Food Anal. Methods (2015) 8:1079–1087
DOI 10.1007/s12161-014-9991-7

Dispersive Micro-Solid Phase Extraction Combined with High-Performance Liquid Chromatography for the Determination of Three Penicillins in Milk Samples

Noorfatimah Yahaya · Mohd Marsin Sanagi · Takahito Mitome · Norikazu Nishiyama · Wan Aini Wan Ibrahim · Hadi Nur

Received: 26 April 2014 / Accepted: 2 September 2014 / Published online: 11 September 2014
© Springer Science+Business Media New York 2014

Abstract A simple, rapid, and environmentally friendly microextraction termed COU-2-D-μ-SPE has been developed for the analysis of three penicillins (i.e., oxacillin, cloxacillin, and dicloxacillin) in milk samples. An ordered mesoporous carbon, COU-2, was synthesized and used as the sorbent in dispersive micro-solid phase for the extraction of selected penicillins prior to high-performance liquid chromatography-ultraviolet detection (HPLC-UV). Several important parameters, namely, pH value, salt addition, desorption solvent, extraction time, desorption time, and amount of COU-2, were investigated and optimized. Under the optimum extraction conditions, the method showed good linearity in the range of 10–5,000 μg L^{-1} ($r^2 \geq 0.9994$), low limits of detection (2.0–3.3 μg L^{-1}), acceptable reproducibility (relative standard deviation (RSD) 6.2–8.8 %, $n=9$), and satisfactory relative recoveries (80.3–99.5 %) for studied penicillins in milk. The proposed COU-2-D-μ-SPE method has been successfully applied to six commercial milk samples, and the extraction of blank samples indicated that all samples were free from selected penicillin contamination.

Keywords Liquid chromatography · Penicillins · Microextraction · Dispersive micro-solid phase extraction · Mesoporous carbon · Milk

Introduction

β-Lactam antibiotics have been widely used for more than 80 years and still constitute the most important group of antibiotics as antimicrobial drugs (Benito-Peña et al. 2006). β-Lactams, including penicillins and cephalosporins, are frequently used to treat mastitis, a disease which produces significant economic losses that very often occur in animal with high milk production (Bailón-Pérez et al. 2009; Karageorgou et al. 2012). However, drug residues from the excessive use of antibiotics may cause allergic reactions in hypersensitive individuals or they may lead to drug-resistant bacteria (Karageorgou and Samanidou 2011). European Commission has established the maximum residue limits (MRLs) for three penicillins in this study, namely, oxacillin (OXA), cloxacillin (CLOX), and dicloxacillin (DICLOX), in milk product that is 30 μg L^{-1} (Commission Regulation (EU) No 37/2010 of 22 December 2009). Therefore, it is important to develop a sensitive, rapid, and reliable sample preparation method to satisfy the need for the determination of the selected penicillin residues in milk samples. The chemical structures, pK_a, molecular weight, and MRLs of selected penicillins are shown in Table 1.

Penicillins have been mostly determined by liquid chromatography with mass spectrometry or tandem mass spectrometry (HPLC-MS/HPLC-MS/MS) (Pozo et al. 2006; Turnipseed et al. 2008; Zhang et al. 2010; Freitas et al. 2012), high-performance liquid chromatography with photodiode array

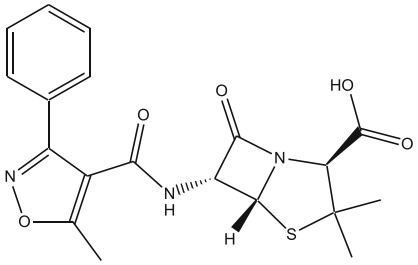
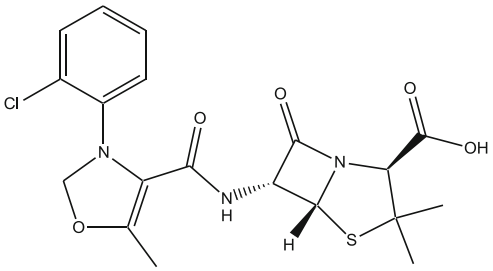
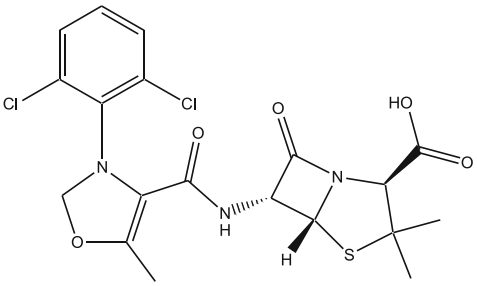
N. Yahaya · M. M. Sanagi · W. A. W. Ibrahim
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

N. Yahaya
Advanced Medical and Dental Institute, Universiti Sains Malaysia, No. 1-8, Persiaran Seksyen 4/1, 13200 Bandar Putra Bertam, Kepala Batas, Pulau Pinang, Malaysia

M. M. Sanagi (✉) · H. Nur
Ibnu Sina Institute for Fundamental Science Studies, Nanotechnology Research Alliance, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia
e-mail: marsin@kimia.fs.utm.my

T. Mitome · N. Nishiyama
Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan

Table 1 Chemical structures and pK_a values of selected penicillins

Name	Structure	pK_a
Oxacillin (OXA)		2.72
Cloxacillin (CLOX)		2.78
Dicloxacillin (DICLOX)		3.75

Source: 'The Drugbank Database' http://www.drugbank.ca (Accessed on 16th May 2013) and Commission Regulation (2010).

