European Food Research and Technology (2022) 248:437–445
https://doi.org/10.1007/s00217-021-03889-6

ORIGINAL PAPER

Application of cloud point extraction for residues of chloramphenicol and amoxicillin in milk samples by HPLC–DAD

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Received: 3 August 2021 / Revised: 3 October 2021 / Accepted: 18 October 2021 / Published online: 1 November 2021

Abstract

A pre-concentration and sensitive determination method for residues of Chloramphenicol (CLP) and Amoxicillin (AMX) in milk samples were developed based on cloud point extraction (CPE) and HPLC–DAD analysis. CLP and AMX molecules were extracted to surfactant phases of polyethylene glycol-6000 (PEG-6000) in the presence of pH 7.0 buffer and high electrolyte concentration. Experimental variables were examined and optimized such as concentration of electrolyte and surfactant, pH, incubation time, and diluting solvent. In the developed method, determination of CLP and AMX using HPLC system was carried out by isocratic elution of water:acetonitrile (30:70) mixture. CLP and AMX antibiotic molecules were analyzed by considering peak area obtained by DAD detector at 273 nm and 276 nm, respectively. After optimization experimental variables, analytical signals were linear in the range of 10–900 ng mL^{-1} and 25–1000 ng mL^{-1}, respectively. The limit of detection values was calculated as 2.98 and 7.46 ng mL^{-1} while relative standard deviations (RSD %) were lower than 4.20% for 100 ng mL^{-1}. Finally, the developed method was successively applied to cow milk samples.

Keywords Amoxicillin · Chloramphenicol · PEG-6000 · HPLC · Cloud point extraction · Milk samples

Introduction

Determination of pharmaceutical substances and biologically active molecules in the biological matrix is crucial in various fields of medicine and pharmacy, e.g., in pharmacokinetic studies, development of new drugs or therapeutic drug monitoring [1]. Antibiotic molecules are proposed for treating and preventing of bacterial infections since penicillin was discovered in 1928. Like most of synthetic drug, their consumption is gradually growing in the world. As one of the most used group of Pharmaceutical and Personal Care Products (PPCPs), antibiotics are commonly used in veterinary medicine, human therapy and aquaculture [2]. Chloramphenicol (CLP) is used for the treatment of bacterial infections and it is one of the first antibiotic synthetically manufactured on a large scale. As a broad-spectrum antibiotic, it shows activity against both Gram-positive, Gram-negative bacteria and also other microorganism groups. Although it has beneficial properties in the prevention of diseases and bacterial growth, serious toxic effects of CLP molecule on human health is also known such as bone marrow depression, fatal aplastic anemia and other blood disorders [3]. Amoxicillin (AMX) is β-lactam group antibiotic that belongs to the group of penicillin. The molecular structure of penicillin comprises a thiazolidine ring fused to β-lactam ring with a side chain. Amoxicillin presents in side chain a primary amine group, that does not exist in any other penicillin except epicillin and bacampicillin. AMX is a fairly active molecule against both Gram-positive and Gram-negative organisms, including several pathogenic enteric organisms [4].

As well known, excessive and uncontrolled use of antibiotics causes the potential risk for residues of drugs in the environment and animal food such as milk, egg, and meat, because, residues of antibiotics can cause allergic reactions in hypersensitive individuals, or they may result in drug-resistant bacteria [5]. Thus, trace analysis of antibiotics is an important challenge to check food safety and potential

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toxicity risk [6]. As known, some antibiotics can be metabolized fairly, although many of them are largely excreted intact. Therefore, antibiotic residues can be transferred in environments for long periods and as a result of this circumstance, antimicrobial resistances are seen for the bacteria [7]. The existence of antibiotic residues in milk causes negative effects not only on human health and also adverse consequences in the dairy industry because it inhibits the formation of bacterial cultures. Therefore, the yield of production of fermented milk products decreases dramatically [8]. The presence of veterinary drug residues such as antibiotics in milk diminishes its nutritional value to a great extent [9].

