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High performance liquid chromatography for the simultaneous analysis of penicillin residues in beef and milk using ion-paired extraction and binary water–acetonitrile mixture

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ABSTRACT

An ion-paired extraction (IPE) has been developed for the analysis of penicillin antibiotics (penicillin G, oxacillin and cloxacillin) in beef and milk samples using tetrabutylammonium bromide (TBABr) as ion-pairing agent and binary water–acetonitrile as extractant. The factors affecting the IPE efficiency were optimized including solution pH, volume of acetonitrile (ACN), concentration of TBABr and electrolyte salt $(NH_4)_2SO_4$. The optimum IPE conditions were 10 mmol L^{-1} phosphate buffer pH 8, 2 mL of ACN, 6 mmol L^{-1} of TBABr and 2.5 mL of saturated ammonium sulfate. Under the HPLC condition: an Xbridge^{TM} C18 reversed-phase column, isocratic elution of 5 mmol L^{-1} phosphate buffer (pH 6.6) and acetonitrile (75:25, v/v) and a flow rate of 1 mL min^{-1}, with UV detection at 215 nm, the separation of three penicillins was achieved within 10 min. Under the selected optimum conditions, the enhancement of 21–53 folds compared to that without preconcentration and limits of detection (LODs) of 1–2 ng mL^{-1} were obtained. Good reproducibility was achieved with RSD < 2% for retention time and <5% for slope of calibration curves. The average recoveries higher than 85% were obtained. The proposed IPE-HPLC method has shown to be high efficient preconcentration and analysis method for penicillin residues in beef and milk with LOD lower than the maximum residue limits.

1. Introduction

Veterinary antibiotics are chemical substances which are extensively used in livestock during the animal breeding for both excessive therapeutic and prophylactic purposes [1]. Their usages are to kill or inhibit bacterial growth [2], promote animal growth [3,4] and also maintain animal health. Penicillin (benzylpenicillin) is the oldest antibiotic discovered by Sir Alexander Fleming in 1928 [2]. Penicillins belong to a β-lactam class of antibiotics [3] and consists of a thiazolidine ring attached to a β-lactam ring forming 6-aminopenicillanic acid and a side chain R_1 in 6-position.

The systematic use of penicillins may result in the presence of residues in edible animal products including meat and milk. The residues may cause the undesirable effects on consumer health such as allergic reactions and drugs-resistant strains in some sensitive people [5–7]. In order to protect human health from the abundant of penicillins, Food and Drug Administration (FDA) and European Council Regulation (ECC) 2377/90 have enacted the maximum residue limits (MRLs) for the presence of penicillin residues in meat and milk product [6,8,9]. The MRLs for antibiotic residues in meat and milk are regulated in the range of 50–300 ng g^{-1} and 4–125 ng mL^{-1}, respectively, depending on the specific type of antibiotic.

Convenient and sensitive method is therefore needed to monitor residual penicillins in human food including meat and milk. Routinely, microbial assays are used to perform testing of animal product but it fails to identify of individual residue [8,10]. Thus, the chromatographic methods including high performance liquid chromatography (HPLC) with photodiode array (PDA) or UV detection are used as an alternative choice to solve this problem. It has been accepted as a sensitive method for the analysis of antibiotics and metabolites in challenging biomatrices [6,8,9,11–18].

To obtain a reliable and accurate method for penicillin analysis in complex matrices, suitable sample preparation methods are required to eliminate the large number of matrix interference prior to chromatographic determination [19,20]. The most popular strategies for the antibiotic extraction from their original matrices include liquid–liquid extraction (LLE) or solvent extraction [13,21,22], solid-phase extraction (SPE) [23–28], dispersive solid-phase extraction (DSPE) [29,30] and pressurized liquid extraction (PLE) [9,13].

