

Limits of detection (LOD) and limits of quantification (LOQ) was evaluated using the spiked samples spiked at the MRPL level. The LOD and LOQ of AMOZ was calculated by signal-to-noise ratio of 3 and 10 (the ratio between intensity of signal of each compound obtained and intensity of noise in a spiked sample).

Accuracy

Accuracy was evaluated by determining recoveries of AMOZ in spiked samples using six replicates. According to the 2002/657/EC Decision, for AMOZ spiking levels were 0.25, 0.5 and 0.75 µg/Kg that represents 0.5 MRPL, 1.0 MRPL and 1.5 MRPL, respectively were employed.

Statistical analysis

The recovery and precision data were evaluated with an in-house statistical software program making use of Snedecor and Cochran concepts (Snedecor and Cochran, 1989) [14].

Table 1: Chromatoghraphic conditions

Mobile phase A	**0.01mM Ammonium Format (0.1% formic acid)**
Mobile phase B	Methanol
Column	Symmetry C_{18}, 2.1 x 100 mm, 1.7 µm at 45 °C
Flow rate	0.3 mL/min
Injection volume	10 µL

Table 2: Gradient Elution Programme

Time 0 min	**90% A**	**10% B**
Time 0.5 min	90% A	10% B
Time 7 min	10% A	90% B
Time 8 min	10% A	90% B
Time 8.1 min	90% A	10% B
Time 10 min	90% A	10% B

Table 3: MS Conditions

Ionization Mode	**Electrospray**
Polarity	positive
Capillary voltage	0.5 kV
Source temperature	150 °C
Desolvation temperature	650 °C
Cone gas flow	50 L/hr
Desolvation gas flow	1000 L/hr

Table 4: MS/MS parameters

Analyse	MRM transition	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
AMOZ	335>291	0.005	14	12
	335>127	0.005	14	20
	335>262	0.005	14	16
Internal standard d_5- AMOZ	340.1>101.9	0.005	35	30

Results and Discussion

The proposed procedure entails liquid-liquid extraction of dried meat powder with ethyl acetate after 2-NBA derivatization as discussed in extraction method. LC conditions were optimised (Table.1), good peak separation was achieved on a BEH C_{18} column (100 mm × 2.1 mm, 1.7 µm) column with gradient elution mode (Table.2) using mobile phases consisting of methanol and 2mM ammonium formate. The retention time of AMOZ and internal standard (D5-AMOZ) was 3.3±0.1 min. The optimized mass spectrometry parameters (Table.3) were performed by injecting the AMOZ standard solutions into the electrospray ion source is in line with Guichard *et al*. (2020) [9] and Leitner *et al*. (2008) [10]. Using electrospray LC–MS/MS with multiple reaction monitoring (MRM), identification and quantification of the AMOZ were performed (Table.4) based upon the intensities of mass fragments from the respective precursor ions: MRM of AMOZ: 335 > 291, 335 > 127; MRM of D_5-AMOZ - 340.1 > 101.9, respectively. The results were in accordance with EU document (EC/1871/2019) on confirmation for compounds with an MRPL, two MRM transitions were monitored (Figure 1) satisfying the confirmatory criteria laid in CD 2002/657/EC.

LODs and LOQs studied by spiking at MRPL. The LOD and the LOQ for AMOZ is 0.04 and 0.13 (µg/Kg), respectively. The obtained LOQ values was 0.13 µg/Kg lower than the EU MRPL (0.5 µg/Kg) established for the AMOZ (EC/1871/2019).

Following that performance parameters were studied to demonstrate that this optimized method complies with the criteria applicable for the relevant performance characteristics was carried out. The performance parameters demonstrated the adequacy of the method for determining the residues of AMOZ, in the dried meat powder keeping in view CD 2002/657/EC**.** Specificity was demonstrated beyond doubt by running blank samples and checked for any interference at the retention time (RT) of AMOZ. Analysis of blank muscle samples demonstrated that there were no interfering compounds at the RT (3.3 minutes) of AMOZ (Fig.1), demonstrating the selectivity of the method in compliance with EC regulation (CD 2002/657). Linearity accuracy was studied by constructing a (0.1, 0.2, 0.5, 1.5,1,2 and 5 µg/Kg) matrix-matched calibration curve in the concentration range 0.1 to 5 µg/Kg that corresponds to 0.2 to 10 times the MRPL. The assay was linear from 0.1 to 5 µg/Kg (Fig.2). The coefficients of determination (R^2) values of the calibration curves were higher than 0.99, complying the (CD 2002/657) EC guidelines.

Also, the recovery percentage was investigated by fortifying meat powder at three levels *viz*. 0.25, 0.5 and 0.75 µg/Kg that corresponds to 0.5,1 and 1.5 times the MRPL (0.5µg/Kg) and analyzed six replicates at each level. The percentage recovery in powdered meat matrix was quite adequate for quantitation purpose, it ranged from 81-108% (Table.5). The recovery percentage is well within the acceptance range (80-120% with RSD values ≤ 20%) as documented in acceptance criteria of CD 2002/657/EC. The intraday variation was studied by arriving at the RSD % of the mean yield fortified at 0.5,1 and 1.5 times MRPL (Table 6.) was found not exceeding 6% which is on par with the results obtained by Verdon *et al*. (2007) [16]. The applicability of the optimized LC–ESI-MS/MS method for AMOZ was also checked by participating in Inter-Laboratory Comparison (ILC) conducted by National Food Laboratory, Kolkata by analyzing the blind samples of meat powder and secured a z- score of 0.26. Our results demonstrated that this optimized liquid chromatography-tandem mass spectrometric method is quite adequate for analyzing AMOZ residues in meat powder at and below its MRPL.