The amount of antibiotic residues in food samples is very low and the other components in the sample are complex for directly conventional determination approaches without any treatment. To avoid these circumstances, it is proposed to develop more convenient and effective pretreatment methods for the requirements of modern detection technology [10]. The determination of drug residues in complex matrices is mainly divided into two steps: pretreatment and determination. In the first step, it is aimed to separate the target molecules from the complex medium and increase their concentration till the detection limit level of an instrumental system [11]. In the second step, target molecules are ready for determination with a suitable detector system by considering the molecular structure of target drugs. An ideal sample preparation approach should have some characteristics: avoiding of toxic organic solvents (or at minimum level), easy accessible equipment in every laboratory, high accuracy, fast speed, good repeatability, and high sensitivity, low cost, and availability for automation [12].

An ideal sample preparation approach typically includes isolation and enrichment of target analytes from a sample mixture. Conventional well-known extraction approaches have many disadvantages such as large amounts of volatile organic solvents, time-consuming, and labor-intensive [13]. Some useful extraction methods such as microwave-assisted extraction, membrane processes, or supercritical fluid extraction are not mostly preferred because of expensive equipment or high energy demands [14, 15].

HPLC-based methods are fairly preferred for separation and pre-concentration antibiotic molecules such as diode array Ultraviolet (DAD) detection [6] or mass spectrometry (MS) [16]. Before chromatographic determinations, generally, a pretreatment approach should be needed for the effective isolation of antibiotic molecules from the complex sample matrix. Mostly used sample preparation approaches aim to extract target analytes molecules by solid-phase extraction [17], solid-phase micro-extraction [18] and liquid–liquid extraction [19]. Cloud point extraction (CPE) is a sample pretreatment procedure in which the extraction of organic/inorganic compounds from chemical or biological systems using various surfactants. All the experimental details of CPE are well known and studied in the previous studies [20–22]. This technique is sometimes called micelle-mediated extraction because surfactant molecules form micelles in the presence of certain experimental conditions. Extraction of target molecules from aquatic phases is mainly provided by micelles [23]. The main advantages of CPE are less solvent consumption, higher recoveries, and better reproducibility, repeatability, and lower detection limits [24, 25].

In the present work, a cloud point extraction technique combined with HPLC–PDA was developed for simultaneous determination of Chloramphenicol and Amoxicillin molecules at trace levels in milk samples.

Experimental section

Reagents and standard solutions

Analytical grade chemicals were used throughout all the experiments. MES Minipure Dest Up (Ankara, Turkey) water purification system was used to obtain ultrapure water with a resistivity of 18.2 MΩ. HPLC solvents were from Sigma (St. Louis, MO, USA). Amoxicillin, Chloramphenicol, and Polyethylene Glycol (PEG-6000) were purchased from Sigma (St. Louis, MO, USA). PEG-6000 stock solutions were prepared by dissolving in ultrapure water. pH of solutions was controlled employing Britton Robinson (BR) buffer (10 mM Glacial acetic acid, 10 mM Boric acid and 10 mM Phosphoric acid) in the range of pH 2.0–10.0.

Instrumentation

The chromatographic system (Shimadzu, Tokyo, Japan) used is equipped with a pump (LC20-AD), a thermostatic oven (CTO-10 AS), autosampler (SIL-20AC) and PDA detector model (SPD-M20A). Evaluation of chromatography data were carried out via software (LC Solution) provided by Shimadzu. A Luna Omega C18 (250 × 4.6 mm, 5 µm) column was used for chromatographic separation. A pH meter with a glass-calomel electrode (Selecta, Spain) was used to measure the pH values. An ultrasonic water bath (Kudos, China) was used for sample preparation. All the solvents used in the chromatographic system were filtered through a 0.45 µm PDFA membrane filter (HNWP, Millipore) using a vacuum pump (Buchi, Switzerland) and degassed for 10 min in an ultrasonic bath.

