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A COMPARATIVE STUDY IN THE CONFIRMATORY METHOD DEVELOPMENT AND VALIDATION OF FLUBENDAZOLE IN FISH AND SHRIMP MUSCLES BETWEEN HPLC-MS/MS (Q MICRO) AND UPLC-MS/MS (TQD)

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ABSTRACT

A confirmatory method for determination of flubendazole was developed in ultra performance liquid chromatography–tandem quadrupole mass spectrometry (AQUITY UPLC–TQD) and in HPLC-MS/MS (Quattro micro) at same time. The method was validated in Bagda shrimp (*Penaeus monodon*) and Tilapia (*Oreochromis niloticus*) fish. The liquid chromatographic separation was done with gradient elution by using (Atlantis® dC18, 3µm, 4.6x100 column in case of HPLC system whereas AQUITY UPLC BEH C18, 2.1x50mm, 1.7µm, Column in UPLC system) same mobile phase of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Mass spectral acquisition was done using electrospray ionization in the positive ion mode applying multiple reaction monitoring (MRM) of two diagnostic transition reactions for flubendazole and for flubendazole_d3. Shrimp and fish samples were extracted with ethyl acetate and evaporated to dryness and finally reconstituted with acetonitrile. The method validation was carried out according to the criteria of Commission decision 2002/657/EC. The calibration curve showed a good linearity in the concentration range from 1 to 20 ng/g with the correlation coefficient of >0.996 in all cases. In case of shrimp samples the decision limit (CCα) and the detection capability (CCβ) were 5.8µg/kg and 6.5µg/kg respectively in system1 and 5.6µg/kg and 6.2µg/kg in system2. In case of fish sample CCα values were 6.00µg/kg and 5.4µg/kg in system1 and system2 and CCβ values were 7.1µg/kg and 5.8µg/kg respectively for

flubendazole. The precision of the method, expressed as RSD values for the within laboratory reproducibility at the three levels of fortification (2.5, 5.0 and 7.5) µg/kg was less than 15%. The mean recoveries were in the range of 90–107% for shrimp and that of 90-109% for fish.

Keywords: UPLC-TQD, HPLC-Quattro micro, Flubendazole, Shrimp, Fish and Validation.

INTRODUCTION

Flubendazole (Fig.1) is a benzimidazole anthelmintic. It is the fluoro- analogue of mebendazole and has many similar properties. Flubendazole is listed in Annex I of Council Regulation (EEC) No. 2377/90 for turkey, chicken, game birds and porcine species as shown in the Table1.[1] Flubendazole is a broad-spectrum anthelmintic, widely used in veterinary medicine in order to treat diseases in agriculture and aquaculture and also in human medicine.[2] Being excreted from the body with faeces and urine, they reach environment via different routes. According to literature, residues of flubendazole (FLU) was found in the leachate from agricultural manure to drainage waters reaching values of up to 300 ng L−1[3] as well as in influent (19.9– 89.7 µg L−1) and effluent (55.0–671.0 ng L−1) wastewater from the pharmaceutical industry.[4] Moreover, they were also detected in the surface waters (the Llobregat River, Spain) at the concentrations up to 1.32 ng L−1.[5] Hence, this compound as well as other pharmaceuticals has been classified as emerging environmental contaminants for almost 15 years now. The chemical structure of this compound has a specific mode of action which binds to β-tubulin and inhibition of microtubule formation in the intestinal cells inducing a decreased glucose uptake and starving of the parasites.[6] Since microtubules serve a variety of important functions in animal, plant, fungi and some bacterial cells make FLU to be evaluated for potential effects on aquatic flora and fauna. In fish it is used for controlling a number of organisms, including hydra, intestinal parasites (Heximata, gill flukes and Camallanus) possible by adsorption through the fish's skin.

