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HPLC-Fluorescence Detection Method for Quantitative Determination of Tetracycline Antibiotic Residues in Honey

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ABSTRACT

A high performance liquid chromatography method utilizing fluorescence detection was optimized and validated to determine tetracycline residues in honey. The separation of three tetracycline residues; oxytetracycline, tetracycline and chlortetracycline was carried out on a reverse–phase C_8 column with a gradient elution. A mobile phase system consisted of 50% (v/v) methanol and 25 mM sodium acetate buffer (containing disodium ethylenediamine-tetraacetic acid and calcium chloride, pH 8.10). Fluorescence detection was observed at 518 nm (excitation wavelength at 393 nm) with 20 minute analysis time. The extraction with disodium ethylenediaminetetraacetic acid (Na_2EDTA)-McIlvaine buffer pH 4 was performed and followed by HLB cartridge clean up step. Calibration curves for oxytetracycline, tetracycline and chlortetracycline showed good linearities ($r^2 > 0.998$) at concentrations ranged from 5 to 1000 ng/mL. The limits of detection (LODs) and quantifications (LOQs) for oxytetracycline, tetracycline and chlortetracycline were found to be 3.40, 0.29, 4.69 μg/kg, and 11.35, 0.96, 15.62 μg/kg, respectively. The recoveries of oxytetracycline, tetracycline and chlortetracycline, at 50, 100 and 200 μg/kg spiked samples were higher than 80% for all compounds. The analytical method was successfully applied to honey samples collected from northern part of Thailand.

Keywords: tetracyclines, honey, fluorescence detection, HPLC

INTRODUCTION

Tetracyclines are broad-spectrum antibiotics used routinely in veterinary medicine for the treatment and prevention of some infectious animal diseases (Aranda *et al*., 2006). Tetracyclines have been used worldwide as well as in Thailand for prevention and control of bacterial brood diseases (American and European), in honeybees, because of the easy availability and relatively low cost of tetracyclines, this method has been widely adopted by beekeepers.

The occurrence of antibiotic residues in human food, arising from its veterinary use, is a cause of concern to consumers worldwide, because of possible toxic or allergic reactions and the possibility that pathogenic organisms could become resistant to these drugs (Saenz *et al*., 2001). The application of the law in relation to these antibiotics is not harmonized across all member states of the European Union. The Commission of the European Union laid down the procedure for establishing maximum residue limits (MRLs) of veterinary medical products in foodstuffs of animal origin (ECC, 1990). However, no MRLs have been fixed for using with bee products; nevertheless, some countries, such as Switzerland, have set

MRLs for the TCs in honey at 20μg/kg. In Japan, base on microbiological research, a value of 0.1 mg/kg was introduced as the allowed residual quantity of tetracycline in honey (Dinkov *et al*., 2005). In Thailand, there is no law about the control of these drugs in honey.

Determination of tetracycline residues in honey were normally employed 2 steps; screening and quantifying steps. Screening of antibiotics in honey is carried out by the Charm test (Reybroeck, 2003; Morlot and Beaune, 2003) or enzyme-linked immunosorbent assay (ELISA) (Jeon and Rhee-Paeng, 2008; Pastor-Navarro *et al*., 2007) prior to quantify the residues of the positively tested samples mostly by using HPLC. Tetracyclines can be successfully determination in various biological matrices. HPLC in a reverse-phase mode, with different detection modes, such as UV (Clinquina *et al*., 2003; Furasava, 2003; Solkol and Matisova, 1994), fluorescence (Schneider *et al*., 2007; Blackwell *et al*., 2004; Kuhne *et al*., 2000), chemiluminescence methods (Pena *et al*., 2000; Wan *et al*., 2005; Santiago *et al*., 2007) and mass spectrometry (Wang *et al*., 2004; Reverte *et al*., 2003; Carrasco-Pancorbo *et al*., 2008) have also been reported. The UV detection has lower sensitivity than mass spectrometry. In general, fluorescence detection is highly sensitive and selective with relatively lower cost than mass spectrometry. Hence, fluorescence detection is a detection of choice.

In this research, the extraction and chromatographic detection of three tetracyclines; oxytetracycline, tetracycline and chlortetracycline (see Figure 1) were optimized and validated for the determination of tetracycline residues in honey at LODs below 10 μg/kg. The separation of three tetracycline residues was observed on a reversed phase C_8 column with a gradient elution. The tetracycline residues was extracted with buffer and cleaned up by using solid phase extraction (SPE), detected on a high performance liquid chromatography method utilizing fluorescence detection. Honey production mainly in the northern part of Thailand, has not been reported on tetracycline residues. Hence the developed method was applied for the determination of tetracycline residues presence in honey samples collected from the North of Thailand.

