Meat Science 106 (2015) 61–68

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

# A phase separation method for analyses of fluoroquinones in meats based on ultrasound-assisted salt-induced liquid–liquid microextraction and a new integrated device

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ARTICLE INFO

*Article history:*
Received 27 September 2014
Received in revised form 24 March 2015
Accepted 26 March 2015
Available online 3 April 2015

*Keywords:*
Sample extraction
Antibacterial agent analysis
Fluoroquinolones
Ultrasound-assisted
Salt-induced
Liquid–liquid microextraction
Meat

ABSTRACT

Herein, we developed a novel integrated device to perform phase separation based on ultrasound-assisted, salt-induced, liquid–liquid microextraction for determination of five fluoroquinones in meats by HPLC analysis. The novel integrated device consisted of three simple HDPE (high density polyethylene) parts that were used to separate the solvent from the aqueous solution prior to retrieving the extractant. The extraction parameters were optimized using the response surface method based on central composite design: 589 μL of acetone solvent, pH 2.1, 4.1 min extraction time and 3.5 g of $Na_2SO_4$. The limits of detection were 0.056–0.64 μg $kg^{-1}$ and recoveries were 87.2–110.6% for the five fluoroquinones in muscle tissue from fish, chicken, pork and beef. This method is easily constructed from inexpensive materials, extraction efficiency is high, and the approach is compatible with HPLC analysis. Thus, it has excellent prospects for sample pre-treatment and analysis of fluoroquinones in meat samples.



## 1. Introduction

Fluoroquinolones (FQs) are widely used as antibacterial agents in human and veterinary medicines due to their broad spectrum activity against both Gram-positive and Gram-negative bacteria through inhibition of DNA gyrase (Gao et al., 2011). Fleroxacin (FLE), ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CIP) are third-generation FQs used in treating human and animal diseases, while enrofloxacin (ENR) is used only for treating animal diseases. With the overuse of these FQs in animal husbandry and aquaculture, they are widely detected in all kinds of matrices, especially in meat such as fish (Huet et al., 2008; Huet, Charlier, Weigel, Godefroy, & Delahaut, 2009), chicken (He, Lv, Yu, & Feng, 2010; Lee, Kim, & Kim, 2013; Tian et al., 2014), pork (Li et al., 2009; Lee et al., 2013) and beef (Lee et al., 2013; Sheng et al., 2009). Because of the complex matrix interferences in meat, the previously reported analytical methods often require extensive sample preparation (Vazquez, Vazquez, Galera, & Garcia, 2012; Ebrahimpour, Yamini, & Moradi, 2012). Accordingly, there is considerable interest in developing a cost-effective, efficient and reliable extraction method for the analysis of complex samples prior to FQ quantification.

In recent years, some novel liquid-phase microextraction (LPME) techniques have been developed such as dispersive liquid–liquid microextraction (DLLME), ionic liquid-based homogeneous liquid–liquid microextraction (IL-HLLME) (Gao et al., 2011) and ion pair-based surfactant-assisted microextraction (IP-SAME) (Ebrahimpour et al., 2012). However, a major drawback for the use of non-polar, water-immiscible, organic solvents in all types of LPME is their low dielectric constant, making extraction of polar solutes relatively poor (Gupta, Archana, & Verma, 2009). More polar solvents, such as acetonitrile and ethanol, which provide solubility for polar to non-polar compounds, are frequently water-miscible and thus can't be used in conventional LPME. Salting-out is a process of electrolyte addition to an aqueous phase in order to increase the distribution ratio of a particular solute. The term also connotes reduction of mutual miscibility of two liquids by addition of electrolytes. Weak intermolecular forces, e.g., hydrogen bonds, between organic molecules or non-electrolytes and water are easily disrupted by the hydration of electrolytes. Salting-out assisted liquid–liquid microextraction (SALLME) is based on phase separation of water-miscible organic solvents from the aqueous solutions at high salt concentrations (Tsai et al., 2009). It uses water-miscible organic solvents that, generally, have low toxicity and small amounts of salt that cause little environmental pollution. Additionally, this method has the advantages of simplicity and sensitivity and uses less solvents, and the product is compatible for subsequent

