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Determination of Chloramphenicol and Thiamphenicol Residues in Fish, Shrimp and Milk by ESI-LCMSMS

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Abstract

Chloramphenicol (CAP) and thiamphenicol (TAP) – analogue of CAP are widely used in the growth of animals and marine culture as they are broad spectrum antibiotics. Due to the development of resistance of microorganisms against antibiotics many countries have completely banned the use of them. A Negative Electro spray ionization mode with liquid chromatography and mass spectrometry was used to monitor the contamination of TAP and CAP in shrimp, fish and milk. Transitions 321 → 152, 120.4, 257 for CAP, 326 → 126, 157, 262 for CAP IS and 354 → 184.9, 168.8, 78.8 for TAP were monitored. For extraction of antibiotics ethyl acetate was used, showed recovery batter than 75% in all three matrixes. For timely efficient separation Agilent Eclipse C_{18} column was used with the simple isocratic mobile phase methanol as A (99%) and water: methanol in ratio of (8:2) as a B (1%), which needed 3.5 min to elute out both the compounds. Full validation was done as per EU decision 2002/657. Linearity was greater than 0.99. Coefficient variation was performed at 3 different concentrations and it was not greater than 3% between 6 replicates. CCα and CCβ in different matrices on an average were 0.27 and 0.29 µg/kg for CAP and 51.85 and 53.45 µg/kg for TAP. Accuracy was calculated at three different concentrations and the readings were found between 90 to 110%. Youden approach was used for the calculation of ruggedness and their statistically calculated results were satisfactory. Method was applied to the samples in which 56 shrimp, 18 fish and 8 milk samples were tested and 4 shrimp samples were found positive at MRPL level

so that the method is fit for CAP and TAP analysis of commercial products at MRL compliance.

Keywords: Chloramphenicol, thaimphenicol, LCMS, EU commission.

1. Introduction

Chloramphenicol (CAP) and Thiamphenicol (TAP) are broad spectrum antibiotics hence they are widely used on animal and marine growth [1]. Application of them has been employed to overcome the infectious disease in the hatchery and grow-out phase. CAP ($C_{11}H_{12}C_{l2}N_2O_5$) was synthesized from the bacterium *Streptomyces venezuelae* by David Gotlieb in 1947. This antibiotic is active against a wide range of aerobic and anaerobic bacteria and fungi [2].

Several analytical methods are employed in the quantitative detection of CAP and TAP in sea foods. The methods are categorized as bio-assay method using swab tests, instrumental methods (HPLC and GC) and immunoassays methods (RIA, CLIA, ELISA, etc.). [3]. Immuno assay methods were more laborious as every antibiotic need different kit. In such instance it becomes very difficult to analyze multi antibiotics at a time Hence, such methods are not suitable for screening of commercial samples for regulatory purposes, as they are time-consuming and less confirmatory. In this method effort was made to develop method for CAP and TAP on highly sensitive LCMSMS instrument for marine and milk samples. For constant results Internal standard (IS) was used for CAP as MRPL is set up at 0.3 ppb. Validation of method was done as per EU decision 2002/657 at MRPL (Maximum required performance limit) and MRL (Maximum residue limit) level for CAP and TAP respectively.

2. Experimental

2.1 Chemicals and Reagents

Spectra grade Methanol and Water were purchased from JT Baker (USA). Ethyl acetate solvent and Ammonium acetate buffer were purchased from Merk. Standards like Chloramphenicol, DL chloramphenicol (IS) and Thiamphenicol were purchased from Sigma Ald rich.

2.2 Methodology

Samples like Fish and shrimp were purchased from local market of Maharashtra and Gujarat. They were transported with Ice pack to maintain integrity of sample. Sample was grinded to homogenize matrix. Then this matrix was stored at -17 ^{o}C in deep freezer for further analysis. For spiking negative samples were used. Milk sample were collected from the local market of Mumbai.