Recently, we described mixed micelle-cloud point extraction (MM-CPE) for the analysis of penicillin residues in bovine milk using HPLC [6]. This methodology used mixture of neutral and

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charge surfactant (opposite charge from penicillin ions) to achieve both ideal hydrophobic and non-ideal electrostatic interaction within the same extraction system. The proposed CPE method shows good compatibility between the surfactant and the hydro-organic mobile phase used in HPLC. The disadvantage of this method is dilution effect from the addition of organic solvent to reduce viscosity of the extract prior to injection into the HPLC column. Hence, the detection limit is increased.

The extraction and purification of penicillins from biomatrices into non-polar organic solvent is a challenging task because of their polar properties and the instability of β-lactam ring toward acid and alkali. Liquid–liquid extraction can be modified to extract polar organic compounds like penicillins using ion-pairing agent and water–miscible organic solvent. A phase separation takes place when electrolyte was added into the mixed solvents of water and water–miscible organic solvent like acetonitrile. Salting-out phenomenon occurs owing to the decreasing of solubility of the organic solvent in aqueous solution [31]. Therefore, the solvent can easily extract ion-paired compounds of penicillins. Moreover, the solvents used for extraction have been generally used as mobile phase in HPLC [32]. Thus, the separated phase can be directly injected to HPLC column without any further treatment.

The work described hereafter is aimed at using tetrabutylammonium bromide (TBABr) as ion-pairing agent to achieve a simple and effective pretreatment method for HPLC analysis of complex analytes in biological matrices like beef and milk. Ion-paired extraction (IPE) is possible by the addition of a small amount of the cationic ion-pairing agent, tetrabutylammonium bromide, into the aqueous phase of penicillins prior to extraction. This method is based on the extraction of penicillin–TBABr complexes into an acetonitrile phase by using ammonium sulfate as the salting-out agent, followed by direct injection of the extract into HPLC.

2. Experimental

2.1. Chemical and reagent

All penicillin standards were purchased from Fluka, including penicillin G sodium salt (Austria), 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 water and stored at 4 °C. The working solutions were freshly prepared by diluting the stock solutions with water. Tetrabutylammonium bromide (TBABr) was purchased from Fluka (India). The stock solution of TBABr (100 mM) was prepared in water. Table 1 shows the chemical structures and physical properties of the studied penicillins and cationic ion-pairing agent (TBABr). Ammonium sulfate, $(NH_4)_2SO_4$ (Ajax Finechem, Australia), potassium hydrogen phosphate (KH_2PO_4) (BDH, Belgium), and di-potassium hydrogen phosphate (K_2HPO_4) (BDH, Belgium) were also used. Deionized water with the resistivity of 18.2 MΩ cm from RiO$_s$^{TM} Type I Simplicity 185 (Millipore water, USA) was used throughout. Methanol (MeOH) (RCI Labscan, Thailand) and acetonitrile (ACN) (Merck, Germany) were of HPLC grade.

2.2. Instrumentation

The HPLC system consisted of a Waters 600E Multi-solvent Delivery System, a Waters In-Line Degasser AF and a Rheodyne injector with sample loop of 20 μL equipped with a Waters 2996 Photodiode Array Detector (operated at 215 nm). The Empower Software was used for data acquisition and analysis. An Xbridge^{TM} C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) from Waters (USA) coupled to a guard column was used. A centrifuge (Kokusan Type H-11N, Biomed Group Co. Ltd., Japan) was used for protein precipitation. Moulinex DPA 141 (France) was used for blending beef samples. Rotavapor R-200 (BUCHI Labortechnik AG, Flawil, Switzerland) was also used.

2.3. Ion-paired extraction

Treated sample (from Section 2.4) or standard solutions were mixed with 1.00 mL of phosphate buffer (pH 8) and 600 μL of 100 mmol L^{-1} TBABr. Afterward, saturated $(NH4)_2SO_4$ and 2.00 mL of ACN were added and made up to a final volume of 10.00 mL with water. The mixture solution was well mixed and stood for an adequate time to complete phase separation. The upper phase was directly injected into HPLC using the optimum condition in Section 3.1. The concentrations of the reagents used were optimized (see Section 3.2).