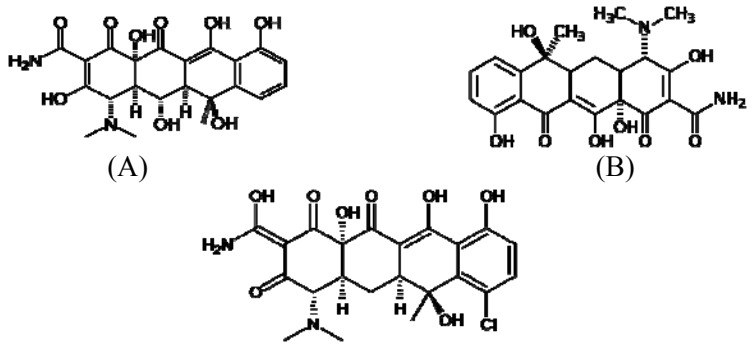
METHODOLOGY

Apparatus

The tetracycline residue separation was carried out on a HPLC system (HP1100, Agilent, USA) with a reversed phase Zorbax C_8 (150 x 4.6 mm I.D., 5μm, Agilent, USA). The fluorescence detector was coupled and set at 380 nm and 518 nm for excitation and emission wavelength, respectively.

Reagent and Materials

Tetracycline, oxytetracycline and chlortetracycline standards were purchased from Merck, Germany. Disodium ethylenediaminetetraacetic acid, sodium acetate, calcium chloride and methanol were analytical-reagent grade (Merck, Germany). The Oasis® HLB extraction (SPE) cartridges, 6 cm^3 (200 mg) were purchased from Waters Corporation, USA



(A) (B)

(C)

Figure 1 Structure of tetracyclines. Oxytetracycline; OTC (A), Tetracycline; TC(B), Chlortetracycline; CTC (C)

Individual stock standard solutions were prepared in 100 mL of methanol containing 100 mg of neat standard in a volumetric flask and were stored at -20 °C in brown glass vials for a maximum period of 1 month. The working standards were mixture of three compounds prepared by serial dilutions of the stocks in 50% (v/v) methanol and 25 mM sodium acetate buffer (containing disodium ethylenediaminetetraacetic acid, pH 8.1). McIlvaine-Na_2EDTA buffer was prepared as described by Pena *et al*., 2005. A mobile phase system was consisted of solvent A (10% v/v methanol and 10 mM sodium acetate buffer, containing disodium ethylenediaminetetraacetic acid and calcium chloride, pH 8.1) and solvent B (50% v/v methanol and 25 mM sodium acetate buffer, containing disodium ethylenediaminetetraacetic acid and calcium chloride, pH 8.1) .

Calibration Procedure

The calibration graphs were constructed with standard solution concentrations ranged from 5-1000 ng/mL. The linearities were evaluated by linear regression analysis, which was calculated by least square regression method.

Honey Samples

Commercial honey samples were purchased from markets in Chiang Mai during 2008. Samples were stored at 4°C in darkness until processing.

Extraction and Clean-up Procedure

A sample of honey (3.0 g) was weighed into a polypropylene tube and 20 mL of McIlvaine-Na_2EDTA buffer was added. The sample solution was mixing for 5 min. After filtration, it was loaded on Oasis® HLB cartridge previously conditioned with 3 mL of methanol, 2 mL of water and 3 mL of McIlvaine-Na_2EDTA buffer, respectively. The cartridge containing the sample was then washed with 10 mL of 10% methanol-water, and tetracyclines were eluted with 3 mL of methanol. The solvent was removed under a nitrogen stream and the residue was reconstructed in 1 mL of 50% (v/v) methanol and 25 mM sodium acetate buffer (containing disodium ethylenediaminetetraacetic acid, pH 8.1) and filtered with a 0.45 μm Nylon filter membrane. An aliquot of 50 μL was subjected to the HPLC-FLD system.

Validation Method

The limits of detection (LODs) and quantification (LOQs) values were estimated by fortifying honey at three levels of 50, 100 and 150 μg/kg, and the SDs of %recoveries were plotted against the concentrations found. The corresponding SDs at the y intercept is a SD_0 corresponding to standard deviation of blanks. The LOD values were calculated of $3SD_0$ and $10SD_0$ for LOQs. The recovery and precision of this method were also evaluated by analysis of three levels of these fortified samples.

