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Analysis of colistin A and B in fishery products by ultra performance liquid chromatography with positive electrospray ionization tandem mass spectrometry

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ABSTRACT

A rapid and simple method for the determination of colistin A and B in fishery products by reversed phase ultra performance liquid chromatography with positive electrospray ionization tandem spectrometry (UPLC–ESI-MS/MS) method was described. The samples were extracted with 1.0 mol/L of hydrochloric acid in methanol–water and then purified on the PLS solid phase extraction columns. Then the eluate was evaporated to less than 1 mL under a gentle stream of nitrogen at 40 °C and formic acid–acetonitrile–water (0.2/10/90, v/v/v) was added to adjust volume to 1 mL final volume. An aliquot (10 μL) was injected onto the LC column for analysis with the mobile phase of 0.2% formic acid in acetonitrile and 0.2% formic acid in water at 0.20 mL min^{-1}. Multiple reaction monitoring was performed using precursor-product ion combinations. Calibration curves were linear from 200 ng/mL to 2000 ng/mL for colistin A and B. Mean recoveries were between 72.9% and 82.9%. The LOD was 10.0 μg/kg and LOQ was 40.0 μg/kg. The intra-day assay precision values for QC samples were between 2.17% and 9.00%, and inter-day values were between 2.80% and 6.97%. The method has merits of simplicity, sensitivity and rapidity, and it can be used for the determination of colistin A and B in fishery products.

1. Introduction

Colistin (also known as polymyxin E) is an important member of the polymyxin group of cationic peptide antibiotics and was isolated from *Bacillus colistinus* in 1950 [1], which comprised mainly of colistin A (PME1) and colistin B (PME2) [1,2] (differ only in the fatty acid side chain), as shown in Fig. 1. Colistin is an old antimicrobial substance, and its use in human medicine has augmented the last decade, largely due to the appearance of multidrug resistant *Pseudomonas*, *Klebsiella* and *Acinetobacter* spp. [3,4]. Colistin is often parenterally used or by nebulization for treating pulmonary and systemic infections [5]. Even though parenteral and intramammary administration occasionally occurs in veterinary medicine, colistin is mainly used in oral preparations. Due to its excellent intrinsic activity against *Escherichia coli*, the low prevalence of acquired resistance and the poor absorption after oral administration, colistin is a frequently used antimicrobial agent for the prevention and treatment of bacterial infections in animals, including fishery products. Colistin is found to be effective to treat the infections caused by Gram-negative bacteria, in particular, for those strains that are resistant to aminoglycosides, β-lactams and fluoroquinolones in aquaculture [6]. However, the residues of colistin A and B in edible fishery products tissues can be absorbed by human through the food chain and be harmful for human consumption.

Colistin was abandoned during the last two decades because of its toxicity [7]. And there was concern regarding its nephrotoxicity and the rate of nephrotoxicity of colistin in recent studies was highly variable, ranging from 0% to 37% which might be due to differences in criteria for renal toxicity [8]. Its systemic use was abandoned also because of toxicity such as neuromuscular blockade and neurotoxicity [9–11]. To safeguard public health, national health authorities worldwide have established respective maximum residue limits (MRLs) in animal milk and tissues for regulating the use of colistin. Japanese Positive List System has established a minimum required performance limit (MRPL) of 200 μg/kg for colistin in fish [12]. Therefore, determination of colistin at sub μg/kg levels is required.

Regarding recent concerns on the health risks associated with the abuse use of the drugs, an increasing number of methods