2.4. Sample preparation and analysis

Beef and milk samples were purchased from a supermarket in Khon Kaen province, northeastern Thailand. Proteins and fats in 10.0 g of blended beef sample were separated by mixing with 20.00 mL of a mixture of acetone and acetonitrile (4:1, v/v). The supernatant was evaporated at 37 °C to eliminate organic solvents after the supernatant was completely separated from beef sample using centrifugation at 4500 rpm for 20 min. The final solution was extracted by ion-paired extraction (see Section 2.3).

For milk sample, proteins and fats were separated using the method in Ref. [6]. The supernatant was evaporated and separated in the same manner as beef samples described above.

For the fortification of samples, series of standards of antibiotics were spiked into the blended beef samples and milk samples prior to protein and fat separation.

3. Results and discussion

3.1. HPLC conditions for analysis of penicillins

The separation of three penicillins including penicillin G (PEN-G), oxacillin (OXA) and cloxacillin (CLO) was performed using isocratic elution of 5 mmol L^{-1} phosphate buffer (pH 6.6) and ACN (75:25, v/v) at a flow rate of 1.0 mL min^{-1}. The UV detection was accomplished at 215 nm. The experiments were carried out at ambient temperature. Under the optimum condition, the studied penicillins were successfully separated within 10 min with the elution order of PEN-G, OXA and CLO, respectively (see Fig. 1). The resolutions of separated penicillins were greater than 1.4.

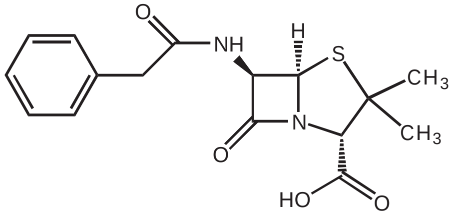
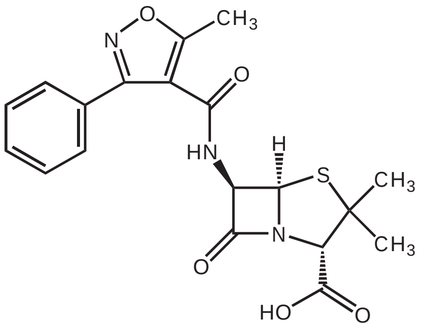
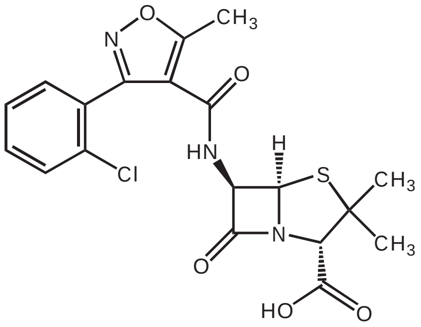
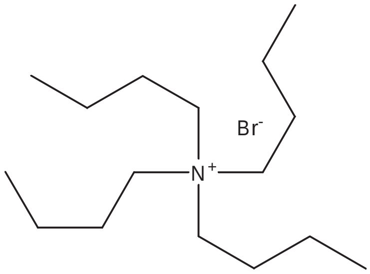
3.2. Optimization of ion-paired extraction

The studied penicillins are quite polar with the pKa_1 (−COOH) ≈ 2.4 [33]. Under neutral conditions, they are anion and highly soluble in aqueous solution, resulting in poor extraction efficiency in LLE. Therefore, the cationic ion-pairing agent, TBABr was used during LLE process. The penicillin–TBABr ion-pair can distribute effectively into the acetonitrile compared to the original polar forms, leading to higher extraction efficiency. Therefore, the pH of solution is the first parameters to be investigated. The other parameters including concentration of TBABr and the amount of ammonium sulfate salt were optimized subsequently. The results are expressed as extraction recovery (ER) using the following equation.

$$ER\% = \frac{C_{ex}V_{ex}}{C_0V_{aq}} \times 100$$

where C_{ex} and C_0 are concentration of the analytes in the extraction phase and initial concentration of the analyte in the aqueous

Table 1
Chemical and physical properties of the studied penicillins and ion-pairing agent.