Chromatographic analysis

The mobile phase composition was 70% ACN at isocratic mode throughout the analysis. The flow rate was 1.0 mL min^{-1}. The absorbance of antibiotic molecules was followed by PDA detector at 273 and 276 nm, respectively.

The column temperature was maintained at 40 °C and injection volume in the autosampler was 10 µL for all determinations. HPLC system was flushed with 50% acetonitrile to remove the residues of surfactants and the other organic compounds for 30 min after each day's work. The peak area of target molecules was calculated by LC Solution software provided by Shimadzu.

Cloud point extraction procedure

Unlike conventional CPE approaches, surfactant-rich phase (SRP) was formed on top of aquatic solution in this method. This is to the density of SRP which is lower than the aquatic phase including high salt concentration.

In this study, 1.0 mL of pH 5.00 BR buffer and 2.0 of mL 20% (w/v) (PEG-6000) were added to the solution including 500 µL of sample and mixed a few times. Then, 11.0 mL of 20% (w/v) Na_2SO_4 was transferred to this mixture and vortexed vigorously. The obtained solution was incubated for 30 min at 40 °C in a water bath. After the cloudy phase was observed, the solutions were centrifuged at 4000 rpm for 5 min and were kept in the refrigerator (+4 °C) for 20 min to facilitate phase separation. The SRP was collected in the upper part of the tube at end of this process, and the lower phase (aquatic medium) was easily separated by a simple injector as explained in our previous study [26]. After phase separation, 500 µL of ethanol was added to SRP and vortexed for homogenization of solution. In addition, finally, samples were filtered through a 0.45 µm and then transferred to HPLC vials.

Sample collection and application of the proposed method

Milk samples were collected from local food stores (Sivas, Turkey). Four fresh milk samples were prepared before MCPE according to the approach proposed by Guan et al. with some minor modifications [27]. Before CPE procedures, the milk samples were stored in a refrigerator and prepared using this procedure: 2 mL of milk samples were transferred into a 10-mL polypropylene falcon tube. The antibiotic mix standard was spiked at two different levels to samples. Then, 5 mL of acetonitrile was added to all samples and the mixture was vortexed for 60 s. Separation of supernatant was carried out by centrifugation for 5 min at 4000 rpm. 1 mL of supernatant was transferred to falcon tube and the proposed CPE method was applied to samples.

Results and discussion

To optimize the analytical procedure, preliminary tests were performed to select experimental conditions. Transferring of molecules to the surfactant-rich phase can be facilitated by optimizing all the experimental parameters. The concentration of surfactant molecules, ionic strength, pH of the medium, and incubation conditions were studied step by step and optimized.

pH of medium

The extraction process can be driven by checking pH of the medium, because it affects interactions between the target molecules and sorbents in solutions [26]. In the low pH values, positively charged ions mainly exist in solution, and also pH affects the molecular structure of surfactant molecules [28]. Optimization pH for extraction efficiency was studied in the range of 2–10. The obtained results are shown in Fig. 1. pKa values of amoxicillin were reported as 2.67, 7.11 and 9.55 [29]. Moreover, pKa value for Chloramphenicol was also given as 5.50 [30]. If pKa values were evaluated by comparing experimental data, Fig. 1 can be evaluated better. As can be seen in Fig. 1, the maximum analytical signal for both molecules was obtained at pH 7.0. In this case, according to the given pKa values, at pH 7.0 the ionized forms of AMX or CLP prevail and the electrostatic interactions with the positively charged composite surface become increasingly important [29–31]. As can be seen in Fig. 1, a deprotonated form of antibiotic molecules did not pass efficiently to SRP and signals decreased with pH.

