Zhejiangsheng Shuichanpin Jiagong Chanye Chuangxin Tuandui Lunwenji (Er)

浙江省水产品加工产业创新团队论文集(二)

戴志远 主编　钱国英 马永钧 副主编

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序

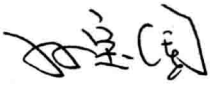
水产食品作为公认的餐桌佳品，人们不但乐于其美味，更见证了它对人们生活和健康的诸多益处。千百年来，人们将“连年有余（鱼）”作为理想的富庶生活，从中即可反映出水产食品在人们生活中的地位。我国拥有丰富的海洋和内陆水产品生产条件，不断发展的渔业为我国广大渔民提供了大量的就业机会，同时也为国家创造了显著的经济效益。当前我国渔业及渔业经济发生了巨大变化，水产品生产总量已连续十多年名列世界首位，水产品出口占据出口农产品首位。

作为海洋大省的浙江，渔业是其传统产业和重点产业之一，在国民经济中占有十分重要的地位。其中，水产品加工由于具有高附加值、高科技含量、高市场占有率、高出口创汇“四高”特点，经济效益和社会效益明显，已连续多年成为浙江省农产品出口的第一大产业，并在全国具有领先地位。

然而，我们必须认识到，虽然我国水产品加工业有了长足的发展，但与发达国家相比，仍存在很多不足，主要体现在基础研究薄弱、加工与综合利用率比较低、加工产品品种少、附加值低、装备落后、标准体系不健全、产品质量不稳定等方面的不足。

为此，在浙江省委、省政府的领导下，浙江省科技厅于2009年批准成立浙江省水产品加工产业创新团队，旨在通过整合浙江省水产品加工的科技与产业资源，加强水产加工基础研究，建设一个集水产加工科技源头创新、成果转化与人才培养为一体的现代水产加工利用和质量安全控制技术创新平台，提高浙江省水产品加工利用的技术创新能力。

本论文集是创新团队成员，尤其是中青年学术骨干围绕各自专长潜心研究多年的最新研究成果，内容涵盖水产品贮藏与加工、水产品质量安全与控制以及水产品营养与功能性水产食品开发。希冀此论文集的问世能够助推我国水产品精深加工领域的科技创新，为我国渔业经济的持续、繁荣发展贡献力量。

中国工程院院士 

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Development of an On-line Matrix Solid-phase Dispersion/Fast Liquid Chromatography/Tandem Mass Spectrometry System for the Rapid and Simultaneous Determination of 13 Sulfonamides in Grass Carp Tissues

Yanbin Lu* Qing Shen Zhiyuan Dai** Hong Zhang Honghai Wang

1 Introduction

Sulfonamides (SAs) are N-substituted derivatives of sulfanilamide and compete with p-aminobenzoic acid in enzymatic synthesis of dihydrofolic acid. Because of broad-spectrum antibacterial action, SAs have been widely used as bacteriostatic reagents for treatment of humans (inhibition of folic acid synthesis in bacteria) and as growth promoters in animals[1]. However, the possible presence of SA residues in the products of animal origin became a public health concern, due to a possibility that exposure to these drugs could reduce the effectiveness of human therapeutic drugs and some of the compounds may lead to many diseases such as thyroid cancer[2], anaphylactic reaction and resistance to drugs[3]. Therefore, a joint meeting of Food and Agriculture Organization (FAO) and World Health Organization (WHO) and Office International des Epizooties (OIE) has categorized SAs as critically important antimicrobials. Nevertheless, due to their steady antibiosis effectiveness, these drugs are still illicitly used in aquatic products. In order to identify the potential risk of SAs to consumers related to the consumption of food, specific and sensitive analytical methods are thus required for a concrete monitoring of SAs at residual levels, which must be in compliance with the criteria of the Commission Decision 2002/657/EC (The combined total residues of all substances within the SA group should not exceed 100 $\mu g\ kg^{-1}$).

The quality of sample preparation is a key factor in residue analysis[4] and thus there is a considerable interest in developing new selective and sensitive methods for extracting and isolating components from complex samples. During the years, the classical solid-phase extraction (SPE) technique has evolved to meet the need of monitoring several classes of

[Resume] College of Food Science and Biological Engineering, Zhejiang Gongshang University, Hangzhou 310035, China. * Corresponding author.

