Quantitative detection of tetracycline residues in honey by a simple sensitive immunoassay

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Abstract

Tetracyclines (TCs) are widely used for prevention and control of infectious diseases and have a great activity against variety of Gram-positive and Gram-negative bacteria. Due to the widespread use of TCs in animal husbandry, it can lead to an increase the risk of TCs remaining in human food. To protect consumers, many countries have set acceptable tolerance levels for these drugs. Therefore, it is necessary to establish a suitable analytical technique with specificity, sensitivity and simplicity.

The biotin–avidin mediated ELISA method was performed to determine TC residues in honey quantitatively. By using PBS-EDTA assay buffer at pH 7.2, a honey solution of TC standard was prepared and diluted. And no additional pre-treatment of sample was required in this method. The limit of detection and limit of quantitation of the optimized method were $3.98 \times 10^{-10}$ M (0.19 $\mu$g L$^{-1}$) and $7.94 \times 10^{-10}$ M (0.38 $\mu$g L$^{-1}$), respectively, and the dynamic range was from $1.52 \mu$g L$^{-1}$ to $152 \mu$g L$^{-1}$ of TC in honey. No cross-reactivity was observed with the structurally similar compounds, and mean percent recoveries of TC spiked in honey ranged from 95% to 101%. Compared to other methods, this method was superior in terms of detection limit, dynamic range, and % recovery with simple sample-preparation.

1. Introduction

Tetracyclines (TCs) are broad spectrum antibiotics, and show a great activity against variety of both Gram-positive and Gram-negative microorganisms. Due to their broad spectrum activity and cost-effectiveness, TCs are widely used in animal husbandry as veterinary drugs [1]. The action of TC is inhibition of protein synthesis by binding to the small ribosomal subunit at the A site which binds with the aminoacyl tRNA [2]. In beekeeping, TC antibiotics are used to treat bacterial brood diseases such as American Foulbrood caused by Paenibacillus larvae and European Foulbrood caused by Melissococcus pluton [3,4]. As these drugs have been widely used for prevention and treatment of diseases, and often have not been followed the label direction in its use, its residues often remain in food.

Due to these antibiotic residues, it can lead to increased drug-resistance of microbial strains in consumers and it can cause allergic or toxic reactions in some hypersensitive individuals [5].

Arising from concerns about these drug residues in food and environment, several countries have set the maximum residue levels (MRLs) for controlling the safety of food. The Codex has set the MRLs for TCs group including chlortetracycline (CTC), oxytetracycline (OTC) and TC: 200 $\mu$g kg$^{-1}$ in muscle (cattle), 100 $\mu$g kg$^{-1}$ in milk (cattle, sheep) and 1200 $\mu$g kg$^{-1}$ in kidney (cattle), respectively. The MRLs for TCs in honey have not been established by Codex yet, however, KFDA (Korea Food & Drug Administration) has set the MRL for OTC in honey at 300 $\mu$g kg$^{-1}$.
There are several chromatographic methods [6,7] to detect residual TC levels including liquid chromatography (LC) [2,8–10] and high-performance LC (HPLC) [1,11–13]. Viñas et al. [8] performed reversed-phase LC for analysis of TCs residues in honey, and limit of detection (LOD) of 15-30 ng g\(^{-1}\) was obtained. Cinquina et al. [12] performed HPLC with diode-array detection (HPLC-DAD) for determination of TCs in milk and muscle. The detection capability was in the range of 117.2–131 ng g\(^{-1}\) in milk and 115–133 ng g\(^{-1}\) in muscle. Although these methods have an advantage of determining individual TCs simultaneously, accurately with a highly sensitivity, however, they cannot detect TCs directly in sample as it was necessary to perform a lengthy sample extraction. Sample matrices such as milk, honey, egg, fish, meat, urine and plasma contain lots of substances which can interfere with analysis for TCs. Thus, these chromatographic analyses require time consuming sample cleanup and extraction process. The common techniques for those are liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [2]. TCs have a high affinity to interact with metal ions and bind with proteins. Thus, sample extraction methods often utilize disodium ethylenediaminetetraacetate (Na\(_2\)EDTA) under mildly acidic conditions.