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http://dx.doi.org/10.1016/j.meatsci.2015.03.023
0309-1740/

analysis by HPLC (Myasein, Kim, Zhang, Wu, & Tawakol, 2009; Cai et al., 2007). In SALLME, a glass centrifuge tube is often used as the extraction device. However, collection and measurement of microliter volumes of organic phase are difficult because the thin layer of extract is difficult to retrieve from the wide diameter glass tube increasing extraction time. To solve the above-mentioned problem, a few approaches have been reported for introducing extraction apparatuses into the microextraction process that allows for the use of low-density solvent, either by using a narrow-necked tube (Ye, Zhou, & Wang, 2007) or by using a sample vial (Cheng, Matsadiq, Liu, Zhou, & Chen, 2011). Two narrow open necks were specially designed to be equipped in a round-bottom flask, among which one had a capillary tip making the collection step more convenient (Zhang, Shi, Yu, & Feng, 2011). Chen et al. utilized a plunger plug to push the upper layer solvent into a capillary tip, which made the final collection step rapid (Chen, Liu, Lin, Ponnusamy, & Jen, 2013). However, all of the glass apparatuses used to collect the low-density extractant have some prominent drawbacks. For example, the narrow-necked glass tube is easily broken, requires special design and is costly. Consequently, there is limited commercial availability for these specially designed glass tip tubes (Wang, Cheng, Zhou, Wang, & Cheng, 2013).

In recent years, many researchers used a polyethylene plastic tube as an extraction device for extracting low-density solvents in the microextraction procedure (Hu, Wu, & Feng, 2010; Guo & Lee, 2011). A polyethylene dropper and a sample vial were integrated to conduct microextraction of organic pollutants in a single step (Cheng et al., 2011). The plastic tube has advantages of low cost, use of easily available materials, ease of operation and avoidance of carryover problems (Wang et al., 2013). However, the major drawback of this device is that the organic phase was difficult to completely retrieve because the organic phase and aqueous solution were not separated prior to the collection of the extractant. The repartitioning of the extractant into the aqueous phase may occur over a long retrieval time, which will possibly result in low extraction efficiency.

To overcome the above-mentioned limitations of current methods, this study developed and optimized a novel integrated device and methodology for extraction of FQs by means of a phase separation method based on ultrasound-assisted, salt-induced, liquid–liquid microextraction (PS-USLM). The proposed PS-USLM method was optimized for major operational factors (extraction time, pH, salt kind and volume, solvent kind and volume, and centrifugation time) using a response surface method (RSM) based on central composite design (CCD). The optimized method was compared with other commonly used LPME methods to evaluate its advantages and feasibility for determining trace levels of FQs in fish, chicken, pork and beef. To the best of our knowledge, this integrated device, designed to completely and rapidly separate the organic and aqueous phases prior to collection of the extractant, is the first reported use of this approach for determination of FQs in meat.

## 2. Experimental

### 2.1. Reagents and materials

Analytical standards for fleroxacin (FLE), ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP) and enrofloxacin (ENR) were purchased from J&K Chemical Corporation (Shanghai, China) and used when received. The chemical structures and molecular weight of the five FQs are shown in Supplementary Fig. 1, and they have a common 4-oxo-1,4-dihydroquinoline skeleton, where the pharmacophore unit consists of a pyridine ring with a carboxyl group, a piperazinyl group and a fluorine atom placed at positions 3, 6 and 7 (Gajda, Posyniak, Zmudzki, Gbylik, & Bladek, 2012). HPLC-grade ethanol, methanol, ethyl acetate, acetonitrile and acetone were sourced from Merck Corporation (Shanghai, China). Salts (magnesium sulfate ($MgSO_4$), sodium sulfate ($Na_2SO_4$), ammonium sulfate ($(NH_4)_2SO_4$) and ammonium acetate ($CH_3COONH_4$)) with purities ≥99% were obtained from Aladdin Industrial Co. Ltd. (Shanghai, China).