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substances in samples of different origin, often present at trace levels[5]. But as we know, the SPE procedures is time-consuming and cumbersome to perform, often requiring many steps before reaching a concentrated extract suitable for instrumental analysis, of which only a small portion is actually injected onto the chromatographic column[6]. Recently, the on-line SPE technique was widely used, which significantly decreased the analysis time and met the increasing demand for automation and high-throughput analysis. But the complicated pretreatment steps for solid, semi-solid and viscous samples were still unavoidable including solid-liquid extraction (SLE), centrifugation, supernatant evaporation and residue re-dissolution. Matrix solid-phase dispersion (MSPD), which was first introduced in 1989 by Barker et al.[7—8], was established as an effective technique for the analysis of trace substances, both exogenous (i. e. drugs, pollutants, pesticides) and endogenous ones (i. e. food and bacteria components, etc.) from solid, semi-solid, and viscous matrices (animal tissues, blood, milk, bacteria, fruits, vegetables, etc.)[9—11]. This technique has four main steps: (I) The sample is blended with the dispersant material in a mortar with a pestle and the homogenized powder is transferred in a solid-phase extraction cartridge; (II) Target compound is eluted with a suitable solvent or solvent mixture; (III) The elution is evaporated to dryness, and the residue is dissolved in appropriate amount of HPLC mobile phase for further analysis. In these studies, the second and third steps cost much time and may induce analyte loss and contamination. Kashida et al.[12] had developed a method using MSPD and HPLC for the simultaneous determination of six SAs in chicken with total time nearly 1.5 h. However, if the MSPD method could be performed in an automated on-line system, many obvious benefits would be achieved, such as acceleration of analytical process, together with reduction in manual handling, risk of contamination, loss of analytes and sample consumption.

Recently, several analytical methods have been developed for the analysis and determination of SA residues. Enzyme-linked immunosorbent assay (ELISA) is the most representative method for the fast screening analysis and batch determination of SAs[13], thin-layer chromatography (TLC) can be used for the semi-quantification of SAs[14]. High-performance liquid chromatography (HPLC) method has been frequently used because of its high sensitivity and broad linear range. HPLC with electrospray tandem mass spectrometry (ESI-MS/MS)[15—16] has been favored by many analysts due to their higher sensitivity and their ability to provide compound confirmation. However, analysis performed on conventional HPLC columns was always time consuming and organic solvent costing. Dasenaki et al.[17] presents rapid and effective separation for the determination of SA residues using ultra-high performance liquid chromatography (UHPLC) coupled with MS/MS. However, expensive ultra-high-pressure instrumentation and new laboratory protocols were needed. Therefore, none of the above-mentioned methods have the required characters of fast, simplicity, sensitivity to unequivocally confirm a positive result. The development of fused-core particles was considered as a breakthrough in column

technology aimed at reducing analysis times while maintaining column efficiencies and requiring relatively low back pressures[18—19]. With a 1.7 μm solid silica inner core surrounded by a 0.5 μm porous silica shell[20], the material provides a high diffusion path which allows for rapid mass transfer and thus reduce axial dispersion and peak broadening. More importantly, 2.7 μm fused-core particles produce only approximately half the back pressure of the 1.8 μm particles, which makes it possible to use fused-core columns on conventional HPLC systems[21]. Comparing to the sub-2 μm particles, similar efficiency separations could be achieved using the fused-core particles. Meanwhile, the low pressure allows fast separations to be performed routinely on conventional HPLC systems, but demands neither expensive ultra-high-pressure instrumentation nor new laboratory protocols[22—24]. To reduce analysis time and save solvents, the integration of fused-core columns into on-line MSPD-HPLC-MS/MS systems is generally recommended.

Therefore, in this paper, a novel protocol based on interfacing on-line matrix solid-phase dispersion (MSPD) with fast liquid chromatography-tandem mass spectrometer (LC-MS/MS) was established aiming at improving and simplifying the process of veterinary drug residues in aquatic products. The grass carp tissue was chosen as sample and SA residues was used as representative indicator compounds for testing the accuracy and precision of the on-line MSPD-HPLC-MS/MS method. The effects of several extraction parameters, such as extraction solvent polarity, extraction flow rate and duration time were tested in order to improve recovery and sensibility. Compared to traditional MSPD method, the proposed on-line MSPD technique significantly decreases pretreatment time consumption, loss of analytes and sample contamination. Meanwhile, this system permits to extract one sample while another one is being analyzed by MS/MS. Thus, sample throughput is greatly increased.

2 Experiment

2.1 Reagents and Materials

The grass carp samples were purchased from a local supermarket (WuMart, Hangzhou, China). The standards of 13 sulfonamides, i. e. sulfadiazine (SD), sulfathiazole (STZ), sulfamerazine (SM), sulfamethazine (SMZ), sulfameter (SME), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP), sulfamonomethoxine (SMM), sulfamethoxazole (SMX), sulfadoxin (SDO), sulfisoxazole (SSA), sulfadimethoxine (SDM) and sulfaquinoxaline (SQX), were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures of 13 SAs were shown in Figure 1.

