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Short communication

# Simultaneous determination of thiamphenicol, florfenicol and florfenicol amine in eggs by reversed-phase high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A specific, sensitive and widely applicable reversed-phase high-performance liquid chromatography with fluorescence detection (RP-HPLC-FLD) method was developed for the simultaneous determination of thiamphenicol (TAP), florfenicol (FF) and florfenicol amine (FFA) in eggs. Samples were extracted with ethyl acetate–acetonitrile–ammonium hydroxide (49:49:2, v/v), defatted with hexane, followed by RP-HPLC-FLD determination. Liquid chromatography was performed on a 5 μm LiChrospher $C_{18}$ column using a mobile phase composed of acetonitrile (A), 0.01 M sodium dihydrogen phosphate containing 0.005 M sodium dodecyl sulfate and 0.1% triethylamine, adjusted to pH 4.8 by 85% phosphoric acid (B) (A:B, 35:65 v/v), at a flow rate of 1.0 mL/min. The fluorescence detector of HPLC was set at 224 nm for excitation wavelength and 290 nm for emission wavelength. Limits of detection (LODs) were 1.5 μg/kg for TAP and FF, 0.5 μg/kg for FFA in eggs; limits of quantitation (LOQs) were 5 μg/kg for TAP and FF, 2 μg/kg for FFA in eggs. Linear calibration curves were obtained over concentration ranges of 0.025–5.0 μg/mL for TAP with determination coefficients of 0.9997, 0.01–10.0 μg/mL for FF with determination coefficients of 0.9997 and 0.0025–2.50 μg/mL for FFA with determination coefficients of 0.9998, respectively. The recovery values ranged from 86.4% to 93.8% for TAP, 87.4% to 92.3% for FF and from 89.0% to 95.2% for FFA. The corresponding intra-day and inter-day variation (relative standard deviation, R.S.D.) found to be less than 6.7% and 10.8%, respectively.



## 1. Introduction

Thiamphenicol (TAP) and florfenicol (FF) are all synthetic and broad-spectrum antibiotics belonging to the fenicol drug family which have been widely used in veterinary medicine for treatments of various infections [1–6]. In various types of animal farm, abuse of TAP and FFA will leave undesirably large concentrations of residues in the product. TAP and FF have been found in various foods, including fish, liver, milk, poultry, even honey, because of their widespread availability and low cost. Because of concerns related to drug residues in animal tissues and the potential for emergence of drug resistant bacteria, their clinical applications are strictly controlled in many countries including China, USA and member states of European Union (EU) [7–10].

FF is partly transformed into FF amine (FFA), FF oxamic acid, and FF alcohol in animal bodies after administration. Florfenicol-amine is the longest-lived major metabolite in the liver from cattle [11]. Although the ratio of them is different in different species, FFA is always the largest in all metabolites in mostly food animals [12–14]. Therefore, FFA was defined as one of the FF residue markers by many countries and organizations. There are various analytical methods have been reported for the determination of TAP, FF, and FFA in animal tissues, such as gas chromatography (GC) [15,16], liquid chromatography (LC) [17–20], GC–MS [21–23], LC–MS [24], and LC–MS/MS [25–28]. However, no public reports describe the method for the simultaneous determination of TAP, FF, and FFA in eggs right now. Therefore, a specific and sensitive

*Abbreviations:* TAP, thiamphenicol; FF, florfenicol; FFA, florfenicol amine; LODs, limits of detection; LOQs, limits of quantitation; R.S.D., relative standard deviation; RP-HPLC-FLD, reversed-phase high-performance liquid chromatography with fluorescence detection; GC–MS, gas chromatography/mass spectrometry; LC–MS, liquid chromatography/mass spectrometry; MRPL, minimum required performance limit.

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method for the simultaneous determination and confirmation of TAP, FF, and FFA residues in eggs is urgently needed.

The aim of the present study was to describe a specific, sensitive and widely applicable RP-HPLC-FLD method for the simultaneous determination of TAP, FF, and FFA in eggs.

## 2. Materials and methods

### 2.1. Chemicals and reagents

TAP (100% standard) and FF (99.5% standard) were obtained from China Institute of Veterinary Drugs Control (Beijing, China). FFA (100%) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Acetonitrile of HPLC grade was purchased from Merck KGaA (Darmstadt, Germany). HPLC-grade triethylamine was the product of Tedia Company Inc. (Fairfield, OH, USA). Other reagents of analytical grade were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Water was purified using a PURELAB Option-Q synthesis system from ELGA LabWaters (High Wycombe, Bucks, UK).

