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# High-performance liquid chromatographic determination for bile components in fish, chicken and duck

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**Abstract**

A HPLC procedure for the determination of 13 bile acids and cyprinol sulfate in animals was developed. The mobile system 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v) 10 min→(68:32) 10 min→(50:50) 10 min was available for separating all 14 bile components, except for deoxycholic and glycodeoxycholic acids, which could be further separated with 0.3% ammonium carbonate solution–acetonitrile (73:27). After applying this method, grass carp and common carp bile was found to contain mainly cyprinol sulfate, while the other 12 fish species bile contained mainly taurocholic, taurochenodeoxycholic and cholic acids. Chicken bile was mainly composed of glycolithocholic and taurocholic acids, but duck bile was mainly composed of taurochenodeoxycholic, cholic and ursodeoxycholic acids. 

*Keywords:* Bile acids; Cyprinol sulfate

## 1. Introduction

Some poisoning cases occurred in Asian countries due to ingesting animal bile because some people believe that ingestion of animal bile, such as grass carp, common carp, snake and chicken, may improve their visual acuity [1–11]. The bile of grass carp and common carp induced most cases of these incidents. The toxic substances of animal bile were considered as some special bile alcohols or acids [11–13]. Among these animal biles, their toxic effects on rats were quite different from each other [3,11]. Furthermore, the toxic effects of animal bile on rats and victims included renal failure, hemolysis, liver dysfunction and cardiovascular and gastrointestinal impairment [14–16]. It indicated that the toxic components of animal bile might be different from each other and might be composed of multiple substances.

Bile acids have been the subject of clinical research for many years, especially in the last 3 decades, when it was found that some of them could be used for dissolving gallstones [22]. Chenodeoxycholic [21] and ursodeoxycholic acids [23] were proven to be the most suitable for this purpose. Both these bile acids are now used as important medicaments in human medicine. Further, the bile acids have been studied in the bile of terrestrial animals [19,20]. It was found that the pattern of bile acids, including chenodeoxycholic, ursodeoxycholic,

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cholic, deoxycholic and lithocholic acids, in animal bile was quite different depending on species and nutrition type. On the other hand, the food poisoning cases due to ingestion of animal bile such as grass carp and common carp bile have occasionally occurred in Asian countries. Therefore, the bile acids and cyprinol sulfate in common carp were also investigated [18], and cyprinol sulfate was suggested as the causative agent of common carp bile [17,18].

Until now, the high-performance liquid chromatographic (HPLC) determination of bile acids concerning humans such as cholic, chenodeoxycholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic acids have been developed [24,25]. However, there are different multiple components in different animal biles. To elucidate the different profiles of bile components in different animal biles, the aim of this study was first to set up a mobile system with HPLC to determine 14 bile components including cyprinol sulfate and bile acids. Then we identified the bile acid components in the bile juices of some animals.

## 2. Materials and methods

### 2.1. Agents

Standard bile acids, including cholic, chenodeoxycholic, deoxycholic, lithocholic, ursodeoxycholic, glycocholic, glycochenodeoxycholic, glycodeoxycholic, glycolithocholic, taurocholic, taurochenodeoxycholic, taurodeoxycholic and taurolithocholic acids, were obtained from Sigma (St. Louis, MO, USA). Methanol (LC grade) and other reagents were from Merck (Darmstadt, Germany). Crystallized 5α-cyprinol sulfate was isolated from the bile of common carp according to the method of Mohri et al. [18] and identified by $^{1}$H- and $^{13}$C-nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GC–MS) in this laboratory.

### 2.2. Materials

The numbers of animal bile tested were as follows: grass carp *Ctenopharyngodon idellus*, 10; common carp *Cyprinus carpio*, 10; rainbow fish *Bodianus oxycephalus*, 3; rock trout *Salmo salar*, 2; Japanese eel *Anguilla japonica*, 6; Spanish mackerel *Aixos maru*, 3; right eyed flounder *Pleuronectes cornutus*, 2; rudder fish *Girella punctata*, 4; black scraper *Thamnaconus modestus*, 3; tongue sole *Paraplagusia bilineata*, 4; Japanese jack mackerel *Trachurus japonicus*, 3; mackerel *Scomberomorus commerson*, 3; mullet *Liza macrolepis*, 5; ayu fish *Plecoglossus altirelis*, 5; chicken *Pectoralis major*, 6; and duck *Anas platyhynchos* var. *domestica*, 3. These samples were freshly collected from the markets. When the market vendor slaughtered these animals, we immediately collected the gall bladder and transferred them to the laboratory with ice. The gall bladder was then stuck, and the bile juice was collected into a sample bottle and stored at −20°C until assayed.