Source: "The Drugbank Database" http://www.drugbank.ca (accessed on 16th May 2013) and Commission Regulation (EU) no. 37/2010 of 22 December (2009)

(HPLC-PDA) or ultraviolet detectors (HPLC-UV) (Oliveira et al. 2007; De Baere and De Backer 2007; Bovee and Pikkemaat 2009; Bailón-Pérez et al. 2009), and capillary electrophoresis with ultraviolet detector (CE-UV) (Santos et al. 2007). Beside the classical liquid–liquid extraction (LLE) (Gramse and Jacobson 2005; Douša and Hosmanová 2005; Kantiani et al. 2009; Jank et al. 2012) and solid phase extraction (SPE) (Bruno et al. 2001; Marchetti et al. 2001; Riediker and Stadler 2001; Benito-Peña et al. 2006; Bailón-Pérez et al. 2009; Stolker et al. 2008; Maggi et al. 2012), several other popular strategies of sample preparation for penicillins have been reported including pressurized liquid extraction (PLE) (Carretero et al. 2008), mixed micelle cloud point extraction (MM-CPE) (Kukusamude et al. 2010), magnetic molecularly imprinted polymer (MMIP) (Zhang et al. 2010), dispersive SPE (DSPE) (Karageorgou et al. 2012; Rezende et al. 2012), ion pair extraction (IPE) (Kukusamude et al. 2012), and stir bar sorptive extraction (SBSE) (Huang et al. 2013). However, to the best of our knowledge, the development of microextraction methods to extract penicillins from milk is still limited. Recently, another attractive sorbent-based microextraction technique termed dispersive micro-SPE (D-μ-SPE) has been successfully applied to a range of analytes including polycyclic aromatic hydrocarbons (PAHs) (Galán-Cano et al. 2011; Jiménez-Soto et al. 2012), nitrosamines (Fu et al. 2012), and synthetic polycyclic musks (Chung et al. 2013) from water samples. D-μ-SPE was another mode of DSPE performed by trapping the analytes of interest into the dispersive sorbent before transferring the analytes to a smaller volume of desorption solvent after discarding most of liquid and drying (Tsai et al. 2009). D-μ-SPE allows the analytes in aqueous sample to interact equally with all the sorbent particles to achieve greater capacity per mass of sorbent used and thus avoiding channeling or blocking which easily occur in conventional SPE column or disks. The advantages of D-μ-SPE procedure over traditional SPE are simple, use of smaller quantity of sorbent and organic solvent, and less time-consuming (Fu et al. 2012).

Porous carbon materials are used extensively as sorbent for separation processes and gas storage, electrode material for batteries, fuel cells and as support for many important catalytic processes. The applications of porous carbon as promising

materials have gained increasing research interest that is directly related not only to their superior physical and chemical properties such as electric conductivity, thermal conductivity, chemical stability, and low density, but also to their wide availability (Liang et al. 2008). Due to their distinct properties, there has been growing interest in the application of porous carbon as sorbent in sample preparation techniques. They have been successfully employed as sorbent in SPE of lead ion from water samples (Moradi and Baniamerian 2011); solid phase microextraction (SPME) coatings for benzene, toluene, ethylbenzene, *o*-xylene (BTEX), and phenols from water samples (Zhu et al. 2010); and SPME coatings for phthalate esters (PAEs) and PAHs from PVC food wrap samples (Sun et al. 2013). A mesoporous carbon designated as COU-2 was synthesized by a direct synthesis using soft-templating method using a triblock copolymer, Pluronic F127 as a soft template for organic–organic self-assembly of RF-F127 composites. COU-2 exhibits a worm-like mesostructures with highly ordered mesopore structure (Jin et al. 2009). In addition, the starting material to synthesize COU-2, Pluronic F127, has a low toxic characteristic which is environmentally friendly (Jung et al. 2010). More recently, a newly developed D-μ-SPE with mesoporous carbon COU-2 was employed in our laboratory to extract CLOX from water samples (Yahaya et al. 2013). Nevertheless, the application of COU-2-D-μ-SPE in complex matrix such as milk sample remains unexplored.

In the present work, COU-2 mesoporous carbon was utilized as dispersive sorbent in D-μ-SPE for the analysis of three penicillins in milk samples prior to their determination by HPLC-UV. Several important experimental parameters affecting the extraction efficiency of COU-2-D-μ-SPE, namely, sample pH, salt addition, desorption solvent, extraction time, desorption time, and amount of COU-2 sorbent, were investigated and optimized. The results revealed that the proposed method can be applied to the determination of studied penicillin residue in milk samples.

Materials and Methods

Chemicals and Reagents

A triblock copolymer, Pluronic F127, was purchased from BASF (Ludwigshafen, Germany). Resorcinol, ethanol, formaldehyde, and hydrochloric acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Sodium hydroxide (NaOH) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). OXA, CLOX sodium salt monohydrate, and DICLOX standards were all purchased from Sigma (St. Louis, MO, USA). Sodium chloride (NaCl) was obtained from Bendosen (Selangor, Malaysia). All chemicals were of analytical reagent grade. HPLC grade acetonitrile and isopropanol were purchased from J.T. Baker (Pennsylvania, USA). Methanol and acetone were obtained from QRëC Asia (Selangor, Malaysia). Milli-Q water (resistance ≥18.2 MΩ) was produced by Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of OXA, CLOX, and DICLOX (1,000 μg mL^{-1}) were prepared in Milli-Q water and stored at 4 °C in amber vials. The stock solutions prepared were stable for at least a month. Working solutions of β-lactam antibiotics were also prepared in Milli-Q water.