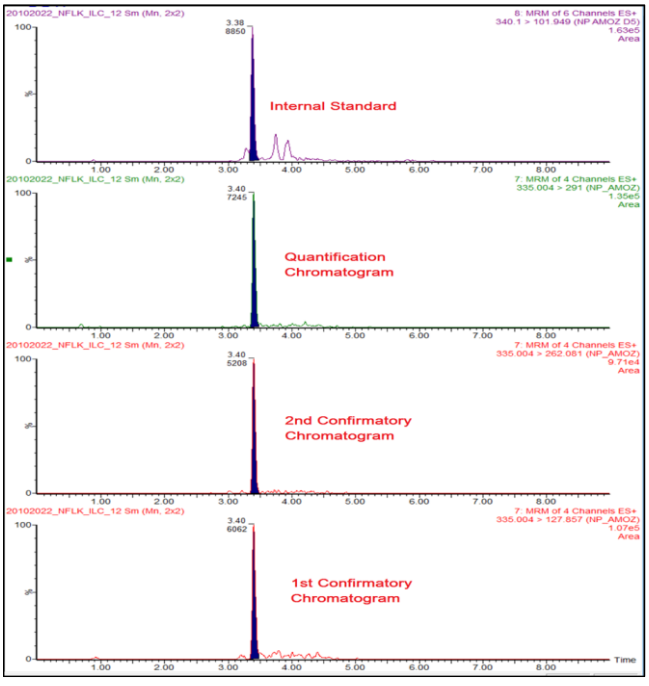

Fig 1: MRM Chromatogram of AMOZ and D5-AMOZ at 0.5µg/Kg.

Table 5: Mean Recovery of AMOZ spiked at three different levels in Meat powder

Spike levels(µg/Kg)	Mean Recovery (%)	% RSD
0.25 (0.5 RPA)	108	6.6
0.5 (1.0 RPA)	97	2.7
0.75 (1.5 RPA)	81	4.8

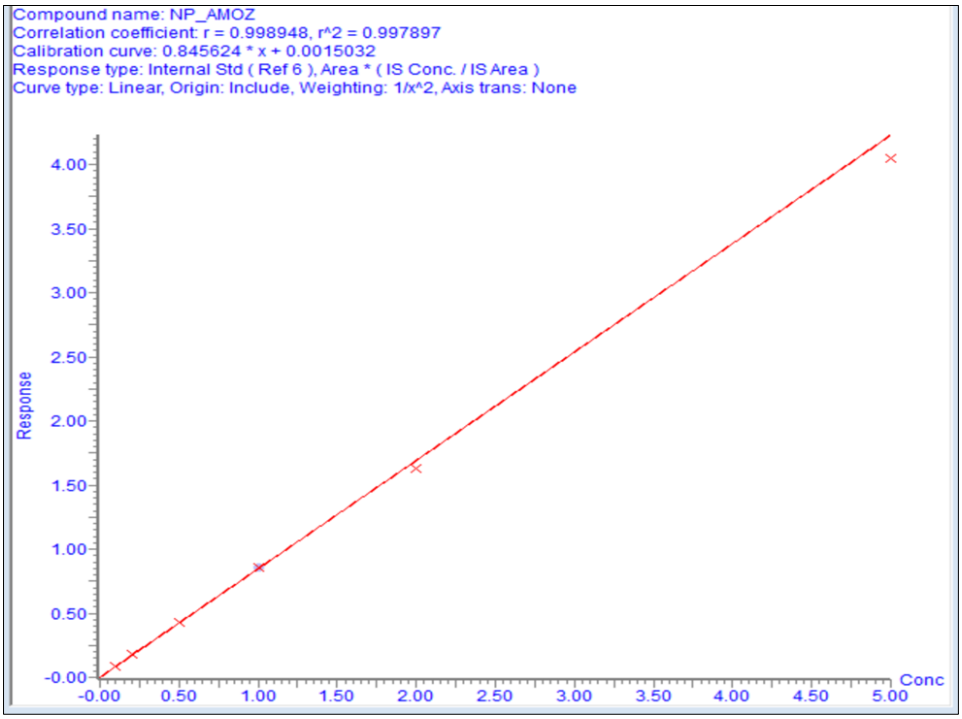

Fig 2: AMOZ matrix-matched calibration curve ranging from 0.1 to 5.0 µg/Kg.

Table 6: Method accuracy of AMOZ

Concentration µg/kg	NP-AMOZ	
	Mean	% RSD
0.1	0.09	4.17
0.2	0.20	3.24
0.5	0.48	6.61
1	1.01	2.38
2	1.97	3.73
5	4.95	2.12

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