### 2.3. Preparation of tested solution

The sample (0.5–1 ml) was extracted three times with 10 ml of methanol. After evaporation of solvent the residue was dissolved in 90% ethanol (5 ml) and the solution was applied to a PHP-LH 20 column (5×2 cm) (Amersham Pharmacia, Sweden). Elution was carried out at a flow-rate of 20 ml/h. The column was first rinsed with 90% ethanol (100 ml). Then the eluting procedure was stepwise performed with 0.1 *M* acetic acid in 90% ethanol (100 ml), 0.2 *M* formic acid in 90% ethanol, and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (100%) to obtain unconjugated, glycine-conjugated and taurine-conjugated bile components, respectively [23]. Each fraction was evaporated under vacuum and the residue was submitted to HPLC as described below.

### 2.4. Recovery test for bile components added to fish and chicken bile

The tested samples were prepared by dissolving approx. 200 or 1000 μg of each bile component in ayu fish and chicken bile (0.5 ml). A bile specimen was extracted with methanol, applied to the PHP-LH 20 column and then determined using HPLC as described below.

### 2.5. Instrumentation

The bile components were determined by using a Hitachi liquid chromatograph (Tokyo, Japan) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector set at 210 nm, and a Model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 25×0.3 cm I.D., E. Merck) was used for separation.

### 2.6. Chromatographic conditions

The mixed solvent of 0.3% ammonium carbonate solution–acetonitrile was used as mobile phase. The system of mobile phase was as follows: the ratio of both solutions from 73:27 for 10 min, 68:32 for 10 min, and then to 50:50 for 10 min. Furthermore, only 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v) was used as mobile phase to determine the level of deoxycholic and glycodeoxycholic acids. The flow-rate was 0.8 ml/min.

### 2.7. Standard curves

Standard curves of 14 bile components were separately prepared in the range of 2.5–32 μg and peak area ($y$) vs. amount of bile component ($x$) was plotted. Data for standard curves were subjected to linear regression analysis. The correlation coefficients ($r$) and linear regression coefficients for each bile component were as follows: ursodeoxycholic acid, $y=0.08535x+0.04948$ ($r^2=0.99594$); cholic acid, $y=0.08752x-0.03372$ ($r^2=0.99450$); chenodeoxycholic acid, $y=0.11113x-0.43268$ ($r^2=0.99848$); deoxycholic acid, $y=0.08681x+0.08770$ ($r^2=0.99554$); lithocholic acid, $y=0.09952x-0.20032$ ($r^2=0.99547$); glycocholic acid, $y=1.17443x-1.15603$ ($r^2=0.99367$); glycochenodeoxycholic acid, $y=0.78870x-0.73754$ ($r^2=0.99319$); glycodeoxycholic acid, $y=1.02115x-0.87664$ ($r^2=0.99434$); glycolithocholic acid, $y=1.15806x-0.5152$ ($r^2=0.99471$); taurocholic acid, $y=0.62856x-0.7696$ ($r^2=0.99560$); taurochenodeoxycholic acid, $y=0.59444x-0.84724$ ($r^2=0.99509$); taurodeoxycholic acid, $y=0.76263x-01.01000$ ($r^2=0.99451$); taurolithocholic acid, $y=0.64853x-0.53172$ ($r^2=0.99250$); cyprinol sulfate, $y=39.1271x-0.26439$ ($r^2=0.99920$). The correlation coefficient in every curve was better than 0.99. This indicated a definite linear relationship between bile component concentration and detector response.