Synthesis and Characterization of COU-2

For synthesis part, COU-2 was synthesized using molar compositions and synthesis conditions as described in the previous report (Jin et al. 2009). The molar composition of the starting solution was 0.00792, F127 14.5, EtOH 6.5, water 1, resorcinol 1.2, and formaldehyde 0.25 HCl. For COU-2 preparation, 8 g of Pluronic F127 was dissolved in 160 g of ethanol solution, and then, 26.4 g of resorcinol (R) was added to the solution. The mixture was then stirred for 30 min. After complete dissolution of F127 and resorcinol, 24 g of formaldehyde (37 wt%) (F) was added to the solution, and the solution was stirred for 30 min. Last, 12 mL of HCl solution (5 N) was added to the solution as a catalyst for polymerization. On stirring at room temperature for 72 h, the precursor solution was separated into two phases where the transparent upper phase was ethanol–water-rich and the yellow lower phase was polymer-rich precipitate. The precipitate consisting of RF-F127 self-assembly was separated from the solution by filtration and then dried in an oven at 90 °C for 24 h. The RF-F127 composite was carbonized under a nitrogen atmosphere at 400 °C for 3 h at a heating rate of 1.3 °C min^{-1}, followed by further carbonization at 800 °C for 3 h at a heating rate of 1.7 °C min^{-1}. For characterization part, the nitrogen adsorption/desorption isotherm of the mesoporous carbon was measured at −196 °C using an AUTOSORB-1 instrument (Quantachrome Co.). The total surface area was calculated using the Brunauer–Emmett–Teller (BET) method, while the pore volume was calculated using the Barrett–Joyner–Halenda (BJH) method based on previously reported methods (Jin et al. 2009).

Sample Collection and Pretreatment

Milk samples from different sources were randomly purchased from the local market (Johor Bahru, Malaysia). An aliquot of milk sample (1 mL) was transferred into 15-mL centrifuge tube and spiked with different concentration levels of mixed standard solutions. The mixture was vortexed (Heidolph, Germany) to ensure complete mixing of contents. Then, the solution was added with 2 mL of acetone–acetonitrile (1:1, *v*/*v*) and subsequently centrifuged (Hettich, UK) at 3,641×*g* force for 10 min to remove proteins and fats from the

milk sample. Finally, the supernatant (~3 mL) was transferred to a 50-mL centrifuge tube and then diluted with 2 mL of 100-mM phosphate buffer (pH 2) and 15-mL distilled water to a total volume of 20 mL.

D-μ-SPE Procedure

The extraction procedure consists of several simple steps. First of all, diluted milk sample (20 mL) containing the analytes was placed in a 50-mL centrifuge tube containing 75 mg of COU-2 sorbent and 1 g of sodium chloride (NaCl). Then, the mixture of sorbent, salt, and sample was agitated using a vortex (2,500 rpm) for 1.5 min to facilitate the dispersion of COU-2 in the sample with 5 % *w*/*v* salt addition. After extraction, the mixture was then centrifuged at 1,790×*g* force for 2 min to form a separate layers of aqueous (sample solution) and solid (COU-2 sorbent) phases. The aqueous phase was discarded, and only COU-2 sorbent remains in the centrifuge tube. In order to desorb the analytes, methanol (100 μL) was added to the centrifuge tube containing COU-2, and the tube was ultrasonicated for 3 min. The solution was filtered through 0.2-μm nylon syringe filter (Membrane Solutions, China) prior to analysis by HPLC system. Photographs of COU-2-D-μ-SPE are shown in Fig. 1.

Chromatographic Conditions

Analysis was performed on an Agilent 1220 LC System VL (CA, USA) equipped with multisolvent delivery system, degasser, and ultraviolet detector. Chromatographic separation was performed on an Agilent Zorbax SB-C_{18} column (100 mm×4.6 mm, 5.0-μm particle size) (CA, USA). Isocratic elution was used for chromatographic separation in which mobile phase consisted of 5-mM phosphate buffer (pH 6.0) and acetonitrile (75:25, *v*/*v*) at a flow rate of 1.0 mL min^{-1}. The UV detection was measured at 215 nm. A 20-μL aliquot of the clean extract was injected into the HPLC. Under the optimum conditions, the studied penicillins were separated within 10 min with the elution order of OXA, CLOX, and DICLOX, respectively.

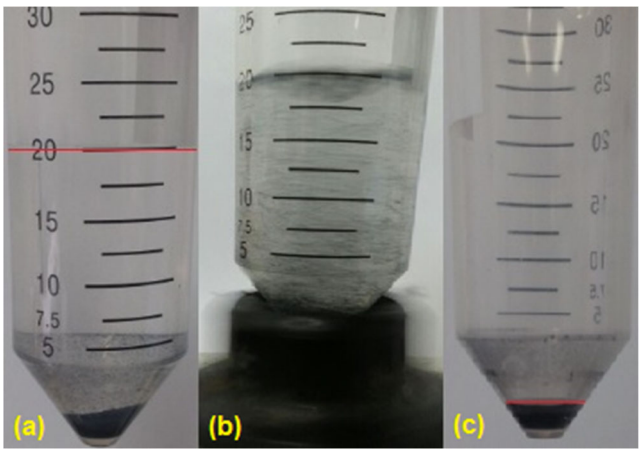


Fig 1 Photographs of COU-2-D-μ-SPE: **a** COU-2 in sample solution, **b** extraction of analytes using vortex system, and **c** desorption of analytes from the COU-2 sorbent (*horizontal lines* indicate the levels of solutions)

Results and Discussion

Characterization of COU-2

The quality of the mesoporous carbon, COU-2, prepared in this study was examined by nitrogen adsorption/desorption isotherm and total pore volume analyses. A transmission electron microscope (TEM) and X-ray diffraction (XRD) analyses of wormhole-like uniform mesopore structure with a 3D interconnected structure of COU-2 have been reported previously (Jin et al. 2009). Nitrogen adsorption/desorption isotherm and total pore volume analyses were in fairly good agreement with the previous work (Jin et al. 2009). N_2 adsorption analysis showed that the BET surface area of COU-2 was 572 m^2 g^{-1} and the total mesopore volume (2<d<10 nm) was 0.49 cm^3 g^{-1}.

