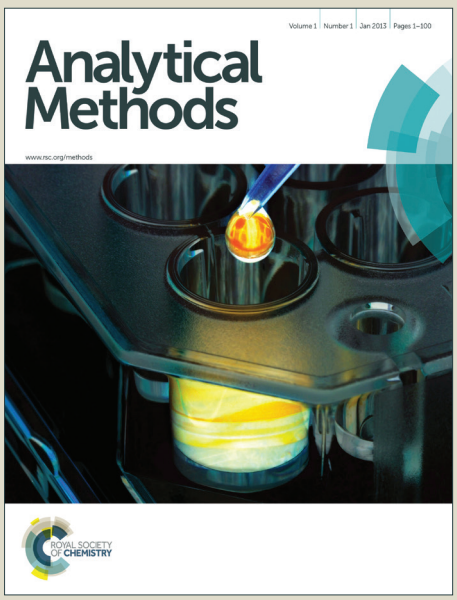
Analytical Methods

Accepted Manuscript



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Salting-out induced liquid-liquid microextraction based on the system of acetonitrile/magnesium sulfate for trace-level quantitative analysis fluoroquinolones in water, food and biological matrices by high-performance liquid chromatography with fluorescence detector

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A list of nonstandard abbreviation used in the paper:

ACN, acetonitrile;

CIP, ciprofloxacin ;

DLLME, dispersive liquid-liquid microextraction ;

DMSPE, dispersive micro-solid- phase extraction;

ENR, enrofloxacin;

FLX, fleroxacin;

FLD, fluorescence detector;

IL-based HLLME, ionic liquid-based homogeneous liquid-liquid microextraction;

IPA, isopropanol;

LOM, lomefloxacin;

LLE, liquid-liquid extraction;

MSPE, magnetic solid-phase extraction;

MeOH, methanol;

NOR, norfloxacinl;

DAD, diode-array detector;

SALLE, salting-out assisted liquid-liquid extraction;

SAR, sarafloxacin;

UA-DLLME, ultrasound-assisted dispersive liquid-liquid microextraction;

UVD, ultraviolet detector.

Running title:

SILLME based on the system of ACN/$MgSO_4$ for analysis of FQs.

Abstract

A convenient, robust and economical salting-out induced liquid-liquid microextraction (SILLME) method coupled with high-performance liquid chromatography/fluorescence detector (HPLC/FLD) for sample preparation, extraction and trace-level quantitative determination of six fluoroquinolones (FQs) in different samples was developed. The critical factors that influence the extraction efficiencies of the target analytes, such as the kind of extraction solvent and salting-out reagent, the ratio of extraction solvent to salt, pH value and extraction time were investigated. The system of acetonitrile/magnesium sulfate showed good extraction efficiencies for the target analytes. Under the optimum conditions, the correlation coefficient (r^2) was obtained within the range of 0.9990-0.9998 by spiking the ultrapure water over the range of 0.002-0.100 μg mL^{-1}. Excellent sensibility was attained with the limits of detection (LODs, S/N=3) ranging from 0.07-0.41 ng mL^{-1}, 0.09-0.62 ng mL^{-1}, 0.48-2.49 μg kg^{-1}, 0.80-5.00 ng mL^{-1}, 0.78-5.58 ng mL^{-1} and 0.40-5.30 μg kg^{-1} for ultrapure water, field water, honey, milk, swine plasma and muscle, respectively. While precision with inter- and intra-day relative standard deviations (RSDs, n=5) for ultrapure water was observed in the range 0.4%-4.0% and 1.3%-6.8%, respectively. Finally, this developed method was successfully applied to all-above mentioned matrices and be a shining method for analysis of FQs.

Keywords: Acetonitrile/magnesium sulfate (ACN/$MgSO_4$); Fluoroquinolones (FQs); High-performance liquid chromatography/fluorescence detector (HPLC/FLD); Salting-out induced liquid-liquid microextraction (SILLME).