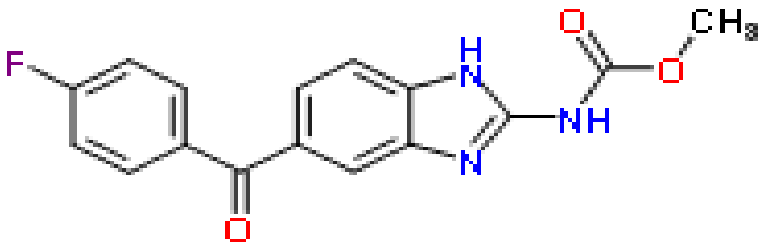

Figure1. Structural formula of flubendazole.[7]

[CAS No.: 31430-15-6, CBNumber: CB2669630, Molecular Formula: C16H12FN3O3, Formula Weight: 313.28, mp: 290°C]

Residues of veterinary medicinal products, as defined by the European Union, are "pharmacologically active substances (whether active principles, excipients or degradation products) and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered". The MRL is the maximum concentration of residue following administration of a veterinary medicine which is legally permitted or acceptable in food under the laws of the EU. This is particularly important in the poultry industry where benzimidazoles are veterinary drugs widely used for prevention and treatment of parasitic infections. The 40th JECFA[8] (The joint FAO/WHO expert committee on food additives) evaluation in 1992 set ADI (acceptable daily intake): 0-12 µg/kg body weight and MRLs for flubendazole residue in some matrices as shown in Table2.

Table1: MRLs of flubendazole listed in annex I of Council Regulation (EEC) No. 2377/90[1]

Pharmacologically active ubstance(s)	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Flubendazole	Sum of flubendazole And (2-amino-1*H*benzimidazole- 5-yl) (4-fluorophenyl)- methanone	Chicken, turkey, game birds and porcine	50 µg/kg 50 µg/kg 400 µg/kg 300 µg/kg	Muscle Skin + fat Liver Kidney	
	Flubendazole	Chicken	400 µg/kg	Eggs	

Tabe2: MRLs of flubendazole in some matrices.

Sl No	Species	Tissue	MRL (µg/kg)	CAC	Notes
1	Pig	Muscle	10	21st (1995)	
2	Pig	Liver	10	21st (1995)	
3	Poultry	Muscle	200	21st (1995)	
4	Poultry	Liver	500	21st (1995)	
5	Poultry	Eggs	400	21st (1995)	

But till to date no MRL has been established in fish and shrimp muscles. However, estimation of flubendazole in shrimp and fish marices accurately is very important to assume the dose limit and test is mandatory for NRCP (national residue control plan) by EU. In our present study we have developed a confirmatory method for the determination of flubendazole in fish and shrimp matrices using HPLC-Q. micro system (Alliance- Quattro micro) and UPLC-TQD system. The method was validated in both matrices according to the criteria provided in 2002/657/EC[9] using both systems to compare the results.

MATERIALS AND METHODS

Instruments

System-1: LC: Alliance Waters 2695 separation module, Detector: Quattro micro API (QAB 1485), Waters, USA.

System-2: LC: AQUITY UPLC, Waters, Detector: TQ Detector, ACQ-TQD#QBB933.

Columns for liquid chromatographic (LC) separation

System-1: Atlantis® dC18, 3µm, 4.6x100 (mm), Waters, USA (Made in Ireland), 186001337

System-2: AQUITY UPLC BEH C18, 1.7µm, 2.1x50(mm), Waters, USA.

Glassware and Apparatus

Syringe filter: 13 mm PTFE, 0.2 µrn, Waters, USA, Micropipette: Eppendorf, Test tubes: IW AKI TE32 pyrex, Asahi, Indonesia, Analytical Balance (4 decimal points): Shimadzu AUY 220, Centrifuge: Nuve, NF 1200, Centrifuge tubes: griner B532, Nitrogen evaporator (Organomation Associates Jne.), Microvials and caps: Waters, USA, mmPTFE 0.45 11m filter (Sartrius stedim, Germany), Column: Acquity UPLC, BEH C18, 1.7 µrn, Polypropylene centrifuge tubes (50 ml) (Griner, B 532), Volumetric flasks: 10 ml (Schoot Duran), Vortex mixer: Barnstead Thermolyne, M 16710-33, Incubator: Kullanma Kilavuzunu, ST 402.

Chemicals: Flubendazole Standard and Flubendazole_d3(internal standard): Sigma Aldrich, Germany, Ethyl acetate HPLC grade, Methanol HPLC grade and MS grade, Acetonitrile HPLC grade and MS grade, n-Hexane, Sodium Hydroxide, Water: Type-I and Type-II.