2.3 Extraction method

Simple and efficient extraction method was used for batter recovery of CAP and TAP. Two gm sample was weighed in centrifuge tube and it was spiked with IS. Here IS was used to nullify the effect of manual and instrumental error. Then 7 ml ethyl acetate was added and vortexed to homogenize with solvent. Then it was followed by centrifugation for 5 min at 10,000 rpm. Supernatant solvent was evaporated under nitrogen evaporator. Care was taken that samples and CAP standard are not exposing to lights or sun light to prevent the degradation of them.

2.4 Standard preparation:

Standard solutions were prepared in methanol as they have a good solubility in methanol and methanol also occupies 99% of mobile phase. Standard stock solution of 1000 mg/kg was prepared by dissolving 10 mg of standard in 10 ml methanol and then serial dilution was made as per requirement.

2.4 Instrument parameters:

For detection of CAP and TAP highly sensitive Agilent triple quadrapole mass spectrometer (6460) was used. MS was set to monitor the following transitions 321 → 152, 120.4, 257 for CAP, 326 → 126, 157, 262 for CAP IS and 354 → 184.9, 168.8, 78.8 for TAP. They were monitored in negative mode with different collision and fragmentor energy. Gas flow and temperature were set at 6 L/min and 330 ^{o}C respectively while sheath gas flow and temperature were 8 L/min and 350 ^{o}C respectively. Nebulizer pressure was set at 30 psi. Capillary and Nozzle voltage were set from 500 to 3500 V. In this method isocratic condition was used. Mobile phase was methanol as A (99%) and water: methanol in ratio of (8:2) as a B (1%). For batter peak shape and fast elution Rapid resolution liquid chromatography (RRLC 1200) with stationary phase Agilent Eclipse C_{18} column of 15 cm length and 4.6 mm id was used.

3. Results and Discussion

HPLC method was developed and optimized by selecting few mobile phases like methanol, acetonitrile and water with buffer ammonium formate or acids like formic acid and tetra fluro acetic acid at 0.1 %. Out of many mobile phase ratio and combination it was found that water:methanol in ration of 8:2 as a B and pure methanol as a A in combination of 1 % and 99 % respectively gave the good peak shapes and validation results (Fig-1).