## 3. Results and discussion

### 3.1. HPLC separation and recovery of bile components

A typical chromatographic profile of the 13 standard bile acids and cyprinol sulfate by the step-by-step elution system was developed (Fig. 1). Except for deoxycholic and glycodeoxycholic acids, the other bile components were well separated in a 30-min total run time with gook peak resolution, sharpness and symmetry. Furthermore, the deoxycholic and glycodeoxycholic acids could be separated by using another mobile phase of 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v) in a 35-min run time (Fig. 2). Thus, the step-by-step system of mobile phase of 0.3% ammonium carbonate solution–acetonitrile along with a single mobile phase was useful for determining the bile acids and cyprinol sulfate in this study. Other buffer compositions were found unacceptable for simultaneously separating 14 bile components. The recoveries of these 14 bile components from ayu fish and chicken bile were higher than 90%, except for lithocholic, taurodeoxycholic, taurochenodeoxycholic and glycolithocholic acids (Table 1). Because the least recovery of bile components was higher than 80%, it indicated that the extraction and purification procedure was available for extracting the bile acids and cyprinol sulfate. These results were similar to those reported by Shaw et al. [24] and Goto et al. [25].

### 3.2. Bile components in fish

A typical HPLC profile of bile acids and cyprinol sulfate in grass carp bile is shown in Fig. 3. The levels of bile components in fish are shown in Table 2. The range of bile components was as follows: cyprinol sulfate 52.1–77.4 (64.5±8.1, mean±SD) mg/g, chenodeoxycholic acid 0.4–2.5 (1.7±0.7) mg/g, taurochenodeoxycholic acid 0.5–0.8

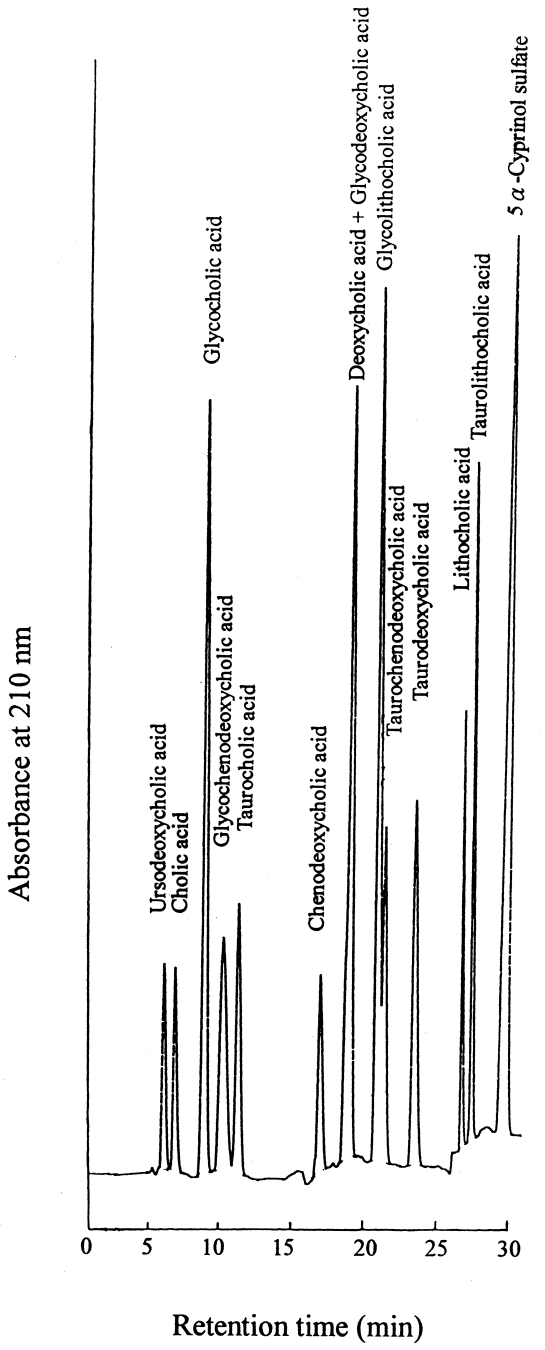


Fig. 1. HPLC profile of authentic bile acids and cyprinol sulfate. Column: LiChrospher 100 RP-18 column (25×0.3 cm), flow-rate: 0.8 ml/min, detection: UV at 210 nm, buffer system: 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v) 10 min→(68:32, v/v) 10 min→(50:50, v/v) 10 min.