Name	Abbreviation	Structure	p*K*a	Molecular weight (g mol^{-1})
Penicillin G	Pen-G		2.5	333.37
Oxacillin	OXA		2.4	400.40
Cloxacillin	CLO		2.4	434.90
Tetrabutylammonium bromide	TBABr			322.37

sample phase, respectively. V_{ex} and V_{aq} are the volumes of the extraction phase and aqueous sample phase, respectively. C_{ex} is calculated from calibration curve obtained from direct injection of the standard analytes.

3.2.1. *Effect of solution pH*

In the case of ionizable organic analytes like penicillins, the analytes trend to ionize and existing in negatively charge forms because of the low p*K*a values of carboxylic group (—COOH) of the penicillins. According to ion-paired extraction strategy, the maximum extraction efficiency is achieved at the pH value where the anionic charge form of the target penicillin prevails. The effect of pH was studied over the range of 2–8.5, using 10 mmol L^{-1} phosphate buffer. The results reveal that pH of solution has strong effect on the extraction of the studied penicillins (see Fig. 2a). The extraction efficiency of all analytes remained rather constant when the pH increased from 2 to 6. Beyond this point, the extraction efficiency of all analytes increased sharply up to pH 8. The extraction efficiency decreased at higher pH. The results indicated that the analytes were fully deprotonated at pH around 8 resulting in the highest extraction efficiency. It was implied that the analytes are suitable for ion-pairing with TBABr and partition in acetonitrile phase at pH 8 with ammonium sulfate induced phase separation. Therefore, the pH of 8 was selected for further studies.

3.2.2. *Effect of concentration of TBABr*

The effect of TBABr concentrations in the range 0.00–20 mmol L^{-1} on the extraction of penicillins was investigated. As the results in Fig. 2b, illustrate clearly that the concentration of TBABr strongly affects the extraction efficiency. The extraction efficiency of all analytes increased sharply with increasing TBABr concentration up to 6 mmol L^{-1}. However, the extraction efficiency

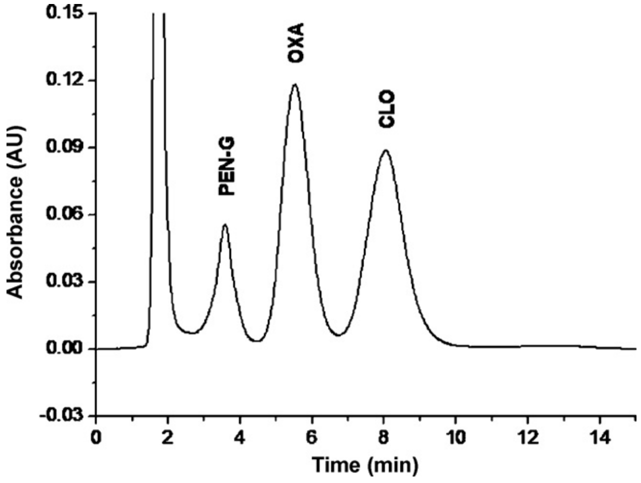


Fig. 1. Chromatogram of standard antibiotics (5 μg mL^{-1} each) under the optimum conditions of IPE and HPLC. IPE conditions: a mixture of solution of 10 mmol L^{-1} phosphate buffer pH 8.0, 6 mmol L^{-1} TBABr, 2.5 mL of saturated ammonium sulfate and 2 mL of ACN. HPLC conditions: an X-bridge^{TM} C_{18} reversed-phase column, isocratic elution of 5 mmol L^{-1} phosphate buffer (pH 6.6) and acetonitrile (75:25, v/v), sample injection of 20 μL, a flow rate of 1 mL min^{-1} and UV detection was set at 215 nm.

of the studied penicillin decreased after 6 mmol L^{-1}, especially for the OXA and CLO which have more bulky groups than PenG. The reduction of extraction efficiency may due to the fact that when large excess amount of TBABr was added, the steric effect caused by its side chains trend to reduce ion-paired forming efficiency of the studied penicillins in the solution. Beside, the excess TBABr itself was existed in ionic form, hence, it competed in dissolving into the binary extraction phase. Therefore, a 6 mmol L^{-1} of TBABr was used throughout.