Introduction

Fluoroquinolone antibiotics (FQs) are derived from quinolones, they are widely used for human and veterinary owing to their broad activity spectrum against Gram bacteria through inhibition of their DNA gyrase and good oral absorption. As veterinary medicine, FQs are commonly and inappropriately used in food-producing animals for the treatment and prevention of diseases and as feed additives to increase the animal mass. Obviously, these probably lead to the undesirable residues in animal derived food products such as honey, milk, body fluids, animal tissues and the likes. Meanwhile, FQs can enter into the ecosystems via different pathways, including human excretion, disposal of waste (unused medicines) into wastewater, direct treatment of aquaculture products, and dispersal of animal faeces on agricultural soil .[1]

In the last years, FQs have been considered as emerging pollutants which are thought to be potential threats to environmental ecosystems, human health and safety. To be sure is that the residues can cause an increased bacterial resistance, as reported in several studies.[2] Generally, the residues of FQs in different kinds of matrices are trace levels. Therefore, reliable and sensitive methods for the determination of FQs residues in food and environment samples are necessary.

SALLE is based on the salt-induced phase separation phenomenon, organic phase is separated from a homogeneous solution and simultaneously the target analytes are extracted into the organic solvent when inorganic or organic salts are added.[3,4] This phenomenon occurs because dissolution of the salt alters the properties of the system, particularly ionic strength and vapor pressure of the individual solvent component.[3] Salting-out assisted liquid-liquid extraction (SALLE) (This) technique was introduced for extraction of metal chelates by Matkovich et al. in 1973.[5] Over four decades later, SALLE (it) has been developed and applied to the determination of various target analytes in water,[6] plant,[7] food[6, 7] and biological matrices.[8-10] These analytes include inorganic elements, polar drugs or hydrophobic drugs dissolving in organic solvent that can be homogenized with water.[10, 11, 12] During

the process of development, researchers have made great efforts to make the method be more high-throughput and automatic for reducing the consumption of reagents and time involved in the method.[10, 13, 14] In a word, this technique has acquired a new momentum. While our knowledge in the field of analysis has grown in leaps and bounds in terms of sample preparation, the application of this process has still been active for analysis, attributed to the method of SALLE integrates sample cleanup and preconcentration in one single step and shares the advantages of the sample pre-treatment technique-QuEChERS.

Compared to traditional liquid-liquid extraction (LLE), SALLE uses low toxicity and small amount of extraction solvent, vigorous mechanical shaking and vacuum distillation are not required to obtain good extraction efficiency and high enrichment factors. Furthermore, the method of LLE is not applicable to higher polarity. In recent years, dispersive liquid-liquid microextraction (DLLME) has also been extensively used for analysis, but most reported applications of DLLME have focused on simple water sample.[15-17] Solid phase extraction (SPE) is a recently developed popular sample pre-treatment technique for separation, purification and concentration. However, commercial SPE column cartridges are relatively expensive. On the other hand, there are potential concerns about batch-to-batch reproducibility of the SPE column cartridges and precision and reliability of the method depends on the product quality of extraction column cartridges.[18] The precision of SALLE depends on the operational quality, which can be precisely controlled by the operator.

Owing to acetonitrile has high miscibility with water/aqueous media, the use of acetonitrile as a LLE solvent has been very limited. However, utilization of the salting-out technique would aid easy phase separation of acetonitrile from biological and food samples would result in an acceptable matrice effect. Since acetonitrile is an organic solvent less harmful than the conventional liquid-liquid extraction solvents used, makes it more favorable within a green chemistry context. And its polarity is favorable to the extraction of a wide range of compounds.[19]

Based on the above advantages, acetonitrile has shown to be the most promising and frequent extraction solvent for SALLE.[9, 10, 20, 21] In this paper, acetonitrile was used as extraction solvent for SILLME.

