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Mixed micelle-cloud point extraction for the analysis of penicillin residues in bovine milk by high performance liquid chromatography

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ABSTRACT

A mixed micelle-cloud point extraction (MM-CPE) has been developed for the analysis of penicillin antibiotics (ampicillin, penicillin G, oxacillin, and cloxacillin) in milk samples using Triton X-114 (TX-114) and cethyl trimethylammonium bromide (CTAB) as the mixed micellar extractant. The parameters affecting the MM-CPE that were investigated including solution pH, CTAB concentration, TX-114 concentration, electrolyte salt, equilibration temperature and incubation time. The optimum MM-CPE conditions were: 10 mmol L^{-1} phosphate buffer pH 8, 0.06% (w/v) CTAB, 1.5% (w/v) TX-114, and 7% (w/v) Na_2SO_4, and 5 min equilibration at 40 °C. The separation of penicillins was achieved within 8 min under the HPLC conditions: a Vydac C_{18} column, isocratic elution of 5 mmol L^{-1} phosphate buffer (pH 6.6) and methanol (55:45, v/v), and a flow rate of 1 mL min^{-1}, with photodiode array detection at 215 and 244 nm. Under the selected condition, the proposed method gave linear calibrations in the range 0.002–10 μg mL^{-1} with correlation coefficients greater than 0.999. Limits of detection (LOD) were 2–3 ng mL^{-1}, and 15–40-fold enhancement compared to that without preconcentration. Good reproducibility was achieved with relative standard deviation <5% for peak area and <3% for retention time. High accuracy, with recoveries higher than 80%, was obtained. The proposed mixed micelle-CPE-HPLC method has shown to be of high potential for the analysis of penicillin residues in milk with LOD comparable to the established maximum residue limits (4–30 ng mL^{-1}).

1. Introduction

Penicillins are β-lactam antibiotics which are widely used in livestock for treatment of bacterial infections caused by Gram-positive and Gram-negative organisms, promote growth and maintain animal health [1]. They include cloxacillin (CLO), ampicillin (AMP), oxacillin (OXA), and penicillin G (PEN-G). CLO is formulated for use in cows at the point of milk production to treat existing mastitis and to protect further infections during the dry period [1]. AMP and CLO are used against both Gram-positive and Gram-negative organisms, especially caused by penicillinase-producing staphylococci [1–3]. OXA is the penicillinase-resistant penicillin, while PEN-G is the only natural penicillin available in the market and is one of the most commonly misused drugs in steers and dairy cows [1]. The systematic use of antibiotics may leave residues in derived, food including milk. The antibiotic residues can have undesirable effects on consumer health such as allergies and the appearance of drug-resistant strains [4]. To protect the health of consumers, the maximum residue limits (MRLs) of antibiotics in milk have been regulated by authorities including the Food and Drug Administration (FDA) and European Council Regulation (ECC) 2377/90 [5,6]. The MRLs for antibiotic residues in milk and muscle are in the range of 4–125 ng mL^{-1} depending on the specific type of antibiotic. Thus, the analytical methods with the following characteristics are in demand: accurate, ease to use, economical in terms of cost and time, and capable of detecting the residues below MRLs.

Several analytical methods have been developed for the analysis of antibiotic residues analysis in dairy products such as microbial assays based on inhibition of microbial growth [7,8], liquid chromatography–mass spectrometry (LC–MS) [9–11], and capillary electrophoresis (CE) [9,12]. However, microbial assays fail to identify and quantify of individual residues, while LC–MS is very expensive and CE when equipped with UV detection is low sensitivity because of its short optical path length. High performance liquid chromatography (HPLC) with photodiode array (PDA) or UV detection is widely used as a sensitive method for the analysis of antibiotics and metabolites in biomatrices [5–9,13–17]. To obtain the reliability and accuracy of the detection in complex matrices, suitable sample preparation methods are required before instru-

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mental analysis. The most popular techniques for the extraction of antibiotics from their original matrices are liquid–liquid extraction or solvent extraction [9,18,19], solid-phase extraction (SPE) [10–12,20–22], dispersive solid-phase extraction (DSPE) [23,24] and pressurized liquid extraction (PLE) [6,9]. Recently, extraction method using surfactants, known as cloud point extraction (CPE), is a promising new separation and preconcentration technique that is being applied to a range of analytes in different sample matrices [25–29]. The advantages of CPE over traditional solvent extraction are good extraction efficiency, low cost, and use of non-toxic reagents, and less toxic organic solvents [30,31]. In addition, CPE shows good compatibility between the surfactant and the hydro-organic mobile phase in the analysis using HPLC [30,31]. CPE provides high extraction efficiency and a large preconcentration factor because a relatively small volume of surfactant-rich phase (SRP) is obtained compared to that of the original aqueous solution (AQ). This methodology is most effective for hydrophobic solutes [25,32].

To apply CPE for hydrophilic analytes such as penicillin antibiotics, a modified condition is needed. Mixed micelle-cloud point extraction has been possible by the addition of a small amount of the cationic cethyl trimethylammonium bromide (CTAB) or anionic sodium dodecyl sulphate (SDS) surfactants into the original non-ionic surfactant CPE system. Mixed surfactants of different charges are used in order to achieve both ideal hydrophobic and non-ideal electrostatic interactions within the same extraction system [33]. Mixed micelle-CPE or mixed micelle mediated extraction has been used for preconcentration of organic compounds [33–35] and metal cations [33,36]. The combined use of cationic surfactant with non-ionic surfactant has been documented to facilitate an increase in the extraction efficiency of polar organic compounds [30,37]. Until now, the application of CPE has never been reported for preconcentration of β-lactam antibiotics.