### 2.2. Standard stock and working solutions

Stock solutions for TAP, FF, and FFA at a concentration of 100 μg/mL were prepared by dissolving TAP, FF, and FFA in acetonitrile, respectively. Working standard solutions at different concentrations for TAP, FF, and FFA were prepared by diluting the stock solutions with acetonitrile–water (35:65, v/v). The stock solutions were stable for 6 months at −20 °C. Fresh working solutions were prepared by an appropriate dilution of the stock solution before use.

### 2.3. Equipment

The following apparatus were used in the sample preparation:

Homogenizer, ART MICCRA D-9 (ART-moderne Labortechnik e.k., Germany); vortex mixer, Model G560E (Scientific Industries Inc., USA); centrifuge, 5810R (Eppendorf, Hamburg, Germany); nitrogen evaporator, N-EVAP 111 (Organomation Associates Inc., Berlin, MA, USA); Ultrasonic Cleaners, KQ-300DE (Kunshan, China).

### 2.4. HPLC instrumentation and conditions

Chromatography was performed on a Waters Alliance 515 LC System and a Waters multi λ 2475 fluorescence detector (Waters Corp., Milford, MA, USA). The separation was achieved on a LiChrospher $C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm; Merck KGaA). The column temperature was maintained at 30 °C. The injection volume was 200 μL manually with a 200 μL quantitative ring. The analysis was carried out using acetonitrile (A), 0.01 M sodium dihydrogen phosphate containing 0.005 M sodium dodecyl sulfate and 0.1% triethylamine, adjusted to pH 4.8 with 85% phosphoric acid (B) (A/B, 35:65, v/v) as the mobile phase, at a flow rate of 1.0 mL/min. The fluorescence detector of HPLC was set at 224 nm for excitation wavelength and 290 nm for emission wavelength.

### 2.5. Sample preparation

3.0 g of homogenized eggs was weight into a 50-mL polypropylene centrifuge tube. The sample was vortexed for 30 s, followed by the addition of 1 mL acetonitrile–water (30:70, v/v). After the addition of 20 mL of ethyl acetate–acetonitrile–ammonium hydroxide (49:49:2, v/v), the mixture was vortexed for 2 min and homogenized ultrasonically for 15 min, then centrifuged for 10 min at 8000 × *g*. The supernatant was transferred to a 20-mL glass test tube and the extraction step was repeated twice. The extracts were evaporated to dryness under nitrogen stream at 50 °C. The residue was reconstituted by 0.5 mL acetonitrile and then vortexed. 8 mL of hexane was added into the tube and the mixture was vortexed. After centrifugation for 5 min at 3500 × g, then the hexane layer was discarded. This de-fatting step was repeated twice. The extracts was evaporated to dryness under nitrogen stream at 50 °C, and then was stable at −4 °C until analysis. Before analysis, residues were reconstituted in 1 mL of the mobile phase, vortexed and poured through a 0.22-μm filter. The resulting solution was centrifuged at 12,100 × *g* for 15 min, then 200 μL of the supernatants was injected manually into the HPLC system.

### 2.6. Method validation

#### 2.6.1. Selectivity and sensitivity

The selectivity of the method was estimated by preparation and analysis of 20 blank and spiked samples. The probable interferences from endogenous substances were assessed by observing the chromatograms of blank and spiked eggs samples.

The sensitivity of the method was assessed by limits of detection (LODs) and limits of quantitation (LOQs). The LODs were defined by the concentration of each of the three analytes in the sample matrix giving a signal-to-noise ratio of 3:1. The LOQs was defined as the lowest point on the calibration curve for each of the three analytes based on a signal-to-noise ratio of 10:1.

#### 2.6.2. Linearity

The calibration curves were prepared on the basis of the peak areas and the working solution concentrations. A series of working standard solutions at concentrations of 5.0, 2.5, 1.0, 0.5, 0.25, 0.10, 0.05, 0.025 μg/mL for TAP; 10.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.10, 0.05, 0.01 μg/mL for FF; 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.01, 0.005, 0.0025 μg/mL for FFA, which were prepared by diluting the stock solutions with acetonitrile–water (35:65, v/v), then they injected into HPLC and analyzed as described above. Triplicate injections were also performed.