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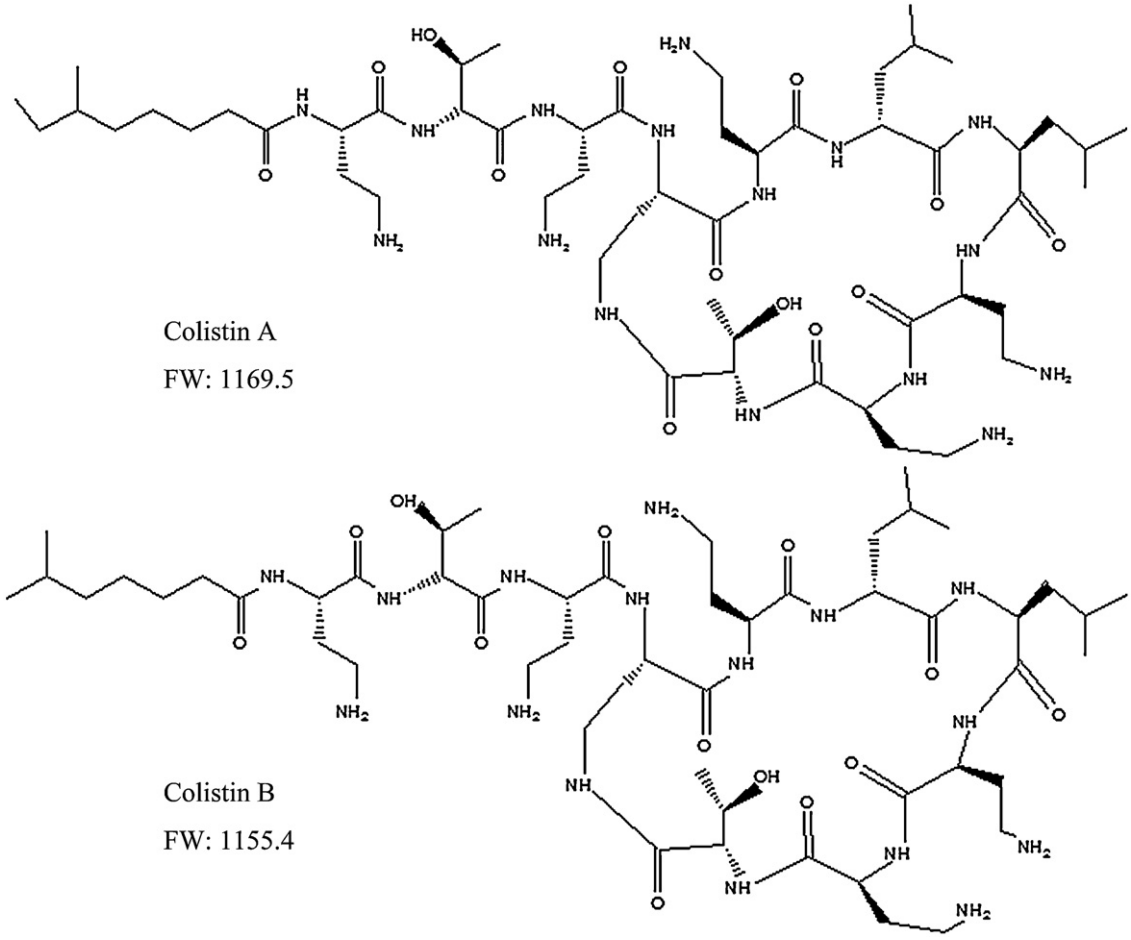

Fig. 1. Chemical structures of colistin A and colistin B.

have been developed for their determination. LC with different detection methods, such as fluorimetric detection, enzyme-linked immunosorbent assay (ELISA) and MS/MS has been developed for quantification of colistin A and B in plasma, milk and animal tissues [13–16]. Li et al. [17] developed and validated a novel HPLC method with fluorimetric detection for the assay of colistin in human plasma, first report of analysis using 9-fluorenylmethyl chloroformate as derivatizing reagent and the LOQ was 0.10 mg/L. Comparing the front method, Cheng [18] avoided using derivatization and SPE process in rat plasma analysis, but using trichloroacetic acid (TCA) for protein precipitation to make better sensitivity and LOQs were 7.3 ng/mL and 5.0 ng/mL for colistin A and colistin B, respectively. Ma [19] reported that HLB solid phase extraction preceded chromatography on a Synergi Fusion-RP column with MS/MS for detection were performed in human urine, getting the LOQs 0.056 μg/mL and 0.032 μg/mL for colistin A and colistin B. Sin [13] developed a method for the determination of colistin in bovine milk samples. Samples were deproteinized and extracted with a mixture of trichloroacetic–formic acid using electrospray LC–MS/MS with time scheduled multiple reaction monitoring (MRM) for detection and employed polymyxin B as the internal standard. The practical quantification limit of colistin A was 50 μg/kg. All the methods cited were based on the extraction with a mixture of organic solvent–acid solution according to the properties of the compound but with different detector.

The purpose of the present study was to develop and validate a general bioanalytical method based on the same principle for the above methods, but with different extraction solvent and SPE column to get a good recovery. And UPLC–MS/MS has the virtue of efficient separation and accurate characterization, becoming the main method of determining residues in recent years. In this study, a method for determining colistin A and B in fishery products has been established. The result is satisfactory after systematized optimization of sample pretreatment methods and instrumental analysis conditions.

2. Experimental

2.1. Materials and reagents

Colistin sulfate (containing colistin A and B) was purchased from Dr. Ehrenstorfer (Germany). Methanol and acetonitrile were all of LC grade and obtained from Merck (Germany). Formic acid and hydrochloride were of guaranteed reagent grade. All other reagents used in the experiment were of analytical grade. Ultrapure water was obtained in a Milli-Q system from Millipore (USA). ProElut PLS solid phase extraction cartridges (3 mL, 60 mg) were obtained from Dikma (China).

