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# Ultrasound-assisted extraction coupled with solid-phase extraction coupled with dispersive liquid–liquid microextraction for determination of enrofloxacin in chicken meat

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**Abstract**

Ultrasound-assisted extraction combined with solid-phase extraction combined with dispersive liquid–liquid microextraction (UAE-SPE-DLLME) has been developed as a new approach for the extraction of enrofloxacin in chicken meat prior to high performance liquid chromatography with UV detection. In the UAE-SPE-DLLME, enrofloxacin was first extracted from 1.0 g chicken meat into the mixture of 10 mL acetonitrile acidified with formic acid buffer (pH=4) and 10 mL EDTEA-McIlvaine buffer (0.1 M) and 20 mL n-hexane as an extracting phase by using ultrasound-assisted extraction. Then, the extract by ultrasound-assisted extraction was used for solid-phase extraction. After clean-up, enrofloxacin was preconcentrated by using DLLME technique. Thus, 1.5 mL methanol extract (disperser solvent) and 200 µL chloroform (extraction solvent) were added to 5.0 mL ultrapure water and a DLLME technique was applied. Under the optimum conditions, the linearity of the method was in the range from 10 to 500 µg $kg^{-1}$ with the correlation coefficient ($r^2$) of 0.9972. The method detection limit was 5.0 µg $kg^{-1}$. The proposed method has been successfully applied to the analysis of the enrofloxacin in chicken meat, and a satisfactory result was obtained.

**Keywords:** Enrofloxacin, Chicken meat, Dispersive liquid-liquid microextraction, Solid-phase extraction, Ultrasound-assisted extraction

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**1.Introduction**

Enrofloxacin (ENR) is a fluoroquinolone with a broad antibacterial spectrum and high bactericidal activity against major pathogenic bacteria found in diseased animals [1-3]. The structure of ENR was shown in the Figure 1. It is a lipophilic molecule with a logP of 4.70 at a pH of 7. The pharmacokinetics of ENR is characterized by good absorption and extensive distribution into various animal fluids and tissues. The widespread administration of these drugs in veterinary medicine represents a potential risk, because their residues may persist inedible animal tissues and may result in the development of drug-resistant bacterial strains or allergies [4,5]. Therefore, the determination of their residues in chicken meat used for human consumption is an important task.

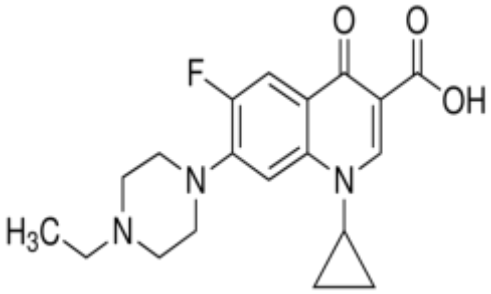



**Figure 1.** The structure of enrofloxacin

Many analytical methods for the determination of fluoroquinolone residue in food-producing animals are described in the scientific literature. Most of these rely on liquid chromatographic (LC) methods using UV [6], fluorescence [7,8] or mass spectrometric (MS) detection [9]. Owing to its specifity, mass spectrometry is a powerful confirmatory technique; it is, however, expensive and thus not available to all laboratories.

Most of these methods involve a preliminary extraction step followed by a second clean-up step with liquid-liquid extraction or solid-phase extraction (SPE). As such, these approaches are complicated, time-consuming, and use large amounts of organic solvents and these are time consuming sample preparation methods.

Dispersive liquid–liquid microextraction (DLLME) is a fairly new method of sample preparation, initially proposed by Assadi et al. [10]. DLLME is a miniaturized LLE that uses microliter volumes of extraction solvent. This method is based on a ternary component solvent system in which the extraction solvent and disperser solvent are injected into aqueous sample by syringe. The mixture is shaken and a cloudy solution is formed in the test tube. After centrifugation, the extract is taken with a micro syringe and analyzed. The advantages of DLLME are simplicity of operation, rapidness, low cost, high recovery and robustness, high enrichment factors, and environmental benignity [11-19]. The main disadvantage of the DLLME is that it is not a selective extraction method. On the other hand, the interferences from matrix co-extractives are often present, especially for the determination of trace analytes in a complex matrix sample such as meat sample. This is the main reason that the most reported applications of DLLME have been focused on simple water samples. Therefore, the exploration of the potential applications of the DLLME technique in more complex matrix samples is desirable. SPE is widely used as a sample clean-up and concentration technique in sample preparations. Assadi and co-workers have reported the combination of SPE with DLLME for the selective determination of chlorophenols in aqueous samples with various matrices [20]. One of the advantages of such a combination is that it can be used for complex matrix samples. SPE-DLLME was used for the extraction of different compounds [21-24].