General Reagents

Mobile Phase

Solvent A: 0.1% Formic acid in Water

Solvent B: 0.1% Formic acid in ACN

0.1 N NaOH: Take 2g NaOH into 500 ml volumetric flask dissolve and make upto mark with water.

Preparation of Flubendazole Standards Solutions

(i) Stock standard FLUB (1000 µg/ml)

Take 10 mg and dissolve with DMSO in a 10 ml amber volumetric flask and make upto mark by methanol, store in a refrigerator for 12 months.

(ii) Intermediate FLUB Standard (10µg/ml):

Take 100µl of stock standard (1000µg/ml) and dilute to volume with methanol in a 10 ml amber volumetric flasks and store in a refrigerator for one month.

(iii) Working standard FLUB (100ng/ml):

Take 100 µl of intermediate standard (10µg/ml) and dilute to ‘volume with methanol in a 10 ml amber volumetric flasks, freshly prepare before use.

Preparation of Flubendazole_d3 solutions

(i) Stock standard FLUB_d3 (1000 µg/ml) in methanol

Take 10 mg and dissolve with DMSO in a 10 ml amber volumetric flask and make upto mark with methanol, store in a refrigerator for 12 months.

(ii) Intermediate FLUB d3 Standard (10 µg/ml)

Take 100µl of stock standard (1000 µg/ml) and dilute to volume with methanol in a 10 ml amber volumetric flasks and store in a refrigerator for one months.

(iii)Working FLUB d3 Standard (l00 ng/ml)

Take 100 µl of stock standard (10µg/ml) and dilute to volume with methanol in a 10 ml volumetric flasks, freshly prepare before use.

Quality Control Samples

(i) Reagent Blank: two sample containing 1 ± 0.05g of Type-I water and carry out through the procedure to check for process contamination.

(ii) Matrix Blank: two matrix samples (usually collected from deep sea, which is free from flubendazole contamination) are carried out through the procedure to check matrix interference and contamination.

(iii) Matrix Blank_IS: two matrix samples spiked with internal standard (flubendazole_d3) only and carry out through process to check any flubendazole contamination in internal standard.

(iv) Spiked Recovery (QC) Samples: two matrix blank samples are spiked with the flubendazole at the 5 ppb and are carried out through the procedure to check the recovery of the method.

Spiking of samples for Preparation of Calibration Curve (Standard Samples)

Matrix based calibration curve was prepared by spiking flubendazole standard and internal standard in 1 ± 0.05g sample for each concentration as shown in Table3.

Table3: Spiking of flubendazole and flubendazole_D3 in matrix for calibration curve.

SL No	Volume FLUB Std (100 ng/ml) in µl	Volume FLUB_D3 (100 ng/ml) in µl	Std Equivalent Concentration (ppb)
1	0	100	0.0
2	10	100	1.0
3	20	100	2.0
4	30	100	3.0
5	40	100	4.0
6	50	100	5.0
7	100	100	10.0
8	200	100	20.0

Extraction Procedure[10]

- Weigh portion (1 ± 0.05 g) of minced tissue into a centrifuge tube for each type of sample separately.
- Add 100µl of flubendazole_d3 working internal standard (100ng/ml) solution to all tubes (except reagent blank and matrix blank).
- Homogenized with 10 ml deionized water
- Add 5 ml 0.1 N. NaOH for alkalization
- Add 20 ml Ethyl acetate
- Shake the mixture for 1 minute and centrifuge for 10 minutes at 5000 rpm
- Transfer the supernatant into new tube
- Re-extract with 10 ml Ethyl acetate
- Combine both supernatant
- Evaporate at 55^0C using vacuum rotary evaporator approximately 5 ml and transfer in graduated tube
- Rinse the flask tube by 2 ml ethyl acetate
- Dry under Nitrogen Evaporator at 55°C
- Residues reconstitute by 1 ml of 50% acetonitrile.
- Filter through 0.2 µm syringe filter and take into LC-MS/MS vial (2ml amber).

