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Preliminary investigation of chloramphenicol in fish, water and sediment from freshwater aquaculture pond

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ABSTRACT: Analytical methods of chloramphenicol in the aquaculture environment have been developed using high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry. The contents of chloramphenicol were determined using high-performance liquid chromatography for sediment and liquid chromatography–tandem mass spectrometry for fish and water collected from a freshwater aquaculture pond in China. Chloramphenicol in the water and sediment were 112.3 ng/L and 0.1957 mg/kg, respectively. The chloramphenicol residues in 3 kinds of fish, including carp, chub and grass carp were different. Only the muscle and head of grass carp were under the minimum required performance limit (0.3 μg/kg) and were safe to eat. The chloramphenicol in other tissues of grass carp, carp and chub exceeded the minimum required performance limit. The highest content of chloramphenicol was in the branchia of carp and the lowest was in the head of grass carp. The results showed the chloramphenicol in the aquaculture environment was serious, although the government of China had banned the use of chloramphenicol in aquaculture a few years ago.

Keywords: *Aquaculture environment; Content analysis; Enrichment; Liquid chromatography–mass spectrometry; High-pressure liquid chromatography; Safe assessment*

INTRODUCTION

In recent years, aquaculture has experienced vigorous development in China. To satisfy demands of food quantity and income generation for aquatic animal products, aquaculture has been undergoing diversification of cultured species and intensification of production systems. Consequently, aquaculture development requires a larger share of natural resources and has a greater environmental impact. But, as aquaculture production intensifies, the feed inputs increase and waste materials, including organic matter, nutrients, suspended solids and antibiotics in ponds increase (Hopkins *et al.*, 1988; Hirsch *et al.*, 1999; Vinodhini and Narayanan, 2008; Wu *et al.*, 2009). The antibiotics used in aquaculture to prevent or control diseases can directly impact the environment when they are improperly used. The concentration of antibiotics in ponds increases, which directly impacts on oxygen depletion and the turbidity in receiving waters (Lorian, 1999; Lalumera *et al.*, 2004; Chakrabarty, 2007; Panjeshahi and Ataei, 2008). One part of antibiotics was deposited to the pond bottom and the others remained in pond water. Chloramphenicol (CAP) is a broad spectrum antibiotic exhibiting activity against both gram-positive and gram-negative bacteria as well as other groups of micro-organisms (Sorensen *et al.*, 2003; Park and Kim, 2006; Shakila *et al.*, 2006). CAP is commonly used in aquaculture as a prophylactic or disinfectant to prevent diseases, or as a chemotherapeutic agent to control diseases. This antibiotic is either spread directly in the aquatic environment or administered through medicated feeds. It may directly expose to the environment by leaching from uneaten feeds or from the aquatic animals' excrement (Nusbaum and Shotts, 1981; Cravedi *et al.*, 1987; Ervik *et al.*, 1994), and also directly enter the environment through pharmaceutical wastewater (Kümmerer and Henninger, 2003). However, CAP has the potential to cause serious toxic effects in humans and animals when it is given by mouth or injection, with non-target effects such as bone marrow depression, aplastic anemia, hypoplastic anemia, thrombocytopenia

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(Robert *et al.*,1979; Anadon *et al.,* 1994), as well as granulocytopenia (Mottier *et al.,* 2003). Antimicrobial residues entering the environment may also establish a selective pressure in favour of antimicrobial-resistant bacteria. The residues of antibiotics in sediment slurries pose a potential risk to public health or may increase the occurrence of antibiotic-resistant bacteria in the aquatic environment (Hatha and Lakshmanaperumalsamy, 1995; Miller, 1998). The use of CAP in farm animals intended for human consumption has been restricted (Munns *et al.*, 1994; Nicolich *et al.*, 2006). CAP has been banned for use in food-producing animals in many countries. Even if CAP was banned, it may still be in use in some developing countries because of its low cost (Mottier *et al.*, 2003; Fergusona *et al.*, 2005; Ye *et al.*, 2008). Although CAP is extensively metabolised by aquatic animals, residues left in the body and direct contamination of the environment may still be a concern. There are several recently published analytical methods for determination of CAP in various food matrixes, such as honey (Chen *et al.*, 2009), milk (Ronning *et al.*, 2006; Rodziewicz and Zawadzka, 2008), equine, porcine (Gantverg *et al.*, 2003), shrimp (Impens *et al.*, 2003), chicken, beef and fish muscle (Takino *et al.*, 2003; Gikas *et al.*, 2004; Santos *et al.*, 2005), and so on, but few reporting on the concentration of CAP in the sediment, water and fish from the same freshwater aquaculture pond. In this study, the contents of CAP in sediment, water and fish from a freshwater aquaculture pond were investigated. This study has been carried out during September, 2006 to June, 2007 in Guangzhou, China.