The purpose of the present work is to develop a new analytical approach based on UAE-SPE-DLLME and demonstrate its applicability for extraction and preconcentration enrofloxacin from chicken meat samples and further determination by HPLC-UV. The influence of several factors on the performance of the analytical methodology were studied and optimized. The analytical performance of UAE-SPE-DLLME-HPLC–UV methodology was evaluated in terms of

method detection limit, repeatability and linear working range. Finally, the procedure was applied for the determination of enrofloxacin in chicken meat samples.

**2.Experimental**

**2.1. Chemicals and reagents**

Stock solution of enrofloxacin (Sigma, St. Louis, MO, USA, >98%) was prepared in methanol at a concentration of 1 mg $mL^{-1}$ and stored at – 18 ºC; working solutions of standards at suitable concentrations were prepared every day from the stock solution. Carbon tetrachloride (>99.5%), chloroform (>99.8%), chlorobenzene (>99%), carbon tetrachloroethylene (>99.6%), acetone (>99.8%), acetonitrile (>99.9%), methanol (>99.9%), ethanol (>99.5%), n-hexane (>99%), formic acid (>98%) and sodium chloride (>99.5%) were obtained from Merck (Darmstadt, Germany).

$Na_2HPO_4$ (>98%), $Na_2EDTA$ (>99%), sodium citrate (>99%) and citric acid (>99.5%) were of analytical reagent grade obtained from Merck. The McIlvaine solution was prepared using 0.2 mol $L^{-1}$ citric acid and 0.2 mol $L^{-1}$ $Na_2HPO_4$ (8:2, *v/v*). 0.1 mol $L^{-1}$ of EDTA-McIlvaine buffer was prepared using 37.2 g $Na_2EDTA$ dissolved in 1 L McIlvaine solution. The solution of 0.1 M citric acid and 0.1 M sodium citrate (18.6:1.4, *v/v*) was used to acidify ACN (pH = 4.0).

The water used was purified on a Nanopure ultra pure water purification system (Nano pure, USA).

**2.2. HPLC system**

An Agilent 1100 series HPLC system including a quaternary pump and a UV detector were used for separation and determination of the analyte. The separation was performed on Zorbax Eclipse XDB-$C_{18}$ (250 mm × 4.6 mm ID, 5µm) column. Water and acetonitrile and trimethyl amine (85:14.5:0.5,*v/v*) were used as mobile phase in isocratic elution mode. The chromatographic data were collected and recorded using ChemStation software. The direct sample introduction was carried out using a Rheodyne manual injector (Rohnert Park, CA, USA) with a 20 µL loop. Column temperature was kept constant at 25 ˚C using a thermostatted column compartment. The flow rate was 1 ml $min^{-1}$ and detection was performed at 277 nm.

**2.3. Preparation of spiked chicken meat samples**

Chicken meat were obtained from Mazandran slaughterhouse (Iran) and were ready for marketing. The samples, typically 200g, were first minced using a kitchen homogenizer (Multi moulinette, Moulinex, France). Then, in separate plastic bags (20 g in each bag) were placed and were frozen at - 20 ºC until the analysis. Chicken meat were repeatedly measured to confirm that no antibiotic was present, was used for preparation of fortified samples. Prior to extraction, an appropriate amount of the homogenized samples of meat were spiked by adding an appropriate amount of the standard solution to give fortification level of 50 µg $kg^{-1}$.

**2.4. Ultrasound-assisted extraction**

In order to enhance the recovery and shorten extraction time, we used ultrasound-assisted extraction. The optimization of the ultrasound-assisted extraction of the antibiotic from chicken meat samples was developed with the samples that free of the antibiotic. For this purpose, extraction of the spiked samples (50.0 µg $kg^{-1}$ fortification level) was carried out with different extraction phases which was shown in the Table 1. Different amounts of samples (1.0 and 5.0 g) were sonicated with the solvents between 5 and 30 min. Results (Table 1) showed that the best recovery was achieved using the mixture of 10 mL acetonitrile acidified with formic acid buffer (pH=4) and 10 mL EDTEA-McIlvaine buffer (0.1 M) and 20 mL n-hexane as an extracting phase. The addition of a quelating agent was performed, EDTA, especially to compete with the antibiotic. It is known that this compound can form complex with the bi-and trivalent cations present in the sample extraction solution which can lead to significant losses of this compound during the procedure. The presence of another compound, as

EDTA, which has similar behavior, is responsible for the improvement of performance of this antibiotic avoiding drastically those losses. n-hexane was used to minimize the lipid content from the meat and thus the potential interferences during analysis.