2.2. Preparation of stock and working solutions

Because colistin A and B are highly soluble in water but poorly soluble in organic solvents, standard solution (100.0 μg/mL) was prepared by dissolving 0.0100 g of colistin sulfate in formic acid–water (0.2/100, v/v) using a 100 mL volumetric flask. Working solutions were diluted with acetonitrile–0.2% formic acid in water (1/9, v/v) on the day of analysis. Each solution was ultrasonicated

Table 1
Program of gradient elution.

Time (min)	Flow rate (mL min^{-1})	A% (0.2% formic acid in acetonitrile)	B% (0.2% formic acid in water)	Entry curve
0.00	0.200	5	95	
1.00	0.200	5	95	6
1.10	0.200	30	70	6
4.00	0.200	30	70	6
5.00	0.200	5	95	1

over a period of 1 min and filtered through 0.22 μm Millipore cellulose filter (USA) before injection.

2.3. Preparation and purification of samples

Samples obtained from a local supermarket were homogenized with bones in a blender and stored at −18 °C. Laboratory samples were analyzed and those found to contain no detectable residues of the analytes were used as negative controls.

Samples (5 ± 0.01 g) were weighed into 50 mL polypropylene tubes. 18 mL of methanol–water (5/4, v/v) was added and the samples were homogenized for 30 s. And 2 mL of 10.0 mol/L of hydrochloric acid was added and samples were shaken for 3 min and ultrasonicated for 10 min at room temperature. The homogenates were centrifuged at 11,963 × *g* for 10 min at 0 °C. Then the supernatant was transferred to a clean 100 mL polypropylene tube. The supernatant was added 18 mL of water waiting for further purified.

Solid phase extraction (SPE) process with PLS columns can be summarized as follows: (1) activated with 3 mL of methanol, 3 mL of water (both steps at 3 mL min^{-1}); (2) loading all the sample at 1.2 mL min^{-1}; (3) rinsed with 3 mL of water for three times; (4) elution with 2 mL of formic acid–methanol–water (0.2/70/30, v/v/v); (5) the eluate evaporated to less than 1 mL under a gentle stream of nitrogen at 40 °C and then formic acid–acetonitrile–water (0.2/10/90, v/v/v) added to give 1 mL final volume and syringe filtered using a 0.22 μm cellulose filter into an autosampler vial. An aliquot (10 μL) was injected onto the LC column for analysis.

2.4. UPLC–MS/MS condition

The UPLC–MS/MS system comprised an Acquity UPLC system connected online with a Quattro Premier tandem mass spectrometry (Waters, USA). The column used in the experiment was ACQUITY^{TM} BEH C_{18} reversed phase column (2.1 mm × 100 mm, 1.7 μm particle size) maintained at 40 °C. Mobile phase was 0.2% formic acid in acetonitrile and 0.2% formic acid in water. The sample injection was 10 μL. The program of gradient elution is shown in Table 1. The entire eluate was electrosprayed, ionized and monitored by MS/MS detection in the multiple reaction mode using positive electrospray ionization. The flow rate and temperature of the drying gas (N_2) were 750 L h^{-1} and 350 °C, respectively. The cone gas flow (N_2) was 50 L h^{-1}. The collision gas (Ar) flow was 0.35 mL min^{-1} and the capillary voltage was 1400 V. The dwell time was set at 100 ms for each transition. The extractor voltage was 4 V and RF lens voltage was 0.4 V. The source temperature was 110 °C. LM1, LM2, HM1 and HM2 resolutions used in the experiment were 15.0, 13.0, 15.0, and 13.0, respectively. The entrance and exit voltages were −1 and 2, respectively. Ion energy and multiplier were set at 1.0 and 650. Smoothing methods was mean, and window size (scan) was ±1 and number of smooths was set at 1. The software version was Masslynx V4.1. All the parameters were optimized to provide the highest sensitivity.

2.5. Validation procedures

Identification of colistin under study was confirmed by the presence of at least two pairs of MRM fragments from the precursor ions at the defined retention time windows, which was set ±5% with respect to that of the closest matching concentration of used matrix-fortified standard solution, and matching of the specific tolerance of the relative abundance of major ions as stated in the Commission Decision 2002/657/EC [20]. Standard calibration curve and QC samples were analyzed in three consecutive analyses. Linearity of calibration curves based on the analyte area as function of the nominal concentration was assessed by weighted ($1/x^2$) least square regression. Line equations, linearity range, and correlation coefficient were also calculated in the experiment.