Acetonitrile (ACN), methanol (MeOH) and formic acid were chromatographic grade and obtained from Merck (Darmstadt, Germany). High-purity water with a resistivity of 18.2MΩ cm^{-1} was obtained from a Milli-Q water system (Millipore, Bedford, MA,

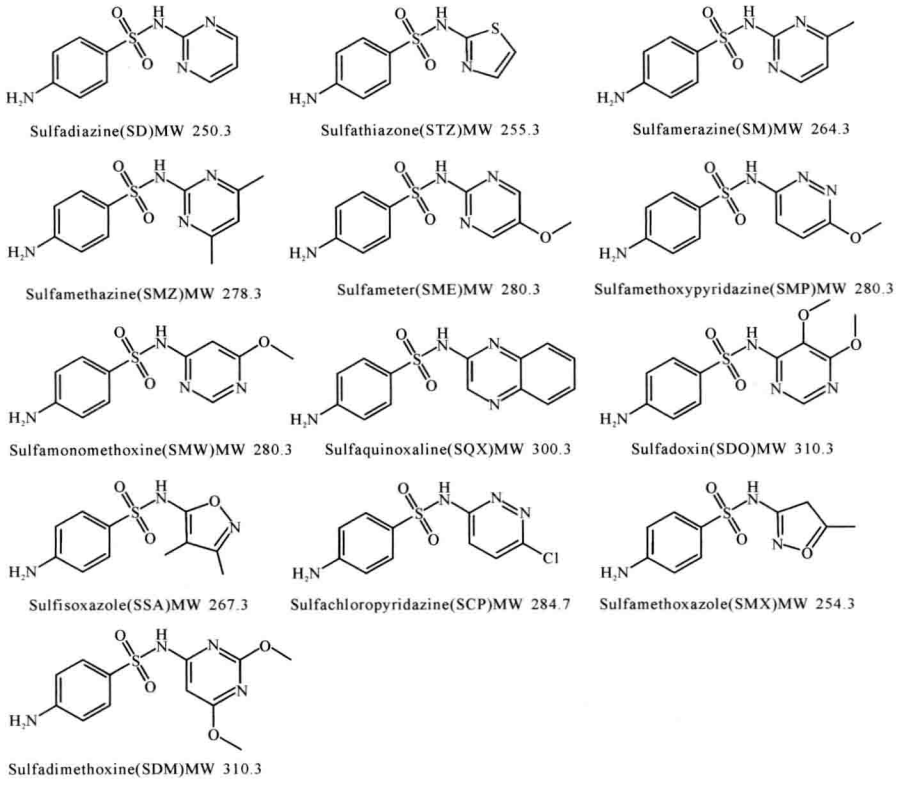

Figure 1 Chemical structures of 13 sulfonamides in the present study

USA). The solid phase material used for MSPD was ODS-A (particle size 50 μm) bought from YMC Corp. (Kyoto, Japan).

2.2 Instrumentation

The proposed on-line MSPD-HPLC-MS/MS system is illustrated in Figure 2. In this system, a Waters 515 pump (Milford, MA, USA) equipped with a 6-port switching valve was used to re-circulate the extraction solvent (ACN/water, 50∶50 v/v). The customized MSPD process was performed by a 25mm × 10mm i. d. extraction vessel (Michrom Bioresources, Auburn, CA) and on-line coupled with LC/MS/MS by a 10-port switching valve (VICI, Schenkon, Switzerland).

Chromatographic analysis was performed on a Waters 2695 LC system (Waters, Milford, MA) which was equipped with a quaternary pump, an autosampler, a vacuum degasser and Empower workstation (Waters, Milford, MA). The separation was achieved on a Halo fused-core C18 silica column (50mm×2.1mm, 2.7μm particle size; Advanced Materials Technology, USA).

A triple-quadrupole linear ion trap mass spectrometer (4000Q-Trap, Applied Biosystems, Foster City, CA) equipped with an electro-spray ionization (ESI) was used in positive ionization multiple-reaction monitoring (MRM) mode. Instrument control, data

acquisition and the processing were performed using the associate Analyst 1.5.1 software.