Enzyme-linked immunosorbent assay (ELISA) is a simple, specific sensitive and powerful tool for the selective detection of substances at trace levels in physiological, biological and environmental samples. Thus, it allows for the determination of target molecules effectively in a complex sample matrix without lengthy extraction steps.

Recently, an ELISA method mediated by biotin–avidin system was developed to detect TCs residues in milk [14]. A dose–response curve was constructed; the LOD and the dynamic range for TC were established to be 0.048 µg L\(^{-1}\) and 0.15–152 µg L\(^{-1}\), respectively. This method presented more sensitive and useful than the one reported by Pastor-Navarro et al. who obtained the LOD of 0.4 µg L\(^{-1}\) and dynamic range of 1.15–38.89 µg L\(^{-1}\), and recoveries between 79% and 108% in honey with cELISA method [15].

In this paper, the biotin–avidin mediated ELISA method was performed to detect TC residues in honey with simplicity without any clean-up or extraction. In general, honey consists of several organic and inorganic constituents—carbohydrate, vitamin, calcium, iron, magnesium, phosphorus, potassium and zinc [16]. Although, the honey matrix is complicated, the proposed method can detect TC antibiotics residue in honey, successfully without sample pre-treatment. Also, this method provides improved LOD and dynamic range in honey with a good reproducibility.

2. Experimental

2.1. Materials and instrumentation

Sheep anti-TC polyclonal antibody (ab30591) was obtained from Abcam (Cambridge, MA, USA). The immunogen of ab30591 is TC-conjugated BSA through amide bond. Affinity purified antibody biotin labeled rabbit anti-sheep IgG was supplied by Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD, USA). Horseradish peroxidase-conjugated avidin was purchased from Pierce (Rockford, IL, USA). Hydrochlorides of TC, OTC, CTC and doxycycline (DC) were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate dihydrate was supplied from Merck (Darmstadt, Germany). Na\(_2\)EDTA, albumin from chicken egg white (OVA), 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) and phosphate–citrate buffer with sodium perborate were purchased from Sigma (St. Louis, MO, USA). Sodium bicarbonate, sodium carbonate, sodium phosphate monobasic, sodium chloride were obtained from Duksan Pure Chemical (Ansan, Gyeonggi-do, Korea). All chemicals were of analytical-reagent grade or better and were used as received.

Enzyme activity was measured on E-max microtiter plate reader (Molecular Device Co., Sunnyvale, CA, USA) at 450 nm. All absorbance intensities reported are the average of at least triplicates and have been corrected for the contribution of the blank. Immuno plate from Nunc (Denmark) was used. The 96 well plates were washed using Multiwasher III (Tricontinent, Grass valley, CA, USA).

All solutions were made by triply deionized water (Milli-Q water purification system, Millipore, Billerica, MA, USA). The coating buffer was a 50 mM bicarbonate buffer (pH 9.6). A 10 mM PBS (phosphate buffered saline) at pH 7.2 was used as the assay buffer. A 10 mM PBS containing 90 mM EDTA (PBS-EDTA) at pH 7.2 was used for honey sample preparation and dilution. The wash buffer was a PBS containing 0.05% Tween\(^{20}\) (PBST). The blocking solution was 1% OVA in PBS. The substrate buffer was a 50 mM phosphate–citrate buffer (pH 5.0). The substrate solution was prepared by adding TMB tablet in 10 mL phosphate–citrate buffer. The stopping solution was 2 M H\(_2\)SO\(_4\). All buffers were filtered through 0.22 µm pore size filter before use.