Preparation of LC-MS/MS Batch for analysis

Two vials for acetonitrile (ACN) to check any contamination in ACN, two vials for water to check any contamination in water, two vial for solvent blank (reconstitute solvent), two reagent blanks, two matrix blanks, matrix blank_IS, standard samples, QC samples and analytes.

UPLC-MS-MS Analysis

Inlet parameters

System-1: Run Time: 7 minutes, Injection volume: 50 µl, column temperature: 35^0C, sample temperature: 20^0C, LC Separation condition: Gradient

System-2: Run Time: 5 minutes, Injection volume: 10 µl, column temperature: 35^0C, sample temperature: 10^0C, LC Separation condition: Gradient.

MS Method Parameters

System-1: API Probe Delay Temp: 20^0C, Number of function: 1, Function 1: MRM of 3 mass pairs, Time 0.00 to 5.00, Type: MRM, Ion Mode: ES+, Inter Channel Delay (sec): 0.020, Inter Scan Time (sec): 0.100, Span (Da): 0.2, Start Time: (min): 0.0, End Time (min): 7.0.

System-2: API Probe Delay Temp: 20^0C, Number of function: 1, Function 1: MRM of 3 mass pairs, Time 0.00 to 5.00, Type: MRM, Ion Mode: ES+, Inter Channel Delay (sec): -1.000, Inter Scan Time (sec): 0.01, Span (Da): 0.0, Start Time: (min): 0.0, End Time (min): 5.0.

Tune Parameters

System-1: Capillary (kV): 3.74, Cone (V): 35.00, Extractor (V): 3.00, RF (V): 0.1, Source temperature (^{0}C): 130, Desolvation Temperature (^{0}C):400, Cone gas flow (L/Hr): 50, Desolvation gas flow (L/Hr): 800, Collision gas flow (mL/Hr): 0.13 to maintain $3.75x10^{-3}$ pressure (mbar, Multiplier (V): 650.

System-2: Capillary (kV): 3.00, Cone (V): 30.00, Extractor (V): 3.00, RF (V): 0.1, Source temperature (^{0}C): 80, Desolvation Temperature (^{0}C): 150, Cone gas flow (L/Hr): 0, Desolvation gas flow (L/Hr): 300, Collision gas flow (mL/Hr): 0.10 to maintain $3.75x10^{-3}$ pressure (mbar), Multiplier (V): 650

Analyzer Settings

System-1: LM1 Resolution 1: 14.00, HM1 Resolution: 15.00, Ion Energy 0:5, MS Mode Entrance: 50.00, MS Mode Collision Energy: 3.00, MS Mode Exit: 50.00, MSMS Mode Entrance: 1.00, MSMS Mode Collision Energy: 20.00, MSMS Mode Exit: 50, LM2 Resolution: 14.00, HR2 Resolution: 14.00, Ion Energy2: 2.00.

System-2: LM1 Resolution: 15.00, HM1 Resolution: 15.00, Ion Energy1: 0.50, MS Mode Entrance: 50.00, MS Mode Collision Energy: 3.00, MS Mode Exit: 50.00, MSMS Mode Entrance: 1.00, MSMS Mode Collision Energy: 20.00, MSMS Mode Exit: 0.50, LM2 Resolution: 15.00, HR2 Resolution: 15.00, Ion Energy2: 0.50.

RESULTS

The detection method for confirmation of flubendazole was firstly developed in LC-MS/MS systems using standard and internal standard. The detectors (Quattro micro and TQD) were firstly operated in positive ESI+ MS mode to select characteristic ions as the precursors of FLUB and its deuterated internal standard FLUB_d3. Both FLUB and FLUB_d3 were then analyzed LC-MS/MS systems in a positive ionization product ion scan mode by selecting precursor ion. The collision- induced dissociation (CID) experiments of these ions, giving rise to daughter ions for FLUB and FLUB_d3 (Figure2). The selected transitions for FLUB and FLUB_d3 for the optimal MS–MS conditions are given in Table4 for both systems. The developed gradient method with 0.1% formic acid in acetonitrile and 0.1% formic acid in water for both systems is shown the Table5.