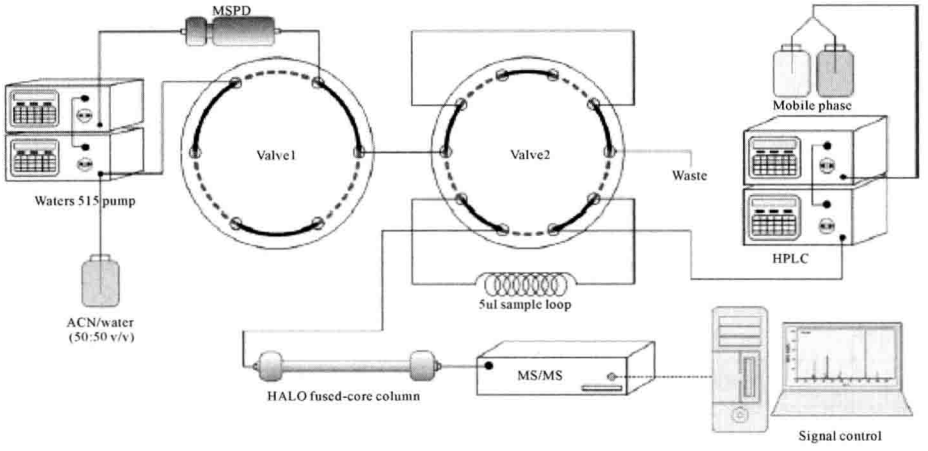

Figure 2 The setup of on-line MSPD-HPLC-MS/MS system

The red lines indicate that the valves are at position A, while the black lines indicate that the valves are at position B. For on-line MSPD procedure, a potion of 5 mL ACN/water (50∶50 v/v) mixture was used as extraction solvent for sulfonamides extraction at a flow rate of 1.0 mL min^{-1} in 5 min. The separation was performed on a HALO fused-core column with linear gradient elutions of water (0.1% formic acid) and ACN. Injection volume was 5 μL. For other details see Section 2.4.

2.3 Preparation of Samples and Standards

The grass carp tissues were first homogenized using an electric blender. Then, a mass of 0.2 g homogenized sample was placed into a glass mortar and gently blended with 0.6 g of C18 (previously washed with 2 mL of ACN and vacuum dried) until a homogeneous mixture was obtained. After dried at room temperature, the MSPD blend was laboratory-packed into the extraction vessel, which was fitted with 10 μm (pore size, 0.75 mm thickness) frits. The vessel was capped tightly preventing the collapse inside.

SA standard stock solutions (5 μg mL^{-1}) were prepared by dissolving individual compound in 50 mL ACN and stored at 4℃. Working solutions at serial concentrations were obtained by combining aliquots of stock solutions followed by subsequent dilution with ACN. For recovery test, three different concentration levels (50, 100 and 150 μg kg^{-1}) of SAs were added to the homogenized tissue sample prior to blending with C18. The resulting samples were operated as described above. Triplicate experiments were performed at each level.

The empty extraction vessel, mortar and pestle were pre-washed with ultra-pure water, acetone, dichloromethane and MeOH. All glassware were cleaned and then heated for 2 h at 500℃.

2.4 On-line MSPD Procedures

The extraction vessel was carefully fixed in the system. Then, 5 mL ACN/water mixtures (50∶50 v/v) was added in a disposable centrifugal tube as extraction solvent. The procedure of on-line MSPD-LC-MS/MS was represented in Table 1.

Table 1 The procedure of on—line MSPD - LC - MS/MS analysis

	Elution	Sampling	Start LC - MS/MS[a]	Analysis completed[b]
Time (min)	Initial - 5 min	5 - 5.1 min	5.1 - 16	16
Valve 1 position	A	B	B	B
Valve 2 position	A	A	B	A
Binary pump	Activated	Activated	Stopped	Stopped
Flow rate	1.0	1.0	-	-
Solvent	ACN/water (50:50 v/v)	Extraction	-	-
HPLC pump	Activated	Activated	Activated	Activated
Flow rate	0.4	0.4	0.4	0.4
Solvent	Initial mobile phase	Initial mobile phase	Gradient elution	Initial mobile phase

All the steps above were programmed and controlled by computer. Sample extraction and analysis can continue to overlap, thus the analysis time can be reduced.

[a] If there was a new sample, it was carried out in a new extraction vessel and started from initial step.

[b] When the analysis of all samples were completed, the system was washed using 50% aqueous ACN.

Step 1: The Valve 1 and Valve 2 were set at position A (red line). The Waters 515 pump was activated and circulated extraction solvent (50% aqueous ACN) through the vessel at a flow rate of 1.0 mL min^{-1} until a stable extraction was obtained. Meanwhile, the HPLC pump was activated for the equilibrium of analytical column by the initial chromatographic mobile phase.

Step 2: When the extraction was completed, the Valve 1 was set at position B (black line) and the extraction solvent was introduced into the sample loop.

Step 3: The Valve 2 was switched to the position B. Thus, the sample solution was transferred onto the analytical column completely. Data acquisition was started simultaneously. Meanwhile, the extraction of the next sample was carried out on a new extraction vessel as described above.

The sampling and analytical procedures were performed automatically, which were previously programmed and controlled by computer. The total analysis time of the initial sample was 16 min. Because sample extraction and LC analysis could be operated simultaneously, the analysis of subsequent samples could be accomplished during 12 min.

2.5 Fast LC/MS/MS Analysis

A gradient HPLC method was employed for separation with the mobile phase A:

0.1% formic acid aqueous solution and mobile phase B: ACN. The gradient profile was carried out as follows: 5% B (initial), 5%～10% B (0～3.5min), 10%～30% B (3.5～8min), 30%～100% B (8～8.5min), 100%～100% B (8.5～9.5min), 100%～5% B (9.5～10min), 5%～5% B (10～11min). The flow rate was 0.4 mL min^{-1}. All of the SAs were eluted within 7 min. During the rest time the column was cleaned, readjusted to the initial conditions and equilibrated. The column temperature was set at 30℃, and injection volume of 5 μL was selected.