Stock standard solutions (1000 μg $mL^{-1}$) for each FQ were prepared by dissolving each compound in methanol and stored at 4 °C. Stock solutions were diluted with methanol to prepare a secondary mixed stock solution of 10 μg $mL^{-1}$. Mixtures of standard working solutions for extraction at different concentrations were prepared by dilution with Milli-Q ultrapure water (Millipore, Bedford, USA).

Fish, chicken, pork and beef muscles (the meat of fish body on both sides, chicken breast tenderloin, pork fillet and beef sirloin) were purchased from local markets in Wenzhou, China. In order to increase the representativeness of the meat samples, we purchased three batches of meat samples on July 14th, 20th and 25th, 2014 in the three local markets. These samples were ground and stored at −20 °C until analysis within one week.

### 2.2. Preparation of meat samples

Fortified samples of fish, chicken, pork and beef were prepared by adding the appropriate volumes of the mixed standards to ground muscle tissues. Prior to sample treatment and analysis, all samples were stirred and allowed to stand in the dark for 30 min at ambient temperature to permit full interaction between the antibiotics and muscle tissue. All samples were prepared in triplicate.

### 2.3. Instrumentation

FQs were analyzed with an Agilent 1260 HPLC equipped with a fluorescence detector (FLD). A Zorbax Eclipse XDB-$C_{18}$ column (150 mm × 4.6 mm, 5 μm particle size) was used and injections were performed manually using a 20.0-μL sample loop. The operating conditions were as follows: mobile phase, methanol–acetonitrile–water (15:5:80, v/v; water consisting of 3.4 mL orthophosphoric acid and 6.0 mL triethylamine per liter); flow rate, 0.8 mL $min^{-1}$; column temperature, 40 ± 1 °C; and excitation and emission wavelengths of 290 and 455 nm, respectively. Solutions were stirred with a model HJ-6A magnetic heater–stirrer with an 8 mm × 4 mm stir bar (Jiangsu Jintan Medical Instrument Factory (Jintan, China)). Centrifugation used a model TDL-50C centrifuge from Anting Instrument Factory (Shanghai, China).

### 2.4. PS-USLM procedure

A schematic of the integrated PS-USLM procedure is shown in Fig. 1. This novel integrated device consists of three parts: (1) a high-density polyethylene (HDPE) centrifuge tube (8 cm × 1.6 cm external diameter, 1.4 cm internal diameter, Fig. 1-A); (2) an inverted cut HDPE dropper (1 cm × 1.4 cm external diameter joined to a 3 cm length of capillary tube); and (3) a "V" HDPE capillary tube (10 cm × 0.5 cm internal diameter). The inverted cut disposable HDPE dropper was inserted into the centrifuge tube, and the "V" tube was easily attached/detached from the inverted HDPE dropper (Fig. 1-G and H).

In the operation, the sample solution was first added to the centrifuge tube followed by the *n*-hexane and extraction solvent, which was water-miscible and had density lower than that of water. After centrifugation, the sedimented proteins, floating fat and other interfering compounds were discarded (Fig. 1-A and B). After that, an appropriate amount of salt was added to the remaining solution (Fig. 1-C). After the salting-out process, the extraction solvent floated on the top of the sample solution following ultrasound and centrifugation (Fig. 1-D–E) (extraction solvent, Fig. 1-E-1; Sample solution, Fig. 1-E-2; Undissolved salt, Fig. 1-E-3). The inverted HDPE dropper was then placed into the sample solution and the extractant was extruded through the tip of the dropper (Fig. 1-F–G). When the extractant was fully transferred into the "V" tube, the "V" tube was detached and the extractant was collected with a microsyringe (Fig. 1-H). The extractant was then dried