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Multi-residual quantitative analytical method for antibiotics in sea food by LC/MS/MS

ASMS 2017 TP 198

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Introduction

Antibiotics are widely used in agriculture as growth enhancers, disease treatment and control in animal feeding operations. Concerns for increased antibiotic resistance of microorganisms have prompted research into the environmental occurrence of these compounds. Assessment of the environmental occurrence of antibiotics depends on development of sensitive and selective analytical methods based on new instrumental technologies.

LC/MS/MS method has been developed for quantitation of multi-residual antibiotics (Table 1) from sea food sample using LCMS-8040, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan. Simultaneous analysis of multi-residual antibiotics often exhibit peak shape distortion owing to their different chemical nature. To overcome this, autosampler pre-treatment feature was used [1].

Table 1. List of antibiotics

Sr.No.	Name of group	Name of compound	Number of compounds
1	Fluoroquinolones	Flumequine, Oxolinic Acid, Ciprofloxacin, Danofloxacin, Difloxacin.HCl, Enrofloxacin, Sarafloxacin HCl Trihydrate, Naldixic Acid	8
2	Sulfonamides	Sulfadimethoxine, Sulfadoxine, Sulfachlorpyridazine, Sulfamethoxypyridazine, Sulfadimidine, Sulfamethizole, Sulfamerazine, Sulfathiazole, Sulfamethizole, Sulfadiazine, Sulfapyridine	11
3	Dyes	Crystal Violet , Leucocrystal violet, Malachite green, Leucomalachite green	4
4	Antihelminthics	Albendazole, Albendazole Sulfone, Albendazole Sulfoxide, Albendazole-2-aminosulfone , Fenbendazole, Flubendazole	6
5	Nitroimidazoles	Ronidazole, Metronidazole, Dimetronidazole	3
6	Phenylbutazone	Phenylbutazone	1
7	Macrolides	Erythromycin, Spiramycin, Tilmicosin, Tylosin Tartarate, Trimethoprim	5

Methods and Materials

Sample preparation

The antibiotic standards procured from Sigma-Aldrich were used for the analysis. All individual standards stock were prepared in the methanol. Further mixture of all antibiotics were prepared in methanol. This stock was serially diluted to prepare calibration levels ranging from 0.5 ppb to 50 ppb in methanol for solvent standard and in matrix for matrix matched standard calibration.
Commercially available shrimp sample were used for analysis. The shrimp was finely crushed by using a sample crushing mixer. Crushed sample was transferred to 50 ml centrifuge tube. To this 10 mL of acidified acetonitrile was used for extraction of anti-biotics from the shrimp sample, because some of the antibiotics require acidic condition for extraction. Solution was then centrifuged at 4°C,8000 rpm for 5 mins. Further dSPE clean-up was given to the supernatant and dSPE cleanup extract was filtered through 0.2 micron filter and injected on LCMS-8040.

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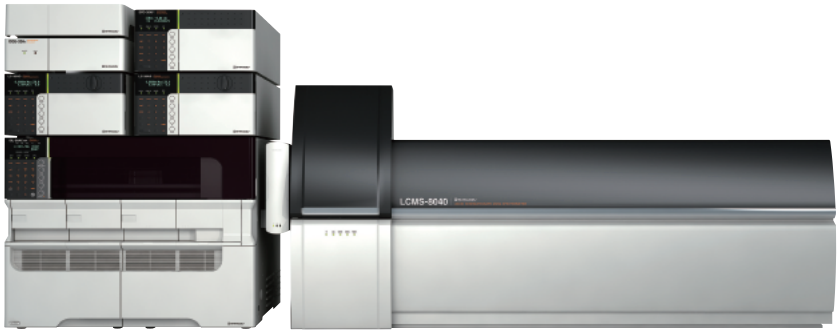

Figure 1. LCMS-8040 triple quadrupole mass spectrometer by Shimadzu

LCMS-8040 triple quadrupole mass spectrometer by Shimadzu, sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 15,000 u/sec (UFscanning) and polarity switching speed of 15 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

LC/MS/MS analysis

All antibiotics i.e. 11 Sulfonamides, 8 Fluoroquinolones, 4 Dyes, 6 Antihelminthics, 3 Nitroimidazole,5 Macrolides and Phenylbutazone were simultaneously analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8040 triple quadrupole system (Shimadzu Corporation, Japan). The details of analytical conditions are given in Table 2.