MS/MS data acquisition was performed in the multiple-reaction monitoring (MRM) mode. According to European Union Decision 2002/657/EC, for the confirmation of the presence of an illegal veterinary drug at least 4 identification points are necessary (1 point was earned with the precursor ion and 1.5 points were earned with each product ions). Therefore, two transitions between precursor ion and the two most abundant product ions were monitored: one for quantitative determination and the other for qualitative analysis. In order to obtain maximum sensitivity for identification and detection of SAs, the ion source temperature (TEM) was set at 450℃, and ion spray voltage (IS) was always set at 5.5 kV. Ion source gas1 (GS1) and ion source gas2 (GS2) were used as the drying and nebulizer gases at a back pressure of 55 psi and 75 psi, respectively. Curtain gas (CUR) was 20 psi. The analyte specific parameters (declustering potential (DP), entrance potential (EP) for precursor ions, collision energy (CE) and collision cell exit potential (CXP) for product ions are shown in Table 2.

Table 2 Optimized MS/MS parameters

Compounds	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	DP[a] (eV)	EP[b] (eV)	CE[c] (eV)	CXP[d] (eV)
Sulfadiazine (SD)	1.17	251.1	156.0	58.10	10.43	23.75	7.00
			108.0	59.83	11.00	33.10	10.00
Sulfathiazole (STZ)	1.61	256.1	156.0	59.84	9.99	22.91	10.00
			92.0	57.11	11.84	39.14	10.00
Sulfamerazine (SM)	1.86	264.9	156.0	58.97	10.28	24.36	10.00
			172	58.44	10.19	24.05	10.00
Sulfamethazine (SMZ)	2.69	278.9	186.0	64.27	10.60	24.83	10.00
			156.0	67.92	10.77	27.28	10.00
Sulfameter (SME)	2.96	281.1	156.0	60.86	11.02	25.42	10.00
			108.0	122.00	10.00	53.00	10.00
Sulfamethoxypyridazine (SMP)	3.27	281.1	156.0	60.86	11.02	25.42	10.00
			108.0	122.00	10.00	53.00	10.00
Sulfachloropyridazine (SCP)	4.23	285.1	156.0	51.38	11.70	22.74	10.00
			92.00	53.04	10.64	40.00	10.00
Sulfamonomethoxine (SMM)	4.25	281.1	156.0	60.86	11.02	25.42	10.00
			108.0	122.00	10.00	53.00	10.00
Sulfamethoxazole (SMX)	4.90	254.0	155.9	61.46	5.10	23.80	10.00
			108.0	59.10	10.62	35.81	10.00

续 表

Compounds	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	DPa (eV)	EPb (eV)	CEc (eV)	CXPd (eV)
Sulfadoxin (SDO)	4.92	311.1	156.0	67.92	10.77	27.28	10.00
			92.0	70.30	11.00	44.73	10.00
Sulfisoxazole (SSA)	5.59	268.1	156.0	51.67	10.04	20.79	10.00
			113.0	56.28	10.23	22.73	10.00
Sulfadimethoxine (SDM)	6.66	311.1	156.0	67.92	10.77	27.28	10.00
			92.0	70.30	11.00	44.73	10.00
Sulfaquinoxaline (SQX)	6.78	301.1	156.0	56.89	11.13	24.64	10.00
			108.0	59.46	10.49	39.99	10.00

Ionization mode: (ESI+); ion spray voltage (IS): 5.5? kV; curtain gas (CUR): 20? psi; ion source gas 1 (GS1): 55? psi; ion source gas 2 (GS2): 70? psi; temperature (TEM): 450?°C; interface heater: on; collision activated dissociation (CAD): medium.

[a] DP: declustering potential.

[b] EP: entrance potential.

[c] CE: collision energy.

[d] CXP: collision cell exit potential.

3 Results and Discussions

3.1 Optimization of On-line MSPD Procedure

In the on-line MSPD procedure, C18-bonded silica (YMC Corp. Japan) was chosen because it presented satisfactory trapping efficiency for SAs, as well as good mechanical strength which was also necessary for MSPD. A sample/sorbent ratio of 1∶3 showed good recovery of target compounds and was adopted in the present work. The optimization process was performed with 0.2 g of sample and 0.6 g of C18 material.

Optimization of the elution sequence was performed using pure ACN and ACN/water mixtures (80∶20 v/v, 50∶50 v/v and 20∶80 v/v) as eluting solvents, respectively. The elution solvent was circulated until SAs were completely extracted. As expected, the SAs could be extracted by all these solvents. However, when pure ACN was used, significant matrix effect was observed, which might arise from its strong dissolution and elution property for a wide range of compounds. By comparing the results obtained using 80%, 50% and 20% aqueous ACN elution, it was concluded that ACN/water mixtures (50∶50 v/v) provided satisfactory recovery and acceptable matrix influence.