Analytical performance: Method validation was carried out according to criteria described in Decision 2002/657/EC. The parameters taken into account were: response, linearity, decision limit (CCα), detection capability (CCβ), reliability and accuracy. The calibration curve showed a good linearity in the concentration range of 1-20 µg/kg with the correlation coefficient >0.996. The analytical performance for validation samples are shown in Table6 and Figure3 for shrimp samples and that of shown in Table7 and Figure4 for fish samples. The Limit of decision (CCα) and detection capability (CCβ) were calculated using the procedure set out in ISO Guide 11843, as described in Commission Decision 2002/657/EC. The validation data were generated (3 levels and seven replicates per level) on each of three days for shrimp and fish matrices separately for both systems and values are shown in Table8 and figure5. The trueness was expressed in terms of recovery rates. The mean recoveries of FLUB spiked samples were in the range of 90-107%.

Table4. Ion monitored and optimal MS-MS condition

Ch	Prnt (Da)	Dau (Da)	Dwell (s)	Cone (V)	Coll (eV)	Delay (s)	Compound	Formula (Da)
System-1	313.75	123.10	0.500	35.00	34.00	0.020	Flubendazole	313.30
	313.75	282.01	0.500	35.00	20.00	0.020		
	317.84	283.10	0.500	35.00	18.00	0.020	Flubendazole_D3	316.30
System-2	314.077	122.899	0.025	48.00	56.00	-1.000	Flubendazole	313.30

	314.077	282.022	0.025	48.00	30.00	-1.000		
	317.077	282.028	0.025	48.00	32.00	-1.000	Flubendazole_D3	316.30

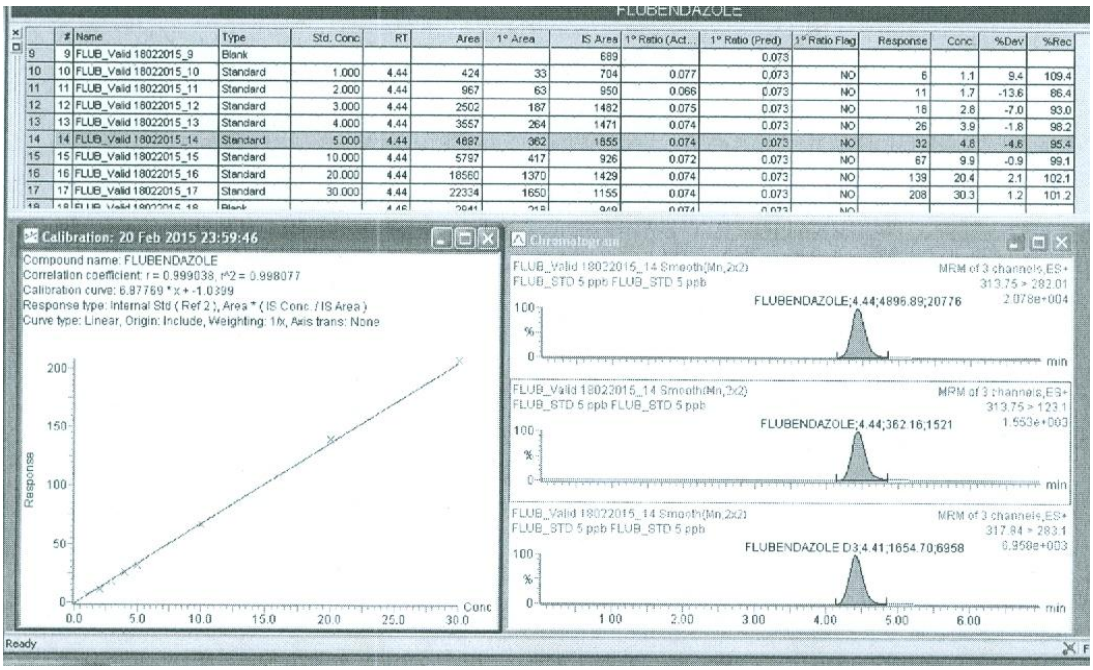

Figure2: Calibration curve of flubendazole showing transitions of FLUB and FLUB_d3

