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Evaluating polymer monolith in-tube solid-phase microextraction coupled to liquid chromatography/quadrupole time-of-flight mass spectrometry for reliable quantification and confirmation of quinolone antibacterials in edible animal food

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ABSTRACT

A simple, rapid, and sensitive method is presented to determine seven trace quinolone antibacterials simultaneously in milk, egg, chicken and fish. This method is based on the combination of polymer monolith in-tube solid-phase microextraction with liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (LC/ESI-QTOF-MS). LC/ESI-QTOF-MS offers the capability of unequivocal identification of target compounds from complex matrices, as well as the possibility of quantitation at low-level concentrations in real samples. The extraction was performed with a poly(methacrylic acid-ethylene glycol dimethacrylate) monolithic column. Under the optimized extraction conditions, good extraction efficiencies for the targets were obtained with no matrix interference in the subsequent LC/ESI-QTOF-MS. Good linearities were obtained for seven quinolones with the correlation coefficients (R) above 0.9951. The limits of detection ($S/N = 3$) for seven quinolones were found to be 0.3–1.2 ng/g in egg, 0.2–3.0 ng/mL in milk, 0.2–0.7 ng/g in chicken and 0.2–1.0 ng/g in fish. The recoveries of quinolones spiked in four different matrices ranged from 80.2 to 115.0%, with relative standard deviations less than 14.5%. The developed method was applied for the determination of quinolone residues in animal-producing food, and the positive samples were confirmed with high number of identification points (IPs) according to the IP system defined by the European Union (Commission Decision 2002/657/EC).

1. Introduction

Quinolones, which act principally by inhibiting DNA-gyrase in bacterial cells, form an important group of synthetic antimicrobial agents with different chemical structures and spectra of activity [1]. Due to their safety with good tolerance and broad antibacterial spectrum, a significant increase in the use of quinolones in human and in veterinary medicine was noted over the last decade. If recommendations for drug withdrawal times are not respected, there is a significant risk for the occurrence of residues in edible animal products, which can be directly toxic or cause resistant pathogens in humans [2]. To protect consumers from risks related to quinolone residues, the European Union has set tolerance levels (maximum residue limits, MRLs) for veterinary medicinal products in foodstuffs of animal origin in different products such as egg, milk, chicken and fish [3,4]. Therefore, sensitive and selective analytical methods are required to monitor the residues possibly present in different animal-producing food.

LC/MS combining the efficient separation capability of HPLC and great power of structural characterization and high sensitivity of MS is becoming a standard tool to analyze trace target compounds in complex food matrices [5–11]. Time-of-flight mass spectrometry (TOF-MS) has distinct advantages over scanning instruments, including the detection of high accuracy in mass measurements, fast acquisition rates, relatively high sensitivity and large mass range [12]. Recent success with the use of LC combined with electrospray ionization (ESI) TOF-MS for characterizing and quantifying a wide variety of compounds in complex food samples [5,13–16] suggests that LC/ESI-TOF-MS might also be a powerful technique in the comprehensive determination of multiple antibacterial residues in complex food matrices. By the accurate mass measurement, LC/ESI-TOF-MS can alleviate the matrix interferences from co-eluting impurities, which are often encountered by conventional LC/MS (with low resolution) methods. In addition, no a priori hypothesis about the presence of certain drugs is required for LC/TOF-MS; its high-resolution and full scan data permit the testing of any a posteriori hypotheses by extracting any desired exact mass chromatogram. However, as to LC/triple-quadrupole (QqQ) MS using

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multiple reaction monitoring (MRM), analyte specific transitions have to be defined before injecting a sample [17]. Furthermore, LC/ESI-TOF-MS offers the capability of unequivocal identification (provided by accurate mass measurements and retention time match) of target compounds from complex matrices, as well as the possibility of quantitation at low-level concentrations in real samples using extracted ion chromatograms (EIC) [17–20].

Due to the complexity of food samples, sample preparation is necessary prior to LC/MS. A recent review offers a good summary of the state-of-the-art on sample preparation methods to determine quinolone residues [21]. Solid-phase extraction (SPE) [6–10,22–24] and liquid–liquid extraction (LLE) [1] are commonly used sample preparation methods. However, the conventional LLE procedures are time-consuming, generally labor intensive and requires large quantities of environmentally unfriendly organic solvents. Meanwhile, SPE technique requires less solvent, but needs the evaporation and reconstitution steps. To overcome those problems, some techniques such as diphasic dialysis [25], on-line microdialysis [26], supercritical fluid extraction (SFE) [27], pressurized liquid extraction [28,29] and hot water extraction [30] have been reported for the extraction of quinolones in biological matrices.

Solid-phase microextraction (SPME) was introduced as an excellent sample preparation technique, since it possesses several attractive features including high sensitivity, solventless extraction, small sample volume, simplicity and easy automation [31–33]. Polymer monolith in-tube SPME is one kind of SPME technique, in which polymer monolith is used as the sorbent [34–36]. Different from the traditional SPME fibers or coated capillaries, the format of the extraction material could be regarded as a multi-channel separation media, which is combined as a whole and will provide sufficient extraction phase. The extraction efficiency is thus expected to improve greatly [35]. Based on poly(methacrylic acid-ethylene glycol dimethacrylate) [poly(MAA-co-EGDMA)] monolith, this technique has been coupled on-line or off-line with HPLC for the determination of several analytes in different kinds of food samples [37–39]. The polymer monolith showed stability within the entire range of pH and exhibited excellent biocompatibility in dealing with biological samples [34].

So far, little attention has been paid to the development of LC and ESI quadrupole (Q) TOF-MS methods for determining quinolone residues in different food matrices and only several works are quoted in the literature for determining quinolones in meat [15], milk [17], urine [18] and pig liver [20]. In the present study, we report on the on-line hyphenation of polymer monolith in-tube SPME with LC/ESI-QTOF-MS for fast and sensitive analysis of quinolone antibacterials in four different animal products. By integrating sample extraction, concentration and introduction into one single step, polymer monolith in-tube SPME offers an effective and cooperative sample preparation approach prior to LC/ESI-QTOF-MS analysis. To our knowledge, there have been no published methods for the determination of quinolones using this technique.

2. Experimental

2.1. Chemicals and reagents

Ethylene dimethacrylate (EGDMA, 98% pure) was purchased from Acros (NJ, USA). Methacrylic acid (MAA), azobisisobutyronitrile (AIBN), dodecanol, toluene, dichloromethane, disodium hydrogenphosphate (Na_2HPO_4) were obtained from Shanghai Chemical Reagent Corp. (Shanghai, China) and all were of analytical reagent grade. Acetonitrile (HPLC grade) was obtained from Fisher Scientific (USA). Purified water was obtained with an Aike apparatus (Chengdu, China).

Ciprofloxacin (CIP), danofloxacin methanesulphonate (DAN), enrofloxacin (ENR), difloxacin (DIF), sarafloxacin (SARA), oxolinic acid (OXO), flumequine (FLU), ofloxacin (OFL) [the selected internal standard (I.S.)] were purchased from Laboratories of Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions of CIP, ENR, SARA, DIF, FLU and OFL were prepared in a concentration of 500 μg/mL stock solution in acetonitrile. Individual stock solution of OXO was prepared in acetonitrile at a concentration of 200 μg/mL and stock solution of DAN was prepared in water at a concentration of 500 μg/mL. The working standard solution was diluted to the desired concentration for experiments. All of the above solutions were maintained at 4 °C. Chemical structures of the quinolones included in this study are shown in Fig. 1.