RESULTS AND DISCUSSION

HLPC conditions

The methodology reported here utilized a Zorbax C_8 (150 x 4.6 mm I.D., 5 μm, column at 30°C. Gradient elution was carried out to the following solvent programs: solvent B was linearly increased from 50 to 100% in 5 min, kept at 100% for 15 min and returned to the initial conditions. The flow rate was 0.5 mL/min and the fluorescence detection had an excitation wavelength of 393 nm and emission wavelength of 518 nm. Under conditions, three tetracycline standards were separated within 20 min and the chromatogram is illustrated in Figure 2(A).

Calibration

A series of standard solutions prepared in 50% (v/v) methanol and 25 mM sodium acetate buffer (containing disodium ethylenediaminetetraacetic acid, pH 8.10) were used in a study of the linear range. Under the optimized experimental conditions, tetracyclines were found good linearities between their concentration and peak area responses ranged from 5-1000 ng/mL with $r^2 > 0.998$. Method validation results were satisfaction with linear regression equations shown in Table 1.

Extraction and clean-up step

The extraction with McIlvaine-Na_2EDTA buffer, a mild acidic solvent containing Na_2EDTA, accepted as a universal extraction method for tetracyclines with Oasis® HLB extraction cartridges, was used.

During method development, the method described by Pena *et al*., 2005 was applied to our control honey sample but high background was observed. The washing step was then modified. Washing with 10 mL of 10% methanol gave higher efficiency in removing the interference, compared to those washed with water and 5% methanol. Following this methodology, higher recoveries more than 80% for all compounds were obtained.

Analytical method validations

The recovery and repeatability of the method were evaluated by analysis of five spiked samples with tetracycline, oxytetracycline and chlortetracycline standard mixture at level 50, 100 and 150 μg/kg, respectively. Under our conditions, the estimated extraction recoveries from spiked honey samples for tetracycline, oxytetracycline and chlortetracycline were higher than 80% for all compounds (Table 2). Figure 2(B) represents the chromatogram obtained from 50 μg/kg fortification assay which contained some interference near tetracyclines. The limits of detection (LODs) and quantifications (LOQs) for oxytetracycline, tetracycline and chlortetracycline were found to be 3.40, 0.29, 4.69 μg/kg, and 11.35, 0.96, 15.62 μg/kg, respectively.

Application to real sample

Six honey samples were purchased from different local markets in Chiang Mai which were different floral origins; lychee, sunflower and longan. Tetracycline residue presences in honey samples are shown in Table 3. An example chromatogram of honey extract is shown in Figure 2C. Tetracycline residues in the honey samples were ranged from 32.53-106.9 μg/kg, nd.-14.01 μg/kg and not detected for oxytetracycline, tetracycline and chlortetracycline, respectively. Honey from Lychee floral was detected oxytetracycline in the highest amount. Sunflower honey was detected tetracycline residues in the lowest concentration compared to another honey from different floral origins.

Table 1 Retention time, regression results[a]

Tetracyclines	Retention time (min)	Regression equation	Correlation coefficient (r^2)
OTC	4.17	y= 0.1388x-0.8840	0.9992
TC	8.09	y= 0.2000x-1.5513	0.9987
CTC	11.20	y= 0.0523x+0.6001	0.9986

[a] Based on three replicates of five concentration in the range 5-1000 ng/mL

Table 2 Average recoveries and standard deviations (SD) of three fortified concentration levels (n=5)

Fortification level (µg/kg)	Recovery (%) ± SD		
	OTC	TC	CTC
50	96.92 ±1.87	96.08 ±1.22	91.27 ±5.15
100	97.25 ±2.55	86.03 ±2.06	81.52 ±3.10
150	93.34 ±3.20	87.77 ±1.69	91.06 ±5.29