Effect of nonionic surfactant

The main actor of cloud point extraction experiments is nonionic surfactants. This process is mainly related to the formation of micelle structures and transferring of target molecules to micelle phases in the presence of certain conditions. Micelle formation takes place when surfactant concentration reach and temperature of the solution to a certain level. Mostly, nonionic surfactants are used in CPE with their capability in micelle formation. A series of Triton and polyethylene glycol are fairly preferred owing to extraction

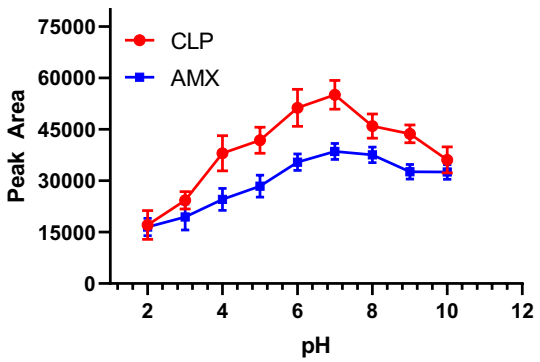

Fig. 1 The pH effect on CPE of AMX and CLP

efficiency and cost-effective properties. The concentration of surfactant molecules in the medium is one of the most important parameters needing to optimize for high efficiency. Because of the concentration of surfactant is small, the formation of micelle structures is not sufficiently realized and the extraction efficiency decreases. If the surfactant concentration is higher than critical micelle concentration (CMC), micelle formation begins and it is getting higher the amount of surfactant-rich phase (SRP). The pre-concentration factor directly depends on the final volume of SRP. If surfactant concentration is not enough level, micelle phases are not observed in the solution. Polyethylene glycol-6000 (PEG-6000) was preferred as a nonionic surfactant with good properties such as cost available sides and low cloud point temperature. A 20% (w/v) of PEG-6000 solution was prepared in water and its concentration was optimized in the range of 0.1–1.1%. The experimental results are presented in Fig. 2. As a result of these processes, the best signal was obtained with 0.7 of (w/v) PEG-6000. Next studies were followed using these concentrations.

Optimization of electrolyte concentration for effective phase separation

Surfactants like the other macromolecules are affected from the ionic strength of the medium. The solubility of these types of macromolecules decreases with the salting-out effect. Some nonionic surfactants have high cloud point temperatures. Micelle formation is observed with a settle of cloudy solution. The presence of ionic salts in the solution causes a decrease in cloud point temperature in the presence of an electrolyte. When the published articles in the literature is examined for this purpose, it will be seen that strength electrolytic inorganic salts are used as in CPE experiments to facilitate the procedure. Therefore, Na_2SO_4 was selected due to high ion contents for each mole. Surfactant rich phase (SRP) consists on the bottom of the aqua phase in conventional CPE experiments because the density of SPR is higher than aquatic solution. If the concentration of the used electrolyte salt is higher than usual conditions, the density of solution increases and SRP consists on top of aquatic phase. Finally, SRP is separated more easily using a syringe [31]. Concentration of Na_2SO_4 was studied and optimized using an increasing series solution in the range of 0.016–0.036% (w/v). As can be seen in Fig. 3, a concentration of 0.030 is suitable for obtaining ideal signal.

Effect of incubation time and temperature for CPE experiments

The temperature needing to consist a cloudy solution is known as cloud point temperature. This value is higher than 60 °C at the standard conditions. Using pre-experiments, the best extraction and phase separation conditions were obtained at 40 °C in the presence of Na_2SO_4 salt. This temperature was also preferred to avoid the degradation of antibiotic molecules at high temperatures. To optimize the effect of incubation time on the cloud point extraction of antibiotic molecules, the experiments were repeated at various incubation times in the range of 0–60 min. As can be seen in Fig. 4, it reached ideal extraction conditions in 30 min. Beyond this point, the signals are getting decreased, especially in AMX. Probably, the stability of drug molecules is effected by time. Therefore, 30 min was determined for incubation at 40 °C.