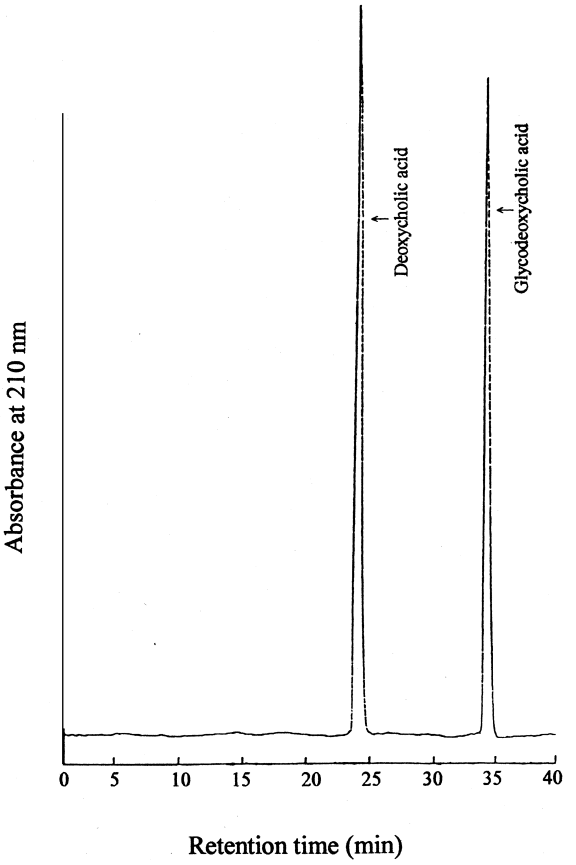


Fig. 2. HPLC profile of authentic deoxycholic and glycodeoxycholic acids. Column: LiChrospher 100 RP-18 column (25×0.3 cm), flow-rate: 0.8 ml/min, detection: UV at 210 nm, buffer system: 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v).

(0.7±0.1) mg/g, taurocholic acid 0.4–0.6 (0.5±0.1) mg/g, cholic acid 0.3–0.8 (0.5±0.1) mg/g and lithocholic acid 0.3–0.5 (0.4±0.1) mg/g in grass carp bile; and cyprinol sulfate 97.6–142.1 (123.8±15.1) mg/g, chenodeoxycholic acid 2.8–4.4 (3.7±0.6) mg/g, cholic acid 2.4–3.7 (3.1±0.5) mg/g, and lithocholic acid 0.3–0.5 (0.4±0.1) mg/g in common carp bile. It was found that the main bile component in grass carp and common carp was cyprinol sulfate with a value of more than 94% of total bile components.

As shown in Table 2, it was found that the other fish bile was composed of few bile components. The amount of taurocholic acid was more than 60% of the total amount of bile components in some fish,

Table 1
Recovery of bile acids and cyprinol sulfate added to the bile of chicken and ayu fish

| Bile component | Recovery (%) | | |
|---|---|---|---|
| | Chicken | Ayu fish | Average |
| Cholic acid | 94±2.8[a] | 96±2.3[a] | 95.0±1.0 |
| Deoxycholic acid | 97±2.5 | 99±2.3 | 98.0±1.0 |
| Lithocholic acid | 82±2.1 | 85±2.6 | 83.5±1.5 |
| Chenodeoxycholic acid | 92±2.3 | 90±1.8 | 91.0±1.0 |
| Ursodeoxycholic acid | 100±2.0 | 96±2.6 | 98.0±2.0 |
| Taurocholic acid | 95±3.1 | 97±2.9 | 96.0±0.5 |
| Taurolithocholic acid | 99±3.0 | 96±2.8 | 97.5±1.5 |
| Taurodeoxycholic acid | 86±2.4 | 83±2.7 | 84.5±1.5 |
| Taurochenodeoxycholic acid | 88±2.1 | 90±2.3 | 89.0±0.5 |
| Glycocholic acid | 96±4.3 | 92±3.1 | 94.0±2.0 |
| Glycolithocholic | 84±2.6 | 87±2.4 | 85.5±1.5 |
| Glycodeoxycholic acid | 89±2.4 | 91±3.1 | 90.0±0.5 |
| Glycochenodeoxycholic acid | 93±2.5 | 95±2.1 | 94.0±1.0 |
| Cyprinol sulfate | 96±3.4 | 94±2.6 | 95.0±1.0 |