2.2. Standard TC in honey solution

Honey consists of metal ions such as calcium, iron, magnesium and zinc [16]. For actual sample analysis, it should be considered that TC has a high possibility of forming chelation complex with these metal ions. Thus, prior to actual sample analysis, honey solution was prepared with 1 g of honey in 4 mL PBS-EDTA at pH 7.2. Honey was purchased from local supermarket. This honey solution was confirmed to be free from TC by HPLC/ESI-MS/MS. By using the honey solution, a stock solution of TC (1.0 × 10\(^{-2}\) M) was prepared and serially diluted to ranging in concentration from 1.0 × 10\(^{-2}\) M to 1.0 × 10\(^{-16}\) M. The resulting standard TCs in honey solution were used in this study.

2.3. Optimization

This competitive immunoassay was performed by varying concentrations of OVA-TC conjugate which was immobilized on the plate and primary antibody because the LOD is dependent on two major factors such as concentrations of competitor and antibody. Selection of appropriate assay buffer, incubation time and pH of buffer was also required for this study. In addition it was necessary to evaluate the effect of the presence or absence of EDTA in assay buffer.
2.4. Development of ELISA

After optimization, the biotin–avidin ELISA mediated method was performed. The selected concentrations of OVA-TC conjugate and primary antibody were $5 \mu g mL^{-1}$ and 1:1000 dilution (9.98 $\mu g mL^{-1}$), respectively. First, OVA-TC conjugate was immobilized on the plate for 3 h. Then, the plate was blocked with 300 $\mu L$ of blocking solution for 30 min. Each 50 $\mu L$ of various concentrations of standard TC solution and primary antibody solution were added to the wells. During the 1 h incubation, free TC and TC immobilized in the plate compete for the TC antibody binding sites. Then, 100 $\mu L$ of 0.5 $\mu g mL^{-1}$ secondary Ab-biotin conjugate was added and incubated for 1 h. A 100 $\mu L$ of 0.4 $\mu g mL^{-1}$ avidin-HRP was followed and incubated for 30 min. Then, 100 $\mu L$ of substrate solution was added. After 10 min, the addition of 2 M sulfuric acid as stopping solution leads to color change from blue to yellow, then absorbance was measured using E-max at 450 nm. Between incubation steps, the assay were performed at RT with constant shaking and wells were washed three times with 330 $\mu L$ PBST.

2.5. Cross-reactivity study

The cross-reactivity study was performed in honey solution, and in the same manner as the studies conducted for TC assay. The percent cross-reactivity was calculated using DC, OTC and CTC which are structurally similar to TC.

3. Results and discussion

3.1. Optimization

3.1.1. Effect of EDTA

TC (Fig. 1) has five ionization states that can be represented by $H_4TC^+$, $H_3TC$, $H_2TC^-$, $HTC^2-$ and $TC^-$. In aqueous environment, the first deprotonation step ($pK_1 = 3.2$) occurs at OH$_3$, generating the zwitterions with negatively charged and positively charged species. The second deprotonation takes place at OH$_{12}$ ($pK_2 = 7.6$). The third deprotonation step ($pK_3 = 9.6$) occurs at NH$_4$, generating a dianion. Finally, fourth deprotonation takes place at OH$_{10}$ ($pK_4 = 12$) [17–19]. Thus, TC has a high possibility for forming chelation complexes with metal ions. The behavior of TC-metal ion binding has been studied using spectroscopic methods including nuclear magnetic resonance spectroscopy, circular dichroism spectroscopy, absorption and fluorescence spectroscopy [17,19,20]. Here, UV–vis spectrophotometric studies were also carried out to examine the interaction of TC with Ca$^{2+}$ and Mg$^{2+}$ ions, which are the physiologically relevant divalent metal ions. The absorption spectra of TC and its complexes with Ca$^{2+}$ ion and Mg$^{2+}$ ion are depicted in Fig. 2. TC shows two absorption bands at 276 nm and 356 nm. In the presence of Mg$^{2+}$ ion, a bathochromic shift of long wavelength absorption band was observed at 356–368 nm, and the band at 276 nm with free TC is shifted to the band at 272 nm. The same trend was observed in the addition of Ca$^{2+}$ ion; the bands are observed at 272 nm and 368 nm, respectively. It means that the TC forms the coordination complex with these divalent cations.