2.2. Preparation of poly(MAA-co-EGDMA) monolithic extraction column

The poly(MAA-co-EGDMA) monolith was synthesized inside a polyether ether ketone (PEEK) tube (1.59 mm O.D. × 0.50 mm I.D.) by a heat initiated polymerization method. The PEEK tube was only required to be washed with methanol and then dried with N_2 without first performing any chemical modification of its internal surface. The pre-polymerization mixture which has been reported in our previous work [35], consisting of MAA monomer (48 mg), EGDMA cross-linker (420 mg), porogenic solvents toluene (110 mg) and dodecanol (860 mg), and AIBN initiator (4.5 mg), was mixed and degassed by ultrasonication for about 10 min. Then the homogeneous solution was filled into a 150 mm length of PEEK tube, then the PEEK tube was sealed with silicon rubber and the polymerization took place at 60 °C for 16 h. Following polymerization, the PEEK tube was washed with methanol to remove the unreacted component and porogenic solvent. Finally, it was coupled to the in-tube SPME/LC/MS system through two PEEK in-line filters (Vici Jour Research, Onsala, Sweden) and stood for extraction. Prior to every extraction, the extraction column was conditioned first by mobile phase and then by phosphate solution.

2.3. LC/ESI-QTOF-MS equipment and conditions

A quaternary pump system from Agilent Technologies (Palo Alto, CA, USA) model series 1200 was coupled to a QTOF-MS system (Bruker Daltonik, Bremen, Germany) with an ESI source. Instrument control was performed using Bruker Daltonics Hystar. The accurate mass data of the molecular ions were processed through the software Data Analysis 3.4, which provided a list of possible elemental formulas by using the Generate Molecular Formula Editor. All mass spectra were acquired in the positive ion mode. Chromatography was performed on a C18 column (250 mm × 2.0 mm I.D., 5 μm) from Shimadzu (Tokyo, Japan) fitted with a C18 guard column (Shimadzu). The column oven temperature was maintained at 30 °C and the flow rate was 0.2 mL/min. A mixture of formic acid solution and acetonitrile was used as the mobile phase. Solvent A was 0.3% formic acid solution and solvent B was acetonitrile. The gradient profile for mobile phase was as follows: t_0, 20% B; t_{12}, 35% B; t_{15}, 80% B; t_{20}, 85% B; t_{30}, 85% B; $t_{30.01}$, 20% B (where t refers to time in min).

Optimal ionization source working parameters were as follows: capillary voltage 4.5 kV; ion energy of quadrupole 5 eV/z; dry temperature 200 °C; nebulizer 1.2 bar; dry gas 6.0 L/min. The analysis was performed in full scan mode; mass range was 100–500 m/z; spectra were acquired by summarizing 30 000 single spectra. During the development of the LC/ESI-QTOF-MS method, external calibration was performed according to Ref. [16]. In brief, numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained by using sodium formate cluster containing 5 mM sodium hydroxide

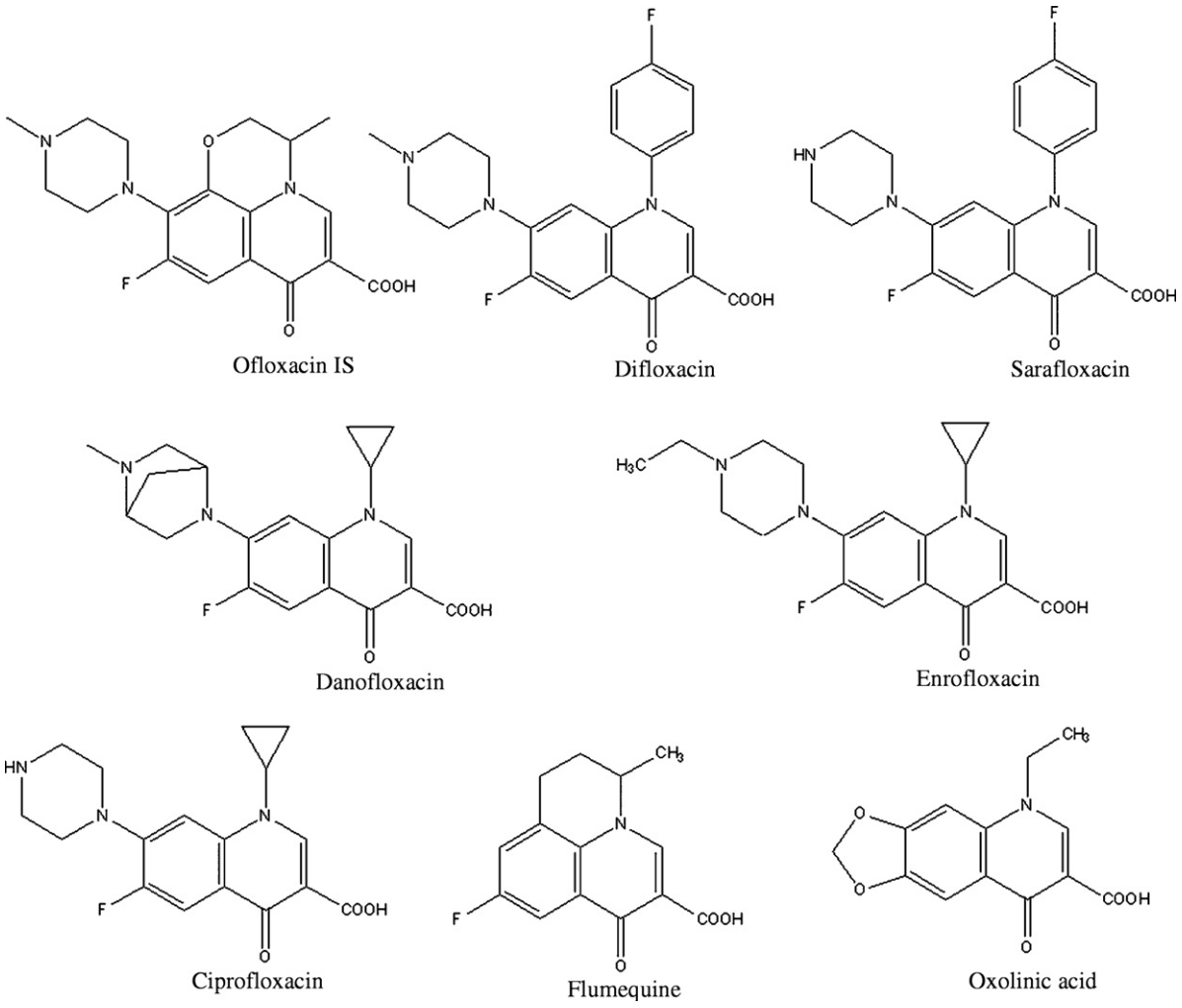

Fig. 1. Molecular structures of selected quinolone antimicrobials.

in the sheath liquid of 0.2% formic acid in water/isopropanol (1:1, v/v).