MATERIALS AND METHODS

Sample collection

Fish, water and sediment samples were collected from a freshwater aquaculture pond in Baiyun District, Guangzhou, China. Sediments were grab-sampled (0 to 3 cm) and immediately transported to the laboratory. They were freeze-dried and then sieved through a stainless wire screen (mesh size: 0.425mm) to remove large debris. The sediment samples were stored in a clean jar until analysis. The moisture content of the samples was determined before analysis and found to be < 2 %. Fish samples included carp, chub and grass carp. Each tissue, including muscle, head, branchia, intestine and scale was separated and minced (0.5cm), then agitated into slurry with whish and stored at -18 ºC in airtight containers until analysis. Water samples were collected randomly from the pond and stored in pre-cleaned, light-preserved bottles.

Treatment and analysis of sediment samples

5g prepared sediment was accurately weighted to a centrifuge tube and then 20 mL extraction solvent (methanol or ethyl acetate) was added to the tube. Ultrasonic extraction was performed for 30 min (or 10 min, 40 min), then centrifuged at 4000 rpm for 10 min and the extraction solvent was decanted into a test tube. Each sample was then extracted a second and third time using 10 mL of methanol extraction solvent for 10 min and was centrifuged at 4000 rpm for 10 min, separately. Following centrifugation the solvents from the second and third extraction step was decanted and combined with the first prior to the clean-up and preconcentration steps. The extraction solvents were concentrated into 2-3 mL by vaporizing with a rotary evaporator in a water bath at 40 ºC. The concentrated solvent was purified through a chromatography column (10 mm × 300 mm, installing: 2cm sodium sulphate anhydrous, 10cm alumina and 2cm sodium sulphate anhydrous). CAP was eluted with methanol (or ethyl acetate). The CAP eluates were evaporated to dryness under a nitrogen stream, redissolved in methanol to a predetermined volume and analysed by high-performance liquid chromatography (HPLC). HPLC-analysis were performed with an Agilent 1100 series. HPLC system was equipped with a quaternary pump on a Hpersil ODS-18 column (250 mm×4.6 mm inside diameter (i.d.), particle size 5μm), and a column thermostat with a column selection valve. The mobile phase consisted of a mixture of acetonitrile and water (60:40, v/v) or methanol and water (70:30, v/v) with a flow rate of 1.0 mL/min. The injection volume was 5μL, 10 μL or 20 μL and the column temperature was 30 ºC. CAP was detected by its UV absorbance at the wavelength 275 nm and quantified by comparison with standards.

Treatment and analysis of water sample

CAP extraction of water samples was performed using a 1000 mL pear-shaped funnel. Ethyl acetate was used as an extraction solvent. 80 mL ethyl acetate was added to the 500 mL water samples and surged 1 min, then allowed to stand to separate into two layers. The ethyl acetate extractions were collected to the rotary evaporator. Using 30 mL ethyl acetate separately, the sample was twice extracted as above, in duplicate. Three extraction solvents were combined and dehydrated with sodium sulphate anhydrous, and then evaporated to almost dryness with a rotary evaporator in a water bath at 40 ºC and redissolved in methanol to a predetermined volume. The methanol solvent was filtered through a 0.22 μm filter membrane and analysed by liquid chromatography–tandem mass spectrometry (LC-MS/