Table5: Gradient Table: A: 0.1% Formic acid in water, B: 0.1% Formic acid in acetonitrile

System-1					System-2				
SL No	**Time**	**Flow Rate (µl/min)**	**%A**	**%B**	**SL No**	**Time**	**Flow Rate (µl/min)**	**%A**	**%B**
1	Initial	0.200	90	10	1	0.00	0.300	10	90
2	0.50	0.200	90	10	2	1.50	0.300	10	90
3	1.50	0.200	10	90	3	2.00	0.300	50	50
4	3.50	0.200	10	90	4	4.00	0.300	50	50
5	4.00	0.200	90	10	5	4.50	0.300	10	90
6	5.00	0.200	90	10	6	7	0.300	10	90

Table6: Summary of flubendazole validation in shrimp matrix using system1 and system2

Overall Summary of Shrimp Validation						
	Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
System1	2.5	2.6	104	4.9	4.3	5.8
	5.0	5.0	100	5.6	4.5	5.6
	7.5	7.6	101	9.4	5.6	10.0
System2	2.5	2.5	99	1.9	2.6	2.5
	5.0	5.0	100	1.6	1.7	2.3
	7.5	7.2	96	2.4	0.3	2.4

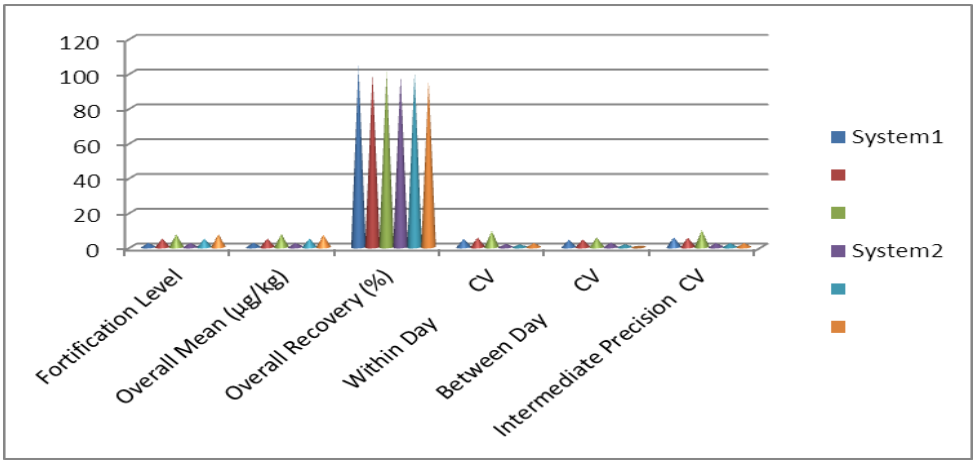

Figure3: Summary of flubendazole validation in shrimp matrix using system1 and system 2.

Table7: Overall Summary of flubendazole validation in fish matrix using system1 and system 2.

Overall Summary of Fish Validation						
	Fortification Level	**Overall Mean (µg/kg)**	**Overall Recovery (%)**	**Within Day CV**	**Between Day CV**	**Intermediate Precision CV**
System1	2.5	2.5	100	3.9	3.6	4.5
	5.0	5.2	104	3.5	4.7	5.3
	7.5	7.5	100	4.3	3.3	5.8
System2	2.5	2.4	96	2.1	1.8	2.3
	5.0	5.0	100	1.6	1.2	2.4
	7.5	7.2	96	2.4	0.3	2.6