**Table 1.** Extraction recovery for different used extraction phases

| Extraction conditions | Extraction recovery (%) |
|---|---|
| 5.0 g sample + 10 mL of ACN acidified with formic acid buffer (pH=4) + 10 mL EDTA (0.1 M) | 8 |
| 1.0 g sample + 10 mL of ACN acidified with formic acid buffer (pH=4) + 10 mL EDTA-McIlvaine buffer (0.1 M) | 25 |
| 5.0 g sample + 20 mL of ACN + 20 mL EDTA-McIlvaine buffer (0.1 M) | 11 |
| 1.0 g sample + 10 mL EDTA-McIlvaine buffer (0.1 M) | 6 |
| 1.0 g sample + 10 mL of MeOH acidified with formic acid buffer (pH=4) + 10 mL EDTA-McIlvaine buffer (0.1 M) | 9 |
| 1.0 g sample + 10 mL of ACN acidified with formic acid buffer (pH=4) + 10 mL EDTA-McIlvaine buffer (0.1 M) + 10 mL n-Hexane | 38 |
| 5.0 g sample + 20 mL of ACN acidified with formic acid buffer (pH=4) + 20 mL EDTA-McIlvaine buffer (0.1 M) + 10 mL n-Hexane | 19 |
| 1.0 g sample + 10 mL ethyl acetate + 10 mL EDTA (0.1 M) | 12 |
| 1.0 g sample + 10 mL Acetone + 10 mL EDTA (0.1 M) + 10 mL n-Hexane | 7 |
| 1.0 g sample + 20 mL of ACN acidified with formic acid buffer (pH=4) + 20 mL EDTA-McIlvaine buffer (0.1 M) + 20 mL n-Hexane | 22 |
| 1.0 g sample + 10 mL of ACN acidified with formic acid buffer (pH=4) + 10 mL EDTA-McIlvaine buffer (0.1 M) + 20 mL n-Hexane | 48 |
| 1.0 g sample + 10 mL of ACN acidified with formic acid buffer (pH=4) + 10 mL McIlvaine buffer + 20 mL n-Hexane | 24 |
| 1.0 g sample + 10 mL citric acid buffer (0.2 M, pH=4.7) + 20 mL n-Hexane | 24 |
| 1.0 g sample + 10 mL McIlvaine buffer (pH=4.7) + 20 mL n-Hexane | 28 |
| 1.0 g sample + 10 mL mixture 85:15 of buffered water (pH=9) and ACN + 20 mL n-Hexane | 33 |
| 1.0 g sample + 10 mL mixture 1:1 MeOH and ACN + 20 mL n-Hexane | 10 |
| 1.0 g sample + 10 mL mixture 3:1 of water and ACN + 20 mL n-Hexane | 4 |

Different amounts of water were used for dilution the extracts. Extracts were collected and brought to 30, 40, 60 and 90 mL. The results show that when the 40 mL was used, the best recovery was obtained and more dilution causes to decrease the extraction efficiency of the analyte. Therefore, extract was collected and brought up to 40 mL with deionized water. This final test portion of 40 mL was passed through the Oasis HLB cartridges using the SPE procedure.

## 3. Results and discussion

In this work, UAE-SPE-DLLME-HPLC-UV was applied to determination of enrofloxacin from chicken meat samples. To achieve a high extraction recovery, the UAE, SPE and DLLME conditions were optimized.

### 3.1. Effect of type and volume of the extraction solvent

Performance of DLLME is mainly determined by the type and volume of extractant. In this work, $CHCl_3$, $CCl_4$, $C_6H_5Cl$ and $C_2Cl_4$ were evaluated as potential extractants. A series of sample solutions were tested using 1.5 mL methanol, containing different volumes of the extraction solvents to achieve about 25 µL volume of the sedimented phase. Thereby, 200.0, 57.0, 55.0 and 52.0 µL of $CHCl_3$, $CCl_4$, $C_6H_5Cl$ and $C_2Cl_4$ were used, respectively. As shown in figure 2, $CHCl_3$ possessed the highest extraction recovery as compared with other extraction solvents.