Various instruments have been developed for the determination of FQs residues, such as spectrofluorimeter,[22] potentiometric titration,[23] capillary electrophoresis,[24] high-performance liquid chromatography (HPLC) combined with fluorescence detector (FLD),[25] mass spectrometry (MS),[26] diode-array detector (DAD)[27] and ultraviolet detector (UVD).[28] For LC/MS/MS, due to its high sensitivity and selectivity, the direct injection of diluted solvent extracted from samples could provide a fast and reliable way to determine the target antibiotics. However, MS is still quite expensive being not available for chemists in most of laboratories. HPLC methods are widely applied owing to their high selectivity, sensitivity and simple sample treatment by using different detection systems. Therefore, HPLC/FLD was chosen for determination of FQs in this study.

The aim of this article is to develop a convenient, robust, economical and selective sample preparation method SILLME for the determination of six commonly used FQs including fleroxacin (FLX), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM), enrofloxacin (ENR) and sarafloxacin (SAR). The critical parameters that influenced the extraction efficiencies in the method of SILLME, such as kinds and amount of extraction solvent and salting-out reagent, pH value and extraction time were investigated. To our best knowledge, it was the first time that the method of SILLME had applied for the determination of FQs in so many kinds of samples. The results showed that the proposed method was exactly feasible for analysis of FQs in above mentioned matrices.

Experimental

Chemicals and Reagents

The standards FLX (0.1 g, 99.0%), NOR (0.1 g, 99.0%), CIP (0.1 g, 95.0%), LOM (0.1 g, 97.6%), ENR (0.1 g, 99.0%) and SAR (0.1 g, 95.0%) were purchased from Dr.Ehrenstorfer (Germany). Stock standard solutions of individual FQs (500 μg mL^{-1}) were prepared in acetonitrile. The working standard solutions containing six FQs

were prepared monthly by mixing and diluting the stock solutions with acetonitrile. All of the standard solutions were protected from light and stored at -20 °C in a freezer, being stable for at least 3 months.

Different salts were tested as salting-out reagents: magnesium sulfate ($MgSO_4$, ≥99%), sodium sulfate (Na_2SO_4, ≥99%), ammonium sulfate($(NH_4)_2SO_4$, ≥99%), ammonium chloride (NH_4Cl, ≥99%) all obtained from Sinopharm Chemical Reagent Co., Ltd. Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) and phosphoric acid (85-90%) in HPLC-grade were purchased from Lark's chemical technology Co., Ltd (Shanghai, China). Acetone (≥99.5%), triethylamine (≥99.0%) and ethanol absolute (≥99%) in analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. Deionized water was purified with a Millipore Milli-Q plus System (Bedford, MA, USA).

Experimental instruments and chromatographic conditions

An Agilent 1200 series HPLC consisting of degasser, solvent quaternary pump, auto sample injector, column oven and fluorescence detector was used to analyze the analytes. Low speed centrifuge (TDL-50C, Anting, Shanghai), pressure blowing concentrator (MTN-2800, Anpu, Shanghai) and vortex device (Vortex-6, Qilinbeier, Haimen) were used for centrifuging, concentrating and mixing samples, respectively. pH values were measured with precise acidity meter (PHS-3E, Leici, Shanghai).

In the HPLC analysis, a Zorbax Eclipse XDB-C18 column (150 mm×4.6 mm, 5 μm particle size) was used to separate the target analytes with the mobile phase of phosphoric acid solution (50 mmol L^{-1}, the pH was adjusted to 2.8 with triethylamine)-MeOH-ACN (82:13:5, v/v/v) with a flow rate of 1.0 mL min^{-1} . The column temperature was 40 ± 1 °C. The analytes were monitored with excitation and emission wavelength (λem and λex) at 280 nm and 450 nm, respectively. The injection volume was 15.0 μL.

Procedure of SILLME method

5.0 mL of ultrapure water (adjusted to pH 1.5 with phosphoric acid) was placed into a 15 mL conical bottom tube and then 1.0 mL of ACN was added into the tube and vortexed evenly. Then added 2.0 g $MgSO_4$ into the mixture and vortexed for 3 min. After centrifuging at 4300 rpm for 5 min, the supernatant was absorbed carefully into another 10 mL glass tube and dried under the nitrogen flow in a 40 °C water bath. The residue was redissolved in 400 μL of mobile phase and vortexed. After filtered though 0.22-μm filter membrane, 15.0 μL was injected into the HPLC/FLD system for analysis.