Optimization of COU-2-D-μ-SPE

In order to obtain the optimum extraction conditions, six extraction parameters, namely, sample pH, salt addition, desorption solvent, extraction time, desorption time, and amount of COU-2 sorbent, were investigated. A blank milk sample that was free from OXA, CLOX, and DICLOX was selected for the optimization procedure. Optimization was carried out in triplicate analyses using a 20.0-mL sample solution spiked with 1 μg mL^{-1} each of the three studied penicillins.

Effects of Sample pH and Salt Addition

The pH of sample solution is one of the important parameters affecting the extraction efficiency of penicillins. The extraction of the analytes in their unionized forms is expected to be easier than the extraction of the analytes in their ionized form (Guo and Lee 2012). Three selected penicillins, namely, OXA, CLOX, and DICLOX, are weak acid compounds which exist in a dissociation form under neutral or alkaline environments. Therefore, the pH of the sample solution should be adjusted to acidic in order to promote the extraction in their unionized form. In this study, the sample pH was varied in the range of 1.5 to 3.0. It was found that the peak areas of studied penicillins increased with decreasing pH from 3.0 to 2.5 and reached a maximum at pH 2.0 and then slightly decreased at pH 1.5 (Fig. 2a). This was probably because the microextraction system has reached its saturation at pH 2 and a further increase in pH resulted in a decrease in peak

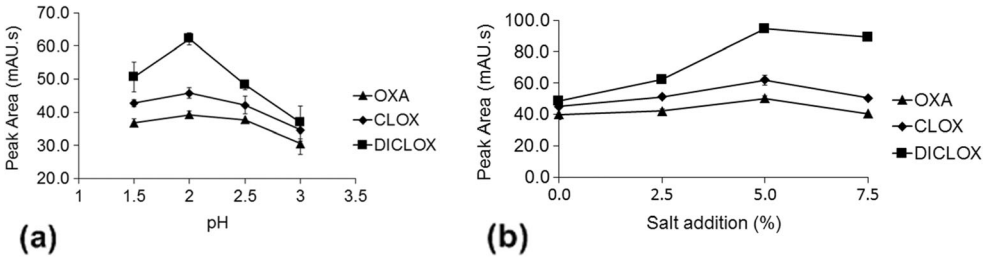


Fig 2 Effect of sample pH (**a**) and salt addition (**b**) on COU-2-D-μ-SPE from spiked milk (n=3 in each case). *Error bars* represent the standard deviations

area as all analytes started to ionize and existed in their conjugated form at sample pH higher than their pKa values. Therefore, pH 2.0 was chosen and used in this study.

The addition of salt to the aqueous sample can reduce the solubility of the polar analytes and improve extraction efficiency due to the salting-out effect. The effect of salt addition was examined by the addition of NaCl in the sample solution at 0.0, 2.5, 5.0, and 7.5 % *w/v*. The results showed that the peak areas of analytes increased when the concentration of NaCl was increased from 0.0 to 5.0 % *w/v* and slightly dropped when the concentration of NaCl was further increased (Fig. 2b). It is probably due to the high salt concentration which increased the solution viscosity, thus, led to the disruption of extraction ability of COU-2-D-μ-SPE (Saaid et al. 2009). Therefore, 5.0 % *w/v* salt addition was selected for further investigations.

Effects of Desorption Solvent and Extraction Time

In general, the selection of desorption solvent relies on its compatibility with the liquid chromatographic system. In order to investigate the effect of desorption solvent, three types of solvents that are compatible with the HPLC-UV system were employed, namely, methanol, acetonitrile, and isopropanol. Methanol and acetonitrile are polar solvent, while isopropanol is less polar. Since the studied penicillins are relatively polar, a polar solvent should give better extraction efficiency than a less polar solvent. As a result, methanol gave the highest peak areas for all analytes and therefore was selected as desorption solvent for further experiments (Fig. 3a).

Since mass transfer is a time-dependent process, it is important to establish the extraction time profiles of target analytes. The effect of extraction time on the extraction efficiency was investigated by agitating the sample solution using maximum speed of vortex at 2,500 rpm for 30, 60, 90, and 120 s. It was found that the extraction efficiency increased to a maximum as the extraction time was increased to 90 s, and after that, the peak areas of analytes slightly decreased when the extraction time was prolonged to 120 s (Fig. 3b). This occurrence might probably be due to the back extraction of analytes from sorbent into sample solution (See et al. 2010). In addition, the standard deviation (SD) values obtained for OXA, CLOX, and DICLOX at 90 s are better than those at 60 and 120 s. Hence, 90 s was selected as the optimum extraction time throughout the experiments in this study.

Effects of Desorption Time and Amount of COU-2

The effect of desorption time was examined by ultrasonication of COU-2 (after extraction) in methanol for a series of desorption times in the range of 1 to 7 min. It was found that maximum desorption time of analytes was obtained within 3 min, and thereafter, the peak areas of analytes were constant (Fig. 4a). Therefore, 3 min was chosen as the optimum desorption time.