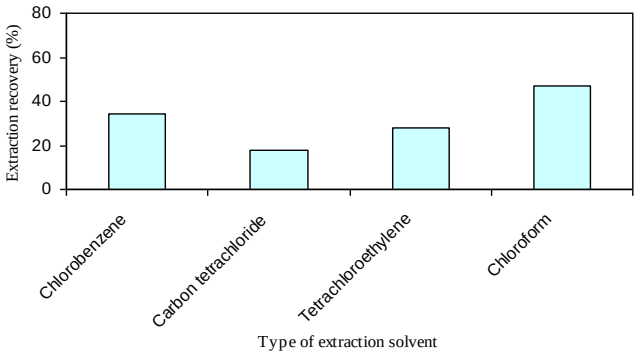



**Fig.2.** Effect of type of extraction solvent on the extraction efficiency. Extraction conditions: disperser solvent (methanol) volume,1.5 mL; extraction solvent volumes, 200.0 µL $CHCl_3$, 55.0 $C_6H_5Cl$, 57.0 $CCl_4$, 52.0 $C_2Cl_4$; flow rate, 6.7 mL $min^{-1}$.

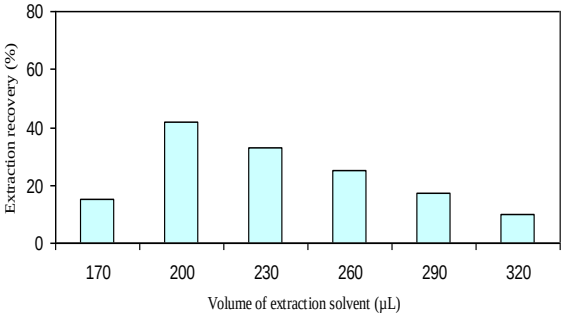



**Fig.3.** Effect of the extraction solvent ($CHCl_3$) volume on the extraction efficiency of the analyte which obtained from UAE-SPE-DLLME. Extraction conditions: disperser solvent (methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volumes, 170.0, 200.0, 230.0, 260.0, 290.0 and 320.0 µL; flow rate, 6.7 mL $min^{-1}$.

To evaluate the effect of the extraction solvent volume on the extraction efficiency, a constant volume (1.5 mL) of methanol containing different volumes of $CHCl_3$ (170.0, 200.0, 230.0, 260.0, 290.0 and 320.0 µL µL) were subjected to the same DLLME procedures. By increasing the volume of $CHCl_3$ from 170.0 to 200.0 µL, the extraction efficiency of enrofloxacin increases, but by increasing the volume of $CHCl_3$ from 200.0 to 320.0 µL, the extraction efficiency of enrofloxacin decreases (Fig. 3). Because the concentration of the analyte in the sedimented phase decreases and dilution effect. On the basis of these results, 200.0 µL of $CHCl_3$ was selected for subsequent experiments.

### 3.2. Effect of type and volume of disperser solvent

The elution solvent in the SPE step is used as the disperser solvent in the DLLME. A series of sample solutions were tested using 1.5 mL of acetone, acetonitrile, ethanol and methanol containing 200.0 µL volume of $CHCl_3$ (as extraction solvent). The results (Fig. 4) indicate that methanol has the highest extraction efficiency in comparison with the other tested solvents. Thus, methanol was chosen as the disperser or eluent solvent for subsequent experiments.

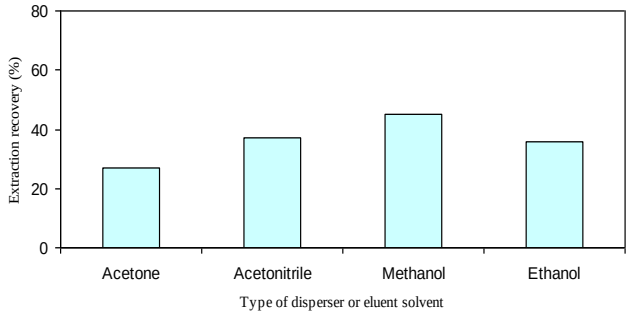



**Fig.4.** Effect of type of disperser or eluent solvent on the extraction efficiency. Extraction conditions: disperser solvent (acetone, acetonitrile, ethanol and methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volume, 200.0 µL; flow rate, 6.7 mL $min^{-1}$.