The propose of this study is to develop CPE based on mixed micellar extractants of Triton X-114 and CTAB for extraction and preconcentration of penicillin antibiotics (ampicillin, penicillin G, oxacillin and cloxacillin) in milk samples prior to analysis by HPLC. The parameters affecting CPE include pH of sample solution, concentration of reagents (CTAB, Triton X-114 and electrolyte salt), equilibration temperature and incubation time, have been optimized.

2. Experimental

2.1. Chemicals and reagents

All the penicillin standards were purchased from Fluka, including the penicillin G sodium salt (Austria), ampicillin trihydrate (Belgium), oxacillin sodium salt (India), and cloxacillin (USA). The stock solutions of the penicillin standards (1000 μg mL^{-1}) were prepared by dissolving each in an appropriate amount in deionized water and stored at 4 °C. The working solutions were freshly prepared by diluting the stock solutions with water. Triton X-114 was purchased from ACROS Organics (USA). The stock solution of Triton X-114 (25%, w/v) was prepared by dissolving it in deionized water. Cethyl trimethylammonium bromide (CTAB) was obtained from Fluka (Denmark). The stock solution of CTAB (2%, w/v) was prepared in deionized water. Table 1 shows the chemical structures and physical properties of the studied penicillins and surfactants. Sodium chloride (NaCl) (APS Fine Chem, Australia) and sodium sulfate anhydrous (anhydrous Na_2SO_4) (Fluka, Switzerland) were also used, along with disodium hydrogen phosphate (Na_2HPO_4) from Merck (Germany). Deionized water with the resistivity of 18.2 MΩ cm from RiO_s^{TM} Type I Simplicity 185 (Millipore water, USA) was used throughout. Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade (Lab-Scan Asia, Co., Ltd, Thailand).

2.2. Instrumentation

The HPLC system consisted of a Waters 600E Multi-solvent Delivery System, a Waters In-Line Degasser AF, a Rheodyne injector with sample loop of 20 μL, and a Waters 2996 Photodiode Array Detector. Empower Software was used for data acquisition and analysis. A Vydac C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm) from Dionex (USA) coupled to a guard column was used. A centrifuge (Kokusan Type H-11N, Biomed Group Co. Ltd., Japan) was used for phase separation.

2.3. Mixed micelle cloud point extraction

Sample or standard solutions (3.00 mL) were mixed with phosphate buffer (pH 8) and CTAB and left for 5 min. Afterward, Na_2SO_4 and Triton X-114 solution were added, and made up to a final volume of 10.00 mL with water. The mixture solution was transferred to a test tube, equilibrated at 40 °C for 5 min, and then centrifuged at 3000 rpm for 20 min. The solution was kept in an ice bath for 10 min. The aqueous phase (lower phase) was then removed by a long-needled syringe. A surfactant-rich phase (600 μL) was mixed with 300 μL of MeOH and ACN (1:1, v/v) to reduce the viscosity before injecting into HPLC. The concentrations of the reagents used were optimized.

2.4. Analysis of milk samples

Commercial milk samples were obtained from a local market in Khon Kaen province, northeastern Thailand, and fresh milk samples were obtained from the dairy shelf of the store at the Faculty of Agriculture, Khon Kaen University. The proteins and fats in 3.00 mL samples were precipitated by shaking vigorously with 6 mL of a mixture of acetone and acetonitrile (5:1, v/v). The solution was then centrifuged at 4000 rpm for 20 min. The supernatant was evaporated in a nitrogen atmosphere to eliminate organic solvents. The residue was dissolved with phosphate buffer to a final volume of 3.00 mL and finally extracted by mixed micelle-cloud point extraction (see Section 2.3).

3. Results and discussion

3.1. HPLC conditions for analysis of penicillins

The separation of four penicillins including ampicillin (AMP), penicillin G (PEN-G), oxacillin (OXA) and cloxacillin (CLO) was carried out using isocratic elution of 5 mmol L^{-1} phosphate buffer (pH 6.6) and MeOH (55:45, v/v) at a flow rate of 1.0 mL min^{-1}. The photodiode array detection was accomplished at 215 nm for AMP and PEN-G and 244 nm for OXA and CLO. Experiments were performed at ambient temperature. Under the optimum conditions, the studied penicillins were separated within 8 min with the elution order of AMP, PEN-G, OXA, and CLO, respectively (see Fig. 1). The resolution of all the studied penicillins was greater than 2.3.

3.2. Optimization of mixed micelle cloud point extraction

Mixed micelle-cloud point extraction was chosen for extraction of the target analytes since the studied penicillin antibiotics are quite polar and pH-sensitive. The pK_{a1} (–COOH) of the studied penicillins is about 2.4 [40]. Under normal conditions, the studied analytes are anions and highly soluble in aqueous solution, leading to poor extraction efficiency in CPE. Consequently, the cationic ion-pair reagent, CTAB, was used in this study forms the ion-pair of penicillin-CTAB before CPE. The penicillin-CTAB ion-pair can transfer effectively into the aggregates of Triton X-114 compared to the original polar forms, leading to higher extraction efficiency. Thus,