Sample preparation

Field water

Field water was sampled from a village of Shaoxing, Zhejiang, China. The field is near to a pig farm. After filtering through 0.45-μm filters, they were stored at 4 °C.

Honey

Honey sample was purchased from a local supermarket. 1.0 g of honey sample was diluted to 10 mL with phosphoric acid solution (pH 1.5) and then spiked with the mixed working solution of FQs. The resulting solution was referred to as sample solution, filtered through 0.45-μm filters, and then stored at 4 °C.

Milk and swine plasma

The milk sample was purchased from a local supermarket. Fresh swine blood sample was obtained from local slaughterhouse. The sample was centrifuged for 15 min at 5000 rpm, and the supernatant was collected and stored at -20 °C.

0.5 mL of milk and swine plasma sample were diluted to 5.0 mL with phosphoric acid solution (pH 1.5)，they were carried out as the procedure of SILLME method.

Swine muscle

Ground fresh swine muscle was obtained from local market and stored at -20 °C until analysis. 0.5 g of swine muscle was measured into a 15 mL conical bottom tube and fortified with the working solution of FQs. Then it was stored overnight at room temperature in the dark, to allow solvent evaporation and FQs adsorption equilibrium to the muscle. Then added 1.2 mL acetonitrile and vortexed for 3.0 min, followed by addition of 5.0 mL of phosphoric acid solution (pH 1.5). Then the subsequent procedure was operated as the procedure of SILLME method.

Results and discussion

Optimization of SILLME

In order to require maximum extraction efficiency of the SILLME method for the analysis of FQs, the parameters influenced extraction efficiency include the selection of extraction solvent, salting-out reagent, extraction solvent volume, amount of salt, sample pH as well as extraction time. The optimization of SILLME conditions was performed using ultrapure water spiked with FQs at the concentration of 80 ng mL^{-1}, each result being obtained from the mean value of three extractions.

Selection of extraction solvent and salting-out reagents

In SILLME, it is important to select the appropriate extraction solvent and salting-out reagents for the extraction and preconcentration of target analytes from aqueous samples. Solvent of MeOH, ACN, ACE and IPA are widely used in SILLME due to their miscibility in water at all proportions, so they were chosen as extraction solvent. $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$ and NH_4Cl were selected as salting-out reagents.

The initial volume of ultrapure water spiked with target analytes and organic solvent were 5.0 mL and 1.0 mL, respectively; 2.0 g each kind of salt was added into the four kinds of organic/water systems respectively to observe the “salting-out” phenomena and calculate the extraction recoveries of the six FQs. No separation occurred when MeOH was used. ACE and IPA were not induced to separate from the mixture except ACN when NH_4Cl was

added. The results showed that good extraction recoveries were obtained when the systems of ACN/$MgSO_4$ and ACE/Na_2SO_4 were used. In terms of the system of ACE/Na_2SO_4, the volume of the upper phase was obviously more than the volume of ACE that was added. When concentrating the supernatant under the nitrogen flow, it was difficult to dry because of the upper phase contained solution of Na_2SO_4, the salt sticked to the tube was observed. However, it was critical to redissolve the residues of FQs with mobile phase for good chromatographic behaviors and signal response. What's more, ACE is not applicable for protein precipitation while ACN is a promising protein precipitation reagent, has favorable polarity to the extraction of a wide range of compounds, good miscibility in water and lower toxicity. For the system of ACN/water, the best extraction efficiencies for target analytes were obtained in the case of addition of $MgSO_4$. ACN was the optimum extraction solvent compared to ACE and IPA when $MgSO_4$ was used. The results were showed in Fig. 1. Therefore, ACN and $MgSO_4$ were chosen as extraction solvent and salting-out reagent respectively.