3. Results and discussion

3.1. Extraction optimization

Colistin has two existing forms in animal tissues; one is free state and the other is combined with phospholipid in cell membrane which are tightly associated with the tissues. So 10.0 mol/L of hydrochloric acid was used for acid hydrolysis and protein precipitation in order to obtain better recoveries for high throughput liquid-liquid extraction methods. If the concentration was too high, the recoveries would be lower when the supernatant was placed for long time. The experiment showed that if the supernatant was placed for 2 h before SPE process, the recoveries were 30–50%. It was also shown that emulsification would occur if adding hydrochloric acid before methanol–water (5/4, v/v), and difficult to centrifugal separation. If methanol–water (5/4, v/v) was added at first, it made centrifugation easier. It was to recommend that the sample extracts were centrifuged as soon as possible.

3.2. SPE optimization

Colistin A and B have primary amines, which can make strong interaction with the remaining silanol groups in C_{18} column. And it was also demonstrated that large diameter packing column and benzene packing column without silanol group can make a better separation effect. So HLB column and PLS column were tested in the experiment using carp and whit prawn samples and the recoveries were 70–90% and 60–80%, respectively, and the reproductivity of PLS column was better. So the PLS column was used in the experiment.

3.3. Elution and reconstituted solution optimization

The strong interaction between the primary amines in colistin A and B and remaining silanol groups in C_{18} column can make the peak shape trailing and abnormal. So pH value of mobile phase was important and adding formic acid in mobile phase can make peak sharp. 0.1% and 0.2% were also compared in the work, and 0.2% had better response in both peak shape and response. So 0.2% formic acid in acetonitrile and 0.2% formic acid in water were chosen for mobile phase. Acetonitrile–water was used for

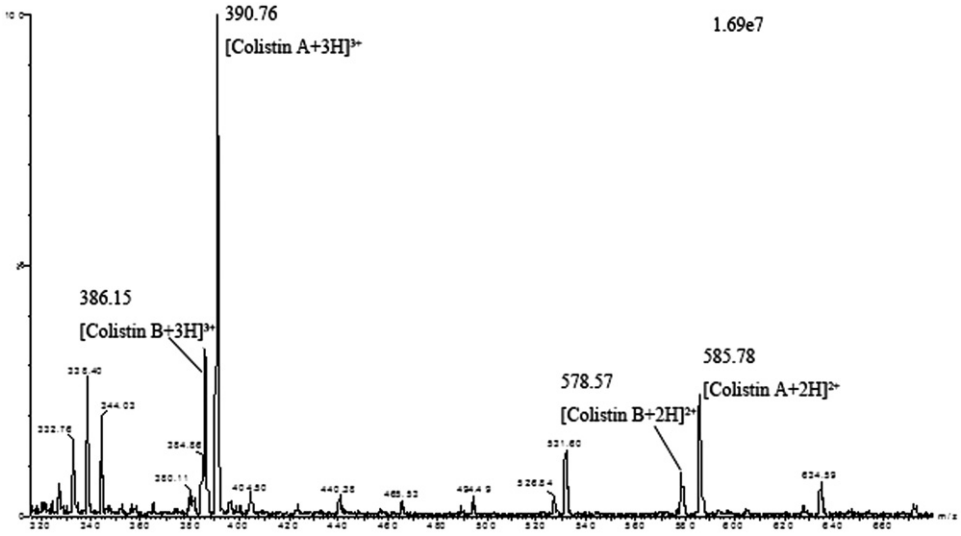

Fig. 2. Full scan mass spectrum of tuning solution (10.0 μg/mL) infused into the triple quadruple mass spectrometer.

reconstituted solution and the proportion was also tested. Adding formic acid can have a better ion abundance response. So formic acid–acetonitrile–water (0.2/10/90, v/v/v) was used for constant solution.