2.4. Sample preparation

Pasteurized whole milk, eggs, chicken and fish were purchased from local retail markets. These samples were homogenized and were stored at −20 °C before use. Preliminary analysis showed they were analyte-free.

For egg and milk samples, I.S. solution (50 μL of 200 ng/mL OFL in H_2O) was added to 0.5 g of egg or 0.5 mL of milk samples which were spiked with known variable amounts of quinolones. 15.0 min was allowed for equilibration at room temperature, after being mixed with a vortex mixer for 2.0 min. These samples were diluted with 25 mM phosphate solution (disodium hydrogenphosphate solution pH 4.0) to 5.0 mL. After being mixed with a vortex mixer for 2.0 min again, the samples were centrifuged at 0–4 °C for 5.0 min at 10 000 rpm (Anting Scientific Instrument Co., Shanghai, China). Then the supernatant was filtered through a 0.45 μm pore cellulose filter prior to in-tube SPME/LC/MS analysis.

For chicken and fish samples, I.S. solution (50 μL of 200 ng/mL OFL in H_2O) was added to 0.5 g of chicken or fish samples which were spiked with known variable amounts of quinolones. 15.0 min was allowed for equilibration at room temperature. After that, 5.0 mL of acetone was added to the above solutions. The mixtures were homogenized with an ultrasonic homogenizer Model HOM-100 (3 mm I.D. chip) (Ningbo Scientz Biotechnology Co., Ningbo, China) for 1.0 min, and then were centrifuged at 0–4 °C for 5.0 min at 10 000 rpm. The supernatant was collected and evaporated to dryness under a mild nitrogen stream by a HGC-12A Gas blowing concentrator (Zhongke-Sanhuan Instrument Co., Beijing, China) at 45 °C. The residue was redissolved with 50 μL of acetonitrile and 950 μL of 25 mM phosphate solution (pH 4.0). The solvent was centrifuged at 0–4 °C for 5.0 min at 10 000 rpm. Then the supernatant was filtered through a 0.45 μm pore cellulose filter prior to in-tube SPME/LC/MS analysis. Blank samples were prepared in the same way as mentioned above but without the compound-spiking step.

For the calibration of this method, variable amounts of the stock quinolone mixtures and a fixed concentration of I.S. solution were added into sample matrices to prepare the spiked working standard solutions and then extracted by in-tube SPME. The matrix-matched calibrations were achieved by measuring three replicates at six different concentrations spiked in four matrices.

2.5. In-tube SPME procedures

The handling of in-tube SPME/LC/MS was pretty much the same as that described in our previous reports [35] except for the automatic switching of two valves can be programmed by a 3012 dual high pressure six-way switching valve model (Shiseido, Tokyo, Japan) (Fig. 2). The program for in-tube SPME procedures is listed

Table 1
Program for in-tube SPME procedures.

No.	Time (min)	Action	Position	Event
1	0.00	Valve A	INJECT	Starting extraction, after sample loop was filled with the sample solution.
2	0.00	Valve B	LOAD	
3	6.00	Valve A	LOAD	Washing the monolithic column with carrier solution for 5.0 min after 6.0 min of extraction.
4	11.00	Valve A	INJECT	Washing the monolithic column with water for 2.0 min.
5	13.00	Valve B	INJECT	Desorption 5.0 min with mobile phase.
6	18.00	Valve B	LOAD	After desorption, the monolithic column was conditioned by carrier solution until next extraction.

The flow rate for extraction, washing and desorption was kept at 0.10 mL/min. Other in-tube SPME conditions were outlined in Section 2.

in Table 1. The monolithic column was mounted at 1 and 4 position of valve B. Before the extraction, the carrier solution, 25 mM phosphate solution (pH 4.0), was driven by a microflow pump (Unimicro Technologies, Shanghai, China) (pump A) to flow through the monolithic column at 0.10 mL/min. At the same time, the sample loop was filled with the sample solution using a syringe. Valve A was switched from the LOAD to INJECT position for a given time interval (6.0 min) in the extraction step and then returned to the LOAD position immediately. Solution driven through the monolithic column was 0.6 mL, which was calculated by the flow rate and the switching time interval. The monolithic column was washed with carrier solution for 5.0 min and then with water for 2.0 min to eliminate the residual sample solution and to remove unretained matrix to waste. Then the extracted analytes were desorbed from the monolithic column to the analytical column by mobile phase (acetonitrile/0.3% formic acid solution 2:8 v/v) at a flow rate of 0.10 mL/min for 5.0 min by switching valve B to INJECT position. After desorption, valve B was switched to the LOAD position, and then the flow rate of the mobile phase was increased to 0.20 mL/min for chromatographic separation. Subsequently, the monolithic column was conditioned by carrier solution for next extraction.

3. Results and discussion

3.1. Optimization of the in-tube SPME conditions

The extraction was optimized with 25 mM phosphate solution spiked with eight quinolones (including I.S.). Several parameters affecting the extraction efficiency such as pH of sample solution, extraction equilibrium profiles, wash and desorption steps were investigated.

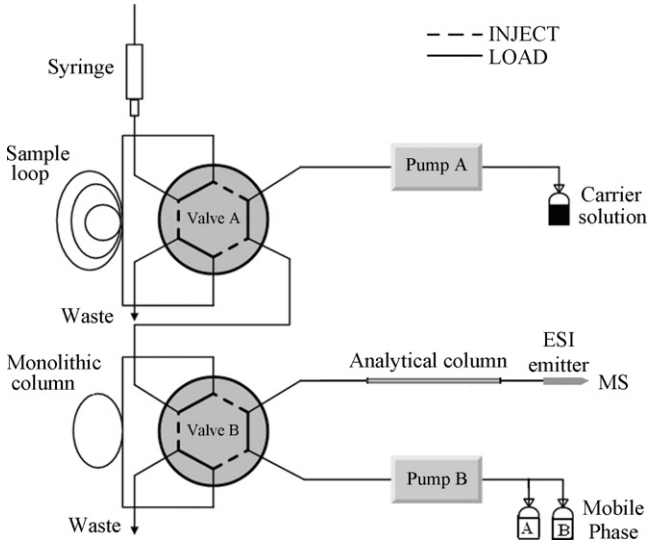

Fig. 2. Construction of in-tube SPME/LC/ESI-QTOF-MS device.

3.1.1. Effect of pH on extraction efficiency

Quinolones possessing a piperazine substituent can be present in aqueous solution as cationic, anionic, or intermediate forms due to the presence of carboxylic group and the charged amino group of the piperazine moiety. Their extraction is therefore pH-dependent. The pK_{a1}, pK_{a2}, and pK_{a3} values are reported to be 5.0–5.5, 6.0–6.5, and 8.0–9.0, respectively. OXO and FLU possess only one ionizable functional group (carboxylic group) in their structure. The pK_a values for these two compounds are determined to be 6.88 and 6.35, respectively [40]. The pH optimization was conducted using 25 mM phosphate matrix solution over the pH ranging from 3.0 to 7.0.