MS). The HPLC equipment consisted of an alliance pump and autosampler (Agilent 1100 series) and HCT plus Bruker mass selected detector (Bruker Daltonics Inc., Germany). The separation was performed on a Hpersil ODS-18 column (150 mm × 4.6 mm i.d., particle size 5µm.). The column oven temperature was set at 30 ºC. The mobile phase consisted of a mixture of methanol and water (70:30, v/v). The flow rate was 0.6 mL/min. The injection volume was10 µL. Operation of the mass spectrometer, fitted with an electro-spray ionisation (ESI) probe, was in negative mode. The following interface parameters were used: 4000 V capillary voltage of ion source, 300,000 µs accumulation time, 7 L/min dry gas N_2, 300 ºC dry temperature, 25V cone voltage, 0.9V extractor voltage. Mass spectra were recorded at a scan range of 50-400 *m/z*. Fig. 1 shows the LC-ESI(-) –MS/MS spectra of CAP. The MS method was a single ion recording (SIR) of four masses (*m/z* 321 , m/z 257, m/z 194 and m/z 152). A typical fragmentation of CAP is shown in Fig. 2.

Extraction and analysis of fish tissue samples

Fish tissue samples were weighed into a 50-mL polytetrafluoroethylene centrifuge tube and added basified ethyl acetate extraction solvent (ethyl acetate: 25 % ammonia=97:3, v/v). Ultrasonic extraction was performed for 20 min, then centrifuged at 4000 rpm for 10 min and the ethyl acetate extraction solvent was decanted into a 50 mL test tube. The remaining sample precipitate was extracted and centrifuged with an additional basified ethyl acetate. Two extraction solvents were combined and evaporated to almost dryness, redissolved in methanol to dissolve the residue. 4% sodium chloride solution was used for a second ultrasonic dissolution, then twice extracted with hexane. The hexane fraction of the centrifuged samples was discarded. The underlying extraction solvent was collected for the wash step. A C18 SPE column was equilibrated with methanol and water using the vacuum pump at minimum pressure. After passing the entire sample through the column it was rinsed with water and methanol: water (30: 70, v/v). The sample was dried at high vacuum pressure, CAP was eluted with methanol: water (60: 40, v/v) and the eluate was collected to a tube. Eluate was redissolved with methanol: water (60: 40, v/v) to a predetermined volume, then filtered through a 0.45µm filter membrane and analysed by LC-MS/MS. The analysis equipment and operation conditions were same as above for water samples.

Reagents and standards

Chloramphenicol was obtained from Sigma Chemical Company (USA, purity 99.9 %). High-pressure liquid chromatography (HPLC) solvents (acetonitrile and methanol) were HPLC grade. All other reagents and solvents were analytical reagent grade. Deionized and bi-distilled water was obtained from a Milli-Q water purification apparatus. SPE columns (Supelco C_{18}, 3 mL cartridge) were purchased from Supelco.

A CAP standard stock solution of 2 mg/mL was prepared by dissolving 200 mg of CAP in 100 mL of methanol. The intermediate standard solutions of 400 µg/mL, 50 µg/mL , 2 µg/mL were prepared by diluting with methanol from the solution and stored at 4 ºC. Suitable working solutions were also prepared as standards for various calibration cures.

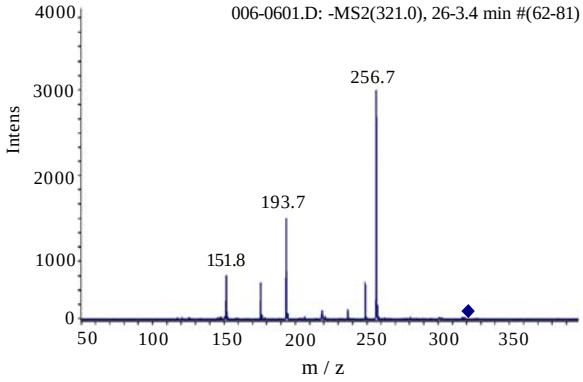

Fig. 1: The pick-up ion chromatograms of CAP (m/z=321)