3.4. Optimization of LC–MS/MS

Colistin A and B belong to polypeptides and can form multiple charged ions in ionization process. The most common was triply charged ions and doubly charged ions. In the experiment, tuning solution (10.0 μg/mL) containing colistin A and B was introduced into the electrospray source by direct infusion (10 μL min^{-1}) during the automatic tuning process. Fig. 2 shows the full scan mass spectrum of tuning solution infused into the triple quadruple mass spectrometer. In contrast to the formation of the charged ions of colistin A and B that have recently been reported [18], it was noted that triply charged ions had better ion response than doubly charged ions in the experiment. Therefore, the triply charged ions were used as the precursors for establishing MRM analysis. The main ions produced in MS and MS/MS were identified in positive ionization modes. The diagnostic fragment ions were selected and all mass spectrometry parameters were optimized to increase sensitivity. Table 2 shows the precursor and daughter ions for colistin A and B as well as the optimum values of MS/MS parameters: voltage of the first quadrupole for isolation of the precursor ion and collision energy for efficient fragmentation. In the study, two daughter ions were routinely monitored. This fulfills the recommendations of the European Union concerning identification [20], since two multiple reaction mode transitions from the ionized molecule of target compound give four points in the scale-a value regarded as sufficient for unequivocal identification. Also the mobile phase was discussed. It has focused on the use of formic acid and its buffer as the acidic medium in the mobile phases which have been applied successfully to separate colistin A and B. So the mobile phase consisted of 0.2% formic acid in both acetonitrile and water. The percentage of acetonitrile and water was optimized to meet the requirements of the method for separation, satisfactory peak performance and high sensitivity, as shown in *UPLC–MS/MS condition* part.

3.5. Matrix effect

Matrix effect is a special phenomenon associated with LC–MS/MS determination of analytes from biological samples such as fishery products. Matrix components extracted along with analytes from fisheries products may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes from LC column. Matrix effect may impair accuracy and reproducibility. For this reason, matrix effect was evaluated under the used experimental conditions. It was evaluated by comparing the peak area of analytes dissolved in blank sample's matrix solution with dissolved in mobile phase. If the ratio was to be <85% or >115%, standard solution diluted by the matrix blank which was operated under the same process as the samples was adopted to calculate the concentration of the drugs. If it was of 85–115%, the matrix effect was absent. In the experiment, when the colistin was infused into the source at a rate to give a constant signal, and then inject tissues blank extracts through the LC, the ionization suppression was found with ratio <85%, shown in Fig. 3, and it can be adjusted with matrix-fortified standard solutions.

3.6. Linearity

Neither colistin A nor colistin B is commercially available at present. Thus it is not practically possible to quantify accurately from known amounts of the pure substances. So the sum of the peak areas of colistin A and B was calculated and used to generate calibration curves. The curve was not forced through the origin. The linearity was determined by five matrix-fortified

Table 2
The optimum potential settings for the analysis of colistin A and B in tandem mass spectrometry.

Compound name	Precursor ion (*m*/*z*)	Daughter ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Colistin A	390.76	385.00[a]	19.00	11.00
		101.10	19.00	19.00
Colistin B	386.15	380.00[a]	19.00	11.00
		101.00	19.00	19.00

[a] Quantification ions.

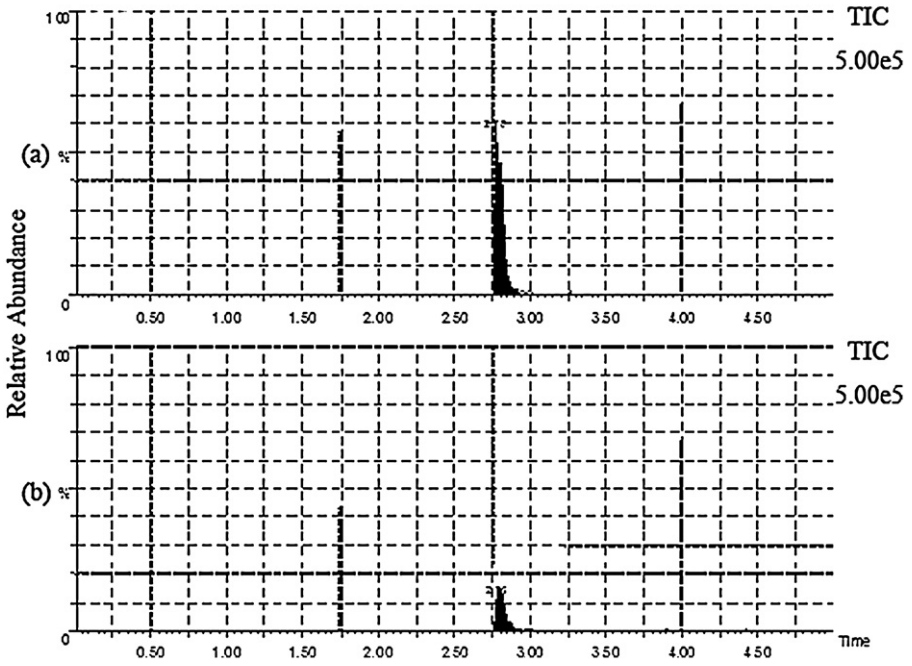

Fig. 3. Matrix effect in common carp. (a) Analyte infused into the source at a rate to give a constant signal and (b) inject tissues blank extracts through the LC when colistin infused into the source at a rate to give a constant signal.

