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Novel method for the determination of tetracycline antibiotics in bovine milk based on digital-image-based colorimetry

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ABSTRACT

Solid-phase extraction was used to increase the intrinsic colour of tetracycline (TC), so that colorimetry could be employed as an analytical method for the detection of TC in milk. Digital-image-based colorimetry, a measurement based on capturing colour of solutions by a webcam camera, was coupled with an artificial neural network learning model for quantitative chemical analysis. The red, green, and blue values were used for data processing and were calibrated using values of standard solutions (from a database). The results were obtained directly as TC concentration. The colour intensity of the extracts was tested for accuracy using samples spiked with three TC concentrations. The average percentage recoveries were favourable in all samples and the relative accuracy of the method was between 91.9% and 105.0%. The method was validated by comparing the results with measurements performed using ultra-violet/visible spectrophotometry. There were no significant difference observed between the two methods.

1. Introduction

Tetracycline antibiotics (oxytetracycline, OTC, tetracycline, TC, chlortetracycline, CTC, and doxycycline, DTC) are broad-spectrum antibiotics that are active against both Gram-positive and Gram-negative bacteria. In dairy farming, TCs are widely used as feed supplements to prevent or treat mastitis and metritis in cows (Jian, James, & Jack, 2012). Due to their cost-effectiveness, good oral absorption, and broad-spectrum activity, TCs are widely used as veterinary drugs. The rate of metabolism of TCs has been estimated to be 25–75% in dairy cows, and a significant amount of TCs is transferred into bovine milk; consequently, they can affect human consumers. The adverse effects on consumers include liver damage, possible allergic reactions, yellowing of teeth, and gastrointestinal disturbance (Jose, Johanan, Karina, Israel, & Jose, 2010). To prevent any health problems in consumers, the European Union (EU) and Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) have established a maximum residue limit (MRL) in milk of 100 μg L^{-1} for TC, OTC, and CTC (EC, 1999; WHO, 1990). The US Food and Drug Administration (FDA) has set the tolerance of TC, OTC, and CTC in milk of 300 μg L^{-1} (FDA, 2005).

The residues of TCs in milk samples have been determined using various techniques such as immunoassays, high performance liquid chromatography (HPLC), capillary electrophoresis, flow injection analysis, electrochemistry, fluorescence spectrometry, and mass spectrometry. Electrochemistry was found to be a suitable technique, which could be performed without sample pre-concentration, when combined with immunosensors that exhibit important advantages such as specificity, and low limit of detection in TC analysis (Felipe et al., 2013, 2014). Nevertheless, there are some disadvantages, including a complicated and a time-consuming procedure. Thus, HPLC is still useful for sensitive, accurate and highly specific TC analysis (Zhao, Zhang, & Gan, 2004). HPLC equipment and various components are often expensive, leading to high purchase and maintenance costs. In addition, this technique requires large amounts of solvents that may be toxic, and it is time-consuming.

Consequently, digital-image-based colorimetry (DIC) was chosen instead of HPLC for the analysis of tetracycline residues in milk samples. DIC is a technique that relies on the solution colour

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photographed by a webcam camera with charge-coupled devices (CCDs) sensor (Stéfani et al., 2013), a digital camera with complementary metal-oxide semiconductor (CMOS) sensor (Aree & Niamh, 2011), and a mobile phone with optical sensor (Sarun, Kosom, & Yuttana, 2014).

Although there are various methods of image data processing obtained from DIC, an artificial neural network (ANN) was chosen in this work. An ANN learning model is a computational model for information processing based on the structure and functions of biological neural networks. Information processing in the ANN happens through neural network changes, or learning, in a sense, based on the flow of information from input to output through the network structure of ANN. The ANN method is widely used in prediction and classification based on learning from examples (Durairaj & Thamilselvan, 2013).

Compared with HPLC, DIC-ANN offers many advantages, including decreased time for analysis (high speed), decreased solvent use, low costs, and no need for a standard curve in every analysis. Nevertheless, both HPLC and DIC-ANN need sample preparation, such as solid-phase extraction (SPE), before analysis. SPE has been applied for isolation of TCs from complex matrices (Hendrik & Herman, 2007; Luciana, Alejandro, & Héctor, 2010; Wang, Yang, & Cheng, 2007).

The objective of this study was to develop the SPE process for preconcentration, clean-up and increasing colour of TC for milk samples before analysis by DIC-ANN, to provide a simple and fast method for routine regulatory analysis of TC residues in bovine milk. Finally, the results from DIC-ANN were compared with those from UV-visible spectrophotometry.

2. Materials and methods

2.1. Chemicals and reagents

A standard stock solution (1000 μg mL^{-1}) of TC (Merck, Darmstadt, Germany) was prepared by dissolving TC hydrochloride in methanol (RCL Labscan, Bangkok, Thailand), and stored in dark glass bottles. The working standard solutions were prepared weekly by dilution of the stock solution with 5.0 M hydrochloric acid in methanol to produce calibration standards of concentration 1.0, 3.0, 5.0, 7.0, and 9.0 μg mL^{-1}.