Selection of the ratio of extraction solvent and salting-out reagent

The ratio of extract solvent and salting-out reagent is also the key point of the method of SILLME. The optimized ratio can obtain the great extraction efficiency and avoid the waste of chemical reagents. The effect of the volume of ACN was evaluated by changing the volume in the range of 0.8-1.4 mL (changed every 0.2 mL) while the amount was $MgSO_4$ was kept the constant at 2.0 g. As shown in Fig. 2, the results indicated that the peak area of the target analytes increased gradually when the volume of ACN increased from 0.8 to 1.2 mL, then almost no further increase. So 1.2 mL was chosen as the optimum volume of ACN for subsequent research.

Various salt concentrations will cause varying degrees of phase separation. Salting-out study was carried out by adding different amounts of $MgSO_4$ (1.0-2.5 g) to the extraction system. The results (Fig. 3) indicated that a 2.0 g portion of the salt for the system of ACN/water was found to prove maximum extraction efficiency in the present method. Additional amounts of salt had not much influence on the extraction recoveries of FQs. The solution was

just saturated when 2.0 g of $MgSO_4$ was added. So the 2.0 g was chosen as the optimum amount of $MgSO_4$ for the following experiments.

Effect of sample pH

FQs have two relevant ionisable functional groups, the 3-carboxyl group and the N-4 of the piperazine substituent. Therefore, FQs have two pKa values and their acid-base behaviour will be significantly affected by physicochemical properties of the solvent.[28] The intermediate form of FQs is a zwitterion.[29] The pH value plays an important role in SILLME method because it affects the ionization status as well as solubility of the analytes. The influence of pH value on the extraction efficiencies of FQs was investigated in the range of 1-8 (adjusted by using phosphoric acid and ammonia solutions). It was noteworthy that best extraction efficiencies were required under the condition of strong acid. As shown in Fig. 4, the target analytes barely could be extracted while pH $\geq$ 3.5, and the optimum extraction efficiencies were obtained at pH 1.5. Under this condition, FQs are in the form of strong cation forms. FOs exsit in the form of amphoteric ion (principal form) and nonionic while the pH are between 6 and 9. However, the main quantity of zwitterion seems not easily dissolve into the organic phase which has low dielectric constant, because organic solvents show lower dissolve capacity for zwitterion than water. The strong alkaline circumstance did not take into consideration because of the occurrence of hydrolytic reaction of Mg^{2+}. Thus, the sample pH was adjusted to 1.5 for all the subsequent experiments.

Validation of the SILLME method

Under the conditions optimized above, the ultrapure water was used for the evaluation of the present method. A series of experiments were performed for obtaining linear ranges, precision, and the limits of detection (LODs). The working curve was constructed by plotting the peak areas measured versus the concentrations over the range of 0.002-0.100 μg mL^{-1} for the six FQs. The good corresponding linearity (r^2) ranged from 0.9990 to 0.9998 were obtained for all the analytes. The LODs were calculated at spiked level of 2 ng mL^{-1} with a signal-to-noise (S/N) of

3 for the six FQs in ultrapure water, ranged from 0.06-0.41 ng mL^{-1} for the six FQs. The accuracy and precision were evaluated by using the ultrapure water spiked at three concentration levels of 10 ng mL^{-1}, 40 ng mL^{-1} and 80 ng mL^{-1}. The intra- and inter-day RSD were in the range of 0.4%-4.0% and 1.3%-6.8%, respectively. The recoveries were in the range of 98.6%-111.7% for all the experiments. The results obtained for all-above mentioned evaluation criterions using ultrapure water were shown in Table 1.

The real blank samples of field water, honey, milk, swine plasma and muscle were also used to evaluate the method of SILLME. To ensure these samples were free of antibiotics, the blank samples were screened for the presence of FQs of interest prior to the study. All the results (Table 2) indicated that the proposed method should be a feasible method in the determination of trace-level FQs in various matrices. Excellent sensibility was attained with the limits of detection (LODs, S/N=3) ranging from 0.07-0.41 ng mL^{-1}, 0.09-0.62 ng mL^{-1}, 0.48-2.49 μg kg^{-1}, 0.80-5.00 ng mL^{-1}, 0.78-5.58 ng mL^{-1} and 0.40-5.30 μg kg^{-1} for ultrapure water, field water, honey, milk, swine plasma and muscle, respectively. Honey, milk and swine plasma formed a floating gelatinous precipitate after centrifuged, this would contribute to absorb the upper phase. Schematic diagram of the presented method SILLME procedure for milk is shown in Fig. 5.