calibration solutions (covering 200 ng/mL, 500 ng/mL, 1000 ng/mL, 1500 ng/mL and 2000 ng/mL) and evaluated by linear regression analysis calculated by the least squares regression method. Good responses over the concentration ranges were obtained. Colistin concentrations (x, ng/mL) and peak areas (y, sum of colistin A and colistin B) were calculated with a weighing factor of $1/x^2$. A typical result of the analysis of standard concentrations (five concentrations in the range between 200 ng/mL and 2000 ng/mL) was $y = 10.118x + 1399.3$, $R^2 = 0.9967$, with coefficient of correlation greater than 0.99.

3.7. Limit of detection and quantification

The limit of detection (LOD) was calculated and defined as signal-to-noise ratio of 3:1. And it was obtained from blank samples spiked with 50 μL of 1.0 μg/mL of standard solution, as shown in Fig. 4. The limit of quantification (LOQ) was also calculated and defined as signal-to-noise ratio of 10:1, which was the lowest concentration of the analytes that could be determined with accuracy, and was obtained from blank samples spiked with 200 μL of 1.0 μg/mL of standard solution. The calibration curves obtained were suitable for the quantification of the analytes in the samples during the intra-day and inter-day validations and stability tests, and were on the day of analysis. According to the concentration factor in the process of preparation and purification of samples, we got the LOD and LOQ, 10.0 μg/kg and 40.0 μg/kg, respectively. The LOD and LOQ were far below the Japanese Positive List System MRPL (200 μg/kg) for colistin in fish.

3.8. Accuracy, precision and recovery

Confirmation of the analytes in fishery products was performed using criteria of two ion transitions and LC retention time of each compound. Specifically, the relative intensities of two major daughter ions of given analytes were unique and they did not appreciably change over the concentration range tested and so were used for the confirmation of the presence of the analytes in the samples. The

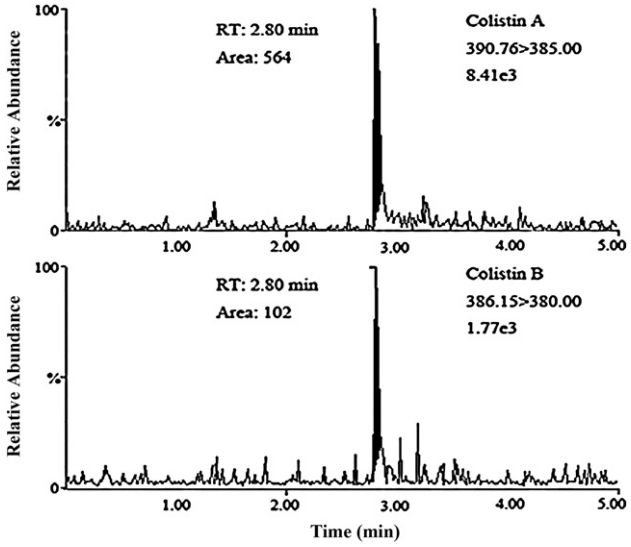

Fig. 4. Quantification ions chromatogram of LOD (10 μg/kg) for colistin A and B in carp.

relative intensities and RSDs of the matrix-fortified standard solutions when injected for 5 times of colistin A and B are tested and the typical results are shown in Table 3. Variation of the relative ion intensities in fishery products within 20% was usually acceptable for confirmation purposes.

Ruggedness can be used for measurement of the analytical result reproductivity in differently normal experiment conditions. The conditions were studied as given: flow rate 0.20 ± 0.02 mL min^{-1}, column temperature 40 ± 5 °C, mobile phase composition 0.2 ± 0.05% formic acid, cone voltage 19 ± 3 V, collision energy 11.00 (19.00) ± 3 eV. The flow rate and column temperature effected a change of the retention time, so can be stable in the experiment. The flow rate also influenced the separation

Table 3
The relative intensities mean values and RSDs of the matrix-fortified standard solutions when injected for 5 times of colistin A and B.

Matrix-fortified standard solution	Colistin A		Colistin B	
	Mean value	RSD (%)	Mean value	RSD (%)
200 ng/mL	0.83	6.96	0.83	6.71
500 ng/mL	0.86	1.33	0.87	4.33
1000 ng/mL	0.84	2.18	0.81	3.11
1500 ng/mL	0.85	2.41	0.83	2.97
2000 ng/mL	0.83	2.90	0.85	2.48

degree of colistin A and colistin B, so flow rate should get a shorter retention time on the premise of satisfactory separation degree, but the fluctuation of the flow rate had no big influence. Formic acid can strengthen ion abundance, but ±0.05% variation had little influence in the experiment. It had been found that cone voltage and collision energy had great effect on sensitivity and so can be optimized according to different instrument and kept constant in the whole study.