Table 2. Optimized LC/MS/MS conditions for antibiotic analysis

Column	:	Shim-pack GIST Phenyl (75mm L X 3.0mm I.D, 2 µm)
Mobile phase	:	A- 2mM ammonium formate + 0.002 % formic acid in water
	:	B- 2mM ammonium formate + 0.002 % formic acid in methanol
Flow rate	:	0.4 mL/min
Gradient program (B %)	:	0.01-1 min → 10 (%); 1-5 min → 10-70 (%); 5-9 min → 70-95 (%) ; 9-11 min → 95 (%); 11-11.5 min → 95-10 (%); 11.5-15 min → 10 (%)
Injection vol.	:	5 µL
Column temperature	:	40°C
MS interface	:	Electro Spray Ionization (ESI)
Nitrogen gas flow	:	Nebulizing gas 2L/min; Drying gas 10L/min
MS temperature	:	Desolvation line 250°C; Heating block 400°C

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Table 3. Details of MRM transitions

Sr.No.	Name of Compound	Quantifier ions
1	Sulfamethoxypyridazine	281.10>156.05
2	Sulfamethizole	271.10>156.00
3	Sulfamerazine	265.15>92.10
4	Sulfadiazine	251.10>156.00
5	Sulfapyridine	250.10>92.10
6	Sulfamethazin	279.15>186.00
7	Sulfamethoxazole	254.10>155.95
8	Sulfadimethoxine	311.10>156.00
9	Sulfadoxine	311.10>156.10
10	Sulfachlorpyridazine	285.10>155.95
11	Sulfathiazole	256.10>156.05
12	Albendazole	266.10>234.00
13	Albendazole sulfone	298.10>159.00
14	Albendazole sulfoxide	282.20>240.00
15	Albendazole 2 aminosulfone	240.10>133.05
16	Fendendazole	300.10>268.00
17	Flubendazole	314.15>282.15
18	Morantel	221.05>111.10
19	Flumequine	262.15>201.95
20	Oxolinic acid	262.15>160.05
21	Ciprofloxacin	332.20>314.20
22	Difloxacin	400.10>382.15
23	Enrofloxacin	360.20>342.25
24	Sarafloxacin	386.15>368.05
25	Nalidixic Acid	233.15>187.05
26	Danofloxacin	358.20>340.20
27	Ronidazole	201.10>140.00
28	Metronidazole	172.20>127.90
29	Dimetronidazole	142.20>96.10
30	Leucocrystal violet	374.20>358.15
31	Malachite green	329.20>313.15
32	Leucomalachite green	331.20>239.10
33	Crystal violet	372.20>356.20
34	Spiramycin II	422.20>101.15
35	Tilmicosin I	435.20>98.90
36	Tylosin tartarate I	916.30>174.20
37	Trimethoprim	291.25>230.20
38	Neo spiramycin II	366.20>174.10
39	Phenylbutazone	309.15>77.10
40	Erythromycin	734.30>576.30

Results

Analysis was performed using aqueous as well as matrix matched standards. The MRM transitions used for these analysis are given in Table 3. Auto MRM optimization feature was used for optimization for MRM transitions. Linearity studies were carried out using external calibration method and linearity results are tabulated in Table 4.

The matrix matched calibration levels were prepared and injected in segmented MRM mode. The calibration curve of all antibiotics are shown in Figure 4 to Figure 6 and the correlation coefficient >0.99 was obtained for all compounds.