Recently, a biotin–avidin mediated competitive assay was developed to detect TC residues in milk [14]. The LOD was 0.048 $\mu g L^{-1}$ and the dynamic range was from 0.15 $\mu g L^{-1}$ to 152 $\mu g L^{-1}$ for TCs. For the TC analysis in milk, sample preparation step was required to remove large molecules such as protein and fat in milk, prior to the assay. However, as mentioned earlier, honey consists of no fat and few proteins, therefore the sample preparation is not needed. However, honey has metal ions such as calcium, iron, magnesium, potassium, sodium and zinc ion [16] so it is necessary to consider the high potential for forming chelating complexes between TC and these metal ions [17,18].

To prevent these interferences, EDTA was incorporated in the assay buffer. EDTA has a greater affinity for the cations than TCs, thus it can block the interaction of TC and cations [6]. So the effect of presence of EDTA in the assay buffer was evaluated by comparing PBS and PBS-EDTA buffers. As shown in Fig. 3, the maximum signal absorbance (0.62) obtained with

![Fig. 1 – Chemical structure of TCs.](image-url)

![Fig. 2 – UV–vis spectra of TC, and TC in the presence of Ca$^{2+}$ ion and Mg$^{2+}$ ion, respectively.](image-url)
3.1.2. Effect of pH
PBS-EDTA buffer was prepared with two different pH conditions, pH 5.0 and pH 7.2. The dose–response curves for TC in honey were performed in both pHs. Fig. 4 demonstrates the results obtained from those; the background signals obtained with PBS-EDTA, pH 7.2 buffer was very low and stable, but the ones with pH 5.0 had relatively high signals.

3.2. Dose–response curve in honey
After a simple dilution of commercial honey (1 g of honey diluted with 4 mL PBS-EDTA, pH 7.2), the dose–response curve was constructed using 5 μg mL\(^{-1}\) of OVA-TC conjugate and 1:1000 diluted antibody (9.98 μg mL\(^{-1}\)). Fig. 5 shows the dose–response curve and calibration curve obtained for TC in honey. The LOD and the limit of quantitation (LOQ) were 3.98 × 10\(^{-10}\) M (0.19 μg L\(^{-1}\)) and 7.94 × 10\(^{-10}\) M (0.38 μg L\(^{-1}\)), respectively. Here, LOD is defined as the concentration corresponding to 3 standard deviations above the mean and the LOQ as the concentration corresponding to 10 standard deviations above the mean. The dynamic range is from 1.52 μg L\(^{-1}\) to 152 μg L\(^{-1}\). The calibration curve in honey was calculated as: Absorbance = −0.446 × Log[TC] − 2.60, R\(^2\) = 0.980.
of TC giving 50% reactivity was calculated:

\[
\text{% cross-reactivity} = \left( \frac{\text{concentration of cross-reactant giving 50\% B}}{\text{concentration of cross-reactant giving 50\% B}} \right) \times 100
\]

B is the ratio of response B, to the maximum response when no analyte is present B0. The 50% B/B0 values were estimated and then their percent cross-reactivity was calculated: % cross-reactivity = (concentration of TC giving 50% B/B0)/(concentration of cross-reactant giving 50% B/B0). B/B0 is the ratio of response B, to the maximum response when no analyte is present B0. The 50% B/B0 value and cross-reactivity for each compound are given in Fig. 6 and Table 1. These results demonstrated that OTC showed 1% cross-reactivity whereas CTC and DC demonstrated less than 1% cross-reactivity with TCs.