[a] Data represent mean±SD ($n$=3).

including rainbow fish *B. oxycephalus*, rock trout *S. salar,* Japanese eel *A. japonica,* Spanish mackerel *Aix. maru*, right eyed flounder *P. cornutus*, rudder fish *G. punctata*, black scraper *T. modestus* and tongue sole *Para. bilineata*, Japanese jack mackerel *Tr. japonicus* and mackerel *Sc. commerson* contained taurocholic and taurochenodeoxycholic acids as two major components. Mullet *L. macrolepis* contained taurochenodeoxycholic acid only. Ayu fish *Pl. altirelis* contained cholic acid as the major component, followed by with taurocholic acid. However, it indicated that the bile acids in fish had several different patterns. The major bile acid in fish bile was the first metabolite of cholesterol such as the conjugate of cholic acid with taurine. Other bile acids, not shown in Table 2, were not detected in these fish biles.

In this study, cyprinol sulfate was detected in the bile of grass carp and common carp only. The average level of cyprinol sulfate was 64.5 and 123.8 mg/g in the bile of grass carp and common carp, respectively. The amount of cyprinol sulfate was more than 90% of the total bile acids and cyprinol sulfate in the bile of grass carp and common carp. Other bile acids were very low (less than 3%). This result was the same as that reported by Mohri et al. [18]. Because cyprinol sulfate was suggested as the causative agent of common carp bile [17,18], it would be reasonable to suppose that cyprinol sulfate might be the causative agent of grass carp bile. The composition of bile acids in other fish was quite different depending on species. The first metabolites of cholesterol including cholic, taurocholic and

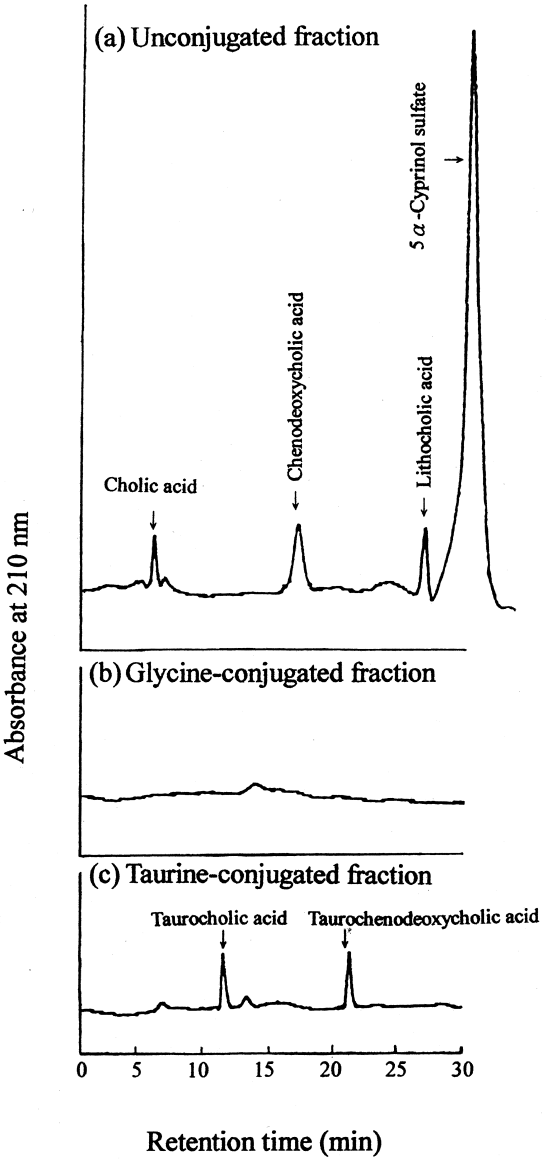


Fig. 3. A typical HPLC profile of bile acids in three fractions obtained from grass carp bile by PHP-LH-20 column chromatography separated with (a) 0.1 *M* acetic acid in 90% ethanol (unconjugated fraction), (b) 0.2 *M* formic acid in 90% ethanol (glycine-conjugated fraction), and (c) 0.3 *M* acetic acid–potassium acetate in 90% ethanol (taurine-conjugated fraction).