As shown in Fig. 3, the highest extraction efficiency could be obtained over the pH range of 4.0–5.0. For the quinolones except OXO and FLU, they exist in bivalent cationic forms (H_2BAH^{2+}), in the pH range of 4.0–5.0. The enhanced extraction efficiency can be explained by the strong ion-exchange interaction between the cationic analytes and the negatively charged poly(MAA-co-EGDMA) material. When pH is increased above 6.0, the quinolone (except OXO and FLU) molecules are transformed to intermediate forms (HB^+A^-), which results in gradually weakened ion-exchange interaction and relative lower extraction efficiency. OXO and FLU showed extraction efficiency independent from pH values. But for CIP and SARA, the extraction efficiencies were less sensitive to the pH of sample solution than those of the other quinolones, in the pH range of 4.0–7.0. It can be explained that both hydrophobic interaction and ion-exchange contributed to the affinity of the analytes for the monolithic column. The changeless extraction efficiency may be attributed to the increased hydrophobic interaction as well as the gradually weakened ion-exchange interaction with the increase of pH. Considering the extraction efficiency and simplicity of the extraction methods, pH 4.0 was selected as the pH of sample solution.

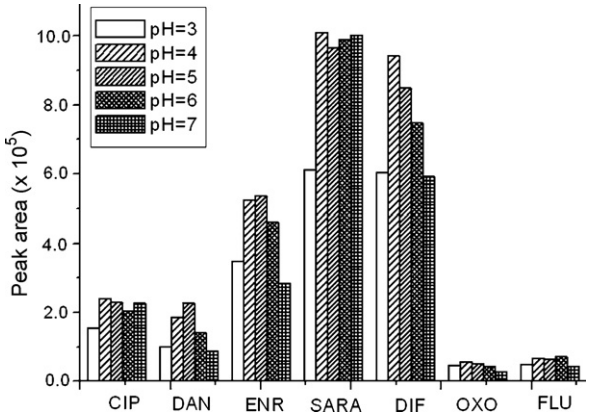

Fig. 3. Optimization of the pH of the sample solution. Sample solutions of seven quinolones spiked at 10 ng/mL were prepared with 25 mM phosphate solution at pH 3.0–7.0. In-tube SPME conditions: extraction time was 5.0 min; elution time was 5.0 min; extraction flow rate was 0.10 mL/min; elution flow rate was 0.10 mL/min. Other in-tube SPME and LC/ESI-QTOF-MS conditions were outlined in Section 2.

Table 2
Enrichment factors[a] and extraction yields[b] ($n = 3$) for eight quinolones from aqueous samples with in-tube SPME.

Compounds	OFL	CIP	DAN	ENR	SARA	DIF	OXO	FLU
Enrichment factors	52.4 ± 4.9	39.6 ± 3.1	59.3 ± 5.5	51.9 ± 3.5	49.9 ± 1.6	55.2 ± 6.5	18.0 ± 1.9	15.6 ± 1.1
Extraction yields (%)	87.4 ± 8.1	66.0 ± 5.1	98.8 ± 9.2	86.6 ± 5.9	83.2 ± 2.7	91.9 ± 10.8	30.0 ± 3.2	26.0 ± 1.8

[a] Calculation is based on the ratio of the peak area obtained with in-tube SPME and without preconcentration. The injection volume was 10 μL for direct injection and 600 μL for in-tube SPME. The concentrations of quinolones were 10 ng/mL.
[b] Percentage of extracted amounts of quinolones over the total amounts loaded (direct comparison of peak areas without I.S. correction) [37].

3.1.2. Equilibrium extraction time profiles

In order to assess the ability of the poly(MAA-co-EGDMA) monolithic column for the extraction of the quinolones, the equilibrium extraction time profile was investigated by increasing the extracting time at constant extraction flow rate (0.10 mL/min) of sample solution. As shown in Fig. 4, the extracted amount of these compounds increased rapidly with prolonged extraction time, indicating the remarkable enrichment ability of the poly(MAA-co-EGDMA) monolithic column towards these quinolones. The equilibrium of extraction was not obtained even up to 12.0 min (corresponding to 1.2 mL sample volume). To achieve sufficient sensitivity within a short time, 6.0 min of extraction was selected for subsequent analysis.

3.1.3. Optimization of the washing and desorption steps

Matrix interference is usually a major problem in the extraction of biological samples. Immediate desorption would result in great difficulty in the following separation of analytes. In this study, the monolithic column was washed for 5.0 min by the carrier solution after extraction to eliminate proteins and other weakly adsorbed components. Subsequently, 0.2 mL of water was used to eliminate the phosphate solution, which was unsuitable for MS detection.

To simplify the manipulation of in-tube SPME, it is important to ensure the selected mobile phase would not only allow the good separation of analytes on the analytical column but also provide effective desorption of the extracted analytes from the monolithic column. In our experiment, the on-line desorption was simply accomplished by driving the optimized mobile phase through the extraction column. The desorption flow rate was set at 0.10 mL/min. The desorption time was also investigated from 3.0 min to 10.0 min. The results showed that the quinolones concentrated in the extraction column can be completely transferred to the analytical column by the mobile phase at a flow rate of 0.10 mL/min within 5.0 min. No carryover was found after 5.0 min of desorption, which was confirmed by the blank analysis performed after extraction and the reproducibility study.

In comparison with the EICs of direct injection, an obvious enhancement of the peak height was observed after extraction, indicating the remarkable preconcentration ability of the monolithic column. The enrichment factors were calculated by comparing the peak area obtained with in-tube SPME and without preconcentration. The extraction yields were based on the percentage of extracted amounts of quinolones over the total amounts loaded. The enrichment factors and the extraction yields were calculated to be 15.6–59.3 and 26.0–98.8% for eight quinolones, respectively (Table 2).

3.2. Performance of ESI-QTOF-MS

The used ESI-QTOF-MS instrumentation permits the extraction of centroid spectra peaks with a width of less than 0.01 Da [18]. There is hardly any loss of signal intensity if the extraction width is reduced from 1 to 0.01 Da. Fig. 5 shows the total ion chromatogram (TIC) and EICs of eight quinolones spiked in fish sample. As indicated in Fig. 5, a significant reduction in noise related to matrix