Additionally, the extraction volume was also optimized, which involved two parameters: flow rate and duration. The flow rate ranging from 0.2 mL min^{-1} to 1.0 mL min^{-1} was evaluated. The results indicated that it had no significant effect on the recovery of each SAs. In order to reduce the extraction duration, the final elution time was established at

5 min at the flow rate of 1.0 mL min^{-1}, with total extraction volume of 5 mL.

3.2 Optimization of Chromatographic Conditions

The optimization of the chromatographic conditions, including the types of column and composition of mobile phase, is a crucial step before applying mass spectrometric detection, because the impurities in the samples can induce the matrix ion suppression and interferences from common product ions that could hinder the specificity or sensitivity achieved by MS/MS. For achieving good resolution and symmetric peak shapes of SAs in a shorter run time, the selection of analytical columns with high separation efficiency is a prerequisite. The HALO C18 silica column (50 mm × 2.1 mm i.d.) packed with 2.7 μm superficially porous fused-core particles appeared to result in the best performance, comparing to a number of conventional fully porous particle packed columns, including (1) Agilent Zorbax SB-C18 250 mm × 4.6 mm i.d., 5 μm; (2) Waters Atlantis T3-C18 150 mm × 2.1 mm i.d., 3 μm; (3) Phenomenex Synergi Fusion-RP 50 × 2.0 mm i.d., 4 μm. The separation efficiency and sensitivity of Halo column was obviously much better due to its special structure (a 1.7 μm solid core particle fused with 0.5 μm porous shell). All the peaks of analyte compounds were sharp and symmetric. The retention time of the last eluted SA in our test was less than 7 min with a flow rate of 0.4 mL min^{-1}. This novel fused-core column packing technique provided a high diffusion path, allowed rapid mass transfer and thus reduced axial dispersion and peak broadening. Comparing to the sub-2 μm particles, similar efficiency separations could be achieved on conventional HPLC systems using this fused-core columns, saving the expensive cost of ultra-high-pressure instrumentation. As a result, the HALO fused-core column was finally chosen in this work.

The analytical sensitivity in condition of samples eluted with MeOH/water, ACN/water, ACN/water (0.1% formic acid), were compared. The results indicated that MeOH gave rise to better selectivity while ACN gave rise to better elution strength and shorter retention time. What's more, ACN generated lower back-pressure than MeOH which made Halo column especially suitable for conventional LC equipment. The addition of formic acid (0.1%) in water solution played an important role in improving chromatographic separation (reduction of peak tailing and better resolution) and promoting the ionization efficiency of mass spectrometry. Results of multiple injections indicated that under such situation nice peak shape and high sensitivity of SAs could be achieved. Therefore, ACN and ultra-pure water (0.1% formic acid) were finally chosen as mobile phases for the simultaneous chromatographic separation. Figure 3 showed the separation of the 13 target analytes (100 ng mL^{-1} standard solution).

3.3 Optimization of MS/MS Parameters

Electrospray ionization (ESI) was tested in both positive-and negative-ion modes. SAs showed much higher response signals using positive-mode ESI than those in the