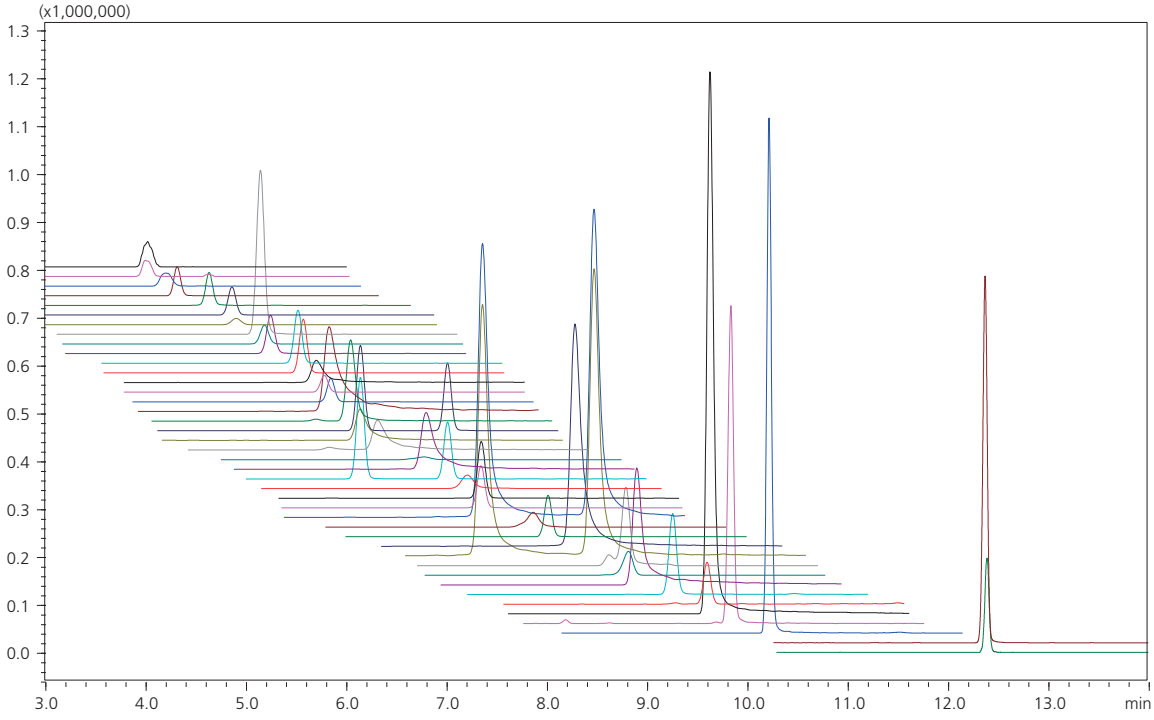

Figure 2. Chromatogram of matrix match standards of 2 ppb

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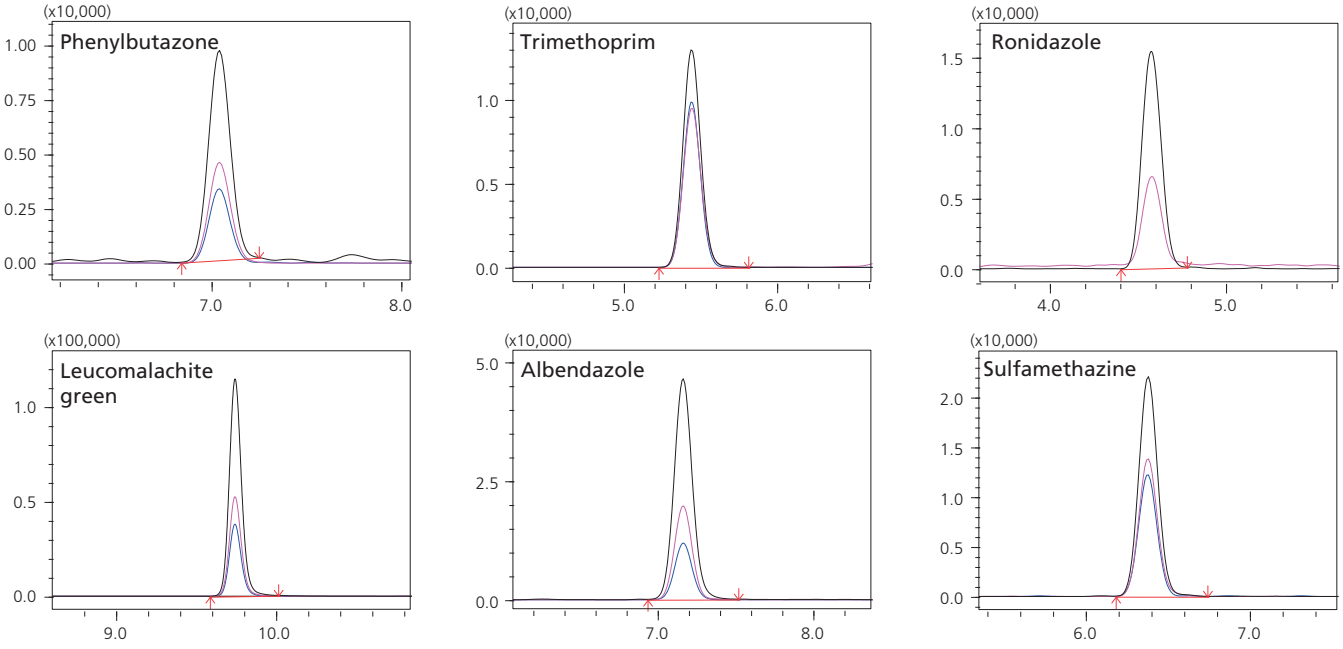

Figure 3. Extracted ion chromatogram of representative antibiotics

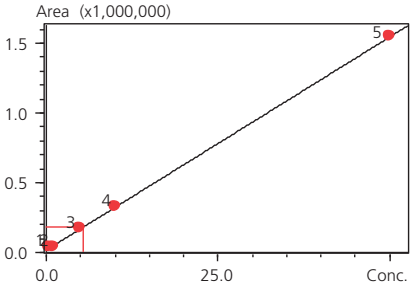

Figure 4. Sulfamethazine

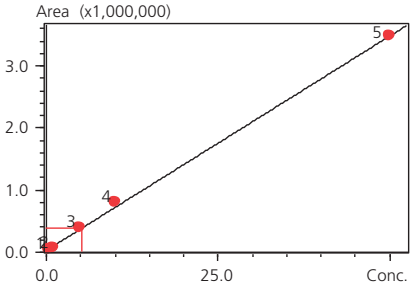

Figure 5. Albendazole

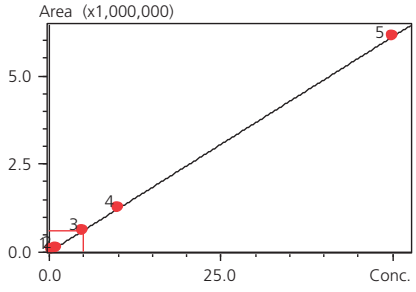

Figure 6. Leucomalachite green

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Table 4. Result table for matrix matched standard calibration and recovery at different level