3.2.3. *Effect of salting-out agents*

Binary extractant with salting-out agents have been demonstrated to be a successful method for extraction of polar organic ion like dihydric phenol from aqueous media [32]. Since the binary extractant (acetonitrile–water) has higher polarity than acetonitrile itself, the ion-paired complex of tris (1,10-phenanthroline) iron (II) chloride was easily extracted [34]. When a binary acetonitrile–water mixture was used as extractant, the separation of the mixture of acetonitrile and water into two phases occurs at concentration of water–ammonium sulfate solution between 16 and 43 wt% [32]. At lower concentrations of salt, phase separation was not occurred [32]. The volume of phase separation depends on individual salting-out agent concentration and initial volume of solvent (ACN). When phase separation is reached, the volume of acetonitrile phase increases linearly with initial volume of ACN [31]. In this work, two types of miscible solvent, acetonitrile and methanol were investigated as extractant. The results confirmed that phase separation was not occurred when using methanol while acetonitrile provides a second phase at a certain concentration of ammonium sulfate. Hence, acetonitrile was selected as miscible solvent for further studies.

Sodium chloride, ammonium sulfate and magnesium sulfate have been popularly used as salting-out agent. The salting-out effect decrease in the following order $Mg^{2+} > Na^{+} > NH_4^{+}$ and $SO_4^{2-} > Cl^{-}$. The salting-out effect is correlated to the hydration energy of these ions. The hydration energy (kJ mol^{-1}) of Mg^{2+}, Na^{+} and NH_4^{+} is 2198 [31], 501.8 [31] and 351.4 [35], respectively. Therefore, the hydration power of Na^{+} ion is higher than NH^{4+} ion. From preliminary experiments, it is found that using $(NH_4)_2SO_4$

