# Solid-phase Extraction and High Performance Liquid Chromatographic Analysis of Fluoroquinolone Antibiotic Residues in Milk Products

# การสกัดด้วยวัฏภาคของแข็งและโครมาโทกราฟีของเหลวสมรรถนะสูงเพื่อวิเคราะห์สารปฏิชีวนะฟลูออโรควิโนโลนตกค้างในผลิตภัณฑ์นม

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## ABSTRACT

A simple solid-phase extraction procedure combined with high performance liquid chromatography was optimized for analysis of fluoroquinolone antibiotics in milk products. A reversed-phase Symmetry C18 column with gradient mobile phase of acetonitrile and formic acid was used for separation. The studied fluoroquinolones, including norfloxacin, ciprofloxacin and enrofloxacin were detected using fluorescence detector at excitation and emission wavelengths of 280 and 450 nm, respectively. Milk samples were treated by preliminary protein precipitation with acetonitrile–formic acid mixture solution followed by solid-phase extraction using Oasis HLB cartridge. Good recoveries were obtained for all analytes and the limits of detection were 15–20 μg $L^{-1}$. The results demonstrated adequate sensitivity and selectivity to allow quantification of fluoroquinolones in various milk products.

## บทคัดย่อ

การหาสภาวะที่เหมาะสมสำหรับการวิเคราะห์สารปฏิชีวนะฟลูออโรควิโนโลนในผลิตภัณฑ์นมโดยวิธีการสกัดด้วยวัฏภาคของแข็งร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูง สภาวะการแยกด้วยคอลัมน์รีเวิร์สเฟส Symmetry C18 โดยใช้การชะแบบเกรเดียนท์ของเฟสเคลื่อนที่ที่ประกอบด้วยอะซิโตไนไตรล์และกรดฟอร์มิก สารฟลูออโรควิโนโลนที่ศึกษา ได้แก่ นอร์ฟลอกซาซิน ซิโพรฟลอกซาซิน และเอนโรฟลอกซาซิน สามารถตรวจวัดโดยใช้เครื่องตรวจวัดฟลูออเรสเซนต์ ที่ความยาวคลื่นกระตุ้น 280 นาโนเมตร และความยาวคลื่นคายแสง 450 นาโนเมตร ก่อนการวิเคราะห์ มีการเตรียมตัวอย่างนมโดยวิธีตกตะกอนด้วยสารละลายผสมอะซิโตไนไตรล์และกรดฟอร์มิก และการสกัดด้วยวัฏภาคของแข็งโดยใช้ตัวดูดซับชนิด Oasis HLB พบว่า ค่าการกลับคืนของการวิเคราะห์อยู่ในช่วงที่ดี และค่าขีดจำกัดต่ำสุดของการตรวจวัดอยู่ในช่วง 15 – 20 ไมโครกรัมต่อลิตร ผลการทดลองที่ได้แสดงให้เห็นว่าวิธีที่นำเสนอมีสภาพไวและความจำเพาะที่สามารถวิเคราะห์สารฟลูออโรควิโนโลนในตัวอย่างผลิตภัณฑ์นมชนิดต่างๆ ได้

**Key Words:** Fluoroquinolone antibiotics, Solid-phase extraction, HPLC

**คำสำคัญ:** สารปฏิชีวนะฟลูออโรควิโนโลน การสกัดด้วยวัฏภาคของแข็ง โครมาโทกราฟีของเหลวสมรรถนะสูง

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## Introduction

Fluoroquinolone antibiotics are synthetic antibacterial compounds widely used in veterinary applications owing to enhanced antibacterial activities against gram-positive and gram-negative organisms. The widespread use of these compounds in agriculture has resulted in the presence of residues in foodstuff of animal origin. It can increase the risk of food-borne antibiotic-resistant pathogenic bacteria and contribute to the exponential increase of microbial resistance problems. The use of fluoroquinolones in lactating breeding animals may leave residues in milk. Because a large amount of milk is consumed all over the world (Vera-Candioti et al., 2010), therefore, the occurrence of fluoroquinolone residues in milk products has drawn great attention. To ensure safety, it is necessary to control/monitor residual levels of these compounds in order to meet regulatory requirements and especially to protect the consumer.