Sr.No.	Class / category	Name of Compound	MRPL in ppb	LOQ achived in ppb	Linearity range in ppb	Recovery		
						0.5 ppb	1 ppb	2 ppb
1	Fluoroquinolones	Flumequine	600	0.5	0.5-50	106.8	88.76	87.5
2		Oxolinic Acid	100	0.5	0.5-50	89.32	97.7	97.03
3		Ciprofloxacin	100	2	0.5-50	NA	NA	105.36
4		Difloxacin.HCL	300	2	0.5-50	NA	NA	84.06
5		Enrofloxacin	100	2	0.5-50	NA	NA	88.52
6		Sarafloxacin	30	1	0.5-50	NA	117.42	86.52
7		Naldixic Acid	Reporting>LOQ	0.5	0.5-50	100.82	105.52	92.6
8	Sulfonamides	Sulfadimethoxine	100	0.5	0.5-50	107.38	104.55	87.9
9		Sulfadoxine		0.5	0.5-50	102.88	89.41	89.54
10		Sulfachlorpyridazine		0.5	0.5-50	80.9	98.12	86.14
11		Sulfamethoxypyridazine		0.5	0.5-50	116.08	108.14	88.95
12		Sulfamethizole		0.5	0.5-50	82.56	87.7	89.78
13		Sulfamerazine		0.5	0.5-50	113.78	78.61	89.88
14		Sulfathiazole		0.5	0.5-50	79.96	83.28	89.46
15		Sulfamethazin		0.5	0.5-50	90.8	90.06	90.47
16		Sulfadiazine		0.5	0.5-50	103.66	91.11	93.93
17		Sulfapyridine		0.5	0.5-50	93.72	84.48	101.36
18		Sulfamethoxazole		0.5	0.5-50	112.68	111.82	96.3
19	Dyes	Leucocrystal violet	Nil	0.5	0.5-50	56.5	62.45	57.75
20		Crystal violet		0.5	0.5-50	94.56	117.22	103.44
21		Leucomalachite green	2	0.5	0.5-50	104.94	99.64	88.48
22		Malachite green		0.5	0.5-50	103.88	73.97	64.352
23	Anthelminthics	Albendazole	50	0.5	0.5-50	79.12	87.79	81.65
24		Albendazole Sulfone	50	0.5	0.5-50	84.74	97.29	98.49
25		Albendazole Sulfoxide	50	0.5	0.5-50	99.56	77.13	84.98
26		Albendazole-2-aminosulfone	50	1	0.5-50	NA	79.08	75.13
27		Fenbendazole	50	0.5	0.5-50	111.62	88.91	88.99
28		Flubendazole	50	0.5	0.5-50	108.34	100.81	97.33
29		Morantel	50	0.5	0.5-50	87.52	95.31	100.6
30		Fenbendazole sulfone	50	0.5	0.5-50	78.44	84.16	93.13
31	Nitroimidazoles	Ronidazole	3	0.5	0.5-50	110.82	95.99	91.68
32		Metronidazole	3	0.5	0.5-50	108.16	96.71	82.89
33		Dimetronidazole	3	2	0.5-50	NA	NA	89.9
34	Phenylbutazone	Phenylbutazone	5	1	0.5-50	NA	107.11	96.11
35	Macrolides	Spiramycin	NA	1	0.5-50	NA	128.75	87.41
36		Tilmicosin	NA	0.5	0.5-50	114.14	89.45	84.68
37		Tylosin Tartarate	NA	0.5	0.5-50	119.98	94.15	105.11
38		Trimethoprim	NA	0.5	0.5-50	89.62	85.55	78.88
39		Neo Spiramycin	NA	5	0.5-50	70.34	75.9	79.23

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Conclusion

- The recovery obtained for most of the compounds are well within the acceptance range of 70-120%.
- This method will improve the overall turn around time of sample analysis, along with reduction in per sample cost.

Reference

[1] Defoirdt, T.; Sorgeloos, P.; Bossier, P. Alternatives to Antibiotics for the Control of Bacterial Disease in Aquaculture. Curr. Opin. Microbiol. 2011, 14, 251–258.

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First Edition: June, 2017

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