In order to examine the effect of disperser solvent volume, the volume of the sedimented phase was kept constant (about 25 µL) and the volume of methanol and $CHCl_3$ was changed, simultaneously. The different volumes of methanol (0.5, 1.0, 1.5 and 2.0 mL) were in concomitant with the corresponding volumes of 175.0, 188.0, 200.0 and 215.0 µL of $CHCl_3$, respectively. It was obvious from Fig. 5 that 1.5 mL of methanol has highest recovery than that of the others. Therefore, 1.5 mL was selected as the volume of methanol.

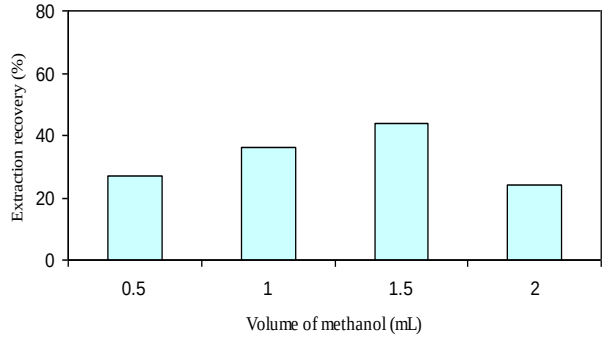



**Fig.5.** Effect of the disperser solvent (methanol) volume on the extraction efficiency of the analyte which obtained from UAE-SPE-DLLME. Extraction conditions: disperser solvent (methanol) volumes, 0.5, 1.0, 1.5 and 2.0 mL; extraction solvent ($CHCl_3$) volumes, 175.0, 188.0, 200.0 and 215.0 µL; flow rate, 6.7 mL $min^{-1}$.

### 3.3. Effect of the flow rate of the sample solution

The flow rate of the sample solution through the solid phase is an important factor, because it controls the time of analysis. The flow rate, on the one hand, must be low enough to perform an effective retention of the analyte. On the other hand, it must be high enough not to waste time. The flow rate influence of the sample solutions from the solid-phase cartridge on the enrofloxacin recovery was investigated in the range of 0.65-8.6 mL $min^{-1}$. It was found that in the range of 0.65-6.7 mL $min^{-1}$, the enrofloxacin recovery by the cartridge was not affected considerably by the sample solution flow rate (Fig. 6). According to the result, 6.7 mL $min^{-1}$ was used as the best sample flow rate.

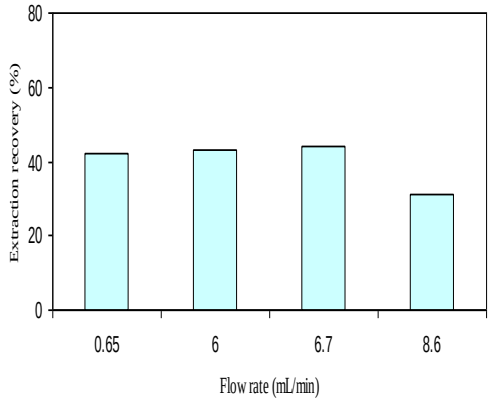



**Fig.6.** Effect of flow rate on the extraction efficiency of the analyte which obtained from UAE-SPE-DLLME. Extraction conditions: disperser solvent (methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volume 200.0 µL.

### 3.4. Analytical performance

The characteristics of calibration curve were obtained under optimized conditions (Table2). The linearity of calibration curve was observed in the range of 10-500 µg $kg^{-1}$. The coefficient of correlation ($r^2$) was 0.9972. The precision of the proposed method was evaluated by carrying out five independent measurements of the studied compound

at 20 µg $kg^{-1}$. The result show that the relative standard deviation (RSD) was 8.6%. The limit of detection (LOD), based on signal-to-noise (S/N) of 3, was 5.0 µg $kg^{-1}$. The limit of quantitation (LOQ), based on signal-to-noise (S/N) of 10, was 10 µg $kg^{-1}$.