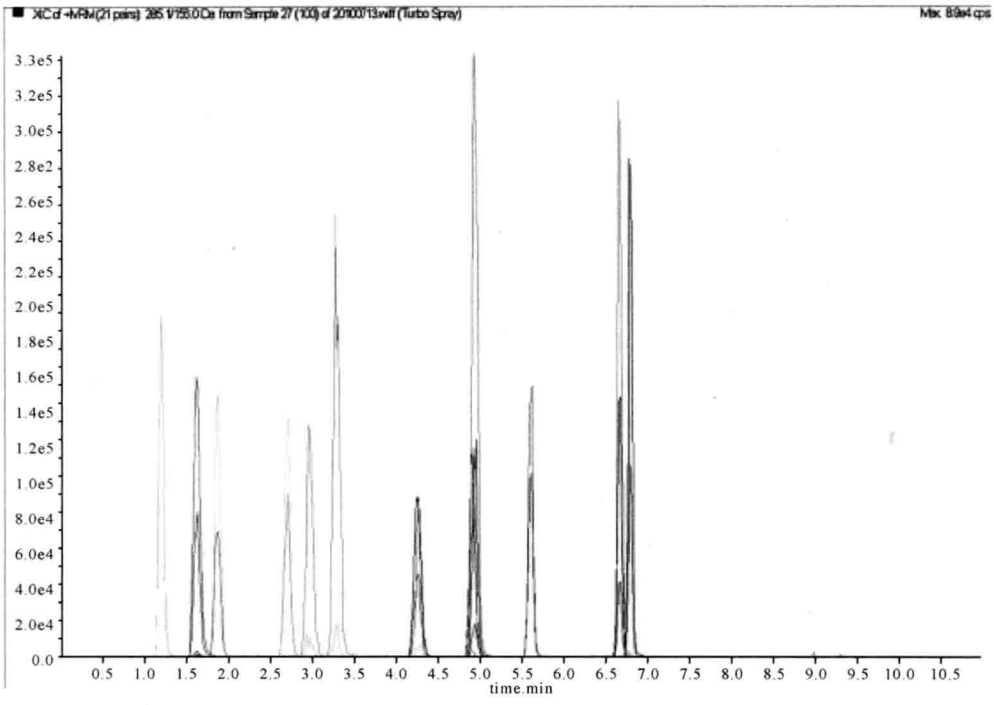

Figure 3 LC-MS/MS chromatogram showing the separation of the 13 target analytes (100 ng mL^{-1} standard solution)

negative mode. Therefore, the ESI source in positive mode was chosen for SAs detection. Acquisition parameters of the mass spectrometer were optimized by direct continuous pump infusion of standard working solutions of the SAs (100 ng mL^{-1}) individually at a flow rate of 10 μL min^{-1} in the mass spectrometer. Full-scan spectra were acquired over the m/z range of 50—400 amu with a circle time of 1.0s and a step size of 0.1 amu for identification of the precursor ions. The selected protonated molecular ion $[M+H]^+$ and product ions were summarized in Table. 2. The declustering potential (DP) was optimized for achieving the highest signal response of $[M+H]^+$. Further identification of the most abundant fragment ions and selection of the optimum collision energies (CE) for SAs was carried out in the product ion scan mode. The fragmentations of m/z 156.0 were comparatively high intensity for most SAs except SMZ. Based on this point, the MRM transition ($[M+H]^+ > 156.0$) was used as a quantifier for 12 SAs, while the mass transition ($[M+H]^+ > 186.0$) was used for SMZ determination. The chromatograms for each SA under optimum conditions were shown in Figure 4.

In order to optimize the gas setting for SAs, flow inject analysis (FIA) method was adopted by introducing 5 μL standard solution of SAs (1 μg mL^{-1}) into the mass spectrometer using the initial chromatographic conditions. The optimum conditions include curtain gas (CUR), ion source gas1 (GS1), ion source gas2 (GS2), temperature (TEM) and collision activated dissociation (CAD).