The trichloroacetic acid (TCA; Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) solution was prepared in deionised water (ELGA Ltd., High Wycombe, Buckinghamshire, England). This solution was used for sample preparation to remove the proteins in the bovine milk samples.

2.2. Milk sample preparation

Milk samples were randomly collected from supermarkets in Phitsanulok, Thailand. The samples were classified into 3 types; UHT milk (ultra-high temperature milk or ultra-heat treated milk) (three different brands), pasteurised milk (three different brands), and sterilized milk (one brand).

Ten boxes of each brand of each type of milk were mixed together and a 5.0 mL sample was transferred into a 15.0 mL centrifuge tube. Five millilitres of 10.0% (v/v) trichloroacetic acid were added to the milk sample followed by shaking for 5 min and centrifugation at (2150× *g*) for 10 min. The supernatant was filtered through a 0.45 μm nylon membrane and passed through a SPE cartridge (VertiPak C18, 6.0 mL, 500 mg Vertical Chromatography Ltd., Nonthaburi, Thailand) which was conditioned by washing sequentially with 4.0 mL of methanol, 4.0 mL of water, and 4.0 mL of 10.0% (v/v) trichloroacetic acid. The sample extract was passed through the C18 SPE tube at an average flow rate of 1.0 mL min^{-1}. The column was dried while drawing full vacuum after washing with 4.0 mL of water. The TC residue was gravity-eluted with methanol, and the extract was evaporated under a flow of nitrogen to 2.5 mL and measured with an UV-visible spectrophotometer and DIC-ANN.

2.3. Digital-image-based colorimetry

DIC using a CMOS camera as a detector [Microsoft (Thailand) Ltd., Bangkok, Thailand] coupled with back propagation neural network (BPNN; Nontawat, Yuthapong, & Prinya, 2013) was applied for the analysis of TC residue in milk samples. This method was based on the red, green and blue (RGB) values of different colour intensities of sample and standard solutions. The coloured solutions were photographed with a webcam camera at a resolution of 1280 × 720 pixels. The brightness of the illuminating white LEDs was 300 lux, which is the optimum condition for photography. The colour pictures of TC standard solutions were used as the learning database and were used for testing the samples. The concentrations of sample solutions are reported in units of μg mL^{-1}.

2.4. Ultra-violet/visible spectrophotometry

All absorption spectral recordings and absorbance measurements were performed on a double beam V-650 UV-visible spectrophotometer (Jasco, Tokyo, Japan) by monitoring the absorbance changes in the wavelength region 200–600 nm. Matched sets of 10-mm cells quartz cuvette were used throughout. The optimum wavelength for TC determination was established as 432 nm. A yellow soluble TC was present in all samples. Sample solutions were measured at 432 nm for all TC concentrations.

3. Results and discussion

3.1. Comparison of solvent for forming colour of tetracyclines

In general, tetracycline analysis by spectrophotometry needs colour-forming reagents such as p-N,N-dimethylphenylenediamine and sodium meta periodate (Elisha, Mohammad, Deepthi, Ben, & Beulah, 2011), chloramine-T (José, Flávio, Mayara, Helena, & Leonardo, 2010), or metal ions (Sultan, Al-Zamil, & Al-Alarfaj, 1988). The complexation between metal ions and TC was used as a basic reaction for the determination of TC by spectrophotometry. However, this method uses chemicals as reagents, which can be environmentally hazardous and add costs to the analysis. Thus, in this study of the application of DIC to detect the TC in milk, the intrinsic colour of TC was used. Solvents used to dissolve TC include hydrochloric acid in water (Chico, Meca, Companyó, Prat, & Granados, 2008), hydrochloric acid in methanol (Zhao et al., 2004), methanol (Luciana et al., 2010), 1.0% (v/v) acetic acid (Naoto, 2003), distilled water (Wang et al., 2007), deionised water (Tsai et al., 2010), and ultrapure water (Hongliang et al., 2013). Hence, 10 different solvents were studied in this work to select the optimal one giving suitable colour of TC that could be taken by the webcam. The solvents were mineral water, deionised water, methanol, 0.1, 1.0, 3.0 and 5.0 M hydrochloric acid in deionised water, 5.0 M hydrochloric acid in methanol, and 1.0 and 10.0% (v/v) acetic acid. The TC standard of 25.0 μg mL^{-1} was dissolved in those solvents and then detected with digital-image-based colorimeter and UV-visible spectrophotometer.

Mineral water (Pranutpong & Wirat, 2012) was used as a green reagent for colour formation with TC because mineral water contains metal ions such as calcium(II), magnesium(II), copper(II), sodium(I), and iron(III). Mineral water could be used as complexing agent; however, the colour of the TC complex is not appropriate to