Preparing of surfactant-rich phase before HPLC determinations

The separation of SRP from aquatic phases is one of the most important processes in CPE experiments. The extraction efficiency is affected directly by the succession of this step. Generally, a Pasteur pipette or a simple syringe was used to remove the aquatic phase. After a cloudy solution is formed in incubation by water bath, samples are centrifuged to facilitate phase separation. At the end of this period, the aqueous phase was easily separated from the surfactant-rich

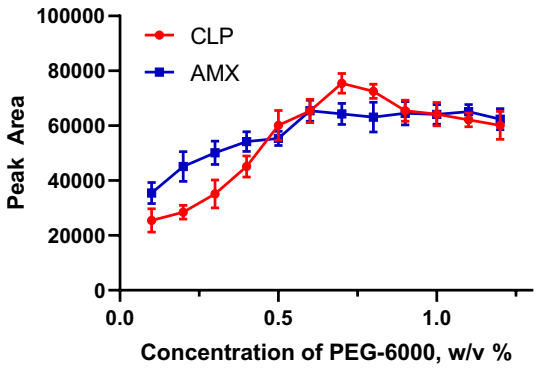

Fig. 2 The effect of PEG concentration on CPE of AMX and CLP

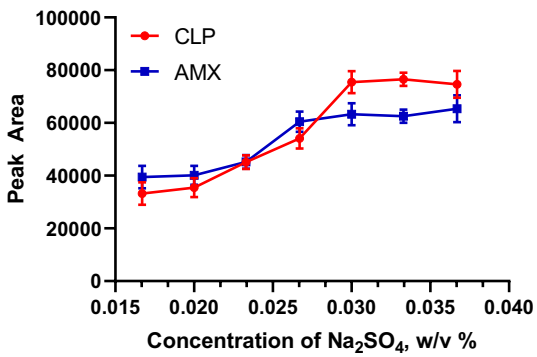

Fig. 3 The effect of salt concentration of CPE on AMX and CLP

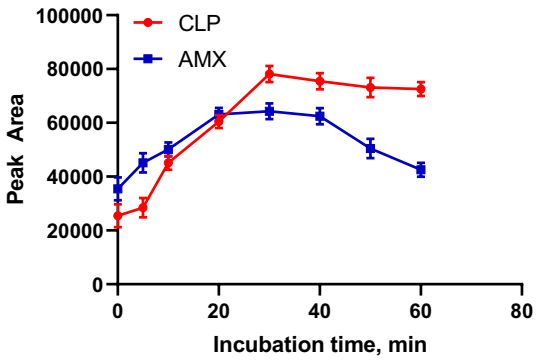

Fig. 4 The effect of incubation time on CPE

phase by a simple injector. After phase separation, SRP should be diluted with a suitable solvent before determination step, because the viscosity of SRP is high to submit for injection to HPLC system. Dilution of SRP with a suitable solvent is required before determinations. Various solvents were studied to optimize the solvent effect before the analysis. The results obtained by adding 1 mL of each solvent are shown in Fig. 5.

As shown in Fig. 5, the highest analytical signals were obtained with ethanol. Therefore, in the subsequent experiments, SRP was dissolved with ethanol. The amount of used solvent for SRP directly affects the pre-concentration factor. To obtain a higher pre-concentration factor, the volume of the solvent should be the lowest if possible. The minimum level for micro-vials is 100 µL. But, it is not easy to solve and filtrate SRP before transferring to HPLC vials. The optimization of solvent volume was carried out in the range of 200–1500 µL of solvent. Figure 6 shows the effect of solvent volume on analytical signals. The analytical signals were not enough at a lower volume, getting higher in an optimal value, and decreasing again due to dilution. As can be seen in Fig. 6, the best signals were obtained with 500 µL and used in further studies.