Table 2
Levels of bile acids and cyprinol sulfate in the bile of fish

| Sample | Sample no. | Cyprinol sulfate (mg/g) | Taurochenodeoxycholic acid (mg/g) | Taurocholic acid (mg/g) | Cholic acid (mg/g) | Chenodeoxycholic acid (mg/g) | Lithocholic acid (mg/g) |
|---|---|---|---|---|---|---|---|
| *Bodianus oxycephalus* (rainbow fish) | 3 | n.d.[a] | 0.4±0.1[b] (3.8)[c] | 133.3±4.2 (96.2) | n.d. | n.d. | n.d. |
| *Salmo salar* (rock trout) | 2 | n.d. | 4.7±1.2 (9.3) | 87.9±2.3 (90.7) | n.d. | n.d. | n.d. |
| *Anguilla japonica* (Japanese eel) | 6 | n.d. | 6.9±2.1 (10.5) | 77.4±3.0 (89.5) | n.d. | n.d. | n.d. |
| *Aixos maru* (Spanish mackerel) | 3 | n.d. | 25.1±1.6 (17.9) | 78.5±4.2 (82.1) | n.d. | n.d. | n.d. |
| *Pleuronectes cornutus* (right eyed flounder) | 2 | n.d. | 33.2±2.5 (23.4) | 96.5±3.7 (76.6) | n.d. | n.d. | n.d. |
| *Girella punctata* (rudder fish) | 4 | n.d. | 29.5±1.6 (20.7) | 108.6±4.6 (79.3) | n.d. | n.d. | n.d. |
| *Thamnaconus modestus* (black scraper) | 3 | n.d. | 26.7±2.4 (23.4) | 97.6±4.6 (76.6) | n.d. | n.d. | n.d. |
| *Paraplagusia bilineata* (tongue sole) | 4 | n.d. | 28.4±2.6 (32.3) | 68.5±2.5 (67.7) | n.d. | n.d. | n.d. |
| *Trachurus japonicus* (Japanese jack mackerel) | 3 | n.d. | 55.9±3.1 (42.6) | 75.4±5.4 (57.4) | n.d. | n.d. | n.d. |
| *Scomberomorus commerson* (mackerel) | 3 | n.d. | 66.3±2.9 (47.6) | 78.6±2.4 (52.4) | n.d. | n.d. | n.d. |
| *Liza macrolepis* (mullet) | 5 | n.d. | 154±5.1 (100) | n.d. | n.d. | n.d. | n.d. |
| *Plecoglossus altirelis* (ayu fish) | 2 | n.d. | n.d. | 34.4±3.4 (36.6) | 89.5±5.1 (63.4) | n.d. | n.d. |
| *Ctenopharyngodon idellus* (grass carp) | 10 | 64.5±8.1 (94.6) | 0.7±0.1 (1.0) | 0.5±0.1 (0.7) | 0.5±0.1 (0.7) | 1.7±0.7 (2.4) | 0.4±0.1 (0.6) |
| *Cyprinus carpio* (common carp) | 10 | 123.8±15.1 (94.5) | n.d. | n.d. | 3.1±0.5 (2.4) | 3.7±0.6 (2.8) | 0.4±0.1 (0.3) |

[a] Not detected.
[b] Data represent mean±SD.
[c] Data in parentheses represent percentage.

taurochenodeoxycholic acids were the major components. Among them, taurocholic acid was the major component of bile acids in some fish including rainbow fish *B. oxycephalus,* rock trout *S. salar,* Japanese eel *A. japonica,* Spanish mackerel *Aix. maru,* right eyed flounder *P. cornutus,* rudder fish *F. punctata*, black scraper *T. modestus* and tongue sole *Para. bilineata*. Taurocholic and taurochenodeoxycholic acids were the two major bile acids for jack mackerel *Tr. japonicus* and mackerel *Sc. commerson.* Taurochenodeoxycholic acid was the major bile acid for mullet *L. macrolepis*. Cholic acid was the major bile acid for ayu fish *Pl. altirelis*. These results were similar to those reported by Goto et al. [26].