Finally, the effect of sorbent amount on the extraction efficiency was evaluated at four different levels (25, 50, 75, and 100 mg) of COU-2 sorbent. It was found that in general, higher peak areas were obtained with increasing amount of COU-2 up to 75 mg due to the availability of the active site in COU-2. However, when higher amounts of COU-2 (100 mg) was employed in the extraction, the peak areas for all analytes remained nearly constant or decreased due to the dilution factor which incurred during the desorption of high amount of COU-2 (from 100 to 120 μL of desorption solvent that was

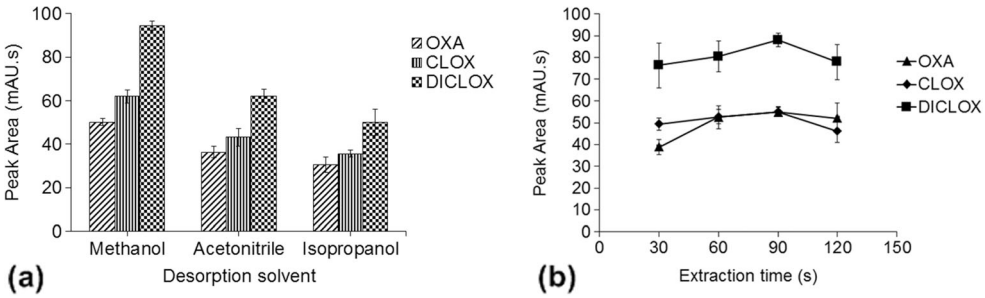


Fig 3 Effect of desorption solvent (**a**) and extraction time (**b**) on COU-2-D-μ-SPE from spiked milk (n=3 in each case). *Error bars* represent the standard deviations

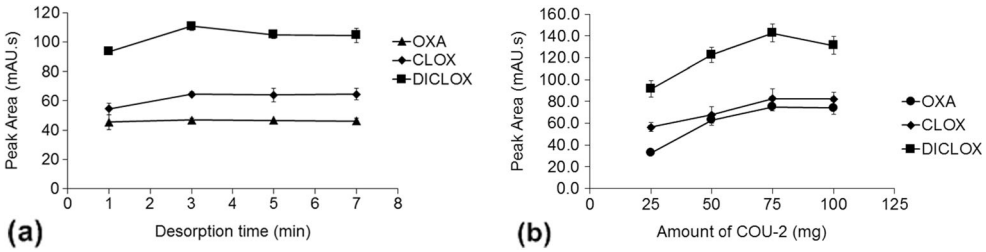


Fig 4 Effect of desorption time (**a**) and amount of COU-2 (**b**) on COU-2-D-μ-SPE from spiked milk (n=3 in each case). *Error bars* represent the standard deviations

required) (Fig. 4b). Based on the experimental data, 75 mg of COU-2 was used for the subsequent experiments.

Method Validation

Under the optimum conditions, the analytical characteristics of the proposed COU-2-D-μ-SPE method were validated in terms of linearity, limits of detection (LODs) and limits of quantification (LOQs), precisions, and recovery study.

Linearity, Limits of Detection, and Limits of Quantification

Prior to method validation, the developed method was applied to the milk samples for the analysis of selected penicillins. As a results, no OXA, CLOX, and DICLOX were detected in the milk samples. Matrix-matched calibration curves were plotted for all analytes in the concentration range of 10–5,000 μg L^{-1} using six spiked concentrations in the studied penicillin-free milk samples. Good linearity was observed for OXA, CLOX, and DICLOX with coefficients of determination, r^2 of 0.9994, 0.9999, and 0.9998, respectively. LOD and LOQ were measured from the equation: $LOD=3.3\times S_{bl}/m$ and $LOQ=10\times S_{bl}/m$, where S_{bl} is the standard deviation of ten blank determinations and m is the slope of calibration obtained from the matrix-matched calibration curve of analyte. The LODs and LOQs for OXA, CLOX, and DICLOX obtained in milk were in the range of 2.0–3.3 and 6.0–10.0 μg L^{-1}, respectively. It is revealed that the proposed method provided lower detection limits than the MRLs established by the European Commission Regulation (EU) at 30 μg L^{-1} (Commission Regulation (EU) No 37/2010 of 22 December 2009). The method has been demonstrated to serve as a useful tool for identification and quantification of the selected penicillins in milk samples. The summarized analytical characteristics of COU-2-D-μ-SPE are tabulated and shown in Table 2.

Intraday and Interday Precisions

Intraday and interday precisions of the method were examined at three different concentration levels, i.e., low (10 μg L^{-1}), medium (100 μg L^{-1}), and high (1,000 μg L^{-1}) spiked in the selected penicillin-free milk samples. Intraday and interday precision studies were carried out with triplicate analyses on the same day (n=3) and over three different days (n=9), respectively. The values for intraday and interday precisions were expressed as percentage of relative standard deviations (RSDs). The results for precision tests are shown in Table 2. Good intraday and interday precisions were obtained for OXA, CLOX, and DICLOX in milk samples, with RSD values in the range of 1.7–7.4 and 6.2–8.8 %, respectively.

Recovery Study

In order to investigate the accuracy of the method, recovery study was accomplished by spiking three different

Table 2 Validation data for target analytes in milk samples

Penicillin	LOD	LOQ	Spiked level (μg L^{-1})	Precision, RSD %		Relative recovery, % (RSD, %) (n=3)
				Intraday (n=3)	Interday (n=9)	
OXA	3.3	10.0	10	7.4	8.8	85.0 (7.4)
			100	2.2	8.5	80.3 (2.2)
			1000	5.0	6.2	98.8 (2.7)
CLOX	2.8	8.4	10	2.2	8.3	82.7 (8.8)
			100	2.6	8.8	98.5 (8.7)
			1000	4.4	7.9	89.9 (7.0)
DICLOX	2.0	6.0	10	2.7	7.3	97.9 (6.4)
			100	1.7	7.6	99.5 (7.2)
			1000	5.7	6.5	91.7 (6.3)