Analysis of samples

To evaluate the applicability of the proposed method, collected samples of field water, honey, milk, swine plasma and muscle were analyzed. The typical chromatograms of the blank spiked samples are shown in Fig. 6 (A) and (B). No significant interference peaks are found at the retention positions of FQs.

In order to establish the accuracy and precision of the method, the above mentioned samples spiked at three concentration levels were studied. Each concentration of all the samples was reduplicated five times by using the optimized method as described in the sections of procedure of SILLME method and sample preparation. Mean recovery and RSDs for all the assaies were summarized in Table 3. As can be seen, good precision was obtained

for all analytes in different samples (RSD≤8.7%). The recoveries of analytes obtained were in the range of 100.6%-108.7%, 88.7%-114.2%, 89.4%-107.4%, 84.0%-107.0% and 61.8%-83.3% for ultrapure water, field water, honey, milk, swine plasma and muscle, respectively. The recoveries for the target analytes in swine muscle were lower than in the other samples. This may be attributed to that the amount of the ACN was not sufficient and the analytes cannot be absorbed sufficiently because of the existence of lipid. The field water was detected to contain the NOR and ENR at 0.72 ng mL^{-1} and 0.28 ng mL^{-1} level respectively. This may probably because the pig farm used the two kinds of antibiotics. The detection results for other samples showed that they were all below detectable level of six FQs, which indicates that these samples are nearly free of FQs contamination.

Comparison of SILLME with other reported methods

The present method for determination of FQs was compared with other methods reported in literatures in terms of the extraction time, recoveries and LODs, such as ultrasound-assisted dispersive liquid-liquid microextraction with liquid chromatography-ultraviolet detector (UA-DLLME-LC-UV),[30] magnetic solid-phase extraction with a variable wavelength UV-vis detector (MSPE-CLC-UV-vis),[31] ionic liquid-based homogeneous liquid-liquid microextraction with high-performance liquid chromatography-photodiode-array detector (IL-based HLLME-DAD),[32] solid-phase extraction with liquid chromatography coupled with ultraviolet detector (SPE-UV),[26] dispersive liquid-liquid microextraction and dispersive micro-solid- phase extraction with high-performance liquid chromatography with diode-array detector (DLLME-HPLC-DAD and DMSPE-HPLC-DAD).[30] The results were shown in Table 4. As can be seen, the LODs of the present method was lower or comparable with other methods applied to the same compounds. Above all, this proposed method integrated pretreatment and preconcentration in one step, which would make the procedure be simple, time-saving and eco-friendly.

Conclusion

In this study, the method of SILLME based on the system of ACN/$MgSO_4$ has been developed and validated as an feasible alternative sample preparation and preconcentration method for simultaneous determination of six FQs in field water, honey, milk, swine plasmas and muscle prior to HPLC/FLD analysis. The parameters effected the extraction efficiencies of the FQs have been optimized. The reliability of the method was evaluated by analyzing the FQs in the real environment, food, and biological samples. The satisfactory recoveries, adequate repeatability, good linearity and relative low detection limits demonstrated that the method is sensitive and accurate for trace-level quantitative analysis of the six FQs in real samples. It also has been proved that the proposed method provided a simple, rapid, inexpensive and eco-friendly procedure to preconcentrate FQs from different samples. Based on the advantages of the method, it would be continually applied for analyzing different analytes in various complex matrices by combining seamlessly with modern techniques and be a shining method.

Acknowledgements

This work was jointly funded by Natural Science Foundation of Zhejiang Province (Y4110244), Analyzing and Testing Foundation of Zhejiang Province (2012C37004), International Cooperation Project of Wenzhou City (H20090079) and National Natural Science Foundation of China (21107083). The authors are also grateful to the anonymous reviewers for their suggestions and critical comments.

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