The most commonly employed analytical techniques for determination of fluoroquinolones are based on high performance liquid chromatography (HPLC) with different detection systems, such as UV (Tsai et al., 2009; Galarini et al., 2009), chemiluminescence (Li et al., 2011), fluorescence (Galarini et al., 2009; Lee et al., 2007; Rodriguez et al., 2008; Herrera-Herrera et al., 2009; Seifrtova et al., 2010) and mass spectrometry (MS) (Lee et al., 2007; Seifrtova et al., 2010; Xiao et al., 2008; Tong et al., 2009; Tang et al., 2009). Fluorescence is found to be suitable for their detection because fluoroquinolones are naturally highly fluorescent compounds.

Fluoroquinolones in milk are often presented at trace concentration levels, but with high amounts of protein and fat. Therefore, a preconcentration step is necessary before analysis. Current methods for pretreatment of milk samples involve a first step protein precipitation using various protein precipitating reagents, i.e. mixture solutions of methanol–trichloroacetic acid (TCA) (Van Hoof et al., 2005), acetonitrile–TCA (Rodriguez et al., 2008). Solid-phase extraction (SPE) using commercially available Oasis HLB polymeric sorbent, that contains lipophilic divinylbenzene units and more hydrophilic *N*-vinylpyrrolidone units, has been used for a second clean-up step and preconcentration of the analytes in many studies (Tong et al., 2009; Vieno et al., 2006; Seifrtová et al., 2008; Speltini et al., 2010)

In the present study, we optimize the chromatographic condition for analysis of fluoroquinolones, including norfloxacin, ciprofloxacin and enrofloxacin, in milk samples. Simple clean-up and preconcentration procedures, based on protein precipitation and SPE, prior to HPLC analysis were also optimized. Applicability of the method aimed for determination of fluoroquinolone antibiotic residues in various milk samples irrespective of the fat contents.

## Materials and methods

### Chemicals and reagents

All fluoroquinolone antibiotic standards (ciprofloxacin, enrofloxacin and norfloxacin) were obtained from the U.S. Pharmacopeia (USA). Stock solutions (1000 μg $mL^{-1}$) of ciprofloxacin and norfloxacin were prepared in 0.1 mol $L^{-1}$ hydrochloric acid. Enrofloxacin stock solution (1000 μg $mL^{-1}$) was prepared in methanol. Working standard solutions were prepared by diluting the stock solution with deionized water. Deionized water resistivity of 18.2

MΩ·cm obtained from a RiOs™ Type I Simplicity 185 (Millipore, USA) was used throughout the experiments. Acetonitrile of HPLC grade was obtained from Lab-Scan Asia (Bangkok, Thailand). Formic acid and hydrochloric acid of analytical reagent grade were obtained from Carlo Erba (Milan, Italy). An Oasis hydrophilic – lipophilic balanced (HLB) SPE cartridge (6 mL, 200 mg) was purchased from Waters.

Pasteurized bovine milk samples (skimmed, full cream and sweetened) and powdered infant formulae were purchased from supermarkets in Khon Kaen province. Powdered infant formulae were prepared in water before analysis.

**Instrumentation**

Chromatographic separation was performed using a Waters HPLC system (Waters, Massachusetts, USA) consisted of an in-line degasser, a 600 multisolvent delivery system, a Rheodyne injector with 20 µL injection loop and a Waters 2475 fluorescence detector. Empower software was used for control the system and data acquisition. An analytical column was a reversed-phase Symmetry C18 (3.9 mm i.d. x 150 mm, 5 µm particle diameter, Waters, Massachusetts, USA) maintained at ambient temperature. The detector was operated at 280 nm excitation and 450 nm emission wavelengths.