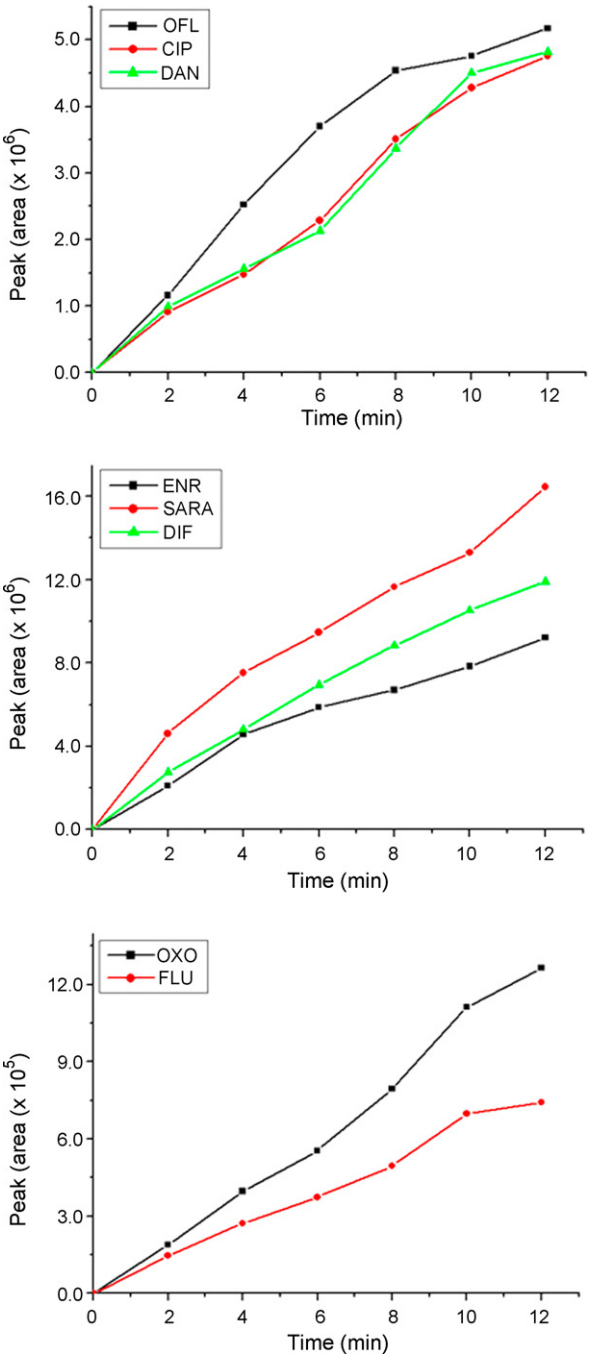

Fig. 4. Extracted sample equilibrium profile of quinolones for in-tube SPME. The sample solution was 25 mM phosphate solution at pH 4.0 spiked with seven quinolones at 50 ng/mL. In-tube SPME and LC/ESI-QTOF-MS conditions are the same as outlined in Fig. 3.

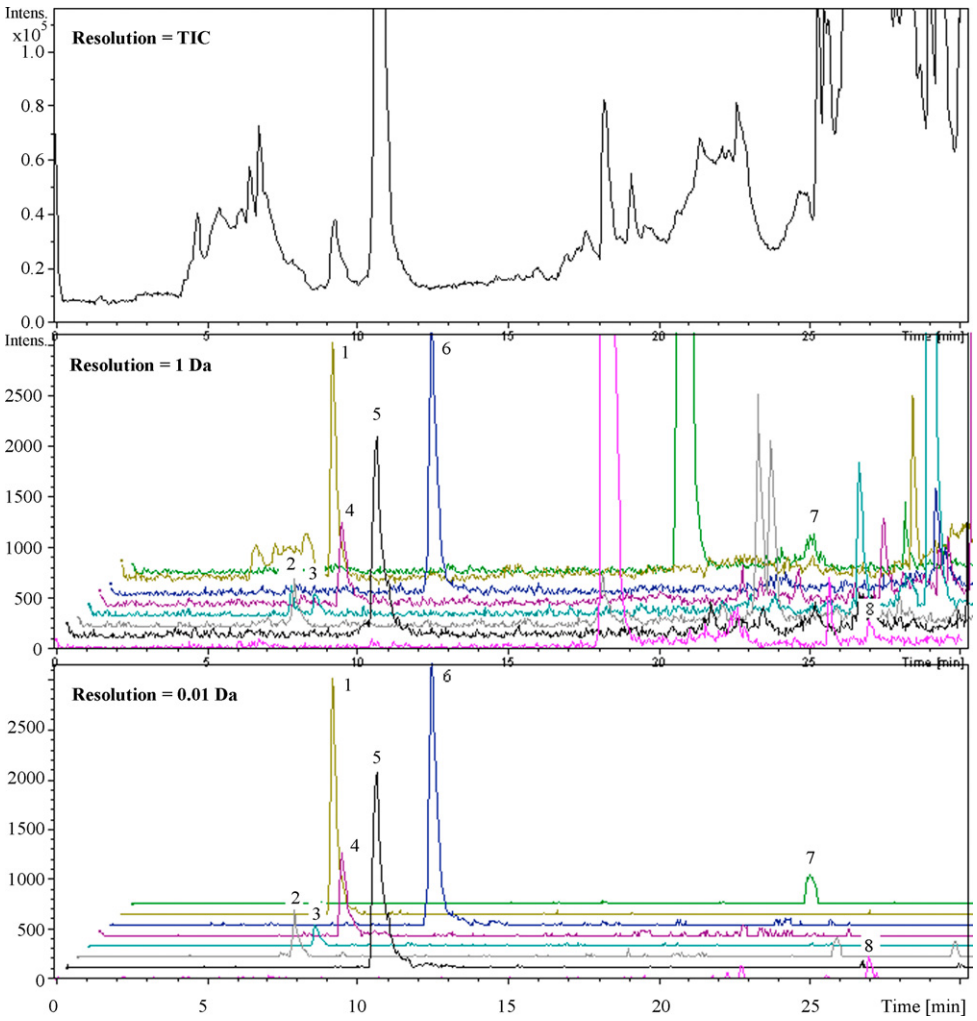

Fig. 5. Effect on selectivity when reducing the mass extraction window width from 400 Da (TIC) to unit resolution (1 Da) and TOF resolution (0.01 Da). The fish sample was spiked with eight quinolones at 4 ng/g. Peaks: 1 = OFL; 2 = CIP; 3 = DAN; 4 = ENR; 5 = SARA; 6 = DIF; 7 = OXO; 8 = FLU.

compounds was obtained when a 0.01 Da resolution was adopted instead of unit resolution with no significant loss in sensitivity. Using too narrow extraction windows could therefore even miss analytes present in the sample. The narrow extraction window of 0.01 Da improves the *S*/*N* ratios of eight analytes as compared to unit resolution, and significantly reduces fish matrix related MS signals.

3.3. Application to edible animal food

3.3.1. Relative recoveries

The relative recoveries were calculated by comparing the peak area ratios of quinolones from the spiked milk, egg, chicken and fish samples to those obtained from the working standard solutions (phosphate solution) at the same concentration. As shown in Table 3, the relative recoveries of eight quinolones (including I.S.) range from 11.6 to 96.4% in four matrices. The results revealed that the determination of quinolones was affected by the interferences from real samples to some extent. On the one hand, protein and fat from the samples would reduce the interaction between analytes and the extraction material. On the other hand, the coextracted components from complex matrices would suppress the MS signals of analytes. Therefore, to provide reliable results, matrix-matched calibration curves were chosen as reference curves throughout this study.

3.3.2. Method comparison

Comparative study of our developed method with other reported sample preparation procedures was performed and the results are presented in Table 4. It can be seen that the developed method is more rapid and sensitive, and requires less amount of sample and organic solvent in the pretreatment process. Furthermore, no elimination of fats and protein in milk or eggs samples was required prior to extraction in this study.

3.3.3. Calibration curves and detection limits

The application of the in-tube SPME/LC/ESI-QTOF-MS method for the determination of seven quinolones was verified using an

Table 3
Relative recoveries ($n = 3$) of eight quinolones (including I.S.) spiked in four different matrices.