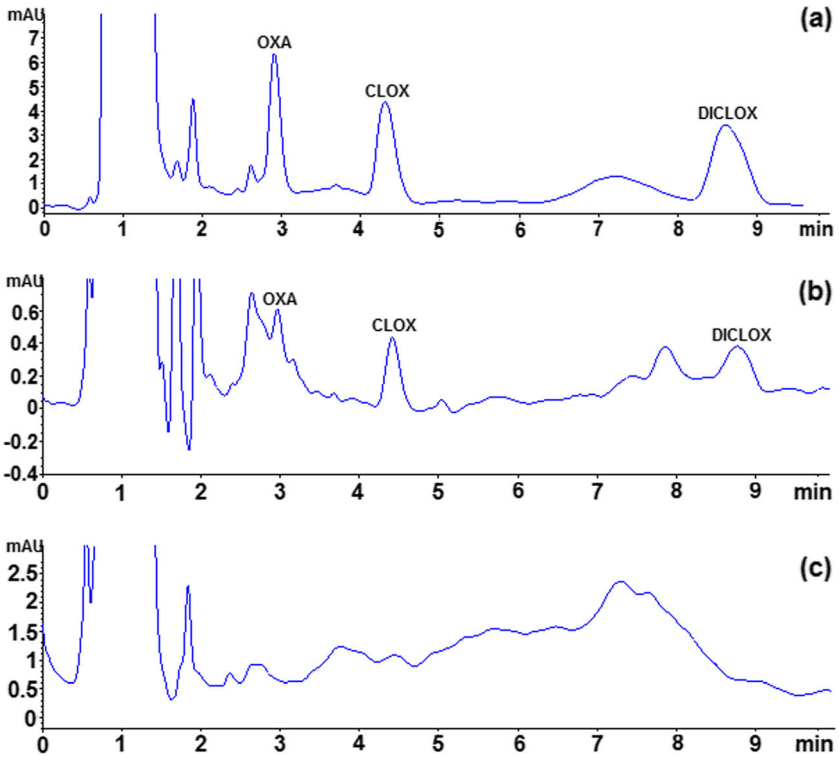


Fig 5 HPLC-UV chromatograms of spiked milk samples with OXA, CLOX, and DICLOX at 1,000 μg L^{-1} (**a**), 10 μg L^{-1} (**b**), and blank milk sample (**c**)

concentrations (10, 100, and 1,000 μg L^{-1}) of studied penicillins in milk samples. The relative recoveries of the method were calculated based on the percentage ratio between the concentration found in the sample and concentration spiked in the same sample of each analyte. The results (Table 2) showed good relative recoveries in the range of 80.3–99.5 % with RSDs ≤8.8 %. Therefore, the proposed COU-2-D-μ-SPE method has been proven to be of highly efficient method for the determination of selected penicillins in milk samples.

Applications to Milk Samples

The developed method was applied to the analysis of OXA, CLOX, and DICLOX in milk samples. Six commercial milk samples (Johor Bahru, Malaysia) were analyzed and showed negative results for all studied penicillins. Figure 5 shows the typical HPLC-UV chromatograms of spiked milk samples with OXA, CLOX, and DICLOX at 1,000 μg L^{-1} (a), 10 μg L^{-1} (b), and blank milk sample (c).

Comparison with Other Reported Methods

The analytical characteristics of the optimized COU-2-D-μ-SPE were compared with other previously reported methods for the extraction of OXA, CLOX, and DICLOX in milk samples, as summarized in Table 3. It can be observed that COU-2-D-μ-SPE gave comparable LODs and allows the

Table 3 Comparison of COU-2-μ-SPE with previously reported works

Instrument	Sample preparation	LOD (μg L^{-1})/ (μg kg^{-1})	LOQ (μg L^{-1})/ (μg kg^{-1})	Precision (% RSD)	Recovery (%)	References
HPLC-UV	COU-2-D-μ-SPE	2.0–3.3		6.2–8.8 (n=9)	80.3–99.5	This work
HPLC-DAD	SPE (HLB Alumina N)	1.0–1.2	3.3–4.0	3.9–5.5 (n=15)	82.9–93.2	Bailón-Pérez et al. (2009)
HPLC-PDA	MM-CPE	2, 3	7, 10	1.1–4.4 (n=15)	91.3–98.0	Kukusamude et al. (2010)
HPLC-UV	IPE	1, 2	3, 7	1.03–4.02 (n=15)	85.3–87.7	Kukusamude et al. (2012)
LC-MS/MS	SPE (C_{18})	–	0.1–0.32	5.8, 6.7 (n=12)	83–102	Riediker and Stadler (2001)
LC-MS/MS	MMIP	1.9	–	8.6–9.8 (n=6)	72.1–90.7	Zhang et al. (2010)
LC-MS/MS	LLE	3.0	7.5	6.3–21.8 (n=15)	73.8–81.3	Jank et al., (2012)
LC-MS	SBSE	0.00030–0.00084	0.001–0.003	0.3–9.1 (n=3)	41.2–108.1	Huang et al. (2013)

quantification of trace levels in comparison with SPE (HLB and Alumina N) (Bailón-Pérez et al. 2009), MM-CPE (Kukusamude et al. 2010), IPE (Kukusamude et al. 2012), MMIP (Zhang et al. 2010), and LLE (Jank et al. 2012). SPE (C_{18}) (Riediker and Stadler 2001) and SBSE (Huang et al. 2013) provided excellent sensitivity for the studied penicillin detection compared to other methods. However, SPE consumes higher amount of organic solvent, while SBSE required longer extraction time of 60 min. In addition, LC combined with MS detectors is generally costly, complicated, and require skilled personnel to operate the system. COU-2-D-μ-SPE uses minimum amount of COU-2 sorbent, and only 100 μL of methanol was employed for each extraction. In addition, COU-2-D-μ-SPE required simple analytical extraction apparatus and ultrasonication system. Thus, COU-2-D-μ-SPE can be a useful alternative approach for "green" microextraction of the three penicillins in milk samples.