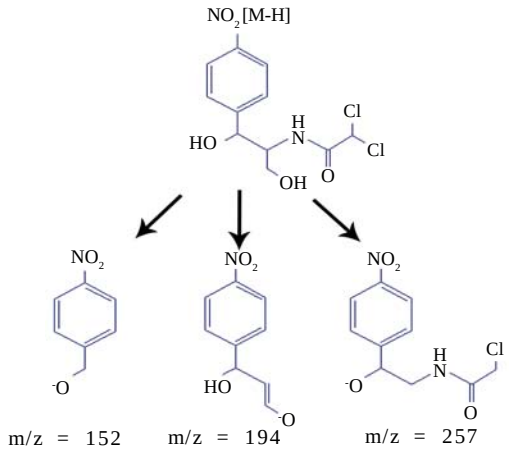

Fig. 2: Typical fragmentation pattern recoded for CAP

Working standard solutions for HPLC analysis of sediment samples were prepared with concentrations of 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 5.00, 7.50 mg/L by diluting suitable intermediate standard solutions in methanol. Working standard solutions for water sample analysis in LC-MS/MS were prepared with concentrations range of 2-400 µg/L in methanol. Intermediate standard solutions were prepared with concentrations of 0.20, 1.00, 2.00, 4.00, 10.0, 20.0 µg/L in methanol, and then diluted with blank fish distilling solution to 0.10, 0.50, 1.00, 2.00, 5.00, 10.00 µg/L in order to eliminating the effect of the fish component for fish sample analysis.

RESULTS AND DISCUSSION

Analysis of sediment samples

Optimization of the analysis method was carried out during the initial stage of research. Extraction solvents, extraction time, clean-up solvents and analytical conditions of HPLC, including mobile phases and injection volumes were studied. Recoveries and chromatograms were compared. It was found that the optimal conditions for obtaining CAP from pond sediment were: ultrasonic extraction over 30 min and 10 min twice with methanol, cleaned up with methanol using solid column, detected by HPLC at 275 nm, with acetonitrile: water = 60: 40 (v/v) as the mobile phase at a flow rate of 1.0 mL/min, 10 µL injection and 30 ºC column temperature.

For the recovery study, blank and spiked sediment samples previously analyzed to confirm the absence of CAP were used. Blank sediment samples were spiked three concentrations: 0.1 mg/kg, 1.0 mg/kg and 4.0 mg/kg. The recoveries for different initial concentration of CAP are shown in Table 1. Acceptable results were obtained and the average recoveries were greater than 88 % with % rsd ranging between 2.6 % and 4.4 % in all the levels tested. The limit of detection is considered to be the quantity yielding a detector response approximately equal to thrice the background noise (Inczedy *et al.*, 1997). Thus, the minimum detectable quantity was found to be 7µg/kg.

Replicate injections (two) of spiked at concentrations of 1.00 mg/kg and 4.00 mg/kg were used for evaluation of the precision in fish pond sediment. The results of the recoveries for different initial concentrations of CAP are shown in Table 2. Acceptable results were obtained and the average recoveries were greater than 90 %, with % rsd ranging between 2.2 % and 4.3% in the two levels tested.

Using the optimal extraction method and analysis conditions to determinate the fish pond sediments, we obtained the results in Table 3. The concentration of CAP was 0.1957 mg/kg. Although CAP had not been directly sent to the pond recently, it probably entered the pond through excrement from the nearby piggery. CAP was probably still present, in equilibrium, from previous use in aquaculture.

Analysis of water sample

Optimization of the present method was carried out during the initial stages of research. Water samples were spiked at three concentrations: 0.10 µg/L, 1.00 µg/L and 10.00 µg/L. LC-MS/MS was used to analyse the CAP in water. The results of the recoveries and precision for different spiked water samples of CAP are shown in Table 4.