#### 2.6.3. Accuracy and precision

The accuracy and precision of the method were evaluated by determining recoveries of TAP and FF in spiked eggs samples at concentrations of 15, 50, 500 μg/kg by sextuplicate analysis; FFA in spiked eggs samples at concentrations of 5, 50, 500 μg/kg by sextuplicate analysis. The recovery of the method was calculated by comparing the determined concentration of spiked samples to the theoretical concentrations.

## 3. Results and discussion

### 3.1. Optimization of HPLC analysis

The chromatographic separation was optimized by testing different mobile phase compositions, such as acetonitrile–water, methanol–water. The mobile phase containing acetonitrile–0.01 M sodium dihydrogen phosphate, gave higher detection sensitivity for the three analytes. 35% acetonitrile was chosen because no interfering peaks appeared in the chromatographys of real samples at the retention time of the three analytes and the peak shape of the three analytes was good, when adjusted acetonitrile percentage from 20% to 50%. The tailing peak appeared because Si–OH of the packing material was bound to FFA, since FFA is an alkaline drug [29]. Meanwhile, ion-pairing agent, such as sodium dodecyl sulfate and tailing reducer, such as triethylamine can reduce the appearance of tailing peak and improve the peak shape. Therefore, the mobile phase composed of acetonitrile (A), 0.01 M sodium dihydrogen phosphate containing 0.005 M sodium dodecyl sulfate and

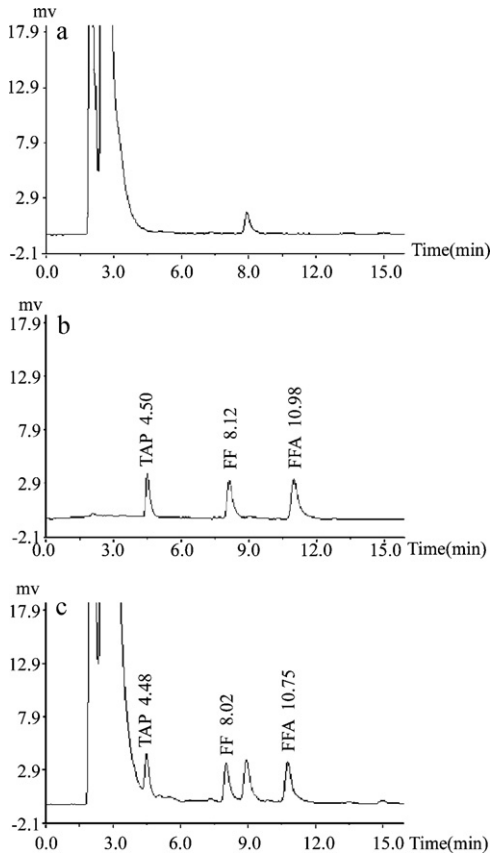


**Fig. 1.** (a) HPLC chromatograms of blank egg, (b) HPLC chromatograms of standard solution containing TAP (0.5 μg/mL), FF (0.357 μg/mL) and FFA (0.125 μg/mL) and (c) HPLC chromatograms of blank egg spiked with TAP, FF and FFA.

0.1% triethylamine, adjusted to pH 4.8 with 85% phosphoric acid (B) (A/B, 35:65, v/v) was finally chosen to separate the three analytes.

### 3.2. Optimization of sample preparation

Acetonitrile, aceton, ethyl acetate, methanol and ethyl acetate–acetonitrile–ammonium hydroxide were used to optimize the efficiency of extraction. Ethyl acetate would permit an efficient extraction as well as high recoveries for TAP and FF, expect for FFA. Basification of ethyl acetate–acetonitrile–ammonium hydroxide was chosen because FFA is a weak base compound and has a better solubility at high pH.

### 3.3. Selectivity and sensitivity

No interference was observed at the retention time of the three analytes. Representative chromatograms of blank samples and spiked samples are shown in Fig. 1.

As shown in Table 1. The LODs of the three analytes were 1.5 μg/kg for TAP and FF, 0.5 μg/kg for FFA. The LOQs of the three analytes, were 5 μg/kg for TAP and FF, 2 μg/kg for FFA.