**Sample preparation**

For recovery experiment, milk sample (3.00 mL) was spiked with different aliquots of standard solution of all the fluoroquinolones studied. Then, the mixture of 20% acetonitrile and 0.5% formic acid (2.5 mL) was added in order to promote protein precipitation. The mixture was added with 10 mL water and centrifuged for 10 min. The supernatant was withdrawn and passed through the HLB cartridge. The analytes were eluted by the solution of 20% acetonitrile in 0.5% formic acid. The eluate was filtered through a 0.2 µm pore size syringe filter before injecting into the HPLC system.

## Results and discussion

**Chromatographic separation**

The HPLC separation of the studied fluoroquinolone antibiotics was performed on a reversed-phase system using gradient mobile phase of acetonitrile and 0.1% formic acid with the flow rate of 0.9 mL $min^{-1}$. A chromatogram of mixture standard is shown in Fig. 1. The resolutions between the adjacent peaks were 0.79 for norfloxacin and ciprofloxacin, and 2.04 for ciprofloxacin and enrofloxacin. It could be observed that norfloxacin and ciprofloxacin peaks were not well resolved. In this work, the first derivative was used. Improving of the chromatographic resolutions was obtained, corresponding to the resolutions of 1.00 for norfloxacin and ciprofloxacin, and 2.39 for ciprofloxacin and enrofloxacin. Quantitative analysis of the individual analyte can be determined using the peak area of the total derivative peaks.

**Solid Phase Extraction**

SPE using Oasis HLB cartridge was selected for preconcentration of fluoroquinolones in this work. Before sample loading, the SPE sorbent was pre-conditioned with 5 mL of acetonitrile. Concerning the retention efficiency of the target fluoroquinolones on SPE sorbent as well as the enrichment factor, experimental parameters including type and volume of eluting solvent, and flow rate were optimized. The sample volume of 10.00 mL was loaded into the HLB cartridge. The minimal eluent volume of

2.00 mL that providing the quantitative recovery of the analytes was selected in order to reach the high sensitivity.

The mixture of acetonitrile and formic acid was optimized as eluting solvent. To maximize the recovery of fluoroquinolones, the influence of eluting solvent compositions was studied by varying the concentration of acetonitrile and formic acid. Acetonitrile contents from 10% to 40% did not have a pronounced effect on the elution efficiencies of ciprofloxacin and enrofloxacin, but higher amount of norfloxacin was eluted from the cartridge when increasing acetonitrile to 20%, see Fig 2. Further experiment was performed by studying the effect of formic acid concentration (0.05–2.00%) in eluting solvent. The highest peak areas of all fluoroquinolones studied were obtained using 0.5% formic acid, as shown in Fig 3. Therefore, the solution containing 20% acetonitrile and 0.5% formic acid was selected as eluting solvent in this work. Because the sensitivity was also affected by the flow rate, therefore, the flow rates of sample loading and eluting steps were studied in the range of 1–3 mL $min^{-1}$, as shown in Fig 4. Using lower flow rate resulted in a higher sensitivity, and in this study, the flow rate of 1.5 mL $min^{-1}$ was selected for both sample loading and eluting steps because it gave high efficiency.