Acceptable results were obtained and the average recoveries were from 96 % to 99 %, with % rsd less than 6.3 % in all the levels tested. The method of analysis for the pond water samples was reliable. The mean concentration of CAP in the water sample was 112.3 ng/L (shown in Table 5). Because there was some stable CAP in the pond sediments, CAP would dissolve into

Table 1: Method facticity and precision of blank sediment for different initial concentration of CAP

Spiked level (mg/kg)	Repeatability		
	Mean recovery (%)	RSD (%)	Replicates
0.10	92.0	4.3	4
1.00	88.6	2.6	4
4.00	92.0	4.4	4

Table 2: Recovery and precision of CAP in fish pond sediment samples (n=5)

Spiked concentration (mg/kg)	Average mensurated concentration (mg/kg)	Average recovery (%)	RSD (%)
1.00	1.077	90.5	2.2
4.00	3.840	91.7	4.3

the water from the sediment clue to inflow of water, even if CAP had not to been sent to the pond recently.

Analysis of fish tissues

Optimization of the present method was carried out during the initial stages of research. Muscle tissue from carp samples were spiked at concentrations: 0.25 µg/kg or 2.50 µg/kg. The other tissue samples were spiked at concentrations: 0.50 µg/kg or 1.00 µg/kg. LC-MS/MS was used to analyse the CAP in fish. The method facticity and precision for different spiked fish musscle samples of CAP are shown in Table 6 and Table 7. The mean recoveries ranged between 86.5% and 102.1%, with reproducibility relative standard deviation from 3.4 % to 6.5 %. The reproducibility was calculated by using the extraction and analysis method.

CAP concentration in different fish

In order to investigate the CAP residue in different fish in the same pond, we found that there are three kinds of fish in the same pond including carp, chub and grass carp. The results were listed in Fig. 3, Fig. 4 and Fig. 5. These results show that the concentrations of CAP accumulated in different families and different tissues varied. In all different tissues, the highest concentrations of CAP was in the branchia of carp (1.51µg/kg) and the lowest was in the head of grass carp (0.21µg/kg). In carp and club, the lowest concentration was in the muscle, but in grass carp, it

Table 3: CAP in fish pond sediments (n=5)

No.	1	2	3	4	5	Averaged
CAP concentration (mg/kg)	0.1862	0.1905	0.1989	0.2025	0.2003	0.1957

Table 4: Method facticity and precision for different initial concentration of CAP in water samples

Spiked level (µg /L)	Repeatability		
	Mean recovery (%)	RSD (%)	Replicates
0.10	97.6	6.3	4
1.00	99.2	5.5	6
10.00	96.2	6.2	4

Table 5: CAP in water samples (n=6)

No.	1	2	3	4	5	6	Averaged	RSD (%)
CAP concentration (ng/L)	112	109	117	105	121	110	112.3	5.1

Table 6: Method facticity and precision for different initial concentration of CAP in carp muscle samples

Spiked level (µg/kg)	Repeatability		
	Mean recovery (%)	RSD (%)	Replicates
0.25	93.0	6.5	4
2.50	88.7	3.4	5

Table 7: Method facticity and precision for different initial concentration of CAP to different tissues in carp (n=4)

Tissue	Spiked level (µg/kg)	Mean recovery (%)	RSD (%)
Head	0.50	86.5	5.9
	1.00	88.2	4.8
Branchia	0.50	90.3	5.4
	1.00	96.5	4.7
Intestine	0.50	102.1	4.5
	1.00	98.2	3.8
Scale	0.50	87.5	4.0
	1.00	91.7	5.3