**Table 2.** Quantitative results of UAE-SPE-DLLME and HPLC-UV method for enrofloxacin

| Linear range (µg $kg^{-1}$) | LOD[a] (µg $kg^{-1}$) | LOQ[b] (µg $kg^{-1}$) | RSD (%)[c] | $r^{2d}$ |
|---|---|---|---|---|
| 10-500 | 5.0 | 10 | 8.6 | 0.9972 |

[a] LOD, limit of detection for S/N=3
[b] LOQ, limit of quantitation for S/N=10
[c]RSD, relative standard deviation (n=5)
[d]coefficient of determination

Table 3 compare the proposed method with other extraction methods for the determination of the target analyte in chicken meat samples. The quantitative results of the proposed method are better than of molecularly imprinted matrix solid-phase dispersion [25] and solid-phase extraction (SPE) [26, 27]. In comparison with molecularly imprinted matrix solid-phase dispersion and solid-phase extraction, the evaporation of the final extraction phase (25 µL) in the proposed method is easier with compare with them. This procedure cause the loss of the analyte and time-consuming. Finally, the proposed method has great potential to determine the selected analyte at trace levels in chicken meat samples.

**3.5. Analysis of samples**

The proposed UAE-SPE-DLLME technique was applied for the determination of enrofloxacin in chicken meat samples. The obtained results are summarized in Table 4. These samples were spiked with the enrofloxacin standard solution at different concentration levels to assess the matrix effects. Figure 7 depict the attained chromatograms before and after the spiked chicken meat samples at the concentration level of 100 µg $kg^{-1}$. As shown in Table 4, the relative recoveries varied between 88-94%, demonstrating that the matrices of the analyzed real samples have little effect on the performance of the UAE-SPE-DLLME method.

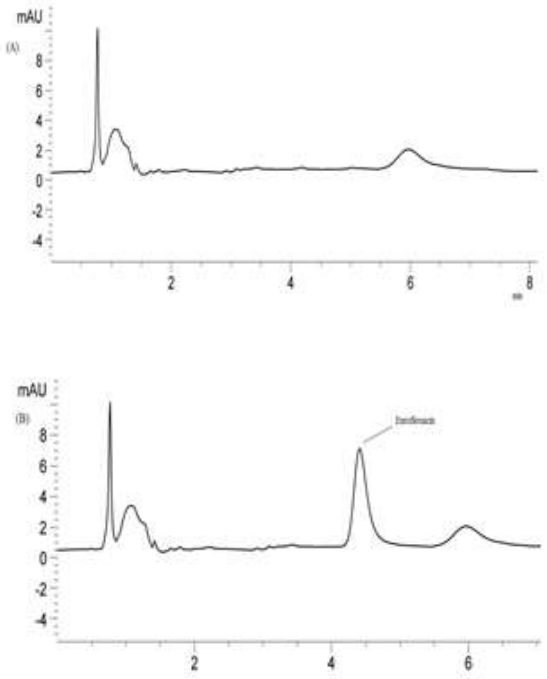
**Table 3.** Comparison of the proposed method with other extraction methods for determination of the target analyte in chicken meat samples

| Methods | R.S.D.% | Dynamic linear range (µg kg $^{-1}$) | Limit of detection (µg kg$^{-1}$) | Extraction time (min) | Ref. |
|---|---|---|---|---|---|
| molecularly imprinted matrix solid-phase dispersion-HPLC-DAD | 5.6 | 30-200000 | 8 | 2 | [25] |
| Solid-phase extraction-HPLC-Fluorescence detector | <4 | 20-2000 | 10 | 5 | [26] |
| Solid-phase extraction-HPLC-Fluorescence detector | <7 | 25-1000 | - | 5 | [27] |
| UAE-SPE-DLLME-HPLC-UV | 8.6 | 10-500 | 5 | A few second | This work |

**Table 4.** Determination of enrofloxacin in chicken meat samples

| Spiking level (µg kg$^{-1}$) | Concentration (µg kg$^{-1}$) | RSD (%) , n=3 | Relative recovery (%) |
|---|---|---|---|
| 25 | n.d[a]. | 16.1 | 88 |
| 50 | n.d. | 13.5 | 91 |
| 100 | n.d. | 11.8 | 94 |

[a] Not detected.




## 4. Conclusions

We have found that UAE-SPE-DLLME-HPLC-UV is an sensitive and reliable method for the extraction and determination of enrofloxacin in chicken meat samples. The analytical technology offered numerous advantages such as ease of operation, high extraction recovery, and low detection limit. Accordingly, the proposed method possesses great potential in the analysis of enrofloxacin in chicken meat samples.

**Fig.7.** HPLC chromatograms of (A) before spiking with the analyte in chicken meat sample, (B) 100 µg $kg^{-1}$ spiked of the analyte in chicken meat sample after extraction via proposed method at optimum conditions.

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