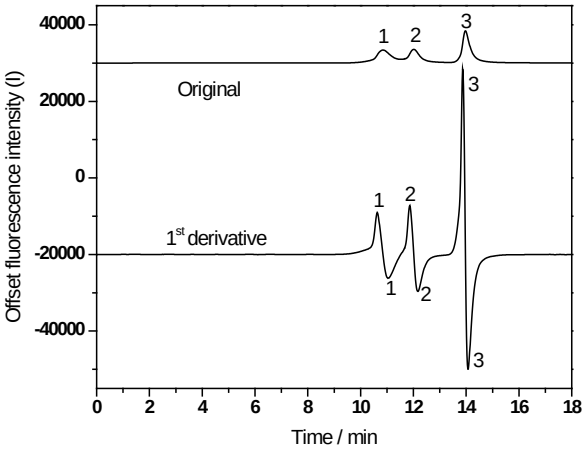


**Fig 1** Chromatograms of fluoroquinolone antibiotics
1, norfloxacin; 2, ciprofloxacin; 3, enrofloxacin

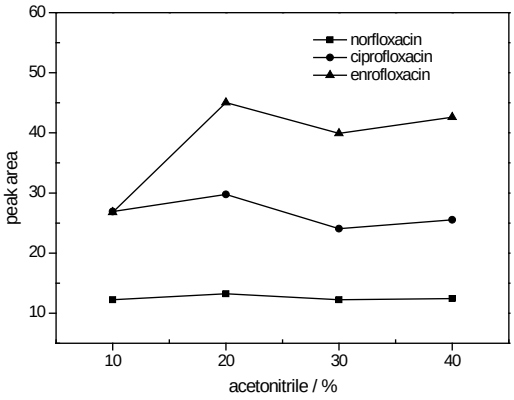


**Fig 2** Influence of acetonitrile concentration on SPE of fluoroquinolones

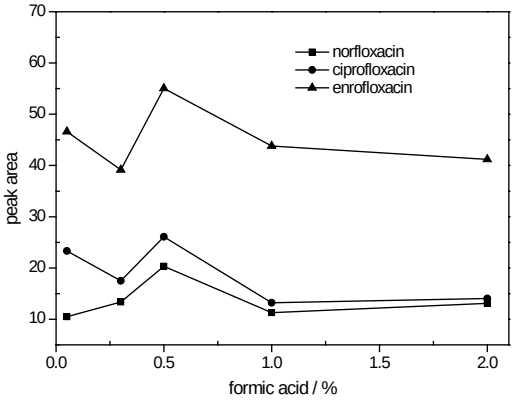


**Fig 3** Influence of formic acid concentration on SPE of fluoroquinolones

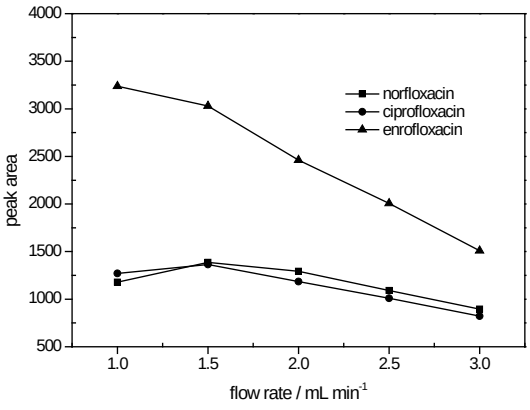


**Fig 4** Influence of flow rate on SPE of fluoroquinolones

**Analytical performance**

The validation parameters were evaluated. Calibration curves of each fluoroquinolone were linear in the ranges of 0.02–0.10 μg $mL^{-1}$ and 0.10–1.00 μg $mL^{-1}$ with the correlation coefficients of greater than 0.98. The limits of detection (LODs) were calculated as three-times the signal-to-noise ratio (S/N = 3). Since the signal was enhanced with the enrichment factor of 10 using SPE procedure, the lower LODs were obtained at 15 μg $L^{-1}$ for enrofloxacin and 20 μg $L^{-1}$ for norfloxacin and ciprofloxacin. The precision of the overall method was evaluated with regard to both the intra- and inter-day reproducibility of the retention time and peak area. By performing replicate analyses of fluoroquinolone standard solutions containing 0.10 μg $mL^{-1}$ each in a day (n = 5, intra-day) and several days (n = 5 x 3, inter-day), the relative standard deviations (RSDs) were lower than 4% and 10% for retention time and peak area, respectively.

**Analysis of milk samples**

It has been known that milk products contain high amounts of proteins and fat, deproteinization and defattening are required for pretreatment of the sample prior to analysis by the proposed method. In this work, we decided to use the mixture of acetonitrile: 0.5% formic acid (20:80) as precipitating solvent because of its compatibility with the mobile phase being used by HPLC. The volume of solvent used for deproteinization was varied in the range of 2.5–7.0 mL. It was observed that using the solvent volume less than 2.5 mL, protein precipitation could not be completed. The solution of 2.5 mL was found to be the minimal volume providing clear extracts, and was selected for further experiments.