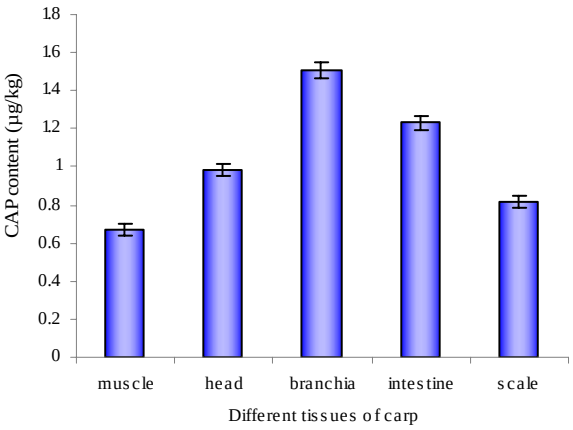

Fig. 3 CAP content in different tissues of carp

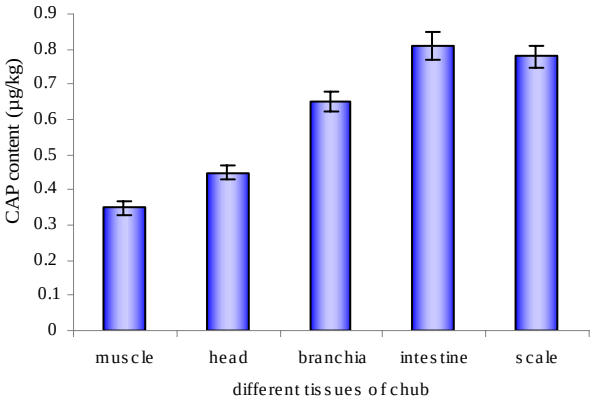

Fig. 4: CAP content in different tissues of chub

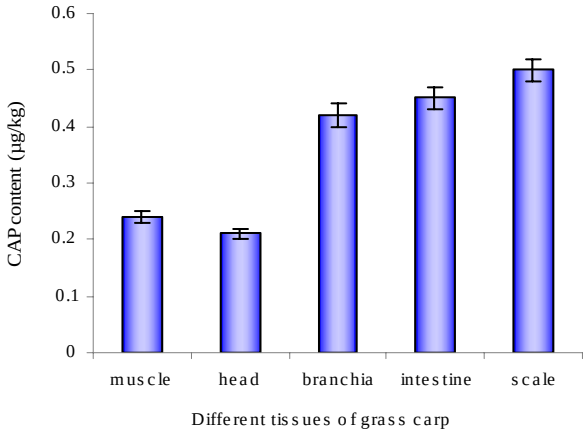

Fig. 5: CAP content in different tissues of grass carp

was in the head. Maybe because the head of grass carp has many unsaturated fatty acid and its ability of enriching CAP is little (Huang *et al.*, 2008). The average concentrations of CAP in each fish was: carp> chub> grass carp.

The minimum required performance limit (MRPL) for CAP determination is 0.3 µg/kg in all food of animal origin (Commission Decision, 2003). To all results of fish , only the concentrations of CAP in the muscle (0.24µg/kg) and head (0.21µg/kg) of grass carp was under the MRPL standard. The others exceeded the MRPL standard. So only the two tissues of grass carp were safe for eating. But all exceeded the least measure limit of 0.1µg/kg set by the European Union (EU) (Gantverg *et al.*, 2003).

Different families and tissues were accumulated various concentrations of CAP. It was likely to be related to the feeding habitat of each species. Carp, which had the biggest content of CAP, likes to live in the bottom of pond and pick up the sludge at the bottom. It feeds with sediments and aquatic organism long-term. As we know that the content of CAP in sediments was higher than in water, so carp absorbed and accumulated more CAP through its food chain than the other fish. Chub lives in the middle and higher levels of pond. It feeds on plankton and the excrements of grass carp, chickens and cattle. Therefore, chub possibly absorbed and accumulated CAP through the food chain. Grass carp likes to live in the middle and top levels of ponds, which have the least content of CAP. It feeds on grass, which has little accumulation of CAP.

CONCLUSION

Satisfactory recoveries of CAP were demonstrated for the developed methods. They demonstrated that the treatments and analyses of samples were attractive, affordable and effective. Analytical methods for the determination of CAP in water, sediment and fish samples have been developed. The results obtained indicate that there was CAP in the water, sediments and fish of the pond. The concentration of CAP in the water and sediment was 112.3 ng/L and 0.1957 mg/kg, respectively. According to the species, three kinds of fish live in the pond and the contents of CAP in the fish differed. Only the CAP in the muscle and head of grass carp was under the MRPL (0.3µg/kg), the other tissues of grass carp and the other fish including carp and chub had CAP concentrations which exceeded the

MRPL. These results also demonstrate that CAP continues to exist in the aquatic environment even though the government of China had banned the use of CAP in aquaculture a few years ago.

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