### *3.3. Bile components in duck and chicken*

The levels of bile acids and cyprinol sulfate in the bile of duck and chicken are shown in Table 3. It was found that the bile of duck and chicken was

Table 3
Levels of bile acids in the bile of chicken and duck[a]

| Bile acid | Chicken | | Duck | |
|---|---|---|---|---|
| | Concentration (mg/g) | % | Concentration (mg/g) | % |
| Cholic acid | 9.6±0.5 | 1.2±0.1[b] | 45.2±2.3 | 17.5±0.5 |
| Chenodeoxycholic acid | 25.2±2.2 | 3.1±0.1 | 28.2±1.6 | 9.7±0.3 |
| Ursodeoxycholic acid | n.d. | n.d.[c] | 43.5±2.1 | 15.5±0.4 |
| Deoxycholic acid | n.d. | n.d. | 31.6±1.9 | 8.4±0.1 |
| Lithocholic acid | 68.7±2.1 | 6.1±0.2 | 37.5±2.1 | 9.0±0.2 |
| Taurocholic acid | 152.6±3.1 | 23.9±1.6 | 16.8±1.5 | 2.4±0.1 |
| Taurochenodeoxycholic acid | n.d. | n.d. | 97.5±3.4 | 37.9±0.7 |
| Taurolithocholic acid | 35.9±0.6 | 3.2±0.7 | n.d. | n.d. |
| Glycolithocholic acid | 228.4±1.6 | 62.5±2.1 | n.d. | n.d. |

[a] Mean of triplicate.
[b] Data represent mean±SD.
[c] Not detected.

inclusive of multiple bile acids. The bile of duck contained mainly taurochenodeoxycholic acid, followed by cholic, ursodeoxycholic, chenodeoxycholic, lithochilic, deoxycholic and taurocholic acids. Other bile acids, not shown in Table 3, were also not detected in duck and chicken bile. The bile of chicken contains mainly glycolithocholic acid, followed by taurocholic, lithocholic, taurolithocholic, chenodeoxycholic and cholic acids. It indicated that the bile acids in terrestrial animals were also different depending on species. The major bile acid in duck was the first metabolite of cholesterol such as chenodeoxycholic acid and its conjugate with taurine, but that in chicken was the secondary metabolite of cholesterol such as lithocholic acid and its conjugate with glycine.

Compared to fish bile components, the bile acids of duck and chicken bile were more multiple. The first metabolite of cholesterol including chenodeoxycholic acid, cholic acid and their conjugates were the major bile acids for duck bile. But the secondary metabolite of cholesterol glycolithocholic acid was the major bile acid for chicken bile. The diversity of bile acids in these animal biles was the same as that reported by Haslewood [19] and Jirsa et al. [20]. The composition of bile acids in chicken bile was very unique in this study because it contained mainly the secondary metabolite of cholesterol glycolithocholic acid. However, chenodeoxycholic acid was well known as the major bile acid of chicken bile [20]. Further, lithocholic acid is transformed from chenodeoxycholic acid and deoxycholic acid by intestinal bacteria. It indicated that the culture conditions would affect the composition of bile acids in the bile of chicken. Further study should be made on the effect of environmental and nutritional factors on the composition of bile acids in the bile of animals. Meanwhile, the order of amount of total bile acids and cyprinol sulfate in animal bile was as follows: chicken, duck and fish.

## 4. Conclusions

A step-by-step HPLC system with a mobile phase of 0.3% ammonium carbonate solution–acetonitrile (73:27, v/v) 10 min→(68:32, v/v) 10 min→(50:50, v/v) 10 min along with a single mobile phase was useful for determining 13 bile acids and cyprinol sulfate. After HPLC analysis, grass carp and common carp bile contained mainly cyprinol sulfate, while the other 12 fish species, duck and chicken bile contained first and secondary metabolites of cholesterol, such as cholic, chenodeoxycholic, lithocholic acids and their salts. The profile was quite different depending on the animal species studied.

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