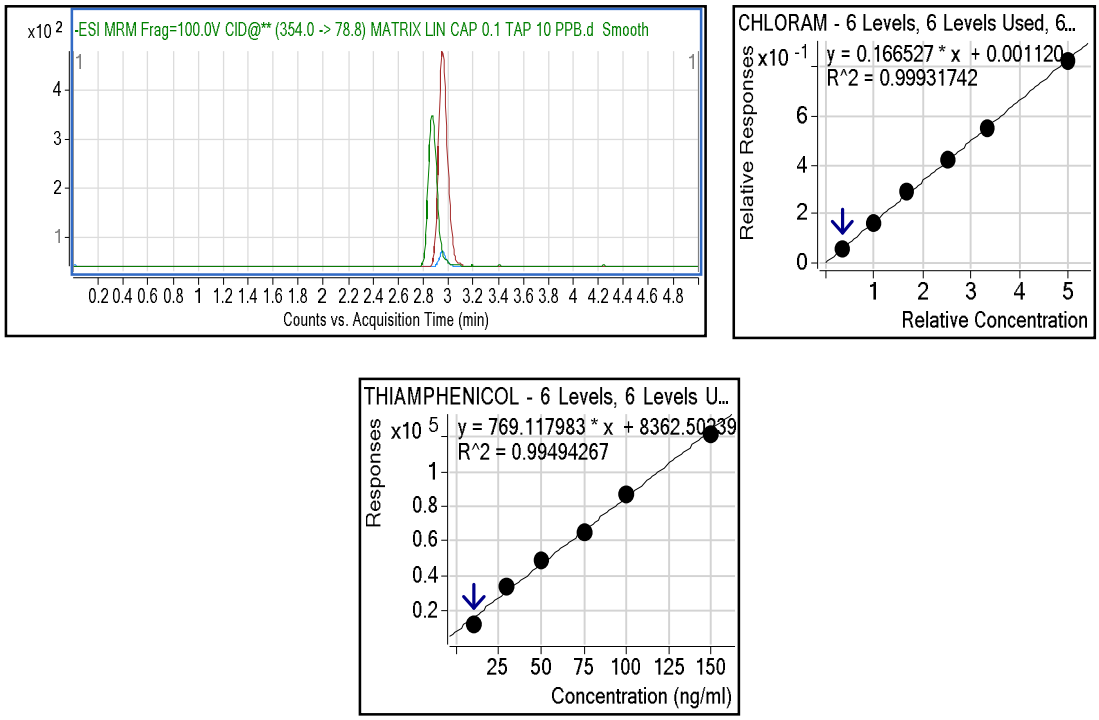

Figure 1: LCMSMS chromatogram and linearity of CAP, TAP and CAP IS.

CAP to CAP IS ratio was used for the calculation of concentration. As these matrices are complex and rich of other interferences more proof needed for the confirmation of antibiotics, hence three parameters were used are Retention time, fragmentation pattern (two qualifier ions) and Ion ratio from quantifier to qualifier. In ion ratio variation was monitored which should not cross ± 20 % (Table 1).

Table 1: Ion ratio between two transitions (Quantifier to Qualifier) of standards.

Antibiotic	Transition	1	2	3	4	5	6	7	8	9	10	SD.	Avg.	RSD
CAP	321-257	72.9	74.7	71.9	70.3	78.1	77.5	75.1	71.6	77.3	72.6	2.75	74.2	3.71
TAP	354-78.8	17.8	17.7	18.6	17.4	17.7	17.8	17.5	18.8	18.3	18.3	0.47	17.99	2.65

Complete Validation of CAP and TAP was done as per EU decision 2002/657 [4] and all parameters were complied with EU requirement as shown in table-2.Trueness, Coefficient variation (CV) and Recovery were calculated at three different concentration as per EU decision i.e. at 0.5, 1 and 1.5 of MRL and 1, 1.5 and 2 of MRPL. CCα and CCβ were calculated at MRPL (0.3 µg/kg) and MRL (50 µg/kg) level

for CAP and TAP respectively in matrix milk, fish and shrimp (Table 2). For CCα and β caculation following equations was used.

CCα = MRL+1.64*SD of 20 Fortified blanks at MRL

CCβ = CCα+1.64*SD of 20 Fortified blanks at CCα

CAP and TAP were validated at a time though they had different MRL. For this, CAP was individually spiked at lower concentration and TAP at higher concentration. Both gave the good validation results in a single extraction and analysis method. In below table average % trueness, % recovery and % Coefficient variation are mentioned.

Table 2: Validation results of CAP and TAP in Milk, Fish and Shrimp.

Antibiotics	**Range in µg/kg**	**(r^2)**	**Con. µg/kg**	**% Trueness**	**% CV**	**% Recovery**	**CCα (µg/kg)**	**CCβ (µg/kg)**
CAP	0.1 - 1.5	>0.99	0.3	102.03	2.51	75 - 82	0.27	0.29
			0.45	98.95	1.77	79 - 85		
			0.6	103.14	1.05	77 - 84		
TAP	10 - 150	>0.99	25	100.23	1.91	78 - 88	51.85	53.45
			50	98.90	1.27	78 - 92		
			75	99.13	2.98	80 - 88		

Samples were analyzed by using the same method. Total 56 shrimp, 18 fish and 8 milk samples were tested and 4 shrimp samples were found positive at MRPL level so that it was proved that the method is fit for CAP and TAP analysis at commercial level.

4. Conclusion

From validation and samples analysis it can be concluded that the following method is very simple, cost effective and selective to analyze CAP and TAP in Fish, shrimp and Milk. It has also proved that the method can be used for simultaneous analysis of CAP and TAP though their MRL are different. As it require less run time and extraction time this method can be used commercially for the analysis of large quantity of samples. This method uses very sensitive and robust instrument LCMS for the analysis of samples so that it can work up to ppt level with high precision and accuracy i.e. fulfill the requirement of EU commission.

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