Application of the proposed method was tested with milk samples containing different protein and fat contents. It was found that no residue of fluoroquinolone antibiotics was observed in the studied milk samples. Typical chromatograms of bovine milk and powdered infant formula with spiked fluoroquinolones are illustrated in Fig 5. It is clearly seen that the sample treatment procedure can remove matrix interference from the sample, resulting clean chromatogram.

Method accuracy was expressed as percentage recovery. Fortification of different milk samples with standard solutions at concentration level of 50 μg $L^{-1}$ treated by the method showed acceptable recoveries in the range of 58–94% for norfloxacin, 44–104% for ciprofloxacin and 52–96% for enrofloxacin, as summarized in Table 2.

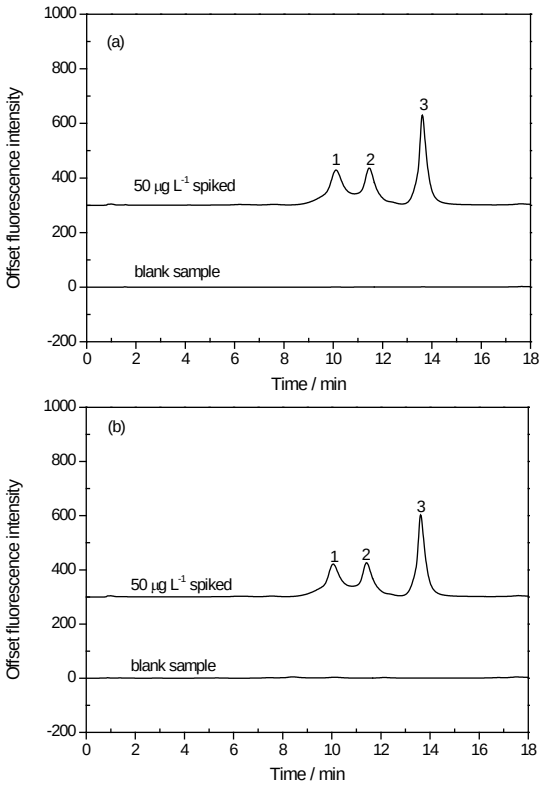


**Fig 5** Typical chromatograms of (a) bovine milk and (b) powdered infant formula: 1, norfloxacin; 2, ciprofloxacin; 3, enrofloxacin

**Table 2** Recovery for determination of fluoroquinolones in spiked milk products

| samples[a] | Recovery (%) | | | samples[a] | Recovery (%) | | |
|---|---|---|---|---|---|---|---|
| | norfloxacin | ciprofloxacin | enrofloxacin | | norfloxacin | ciprofloxacin | enrofloxacin |
| 1 | 90 | 90 | 96 | 9 | 82 | 75 | 88 |
| 2 | 94 | 86 | 92 | 10 | 90 | 64 | 88 |
| 3 | 86 | 104 | 96 | 11 | 90 | 78 | 96 |
| 4 | 90 | 100 | 96 | 12 | 78 | 78 | 82 |
| 5 | 88 | 86 | 92 | 13 | 84 | 70 | 80 |
| 6 | 94 | 82 | 96 | 14 | 82 | 82 | 84 |
| 7 | 78 | 78 | 92 | 15 | 84 | 76 | 80 |
| 8 | 58 | 44 | 78 | 16 | 82 | 73 | 77 |

[a]samples: 1–9, bovine milks; 10–16, powder infant formulae

## Conclusion

In this work, simple SPE and HPLC procedures for determination of fluoroquinolones using signal derivatization were demonstrated. Fluoroquinolones could be determined in a wide concentration range. The method was validated, obtaining satisfactory recoveries and excellent precision, demonstrating its usefulness as an analytical tool in the quality control of the milk products. The methodology could be extended to determine other samples of interest.

## Acknowledgement

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