Analytes	Relative recoveries (%)			
	Milk (100 ng/mL)	Egg (100 ng/g)	Chicken (20 ng/g)	Fish (20 ng/g)
OFL	27.6 ± 5.0	64.3 ± 4.2	43.2 ± 9.2	35.0 ± 5.9
CIP	25.8 ± 5.1	60.0 ± 4.8	11.8 ± 1.5	15.0 ± 2.7
DAN	11.6 ± 1.3	31.9 ± 6.8	12.3 ± 2.5	17.3 ± 2.1
ENR	12.6 ± 2.6	42.2 ± 5.5	27.3 ± 8.2	30.7 ± 3.3
SARA	41.9 ± 4.5	80.3 ± 3.7	23.7 ± 3.1	69.0 ± 2.8
DIF	19.8 ± 5.7	70.8 ± 4.6	27.4 ± 4.6	73.2 ± 7.5
OXO	35.4 ± 4.8	60.3 ± 4.7	69.1 ± 5.0	70.3 ± 8.6
FLU	96.4 ± 5.1	96.4 ± 6.4	60.6 ± 6.0	83.1 ± 6.5

Table 4
Comparison of the sample preparation procedures and LOD between different methods for their application in edible animal food.

Analyte	Sample amount	Extraction solvent	Clean up/organic solvent consumed	Determination	LOD (ng/g)	Method
Chicken, fish	5.0 g	5 mL of 50 mM NaH_2PO_4, 30 mL CH_2Cl_2, 10 mL hexane	SPE (C18), 10 mL MeOH and 2.5 mL MeCN	CE-MS/MS/MS	5–20	A [23]
Chicken	1.0 g	5.0 mL MeCN, 2.0 mL hexane (defat)	On-line SPE (Oasis HLB)	LC–MS/MS	0.4–8.4	B [6]
Fish	1.5 g	10.0 mL MeCN	SPE (Gilson Aspec XL4), 4.0 mL MeOH and 3.0 mL MeCN	LC–MS/MS	1–3	C [7]
Milk	5.0 g	400 μL NH_3 solution	SPE (Oasis HLB), 8.0 mL MeOH	CE-MS/MS	4–6[a]	D [22]
Milk	2.0 mL	2 mL MeCN, 4 mL CH_2Cl_2	SPE (Strata X), 6.0 mL MeOH	UPLC/TOF-MS	<7.0[a]	E [17]
Egg	2.0 g	None	PLE[b] 16 mL phosphate/acetonitrile (50/50) (v/v)	LC-FLD[c]	17–24	F [28]
Egg	1.5 g	None	Hot water extraction, 6.0 mL acid water	LC–MS/MS	0.1–0.6	G [30]
Milk, egg, chicken, fish	0.5 g (mL)	5.0 mL phosphate solution for milk and egg, 5.0 mL acetone for chicken and fish	In-tube SPME [poly(MAA-co-EGDMA) monolithic column]	LC/QTOF-MS	0.2–3.0	This work

[a] The units for milk sample were ng/mL.
[b] PLE: pressurized liquid extraction.
[c] FLD: fluorescence detection.

Table 5
Linear range, LOD and LOQ data for in-tube SPME/LC/ESI-QTOF-MS of the seven quinolones from four different matrices.

	CIP	DAN	ENR	SARA	DIF	OXO	FLU
Egg							
LOD (ng/g)	0.8	0.6	0.6	0.3	0.3	1.2	0.3
LOQ (ng/g)	2.7	2.0	2.0	1.0	1.1	4.0	1.0
Linear range (ng/g)	4–200	2–200	2–200	2–200	2–200	4–200	2–200
Milk							
LOD (ng/mL)	0.2	1.5	0.3	0.2	0.2	3.0	0.5
LOQ (ng/mL)	0.6	5.0	0.8	0.6	0.6	10.0	1.8
Linear range (ng/mL)	2–200	5–200	2–200	2–200	2–200	10–200	2–200
Chicken							
LOD (ng/g)	0.5	0.7	0.3	0.2	0.2	0.6	0.6
LOQ (ng/g)	1.6	2.4	1.0	0.8	0.8	2.0	2.0
Linear range (ng/g)	2–200	4–200	2–200	2–200	2–200	4–200	4–200
Fish							
LOD (ng/g)	0.8	0.7	0.4	0.2	0.2	0.8	1.0
LOQ (ng/g)	2.7	2.4	1.2	0.7	0.6	2.8	3.3
Linear range (ng/g)	4–200	4–200	2–200	2–200	2–200	4–200	4–200

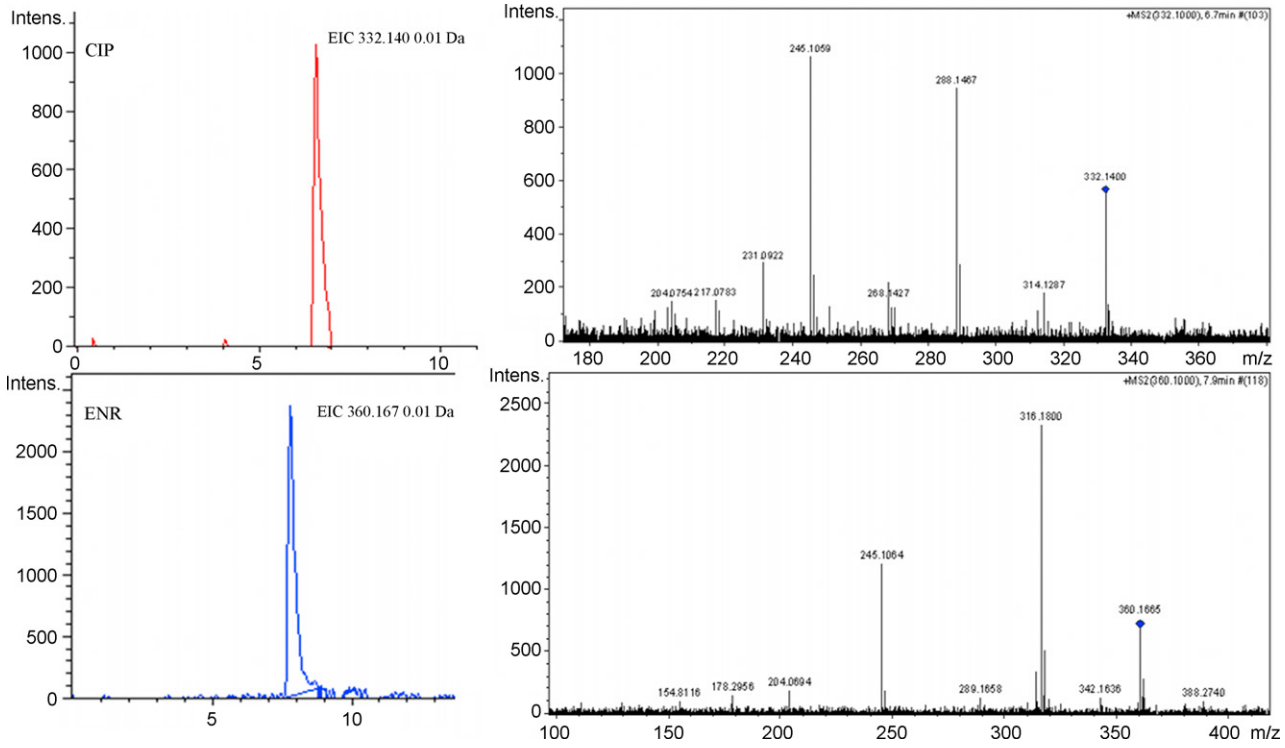

Fig. 6. Chromatograms obtained in the confirmation of CIP (17.1 ng/g) and ENR (7.5 ng/g) in positive samples by in-tube SPME/LC/ESI-QTOF-MS showing the product ion spectra with the accurate mass obtained for each analyte.