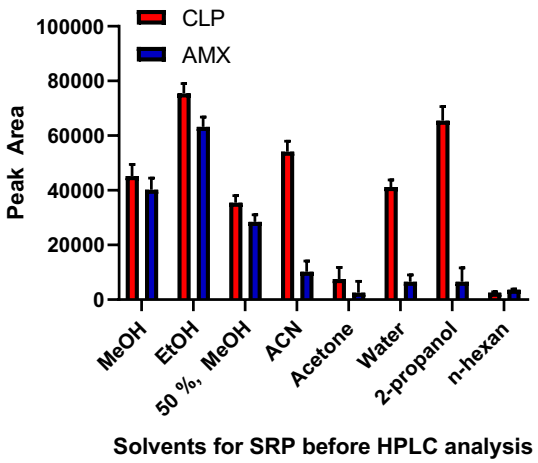

Fig. 5 The effect of solvent for SRP

Vortex time for dissolution of SRP

After phase separation and adding of ethanol, homogenization of SRP was performed using a vortex. The dissolution of the SRP via vortex should be optimized to obtain the maximum yield. Ideal vortex time was studied in the range of 10–120 s. Therefore, under optimized conditions, the surfactant-rich phase including antibiotic molecules was diluted with 500 µL ethanol and the duration of the vortex was studied in the range of 10–120 s. As a result of experimental data, it was optimized that 30 s is ideal and enough to obtain quantitative extraction.

Analytical merits of the proposed method

The optimization of the suggested method was performed under the following conditions: pH: 7.0 utilizing BR buffer, 0.8% PEG-6000, 0.03% Na_2SO_4, Incubation 30 min at 40 °C in a water bath, phase separation: 5 min at 4500 rpm, 500 µL of ethanol. Validation of the method was performed with the final optimized parameters by considering ICH proposals [32–34].

Linearity

Model standard solutions were prepared to check the linear range of the method. Calibration curves were constructed using peak area versus nominal concentrations of the drug molecules and each concentration level in 10 points was studied in triplicate. Slope, intercept, and correlation coefficient or the coefficient of determination values were calculated using least square linear regression analysis. Chromatogram obtained after CPE experiments is given in Fig. 7. The increasing peak area and analytical signals

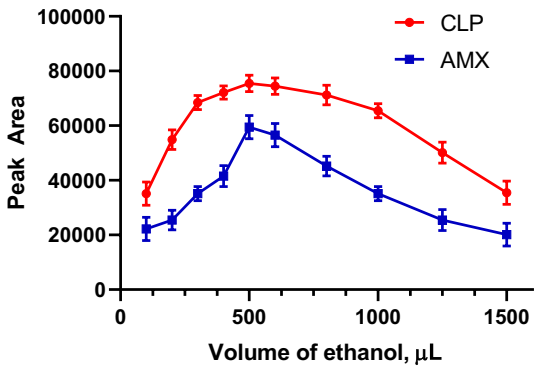

Fig. 6 The effect of ethanol volume on CPE

with a concentration of target molecules can be seen in the chromatogram.

Accuracy of the method by recovery test

Recovery values were calculated by spiking two concentration levels to milk samples. Each concentration level was analyzed in triplicate measurements. The linear regression equation obtained from model solutions was used to determine the nominal contents of antibiotic molecules in spiked samples. The results were calculated and tabulated (in Table 1) as percent recoveries of each component in the milk samples as [mean found concentration/theoretical concentration] × 100.

Precision

Standard deviation (SD) and the relative standard deviation (RSD) values as an expression of repeatability of the developed method were calculated for all the analyses by considering nominal and measured concentrations in three concentration levels.

Selectivity

In CPE techniques, selectivity is mainly provided by certain extraction conditions and separation efficiency of the column. If there are degradation products or impurities in pharmaceutical formulations, they can be evalutaed as a potentialinterfering species. The peak response for each molecule in the same retention times was checked and demonstrated that the method was free of interference from due to examined components only.