Conclusions

A mesoporous carbon COU-2 was successfully synthesized and utilized as dispersive sorbent in D-μ-SPE for the microextraction of OXA, CLOX, and DICLOX in milk samples, prior to HPLC-UV detection. Six commercial milk samples analyzed showed negative results, indicating that the selected commercial milk products are free from the selected penicillin contamination. The proposed COU-2-D-μ-SPE offers advantages such as simple and fast extraction, good linearity, low LODs, acceptable RSD together with minimum consumption of organic solvent and sorbent. The method can be considered as a promising green analytical method for the analysis of OXA, CLOX, and DICLOX in milk samples.

Acknowledgments The authors wish to thank Universiti Teknologi Malaysia and the Ministry of Education Malaysia (MOE) for financial supports through research grant no. R.J130000.7809.4F177 and sponsorship for N. Yahaya through Advanced Medical and Dental Institute, Universiti Sains Malaysia Fellowship Program.

Conflict of Interest Noorfatimah Yahaya declares that she has no conflict of interest. Mohd Marsin Sanagi declares that he has no conflict of interest. Takahito Mitome declares that he has no conflict of interest. Norikazu Nishiyama declares that he has no conflict of interest. Wan Aini Wan Ibrahim declares that she has no conflict of interest. Hadi Nur declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

References

Bailón-Pérez MI, García-Campaña AM, del Olmo-Iruela M, Gámiz-Gracia L, Cruces-Blanco C (2009) Trace determination of 10 β-lactam antibiotics in environmental and food samples by capillary liquid chromatography. J Chromatogr A 1216:8355–8361

Benito-Peña E, Partal-Rodera AI, León-González ME, Moreno-Bondi MC (2006) Evaluation of mixed mode solid phase extraction cartridges for the preconcentration of beta-lactam antibiotics in wastewater using liquid chromatography with UV-DAD detection. Anal Chim Acta 556:415–422

Bovee TFH, Pikkemaat MG (2009) Bioactivity-based screening of antibiotics and hormones. J Chromatogr A 1216:8035–8050

Bruno F, Curini R, Corcia AD, Nazzari M, Samperi R (2001) Solid-phase extraction followed by liquid chromatography-mass spectrometry for trace determination of β-lactam antibiotics in bovine milk. J Agric Food Chem 49(7):3463–3470

Carretero V, Blasco C, Picó Y (2008) Multi-class determination of antimicrobials in meat by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry. J Chromatogr A 1209: 162–173

Chung WH, Tzing SH, Ding WH (2013) Dispersive micro solid-phase extraction for the rapid analysis of synthetic polycyclic musks using thermal desorption gas chromatography-mass spectrometry. J Chromatogr A 1307:34–40

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (http://ec.europa.eu/health/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf).

De Baere S, De Backer P (2007) Quantitative determination of amoxicillin in animal feed using liquid chromatography with tandem mass spectrometric detection. Anal Chim Acta 586:319–325

Douša M, Hosmanová R (2005) Rapid determination of amoxicillin in premixes by HPLC. J Pharm Biomed Anal 37:373–377

Freitas A, Barbosa J, Ramos F (2012) Determination of amoxicillin stability in chicken meat by liquid chromatography-tandem mass spectrometry. Food Anal Methods 5(3):471–479

Fu SC, Tzing SH, Chen HC, Wang YC, Ding WH (2012) Dispersive micro-solid phase extraction combined with gas chromatography-chemical ionization mass spectrometry for the determination of N-nitrosamines in swimming pool water samples. Anal Bioanal Chem 402:2209–2216

Galán-Cano F, Lucena R, Cárdenas S, Valcárcel M (2011) Direct coupling of dispersive micro-solid phase extraction and thermal desorption for sensitive gas chromatographic analysis. Anal Methods 3(4):991–995

Gramse MJ, Jacobson PE (2005) Determination of penicillin G in feeds by liquid chromatography with solid-phase extraction. J AOAC Int 88:679–683

Guo L, Lee HK (2012) One step solvent bar microextraction and derivatization followed by gas chromatography-mass spectrometry for the determination of pharmaceutically active compounds in drain water samples. J Chromatogr A 1235:26–33

Huang X, Chen L, Chen M, Yuan D, Nong S (2013) Sensitive monitoring of penicillin antibiotics in milk and honey treated by stir bar sorptive extraction based on monolith and LC-electrospray MS detection. J Sep Sci 36(5):907–915

Jank L, Hoff RB, Tarouco PC, Barreto F, Pizzolato TM (2012) β-lactam antibiotics residues analysis in bovine milk by LC-ESI-MS/MS: a simple and fast liquid–liquid extraction method. Food Addit Contam 29:497–507

Jiménez-Soto JM, Cárdenas S, Valcárcel M (2012) Evaluation of single-walled carbon nanohorns as sorbent in dispersive micro solid-phase extraction. Anal Chim Acta 714:76–81

Jin J, Nishiyama N, Egashira Y, Ueyama K (2009) Pore structure and pore size controls of ordered mesoporous carbons prepared from resorcinol/formaldehyde/triblock polymers. Microporous Mesoporous Mater 118:218–223

Jung HH, Park K, Han DK (2010) Preparation of TGF-β1-conjugated biodegradable pluronic F127 hydrogel and its application with adipose-derived stem cells. J Control Release 147(1):84–91

Kantiani L, Farré M, Barceló D (2009) Analytical methodologies for the detection of β-lactam antibiotics in milk and feed samples. Trends Anal Chem 28:729–744