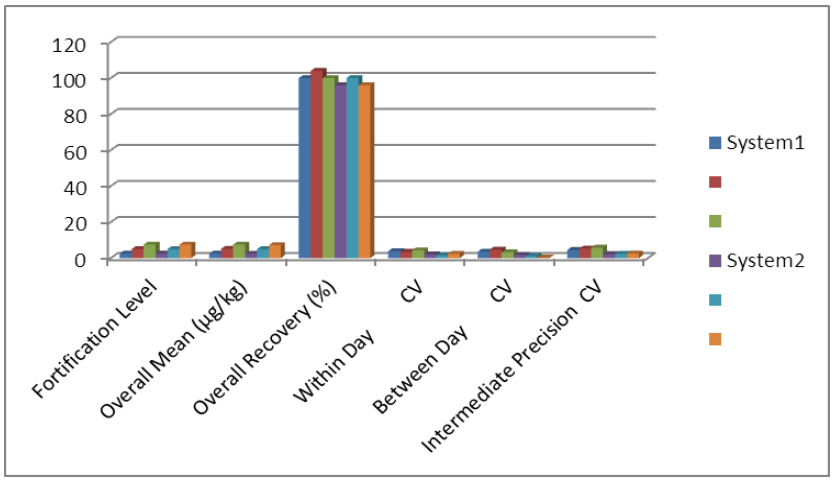

Figure4: Overall Summary of flubendazole validation in fish matrix using system1 and system 2.

Table 8: CCα (µg/kg) and CCβ(µg/kg) of flubendazole in shrimp and fish matrices using system1 and system 2.

	Shrimp	Fish	
System-1	5.8	6.0	CCα (µg/kg)
	6.5	7.1	CCβ(µg/kg)
System-2	5.6	5.4	CCα(µg/kg)
	6.2	5.8	CCβ(µg/kg)

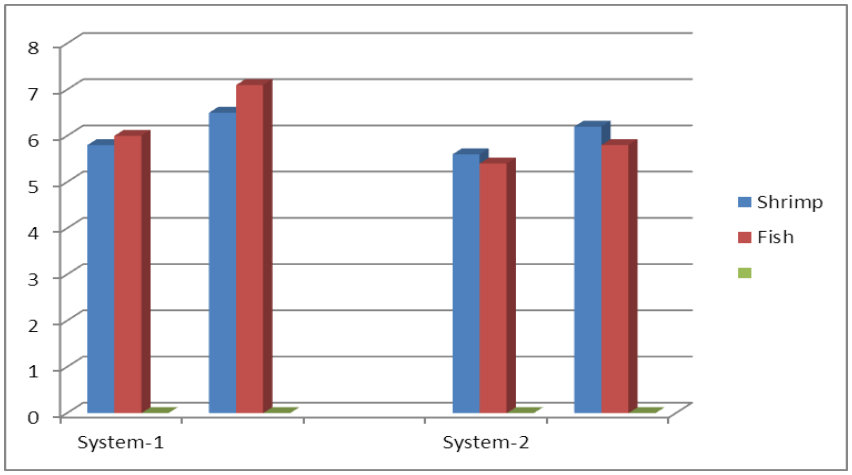

Figure5: CCα (µg/kg) and CCβ(µg/kg) of flubendazole in shrimp and fish matrices using system1 and system 2.

DISCUSSION

The precursor and daughter ions obtained in the result have a good agreement with previous findings,[10, 17-18] which indicates the compound was identified accurately. The selectivity of this method is judged by the use of two transitions for each analyte which count for 4 identification points (IPs), as defined by the EU criteria set out in Commission Decision 2002/657/EC. Consequently our method fulfils this requirement. The instruments were in well and good condition by which our previous findings on chloramphenicol,[11-14] nitrofuran metabolites,[15] dyes[16] were done. The sharp peak of transitions without any contamination (as shown in firure2) was self explanatory for developed gradient method in compound separation in both cases. As shown in the Figure3 there was no peak in chromatogram of a solvent blank, matrix blank (negative sample) and a single chromatogram in case of matrix with internal standard (IS) which indicates no contamination was found in sample and in the extraction process. The sample spiked at 5.0ng/g showed good chromatogram (Figure2) for each transition. The performance characteristics of the method presented in this paper indicate that it may be preferably used to test anthelmintics (flubendazole) in food control by both systems. As shown in the table8 and figure5 the CCα and CCβ values were lower in

system2 than system1 and performance characteristics (as shown in Table6 & 7 and figure3 & 4) indicate UPLC_TQD system exhibited better performance than HPLC-Quattro micro system.

CONCLUSION

The method was developed and validated as per guideline and commission decision 2002/657/EC. The developed confirmatory method for flubendazole analysis in fish and shrimp matrices is good enough for analysis in both systems. But ACQUITY UPLC-TQD system showed better performance in compare to HPLC-Quattro micro system.

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