Sensitivity

Limit of detection (LOD) value directly reflects the sensitivity of a method and means the lowest detectable concentration of a target molecule in a sample. The limit of quantitation (LOQ) is also another reflector of sensitivity of method which means the lowest determinable concentration of analyte in a sample. Both LOD and LOQ values can be calculated using experimental data with acceptable precision and accuracy. For calculating these values, the standard deviation

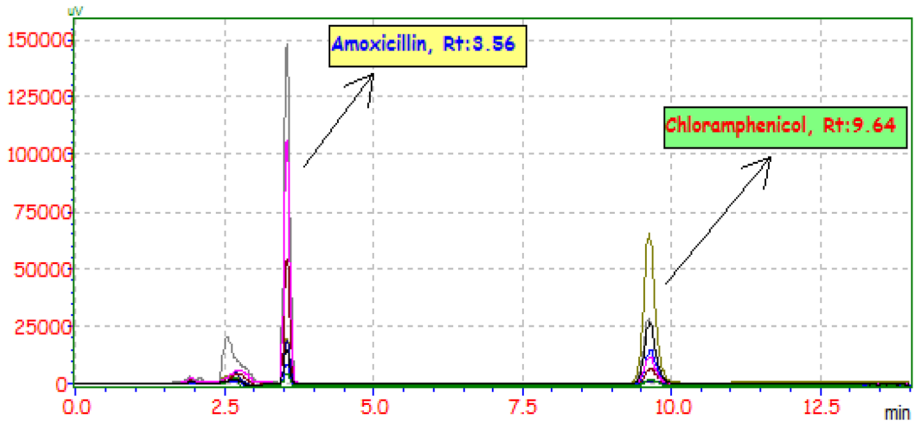

Fig. 7 The obtained chromatogram after CPE

Table 1 Analytical merits of the developed method

Parameter	Before MCPE		After MCPE	
	Chloramphenicol	Amoxicillin	Chloramphenicol	Amoxicillin
Linearity	2.0–50.0 μg mL^{-1}	10.0–100.0 μg mL^{-1}	10.0–900.0 ng mL^{-1}	25.0–1000.0 ng mL^{-1}
LOD	0.57 μg mL^{-1}	2.86 μg mL^{-1}	2.85 ng mL^{-1}	7.14 ng mL^{-1}
LOQ	1.88 μg mL^{-1}	9.43 μg mL^{-1}	9.43 ng mL^{-1}	23.57 ng mL^{-1}
RSD (%)	6.5 (for 2 μg mL^{-1}) 6.4 (for 10 μg mL^{-1}) 7.6 (for 25 μg mL^{-1})	8.4 (for 2 μg mL^{-1}) 8.2 (for 10 μg mL^{-1}) 6.4 (for 25 μg mL^{-1})	4.6(for 50 ng mL^{-1}) 4.2 (for 250 ng mL^{-1}) 3.8 (for 750 ng mL^{-1})	4.1 (for 50 ng mL^{-1}) 3.9 (for 250 ng mL^{-1}) 3.4 (for 750 ng mL^{-1})
M, slope	20.517	1.528	1099.7	64.32
Correlation of determination (R^2)	0.9986	0.9978	0.9910	0.9900
Pre-concentration Factor	–	–	23.3	23.3
Enhancement factor	–	–	53.6	42.1

of the response (Sxy) and the slope of the calibration curve (a) was used by considering this formula: LOD = 3·Sxy/a, and the limit of quantification: LOQ = 10·Sxy/a.

Comparison of developed method was performed by means of LOD values as can be seen in Table 2. Most of similar approaches present similar sensitivities. The proposed method after CPE has good advantages such as selectivity, sensitivity, and simultaneously determination of CLP and AMX. The optimized conditions in CPE and the used HPLC column provide the required selectivity and sensitivity for method. Therefore, this approach can be competed with the other methods.