Karageorgou EG, Samanidou VF (2011) Development and validation according to European union decision 2002/657/EC of an HPLC-DAD method for milk multi-residue analysis of penicillins and amphenicols based on dispersive extraction by QuEChERS in MSPD format. J Sep Sci 34:1893–1901

Karageorgou EG, Samanidou VF, Papadoyannis IN (2012) Ultrasound-assisted matrix solid phase dispersive extraction for the simultaneous analysis of β-lactams (four penicillins and eight cephalosporins) in milk by high performance liquid chromatography with photodiode array detection. J Sep Sci 35:2599–2607

Kukusamude C, Santalad A, Boonchiangma S, Burakham B, Srijaranai S, Chailapakul O (2010) Mixed micelle-cloud point extraction for the analysis of penicillin residues in bovine milk by high performance liquid chromatography. Talanta 81:486–492

Kukusamude C, Burakham R, Chailapakul O, Srijaranai S (2012) High performance liquid chromatography for the simultaneous analysis of penicillin residues in beef and milk using ion-paired extraction and binary water-acetonitrile mixture. Talanta 92:38–44

Liang C, Li Z, Dai S (2008) Mesoporous carbon materials: synthesis and modification. Angew Chem Int Edit 47(20):3696–3717

Maggi L, de Mendoza JH, Zalacain A, Bonetto L, Mocholí FA, Carmona M (2012) On-line solid-phase extraction coupled to liquid chromatography-ion trap tandem mass spectrometry for determination of penicillins in catfish. Food Anal Methods 5(5):1047–1053

Marchetti M, Schwaiger I, Schmid E (2001) Determination of benzylpenicillin, oxacillin, cloxacillin, and dicloxacillin in cows' milk by ion-pair high-performance liquid chromatography after precolumn derivatization. Fresen J Anal Chem 371(1):64–67

Moradi SE, Baniamerian MJ (2011) Applications of chemically modified ordered mesoporous carbon as solid phase extraction sorbent for preconcentration of trace lead ion in water samples. Chem Ind Chem Eng Q 17(4):397–408

Oliveira RV, De Pietro AC, Cass QB (2007) Quantification of cephalexin as residue levels in bovine milk by high-performance liquid chromatography with on-line sample cleanup. Talanta 71(3):1233–1238

Pozo OJ, Guerrero C, Sancho JV, Ibañez M, Pitarch E, Hogendoorn E, Hernández F (2006) Efficient approach for the reliable quantification and confirmation of antibiotics in water using on-line solid-phase extraction liquid chromatography/tandem mass spectrometry. J Chromatogr A 1103:83–93

Rezende CP, Almeida MP, Brito RB, Nonaka CK, Leite MO (2012) Optimisation and validation of a quantitative and confirmatory LC-MS method for multi-residue analyses of b-lactam and tetracycline antibiotics in bovine muscle. Food Addit Contam 29:541–549

Riediker S, Stadler RH (2001) Simultaneous determination of five β-lactam antibiotics in bovine milk using liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Anal Chem 73(7):1614–1621

Saaid M, Saad B, Ali ASM, Saleh MI, Basheer C, Lee HK (2009) In situ derivatization hollow fibre liquid-phase microextraction for the determination of biogenic amines in food samples. J Chromatogr A 1216(27):5165–5170

Santos SM, Henriques M, Duarte AC, Esteves VI (2007) Development and application of a capillary electrophoresis based method for the simultaneous screening of six antibiotics in spiked milk samples. Talanta 71:731–737

See HH, Sanagi MM, Wan Ibrahim WA, Naim AA (2010) Determination of triazine herbicides using membrane-protected carbon nanotubes solid phase membrane tip extraction prior to micro-liquid chromatography. J Chromatogr A 1217(11):1767–1772

Stolker AAM, Rutgers P, Oosterink E, Lasaroms JJP, Peters RJB, van Rhijn JA, Nielen MWF (2008) Comprehensive screening and quantification of veterinary drugs in milk using UPLC–ToF-MS. Anal Bioanal Chem 391:2309–2322

Sun M, Feng J, Qiu H, Fan L, Li L, Luo C (2013) A solid-phase microextraction fiber with carbon nanoparticles as sorbent material prepared by a simple flame-based preparation process. J Chromatogr A 1300:173–179

Tsai WH, Huang TC, Huang JJ, Hsue YH, Chuang HY (2009) Dispersive solid-phase microextraction method for sample extraction in the analysis of four tetracyclines in water and milk samples by high-performance liquid chromatography with diode-array detection. J Chromatogr A 1216(12):2263–2269

Turnipseed SB, Andersen WC, Karbiwnyk CM, Madson MR, Miller KE (2008) Multi-class, multi-residue liquid chromatography/tandem mass spectrometry screening and confirmation methods for drug residues in milk. Rapid Commun Mass Spectrom 22:1467–1480

Yahaya N, Mitome T, Nishiyama N, Sanagi MM, Wan Ibrahim WA, Nur H (2013) Rapid dispersive micro-solid phase extraction using mesoporous carbon COU-2 in the analysis of cloxacillin in water. J Pharm Innov 8:240–246

Zhang X, Chen L, Xu Y, Wang H, Zeng Q, Zhao Q, Ren N, Ding L (2010) Determination of β-lactam antibiotics in milk based on magnetic molecularly imprinted polymer extraction coupled with liquid chromatography–tandem mass spectrometry. J Chromatogr B 878:3421–3426

Zhu F, Guo J, Zeng F, Fu R, Wu D, Luan T, Tong Y, Lu T, Ouyang G (2010) Preparation and characterization of porous carbon material-coated solid-phase microextraction metal fibers. J Chromatogr A 1217:7848–7854