Intra-day accuracy and precision were assessed from six consecutive analyses of the QC samples at three different concentrations in eight kinds of fishery products. Inter-day accuracy and precision were obtained from consecutive analyses of the same batch of QC samples on three separate occasions. The intra-day assay precision (relative standard deviation) values for QC samples were between 2.17% and 9.00%, and inter-day values between 2.80% and 6.97%. All data obtained from eight kinds of fishery products for the analytes were within acceptable limits stated for bioanalytical methods validation, as shown in Table 4.

3.9. Stability

The stability was evaluated by repeated injection of the test solution. The stock solutions would be stable at −18 °C for two months, or will be decomposed almost 100% at 4 °C in two weeks. Studies showed that the lower concentration standard solutions of the analytes were unstable under the conditions used in this work, but the stability will be better in an acid medium. The analytes 0.10–0.20 mg/L in standard solution would degrade half if stayed at room temperature for 1 h, and the peak shape would be furcated. So the prepared standard solution should be determined as soon as possible. But the analytes were stable in matrix at 0 °C for two days. The analytes in the fishery products matrix stored at −18 °C could still be tested in 21 days for credible results in our experiment. It was also found that evaporation to dryness at 40 °C could lead to a relative lower recovery, showing only 30% of the analytes found, so evaporated to 0.8 mL under a gentle stream of nitrogen before SPE process could make better recoveries.

3.10. Application

The method was successfully applied to determine colistin A and B in fishery products, and the applicability of the validated method was tested by analyzing ordinary fishery products without any interference from the matrix. And no interferences between colistin A and B were observed. The results were calculated as a graph of peak area versus analyte concentration, and the mean recoveries for the analytes were between 72.9% and 82.9%. The quantification ion chromatograms of the analytes in carp sample are shown in Fig. 5.

Table 4
Results for intra-day and inter-day accuracy and precision of colistin A and B in fishery products.

Sample name	Spiked level (μg/kg)	Intra-day accuracy and precision						Inter-day accuracy and precision	
		Assay 1		Assay 2		Assay 3		Mean (%)	RSD (%)[b]
		Mean (%)	RSD (%)[a]	Mean (%)	RSD (%)[a]	Mean (%)	RSD (%)[a]		
Common carp	40.0	75.0	4.70	76.3	3.31	76.8	4.17	76.0	3.97
	200	77.6	3.56	79.0	4.67	78.1	3.64	78.2	3.83
	400	79.6	5.40	77.2	2.48	77.2	2.93	78.0	3.90
Eel	40.0	75.6	5.24	74.6	3.24	76.5	4.52	75.6	4.29
	200	74.9	3.69	78.8	5.60	78.7	8.09	77.5	6.24
	400	79.6	5.21	81.7	7.19	77.7	5.01	79.7	5.95
Turbot	40.0	73.0	4.84	73.6	2.25	77.7	3.23	74.8	4.44
	200	76.0	3.59	79.0	3.08	79.4	4.83	78.1	4.20
	400	80.9	3.86	82.4	9.00	78.8	3.44	80.7	6.00
Penaeus vannamei	40.0	72.9	4.41	74.5	2.22	77.5	3.97	74.9	4.35
	200	75.7	3.71	77.9	4.68	78.9	4.24	77.5	4.35
	400	82.9	2.81	82.1	6.28	80.0	4.07	81.7	4.60
Penaeus monodon Fabricius	40.0	74.6	5.14	76.3	3.94	76.5	5.00	75.8	4.58
	200	78.7	2.17	77.8	3.59	78.9	4.22	78.5	3.28
	400	79.7	2.63	81.4	5.72	78.6	5.43	79.9	4.75
Panulirus stimpsoni holthuis	40.0	74.5	6.34	79.5	3.62	79.2	2.57	77.8	5.12
	200	78.4	5.29	78.3	2.89	80.2	7.03	79.0	5.17
	400	80.2	3.14	81.4	4.83	80.4	4.48	80.6	4.02
Chinese soft-shelled turtle	40.0	76.0	6.23	78.8	7.22	79.9	4.51	78.2	6.10
	200	77.5	3.19	80.2	5.90	79.9	5.67	79.2	5.40
	400	81.2	2.61	82.1	2.87	82.6	3.12	81.9	2.80
Scallop	40.0	73.3	5.63	77.1	6.28	82.0	4.32	77.5	6.97
	200	76.8	5.25	82.1	4.37	81.1	4.08	80.0	5.21
	400	80.5	4.19	80.2	4.82	83.0	4.11	81.2	4.42

[a] RSD represented as repeatability ($n = 6$).
[b] RSD represented as within-laboratory reproducibility ($n = 18$).