Analysis of milk samples

The developed method was applied to cow milk samples. Residues of CLP and AMX residues were determined in various milk samples. Milk samples were treated with the procedure explained in Sect. 2.5. All milk samples were spiked using standard solutions to test the accuracy of the method through recovery values at two concentration levels (100 and 300 ng mL^{-1}). Results of analysis and recovery values were presented with together RSD% values in Table 3. As can be seen in Table 3, recovery values are in the range of 93.7–104.9% while RSD% values are in the range of 3.4–6.9%.

Conclusion

Sensitive analysis of antibiotic residues in food samples is a very important task for routine analysis laboratory. Food samples need hard work for analysis due to the complexity of the sample and the low concentration of target molecules. Pre-concentration approaches present very good advantages in accuracy and sensitivity if hybrid instrumental systems are not available for residue analysis. The main objectivities of the proposed method are simplicity, environmentally friendly, easily practicable in almost every laboratory with low cost, a wide linear range for food samples, good sensitivity, and high accuracy with good repeatability. The proposed methodology presents a useful approach for simultaneous analysis of CLP and AMX in the milk samples.

Table 2 Comparison of the new method with other reported methods

Analyte	Pre-treatment procedure	Determination method	LOD (ng mL^{-1})	Samples	Refs.
CLP AMX	SPE	UHPLC-UV–Vis–DAD	0.22 0.29	Surface waters	[35]
AMX	CPE	UV–Vis spectrometry	83.00	Pharmaceuticals	[36]
CLP	-	HPLC–DAD	80.00	Pharmaceuticals	[9]
CLP AMX	-	UPLC	25.00 30.00	Pharmaceuticals	[34]
CLP	SPE	HPLC–DAD	3.02	Milk samples	[37]
CLP AMX	CPE	HPLC–DAD	2.85 7.14	Milk samples	This method

Table 3 Analysis results of real samples by means of the spiked milk

Sample	Added ng mL^{-1}	Found [b] ng mL^{-1}		RSD %		Recovery %	
		AMX	CLP	AMX	CLP	AMX	CLP
Cow milk A	– 100.0 300.0	< LOD 93.5 ± 6.5 288.1 ± 16.7	< LOD 95.6 ± 3.3 292.3 ± 10.4	– 6.9 5.8	– 3.4 3.6	– 93.5 96.0	– 95.6 97.4
Cow milk B	– 100.0 300.0	< LOD 94.9 ± 6.4 314.3 ± 14.2	< LOD 95.4 ± 3.5 307.3 ± 19.3	– 6.7 4.5	– 3.6 6.2	– 94.0 104.7	– 95.4 102.4
Cow milk C	– 100.0 300.0	< LOD 93.5 ± 6.5 294.8 ± 14.7	< LOD 95.6 ± 3.3 298.3 ± 11.4	– 6.9 4.9	– 3.4 3.8	– 93.5 98.2	– 95.6 99.4
Cow milk D	– 100.0 300.0	< LOD 94.9 ± 6.4 305.7 ± 12.2	< LOD 95.4 ± 3.5 287.3 ± 19.3	– 6.7 3.9	– 3.6 6.7	– 94.0 101.9	– 95.4 95.7

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-021-03889-6.

Author contributions BS: methodology, formal analysis, investigation, visualization, and data curation. HİU: writing—original draft, methodology, formal analysis, investigation, visualization, and data curation. SU: investigation, methodology, and formal analysis. SG: conceptualization and validation. ÖD: writing—review and editing.

Funding This study has been supported by Sivas Cumhuriyet University Scientific Research Projects Commission as the research project with the ECZ-048 code.

Declarations

Conflict of interest Sürücü declares that there is no conflict of interest. Halil İbrahim Ulusoy declares that there is no conflict of interest. Songül Ulusoy declares that there is no conflict of interest. Özge Demir declares that there is no conflict of interest. Sümeyra Gülle declares that there is no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects.

Informed consent Informed consent is not applicable.

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