Table 6
The method recoveries and precisions at three different concentrations for in-tube SPME/LC/ESI-QTOF-MS of the seven quinolones from milk (A), chicken (B) egg (C) and fish (D) samples.

Analyte	Intra-day ($n=4$), recovery[a] (%, RSD, %)						Inter-day ($n=3$), recovery[a] (%, RSD, %)					
	5 ng/g		20 ng/g		200 ng/g		5 ng/g		20 ng/g		200 ng/g	
	A[b]	B	A[b]	B	A[b]	B	A[b]	B	A[b]	B	A[b]	B
CIP	86.9 (5.8)	88.0 (7.7)	84.8 (11.1)	85.4 (9.2)	100.1 (4.8)	102.5 (5.5)	94.6 (7.1)	90.0 (7.5)	84.0 (10.1)	83.0 (8.5)	97.8 (2.3)	98.2 (5.9)
DAN	99.2 (12.3)	86.0 (8.9)	91.4 (9.0)	94.4 (7.5)	94.3 (7.3)	110.2 (9.5)	95.0 (7.9)	92.0 (9.1)	97.8 (2.3)	89.8 (10.7)	94.8 (9.0)	107.0 (10.4)
ENR	84.7 (13.3)	100.0 (6.2)	112.2 (8.9)	91.0 (4.3)	95.0 (8.4)	97.8 (4.8)	88.0 (14.4)	94.0 (6.9)	109.8 (11.4)	85.8 (13.7)	94.9 (8.8)	95.5 (3.6)
SARA	99.5 (13.0)	86.0 (5.7)	97.8 (7.1)	88.4 (5.9)	98.4 (8.9)	106.6 (13.3)	109.0 (11.4)	98.0 (9.2)	87.0 (8.6)	90.6 (13.5)	96.1 (9.0)	101.2 (9.4)
DIF	86.4 (12.3)	102.0 (5.7)	113.8 (5.5)	81.2 (11.9)	102.6 (1.3)	95.8 (12.8)	93.0 (12.5)	96.0 (6.9)	114.4 (3.3)	100.4 (14.3)	105.9 (1.5)	88.0 (11.4)
OXO[c]	101.3 (9.8)	86.0 (9.4)	97.6 (7.4)	90.2 (8.6)	99.4 (5.7)	107.9 (11.9)	113.6 (9.4)	88.0 (10.4)	91.4 (9.1)	93.0 (8.5)	96.3 (4.9)	102.6 (8.3)
FLU	86.8 (6.3)	102.0 (9.8)	95.7 (7.2)	109.4 (4.3)	98.3 (1.7)	94.8 (11.1)	93.4 (6.3)	84.0 (9.9)	98.8 (3.9)	103.0 (3.0)	100.4 (2.1)	92.7 (14.5)

Analyte	Intra-day ($n=4$), recovery[a] (%, RSD, %)						Inter-day ($n=3$), recovery[a] (%, RSD, %)					
	5 ng/g		20 ng/g		200 ng/g		5 ng/g		20 ng/g		200 ng/g	
	C	D	C	D	C	D	C	D	C	D	C	D
CIP	92.0 (9.3)	89.4 (12.9)	89.5 (7.7)	84.5 (6.7)	104.2 (9.3)	85.1 (6.5)	84.0 (1.0)	83.5 (5.6)	86.0 (5.1)	90.8 (8.1)	107.6 (7.5)	92.8 (7.9)
DAN	96.0 (6.8)	112.2 (9.5)	88.5 (3.2)	93.6 (9.8)	94.3 (11.7)	103.7 (11.1)	86.0 (12.3)	115.0 (10.8)	85.5 (2.6)	89.4 (10.0)	89.6 (10.5)	108.3 (10.9)
ENR	82.0 (10.6)	90.3 (5.3)	87.0 (6.8)	113.4 (9.3)	101.5 (7.8)	95.9 (7.8)	90.0 (5.7)	89.0 (6.3)	84.0 (4.7)	105.8 (5.9)	113.2 (4.9)	95.9 (9.5)
SARA	94.0 (7.7)	86.2 (12.9)	81.0 (9.3)	80.2 (13.2)	104.4 (7.6)	92.7 (3.9)	92.0 (12.1)	101.6 (13.4)	99.0 (10.1)	82.5 (12.2)	99.5 (5.2)	92.9 (4.7)
DIF	113.0 (5.8)	96.5 (8.6)	104.0 (5.1)	110.2 (5.9)	99.5 (5.3)	97.6 (5.7)	114.0 (4.4)	94.0 (5.3)	103.0 (7.6)	107.2 (5.3)	99.5 (5.2)	94.1 (5.4)
OXO	114.0 (11.6)	81.9 (11.2)	97.5 (9.1)	82.2 (12.9)	98.4 (6.4)	92.4 (7.6)	106.0 (9.9)	97.2 (8.3)	92.0 (7.3)	81.2 (10.7)	90.0 (7.7)	92.6 (8.9)
FLU	90.0 (10.5)	81.5 (6.8)	92.5 (8.6)	104.0 (6.5)	86.4 (4.0)	103.4 (6.0)	92.0 (10.2)	87.6 (4.9)	82.5 (5.2)	106.8 (3.3)	110.5 (4.8)	101.4 (5.8)

[a] The percentage of calculated amounts of quinolones over the total amounts spiked (using I.S. correction factor). The amounts of quinolones were calculated using matrix-matched calibration curves.
[b] The units for milk sample were ng/mL. The in-tube SPME and LC/ESI-QTOF-MS conditions were outlined in Section 2.
[c] The low concentration of OXO spiked into the milk sample was 10 ng/mL.

Table 7
Confirmation of the positive findings.