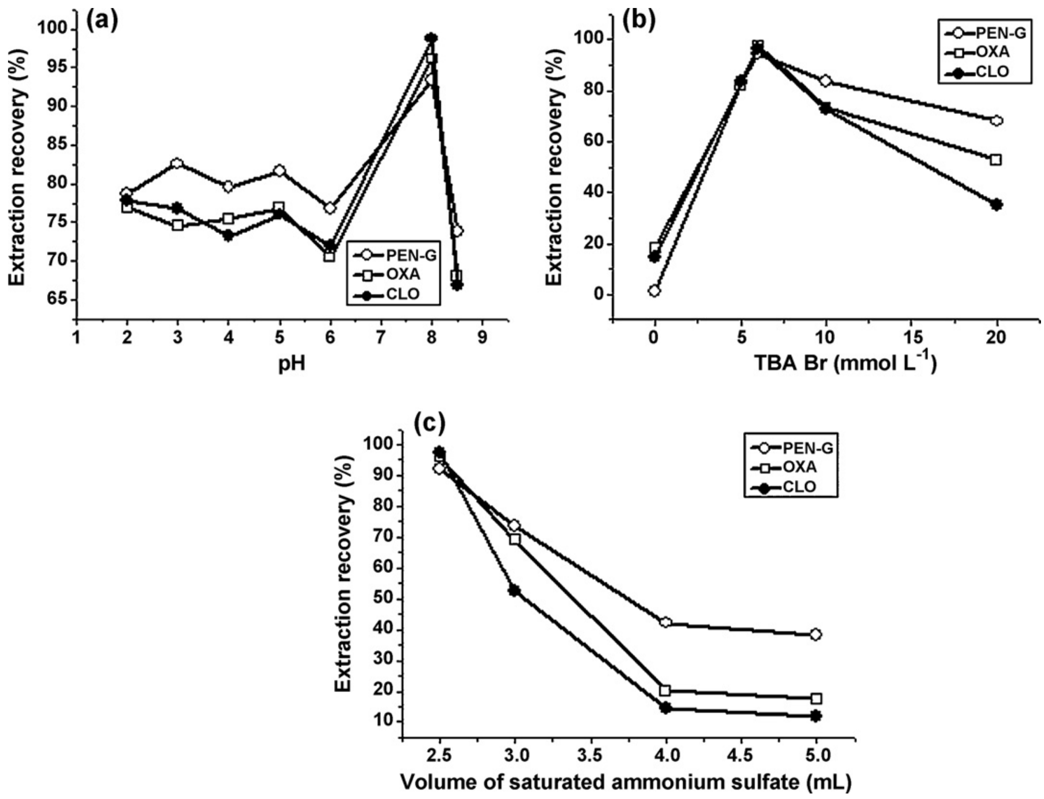


Fig. 2. Influence of (a) pH, (b) TBABr concentration and (c) amount of saturated ammonium sulfate on the extraction efficiency of studied penicillins (5 μg mL^{-1} each). Conditions: (a) 10 mmol L^{-1} phosphate buffer containing 10 mmol L^{-1} TBABr, 3 mL of saturated ammonium sulfate and 2 mL of ACN; (b) 10 mmol L^{-1} phosphate pH 8, 3 mL of saturated ammonium sulfate and 2 mL of ACN; (c) 10 mmol L^{-1} phosphate buffer pH 8 containing 6 mmol L^{-1} TBABr and 2 mL of ACN.

Table 2
Analytical features of IPE-HPLC method compared with direct HPLC method for the standard antibiotics.

Antibiotic	Linear range (μg mL^{-1})	Linear equation	EF	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	MRL[b] (ng mL^{-1})	MRL[c] (ng g^{-1})
Penicillin G	0.001–5 (0.5–10)	$y = 399550x$ ($y = 19037x$)[a]	21	0.9996 (0.9992)	1 (30)	3 (100)	4	50
Oxacillin	0.001–5 (0.5–10)	$y = 1737120x$ ($y = 41061x$)	42	0.9998 (0.9992)	1 (50)	3 (170)	30	300
Cloxacillin	0.002–5 (0.5–10)	$y = 1741666x$ ($y = 33146x$)	53	0.9997 (0.9995)	2 (100)	7 (330)	30	300

[a] The results in parenthesis obtained from direct HPLC method (without IPE).
[b] MRLs (maximum residue limits) in milk samples established by EU.
[c] MRLs (maximum residue limits) in muscle samples established by EU.

as salting-out agent, the volume of separation phase occurred in a smaller volume compared to NaCl electrolyte. This is because Na^+ ion can decrease the solubility of ACN better than NH^{4+} ion. Hence, ammonium sulfate provided higher concentration of analyte in the binary extraction phase.

In order to obtain the optimal volume of acetonitrile and saturated ammonium sulfate used, each 1–3 mL of acetonitrile was mixed with 3 mL of saturated ammonium sulfate. The phase separation of each experiment occurred. The extractant volume from the initial volume of 2 mL acetonitrile was about 1 mL which was enough for 3 replicate injections while that from the initial volume of 1 mL was not enough even for 2 replications. Therefore, 2 mL of acetonitrile was selected for further studies. The volume of saturated ammonium sulfate was studied in the range 1–5 mL (around 6.4–32 wt% water–ammonium sulfate salt solution). It was found that no phase separations were observed when less than 2.5 mL of saturated ammonium sulfate (or 16 wt% water–ammonium sulfate salt solution) was added. As the results shown in Fig. 2c, the maximum extraction efficiency was achieved at 2.5 mL of saturated ammonium sulfate and rapidly decreased when the volume of saturated ammonium sulfate increased up to 3 mL. Beyond this point, the extraction efficiency of all analytes was only slightly affected. Podolina et al. [32] found that the volume fraction of acetonitrile in the extractant phase can be varied from 0.68 to 0.94 by varying the content of ammonium sulfate. Therefore, the lowest concentration of ammonium sulfate that created phase separation will give the lowest fraction of acetonitrile in the extraction phase. Hence, the highest extraction efficiency of the ion-paired penicillin is achieved. Besides, it provided the extraction volume of 200 μL which was still enough for triplicate injections. Therefore, 2.5 mL of saturated ammonium sulfate was selected as the optimum.