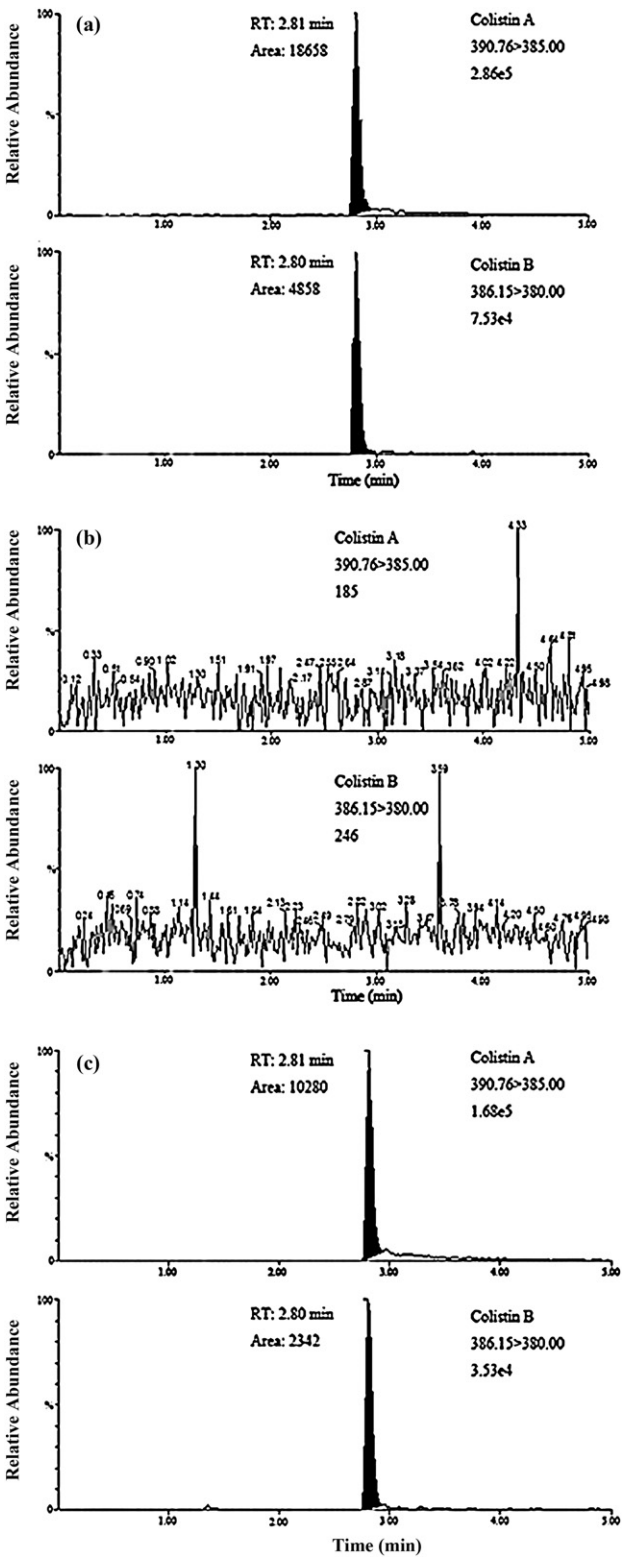

Fig. 5. Quantification ions chromatograms of colistin A and B. (a) Quantification ions chromatogram of standard solution of 2000 ng/mL for colistin A and B; (b) quantification ions chromatogram of negative control carp; (c) quantification ions chromatogram of negative control carp spiked at 200 μg/kg for colistin A and B.

4. Conclusions

A method allowing identification and quantification of colistin A and B in fishery products at μg/kg level has been proposed. Compared to the traditional LC, UPLC has a very short single run time of 5 min per sample, which makes it an attractive procedure in the analysis of the residues and there is no published literature for the determination of colistin A and B in fishery products before. The method can be economical of time, reagent and energy, and no published data have appeared before. It also meets the required sensitivity of the MRPL (200 μg/kg) for colistin in fish.

In short, the proposed method for the determination of colistin A and B in fishery products is reliable (with good response linearity, high recovery and precision, and low detection and quantification levels) and has no interference from the matrix and it is useful for the determination of colistin A and B residues in fishery products.

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