Analyte	Ion	Exact mass (m/z)	Measured mass (m/z)	Deviation (mDa)	Assigned IPs	IPs[a]
CIP	$[M+H]^+$	332.1405	332.1400	0.5	2.0	8.5
	$[MH-H_2O]^+$	314.1299	314.1287	1.2	2.5	
	$[MH-CO_2]^+$	288.1507	288.1467	4.0	2.0	
	$[MH-CO_2-C_2H_5N]^+$	245.1085	245.1059	2.6	2.0	
ENR	$[M+H]^+$	360.1718	360.1665	5.3	1.5	8.0
	$[MH-H_2O]^+$	342.1612	342.1636	2.4	2.0	
	$[MH-CO_2]^+$	316.1820	316.1800	2.0	2.5	
	$[MH-CO_2-C_4H_9N]^+$	245.1085	245.1064	2.1	2.0	

[a] The number of IPs was calculated as shown in Ref. [42].

internal standard for quantification. The internal calibration in milk, egg, chicken and fish samples was performed by plotting peak area ratios (quinolones/I.S.) versus quinolones concentrations. EICs with an extraction window of 0.01 Da were employed for the quantification of quinolones. Matrix-matched calibration curves for four matrices were established with R above 0.9951. Detection limits (LODs) and quantification limits (LOQs) were calculated as the concentration corresponding to a signal 3 and 10 times the standard deviation of the baseline noise, respectively. The concentration level used for the evaluation of the LODs and LOQs was the concentration of LOQ. As listed in Table 5, The LODs for seven quinolones were found to be 0.3–1.2 ng/g in egg, 0.2–3.0 ng/mL in milk, 0.2–0.7 ng/g in chicken and 0.2–1.0 ng/g in fish. The LOQs were found to be 1.0–4.0 ng/g in egg, 0.6–10.0 ng/mL in milk, 0.8–2.4 ng/g in chicken and 0.6–3.3 ng/g in fish.

3.3.4. Accuracy and precision

In this study, the accuracy of the method was measured and expressed as recovery. The precision of the method was accessed by determining intra- and inter-day relative standard deviations (RSDs) of the analysis. Both recoveries and intra- and inter-day RSDs were calculated with seven quinolones spiked at three different concentrations in four different matrices. The recoveries were determined by comparing the calculated amounts of quinolones in the samples (using matrix-matched calibration curves) with the total spiking amounts. The recoveries and RSDs data for quinolones spiked in four different matrices are summarized in Table 6. The intra- and inter-day recoveries were between 84.0–114.4% for milk, 81.0–114.0% for egg, 81.2–110.2% for chicken and 80.2–115.0% for fish. The intra- and inter-day precisions for recoveries of seven quinolones were less than 14.4% for milk, 12.3% for egg, 14.5% for chicken and 13.4% for fish. The results demonstrate that the precision and accuracy of the present in-tube SPME/LC/ESI-QTOF-MS method were acceptable for routine monitoring purposes.

3.3.5. Positive sample determination

The in-tube SPME/LC/ESI-QTOF-MS method validated was applied to the identification and determination of quinolones in ten food samples including milk, egg, chicken and fish for control purposes. In particular, CIP was identified in one batch of milk and ENR was identified in two batches of fish samples. ESI-QTOF-MS provides unique ways to confirm the presence of analytes. The combination of accurate mass and the isotopic ratio pattern (sigma value) of a charged analyte is a powerful tool. Extracting the trace of 332.140 ± 0.01 Da and 360.167 ± 0.01 Da, corresponding to the calculated exact mass of CIP and ENR, produced two single and strong peaks (Fig. 6). The spectra of these peaks were compared to the calculated isotopic pattern of CIP and ENR, respectively, to prove its identity. Running the Generate Molecular Formula Editor which is included in the Data Analysis software, listed the formula for these two targets at the position 3 of the hit list 1 and position 1 of the hit list 2, respectively (Fig. 7). Hits are sorted according to the deviation of the exact mass and the "sigma value", which corresponds to the correlation of measured isotopic patterns with the calculated ratio.

With the aim to add confidence to the identity of the analyte, the criteria established in the Commission Decision of the EU were also used after a further chromatographic run. This Decision proposes a system of identification points (IPs), where at least three IPs are required to confirm a positive finding [41]. The MS/MS spectrograms of positive samples showed two characteristic fragmentation patterns (Fig. 6) matching CIP and ENR, respectively. The accurate mass of the precursor ions and corresponding product ions of CIP and ENR for the positive samples are listed in Table 7, from which the identification points for the positive samples were calculated according to the standard of IP calculation recommended by Hernandez et al. [42]. As shown in Table 7, 8.5 IPs for CIP and 8.0 IPs for ENR were obtained, which is greater than 3.0 IPs. Therefore, the positive samples were further confirmed according to the criteria established.

After confirmation, the quantitation of CIP and ENR identified in the positive samples were performed using matrix-matched cal-

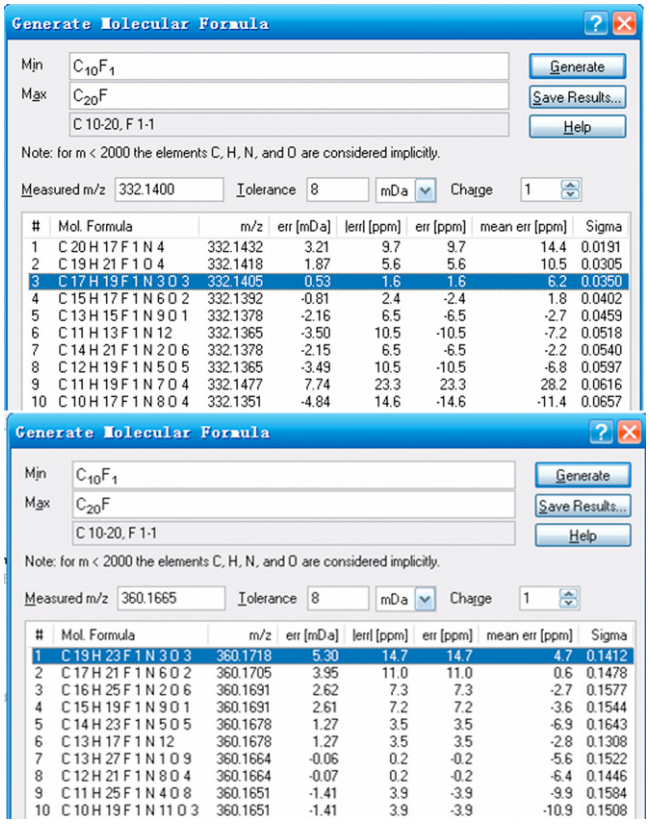

Fig. 7. "Hit list" after investigating the suspected CIP and ENR peaks as shown in Fig. 6. CIP is hit number 3 in list 1 and ENR is hit number 1 in list 2.

ibration curves (Table 5). The concentration of CIP in one milk sample was found to be 17.1 ng/mL. The contamination concentrations of ENR in two fish samples were found to be 2.9 and 7.5 ng/g, respectively. The RSDs ($n = 3$) for the determinations were less than 8.4%. No target analytes have been detected in other animal-producing food.

4. Conclusions

In-tube SPME using a poly(MAA-co-EGDMA) monolithic column on-line coupled to LC/ESI-QTOF-MS provides a simple, fast and sensitive procedure for the determination of seven quinolone residues in edible animal food. The proposed in-tube SPME advocated an environmentally friendly, inexpensive, and rapid sample preparation technique. The sensitivity together with mass accuracy of the ESI-QTOF-MS will allow the unambiguous identification of quinolone residues in positive animal products. The on-line combination of in-tube SPME and LC/ESI-QTOF-MS will provide an alternative practical tool in future antibacterial residues determination as well as the monitoring of prohibited substances from complex foodstuff.

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