3.3. *Analytical features and method validation*

The analytical performance including linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and reproducibility were evaluated under the developed optimum conditions of IPE and HPLC. Accuracy (% recovery) was investigated to verify the capability of the presented method in real samples.

The calibration curves of the studied penicillins were obtained by least-squares linear regression of peak area versus 9 levels of the studied penicillins after IPE. They were linear over the concentration range of 0.001–5 μg mL^{-1} with the coefficient of correlation (r^2) greater than 0.9996. The analytical parameters of the studied analytes are listed in Table 2.

The LOD and LOQ of the proposed method in beef and milk sample matrices were demonstrated. The LOD was evaluated as the concentration giving a signal to noise ratio of 3 (S/N = 3), while the LOQ used S/N = 10. The results are summarized in Table 2. It is revealed that the developed IPE gave low detection limits ranging

Table 3
Precisions and recoveries of the standards spiked in beef and milk samples.

Antibiotic	Spiked level	Precision, RSD (%)				Recovery (%) (mean ± SD) ($n = 3$) beef	Recovery (%) (mean ± SD) ($n = 3$) milk
	(μg mL^{-1} for milk)	Intra-day ($n = 5$)		Inter-day ($n = 3 \times 5$)			
	(μg g^{-1} for beef)	t_R	Slope	t_R	Slope		
Penicillin G	0.003					72.3 ± 2.3	73.4 ± 3.2
	0.03					74.8 ± 2.1	76.5 ± 2.4
	0.05					81.7 ± 2.5	82.6 ± 2.8
	0.1					82.8 ± 2.5	87.3 ± 1.4
	0.5					91.8 ± 3.6	94.6 ± 0.9
	1					94.7 ± 1.8	96.3 ± 0.9
	3					96.8 ± 2.4	97.1 ± 1.7
	Mean	0.46	0.32	1.99	1.24	85.0 ± 9.7	86.8 ± 9.7
Oxacillin	0.003					73.1 ± 3.0	73.5 ± 2.6
	0.03					77.3 ± 2.5	76.8 ± 3.5
	0.05					80.2 ± 2.4	81.0 ± 2.5
	0.1					81.2 ± 2.8	83.8 ± 1.7
	0.5					92.8 ± 4.4	94.3 ± 0.8
	1					95.3 ± 2.6	95.6 ± 1.5
	3					97.3 ± 1.1	97.1 ± 2.1
	Mean	0.28	0.67	1.03	3.45	85.3 ± 9.6	86.0 ± 9.6
Cloxacillin	0.003					73.9 ± 3.3	75.4 ± 2.6
	0.03					76.6 ± 2.9	79.0 ± 2.8
	0.05					81.2 ± 4.1	82.3 ± 2.5
	0.1					82.0 ± 2.6	84.1 ± 1.6
	0.5					94.5 ± 2.7	96.7 ± 1.8
	1					93.9 ± 1.1	97.2 ± 0.6
	3					98.7 ± 0.7	98.9 ± 0.2
	Mean	0.52	1.87	1.52	4.02	85.8 ± 9.7	87.7 ± 9.7