### 3.3. Specificity

Assay specificity indicates the ability of an antibody to generate a measurable response only for the target molecule. The specificity of the antibody is evaluated by cross-reactivity study which is important part of the evaluation of the immunoassay [21]. Here, a study was performed using three structurally similar compounds, cross-reactants, such as DC, OTC and CTC (Fig. 1). The cross-reactivity studies were carried out by a competitive ELISA by adding various free cross-reactants at different concentrations to compete with antigen coated on the surface, to bind with the antibody. Their 50% reactivity was calculated: % cross-reactivity = (concentration of cross-reactant giving 50% B)/(concentration of cross-reactant giving 50% B/B0). B/B0 is the ratio of response B, to the maximum response when no analyte is present B0. The 50% B/B0 value and cross-reactivity for each compound are given in Fig. 6 and Table 1. These results demonstrated that OTC showed 1% cross-reactivity whereas CTC and DC demonstrated less than 1% cross-reactivity with TCs.

### 3.4. Recovery study

The recovery study was performed using two different brands of honey; one brand of honey was made from thousand flowers (brand A), and the other brand was made from acacia flowers (brand B). Both honey brands were purchased from local supermarkets. Free TC (15 µg L\(^{-1}\) and 121 µg L\(^{-1}\)) were spiked in honey solution, and mean percent recoveries were analyzed to obtain the recovery rate of 95% in acacia flower honey (brand B) and 101% in many flowers honey (brand A). The recovery study was performed in ten replicates and the results were quite satisfactory as seen in Table 2.

In summary, this biotin–avidin mediated competitive ELISA method shows a good sensitivity with LOD of 0.19 µg L\(^{-1}\) and wide dynamic range (1.52–152 µg L\(^{-1}\)) in determining TC antibiotics residues in honey with simplicity. Table 3 represents the comparison of analytical data obtained for TCs with other studies. The competitive ELISA method detecting TCs in honey was described by Pastor-Navarro et al. [15]. They obtained the LOD of 0.4 µg L\(^{-1}\) and working range from 1.15 µg L\(^{-1}\) to 38.89 µg L\(^{-1}\) and the recoveries between 79% and 108%. This work was compared to Pastor-Navarro et al.’s work and this work presents more broad dynamic range in MRL and good recovery. The commercially available ELISA test kit from r-biopharm (r-biopharm AG, Darmstadt, Germany) [22] shows good recovery. The commercially available ELISA test kit from r-biopharm (r-biopharm AG, Darmstadt, Germany) [22] shows good recovery. The commercially available ELISA test kit from r-biopharm (r-biopharm AG, Darmstadt, Germany) [22] shows good recovery. The commercially available ELISA test kit from r-biopharm (r-biopharm AG, Darmstadt, Germany) [22] shows good recovery.

### Table 1 – Cross-reactivity (%) of reactants in honey

<table>
<thead>
<tr>
<th>Reactants</th>
<th>50% B/B0 (M)</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>4.0 × 10(^{-6})</td>
<td>100.0</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>3.5 × 10(^{-6})</td>
<td>1</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>7.9 × 10(^{-6})</td>
<td>&lt;1(^a)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>1.8 × 10(^{-5})</td>
<td>&lt;1(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Less than 1%.

### Table 2 – Recovery study performed on ELISA method for TC determination\(^a\)

<table>
<thead>
<tr>
<th>Sample tetracycline added in honey (µg L(^{-1}))</th>
<th>Honey from acacia flower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D. (µg L(^{-1}))</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>24</td>
<td>23 ± 1.8</td>
</tr>
<tr>
<td>121</td>
<td>113 ± 0.257</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample tetracycline added in honey (µg L(^{-1}))</th>
<th>Honey from many flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D. (µg L(^{-1}))</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>15</td>
<td>14.6 ± 0.2</td>
</tr>
<tr>
<td>24</td>
<td>26 ± 1.2</td>
</tr>
<tr>
<td>121</td>
<td>119 ± 10.5</td>
</tr>
</tbody>
</table>

\(^a\) Absorbance values measured at 450 nm (n = 10).
and simplicity and can easily be used for determination of TCs in workplace directly.

Acknowledgements

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References