Table 3 Tetracycline residues in honey samples

Sample code	Origins	Tetracyclines (mg/kg)			
		OTC	TC	CTC	Total
1LOH	Longan	60.61	nd	nd	60.61
2SUH	Sun flower	75.67	nd	nd	75.67
3LYH	Lychee	60.15	7.18	nd	67.33
4LOH	Longan	nd	nd	nd	nd
5SUH	Sun flower	nd	nd	nd	nd
6LYH	Lychee	106.9	14.06	nd	120.9

nd = not detected

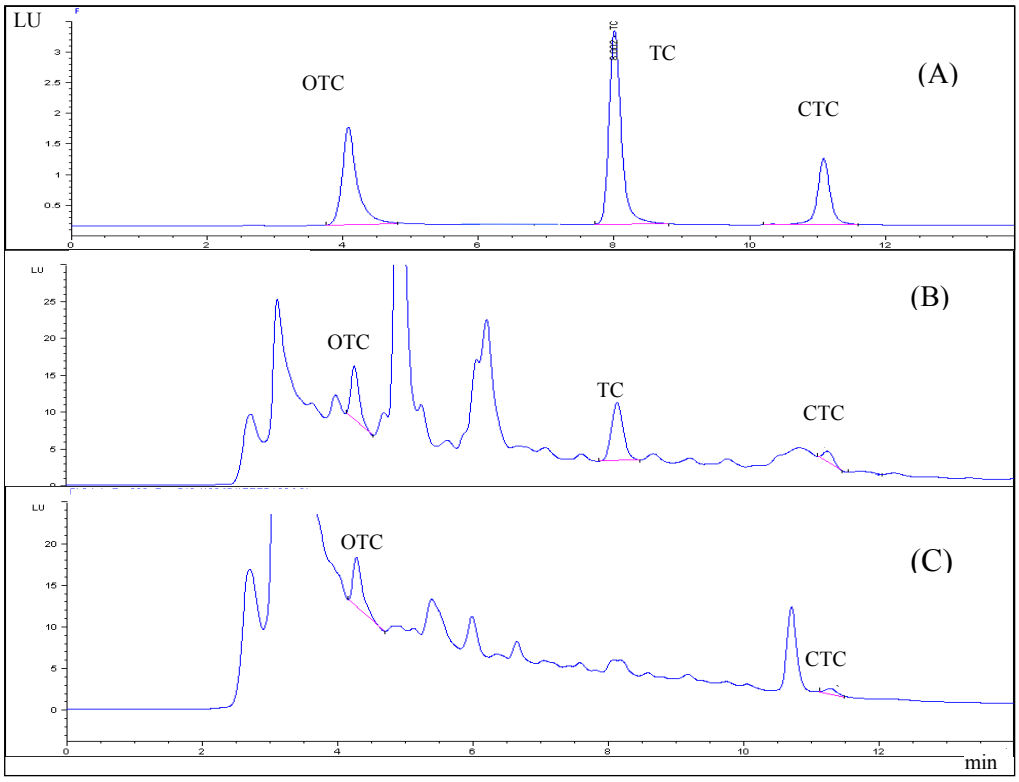


Figure 2 Chromatograms of standard solution of 1 µg/mL tetracyclines (A), fortification assay at 50 µg/kg (B) and longan honey sample (C).

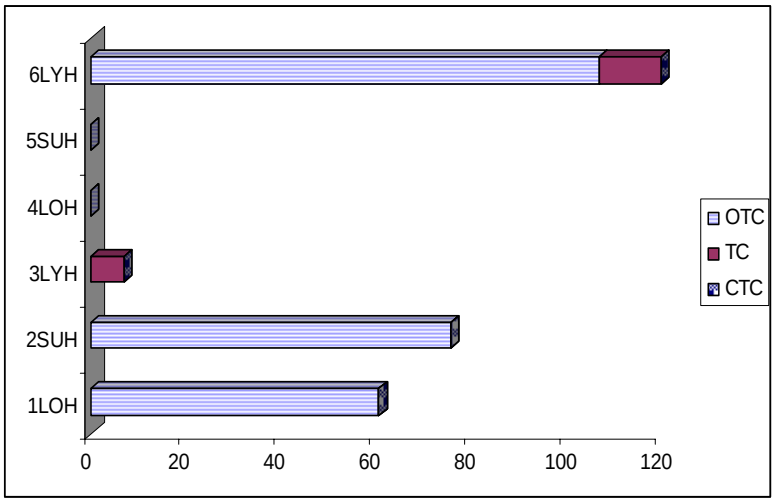


Figure 3 Comparison on tetracycline residues found in honey samples from different floral origins.

CONCLUSION

The developed gradient HPLC-FLD allows rapid and simultaneous determination of three tetracycline presence in honey samples. Low detection and quantitation limits were obtained without post column derivatization requirement.

Thai beekeepers should aware and reduce the use of antibiotics in bee honey in order to control honey quality and for the safety of consumers. Thai honey exporters should consider tetracycline residues that were detected at the higher level than MRL requirement of Switzerland or Japan.

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