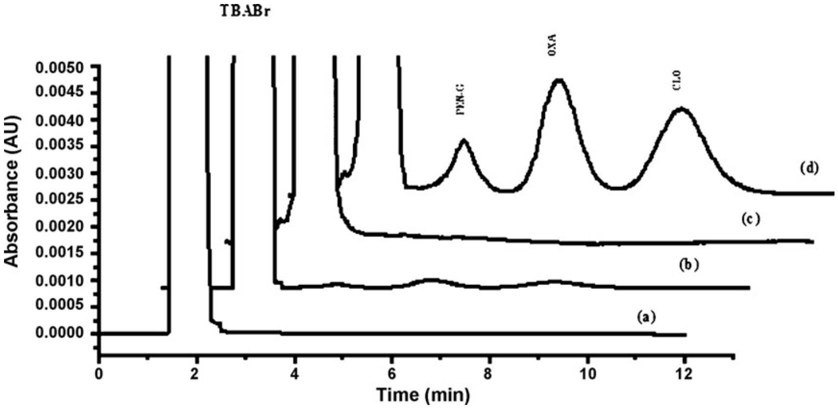


Fig. 3. Overlaid chromatograms of (a) milk sample, (b) spiked milk sample at 0.003 μg mL^{-1}, (c) beef sample and (d) spiked beef sample at 0.03 μg g^{-1}. The conditions are described in Fig. 1.

between 1 and 2 ng mL^{-1} which is lower than the maximum residue limits (MRLs) established by several control authorities at 300 ng g^{-1} (for OXA and CLO in meat), at 30 ng mL^{-1} in milk and at 50 ng g^{-1} (for PEN-G in meat), at 4 ng mL^{-1} in milk [36]. Enhancement factor (EF) is defined as the ratio of slope of calibration curve obtained from IPE method and without IPE. A high EF in the range of 21-fold (PEN-G) to 53-fold (CLO) was obtained.

The intra-day ($n = 5$) and inter-day ($n = 3$ days $\times$ 5) precisions of the developed method were evaluated in terms of relative standard deviation (%RSD) of retention time (t_R) and slope of calibration curve. The results (Table 3) show that good precisions of overall repeatability and reproducibility measurements of t_R and slope were lower than 2% and 5%, respectively.

3.4. Analysis of penicillins in beef and milk samples

The developed IPE-HPLC method was applied to the analysis of the residual antibiotics in beef and milk samples. No contamination of the studied antibiotics was found. Typical chromatograms of beef and milk samples with and without spiked studied penicillins after the IPE are depicted in Fig. 3. The accuracy of the developed method was evaluated in term of percentage recovery. Seven levels (0.003, 0.03, 0.05, 0.1, 0.5, 1, and 3 μg g^{-1} for beef or μg mL^{-1} for milk) of standard mixture of the studied penicillins were fortified into beef and milk samples prior to IPE, the extracts were analyzed with three replicates. The recoveries of each level and mean recovery are summarized in Table 3. The average recoveries were greater than 85% which are in the acceptable range of recoveries for trace analysis enacted by the Association of Official Agricultural Chemists (AOAC) and European commission ($\geq$70% and $\leq$110%) [37]. Therefore, the proposed IPE-HPLC was proven to be of high efficient method for the determination of trace antibiotics in beef and milk matrices. It is expected that the developed method will be effective for multi-residues analysis in other matrices as well.

4. Conclusion

This work demonstrates a simple and rapid extraction and preconcentration of penicillins using ion-paired extraction (IPE). The extraction is based on the formation of ion-pair between anionic analytes and tetrabutylammonium bromide (TBABr). The ion-pair are extracted using extractant of binary water–acetonitrile (creation of the two phase-system using ammonium sulfate). The extracts were injected directly into HPLC without any further pretreatment. The proposed method provided rather high enhancement factor (in the range of 21–53) and low LODs (1–2 ng mL^{-1}). Therefore, this method has excellent potential for determination of penicillin residues in beef and milk samples.

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