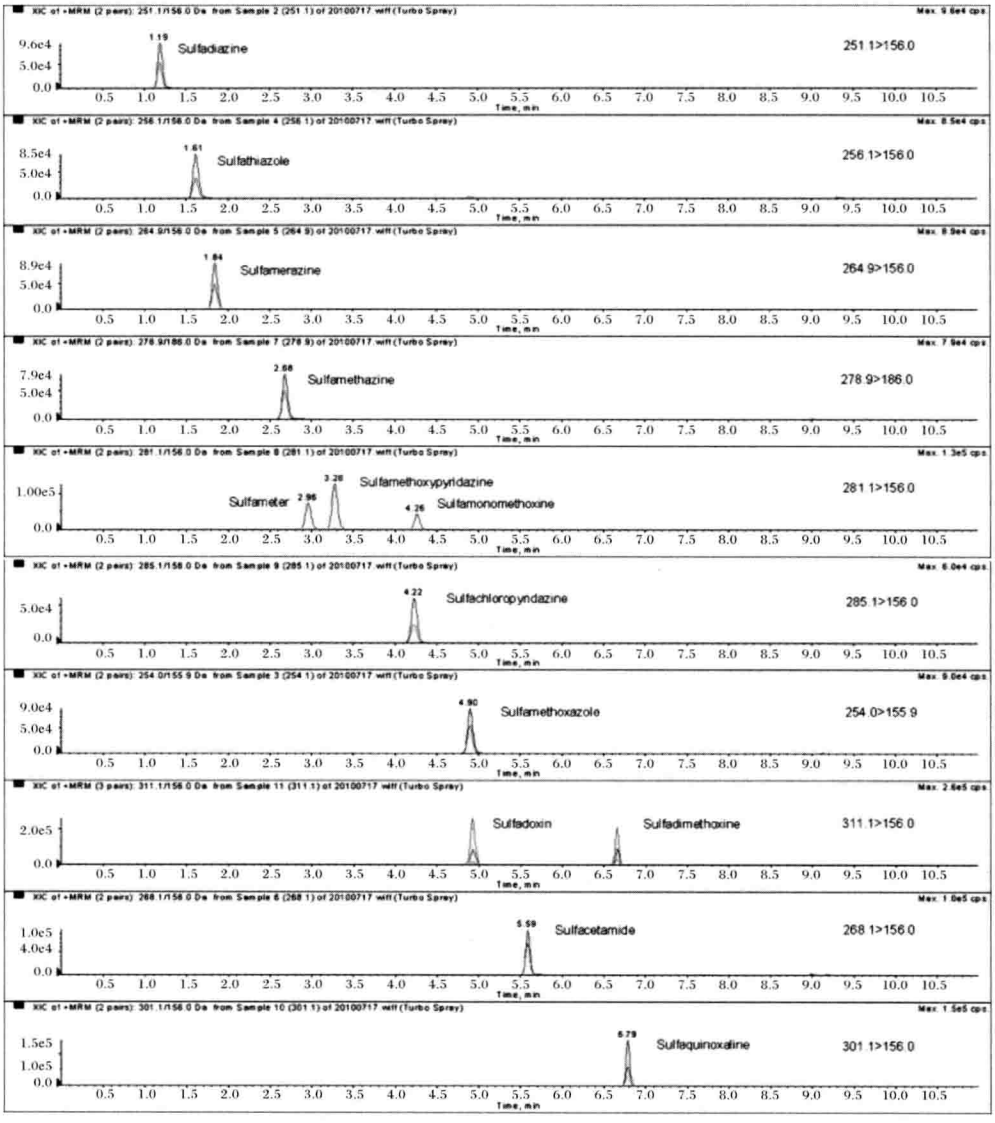

Figure 4 MRM chromatograms for standard of sulfonamides (100 ng mL^{-1}) under optimized On-line MSPD-HPLC-MS/MS conditions

3.4 Method Validation

Parameters of performance of the described on-line MSPD-HPLC-MS/MS method were determined and evaluated according to the considerations proposed in Decision 2002/657/EC using spiked samples at levels of 0, 0.5, 1.0, 1.5 MRL ($\mu g\ kg^{-1}$). The values of the selectivity, recovery, precision, decision limit ($CC\alpha$), detection capability ($CC\beta$), limit of detection (LOD) and limit of quantification (LOQ) were determined.

3.4.1 System suitability. System suitability tests were performed to mass spectrum obtained from standard and test solutions to check parameters such as column efficiency,

retention time, peak asymmetry, and capacity factor of SAs peaks. Results obtained from injection of standard solution showed good system suitability of this new instrumental coupling.

3.4.2 Specificity. Each individual SA was analyzed based on the retention time as well as the two ion transitions selected. Spiked blank grass carp samples from different origins were analyzed for the verification of interference, using the extraction procedure and chromatographic conditions described. The results indicated that no carryover was observed from sample to sample and the SAs were presented at the same retention times.

3.4.3 Selectivity. The interference of other co-extracted substances was evaluated, since it is a very important aspect in the LC-MS/MS analysis of biological fluids, which may affect the results of both qualitative and quantitative analyses. The experiments were performed in two steps. First, spiked grass carp tissues at levels of 0.5, 1.0, 1.5 MRL were analyzed by on-line MSPD-HPLC-MS/MS method in three times. Second, the method was performed using the same conditions at concentrations identical with that of the first step without real sample. The relationship between peak area (y) and analyte concentrations (x, $\mu g\ kg^{-1}$) was calculated. Comparison of the expected concentrations and matrix-based values indicated the ion signal intensities of the SAs were affected by a "negative" matrix effect.

3.4.4 Recovery. Recovery experiments were performed by comparing the analytical results for extracted grass carp tissue samples where the SAs were added at levels of 0.5, 1.0, 1.5 MRL before the extraction procedure, with standards prepared at the same concentration without real sample extract representing 100% recovery. As shown in Table 3, the relative recoveries of thirteen SAs range from 69.0% to 96.3% in three matrices with RSD values were lower than 13.2%. Although the recovery values were not good enough, considering the on-line sample trapping procedure, the present results were acceptable.

3.4.5 Calibration curves. The linearity of the response was determined by using a linear regression model. The matrix-matched calibration curves were analyzed with six levels of concentration, using the peak area of analyte versus the concentration of analyte with a weighting factor of 1/x. Each point of the curves had been injected at least in triplicate. Table 4 shows the results of the calibration curves. Each calibration curve was linear in a concentration ranging from the quantification limit to 100 ng mL^{-1} for each analyte, with satisfactory average correlation coefficients (0.993 ~ 0.998), which indicated good linearity between the peak area ratio (y) and investigated compound concentration (x, ng mL^{-1}).

3.4.6 Precision, accuracy and reproducibility. Precision of the method was evaluated as intra-day and inter-day precision by measuring corresponding relative standard deviations (RSDs). The intra-day precision was evaluated by repeated analyses of thirteen SAs at three fortified concentrations (0.5, 1.0 and 1.5 MRL) on three sequential runs in six replicates. The intra-day precision and reproducibility were obtained in the same laboratory, but conducted by different operators over six separate days. As shown in