**Table 1**
Limits of detection (LODs) and limits of quantitation (LOQs).

| Analyte | LODs (μg/kg) | LOQs (μg/kg) |
|---|---|---|
| TAP | 1.5 | 5 |
| FF | 1.5 | 5 |
| FFA | 0.5 | 2 |

**Table 2**
Accuracy and precision data of the method ($n = 6$).

| Analyte | Spiked level (μg/kg) | Mean result (μg/kg) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| TAP | 15 | 12.95 ± 1.02 | 86.4 ± 6.8 | 7.9 |
| | 50 | 44.17 ± 1.86 | 88.3 ± 3.7 | 4.2 |
| | 500 | 468.80 ± 31.22 | 93.8 ± 6.2 | 6.7 |
| FF | 15 | 13.11 ± 0.70 | 87.4 ± 4.7 | 5.4 |
| | 50 | 44.82 ± 2.16 | 89.6 ± 4.3 | 4.8 |
| | 500 | 461.45 ± 16.20 | 92.3 ± 3.2 | 3.5 |
| FFA | 5 | 4.45 ± 0.20 | 89.0 ± 4.0 | 4.5 |
| | 50 | 45.59 ± 2.61 | 91.2 ± 5.2 | 5.7 |
| | 500 | 475.75 ± 20.36 | 95.2 ± 4.1 | 4.3 |

**Table 3**
Performances of the method in term of precision ($n = 6$).

| Analyte | Spiked level (μg/kg) | Intra-day R.S.D. (%) | Inter-day R.S.D. (%) |
|---|---|---|---|
| TAP | 15 | 4.8 | 9.4 |
| | 50 | 5.5 | 8.9 |
| | 500 | 6.6 | 10.8 |
| FF | 15 | 6.7 | 10.5 |
| | 50 | 5.7 | 9.3 |
| | 500 | 4.7 | 7.9 |
| FFA | 5 | 5.8 | 10.5 |
| | 50 | 4.8 | 9.2 |
| | 500 | 4.7 | 8.2 |

### 3.4. Accuracy and precision

As shown in Table 2, from fortified eggs samples at levels of 5–500 μg/kg, the mean extraction recoveries ranged from 86.4% to 93.8% for TAP, 87.4% to 92.3% for FF and from 89.0% to 95.2% for FFA. The corresponding intra-day and inter-day variation (relative standard deviation, R.S.D.) found to be less than 6.7% and 10.8%, respectively (Table 3).

### 3.5. Linearity

The calibration curves calculated by linear regression method: $Y = aX + b$, where $Y$ is the peak area of the analytes, $X$ is the concentration of the analytes (μg/mL), $a$ is the slope and $b$ is the $y$-intercept. Typical values for the regression parameters $a$, $b$, $r^2$ (determination coefficient) and the linear range of TAP, FF, and FFA are shown in Table 4.

### 3.6. Methods comparison

Comparison of the public methods to the present study; the LODs and LOQs of TAP, FF, and FFA were higher than other methods in the reports of Shen et al. [23] and Luo et al. [28], however, the present procedure of the extraction was more simple and low cost. The extracts were only purified by liquid–liquid extraction with ethyl acetate–acetonitrile–ammonium hydroxide (49:49:2, v/v), defatted with hexane. In addition, the LOQs of the three analytes meet the minimum required performance limit (MRPL) established by European Commission and China. Besides, in comparison with

**Table 4**
Regression lines, determination coefficients ($r^2$) and calibration range of TAP, FF, and FFA.

| Analyte | Regress lines | Determination coefficients ($r^2$) | Calibration range (μg/mL) |
|---|---|---|---|
| TAP | $Y = 0.0198X + 0.0055$ | 0.9997 | 0.025–5.0 |
| FF | $Y = 0.0031X - 0.0447$ | 0.9997 | 0.01–10.0 |
| FFA | $Y = 0.0008X + 0.0063$ | 0.9998 | 0.0025–2.50 |

GC–MS and LC–MS, LC with FLD detection is chiefly characterized by its simplicity of operation, inexpensive and widespread.

### 3.7. Application to real samples

In order to evaluate the applicability and reliability of the proposed method, the three analytes was determined in real samples. A total of 50 eggs samples from local supermarket were analyzed by the described method. One incurred egg sample was analyzed by the described method and 19 μg/kg of FF and 36 μg/kg of FFA were detected. In addition, none of the other eggs samples analyzed showed residues of targeted compounds at detectable levels.

## 4. Conclusion

In the present study, a specific, sensitive and widely applicable RP-HPLC-FLD method for the simultaneous determination and confirmation of TAP, FF, and FFA residues in eggs was developed. This method is accurate, precise and sensitive, hence it could possibly, fulfills the requirement of the confirmatory criteria according to European Commission. Besides, this method can be performed in a relatively short period, utilizes common, inexpensive reagents and supplies.

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