Rapid Detection of the Antibiotic Sulfamethazine in Pig Body Fluids by Paper Spray Mass Spectrometry

Surased Suraritdechachai,[a,b,†] Chayan Charoenpakdee,[a,c,†] Iain Young,[d] Simon Maher,[e] Tirayut Vilaivan,[a,c] and Thanit Praneenararat[a,b,*]

[a]Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd., Pathumwan, Bangkok, 10330, Thailand.

[b]The Chemical Approaches for Food Applications Research Group, Faculty of Science, Chulalongkorn University, Phayathai Rd., Pathumwan, Bangkok, 10330, Thailand. Email: Thanit.P@chula.ac.th.

[c]Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd., Pathumwan, Bangkok, 10330, Thailand.

[d]Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, United Kingdom.

[e]Department of Electrical Engineering & Electronics, University of Liverpool, Brownlow Hill, Liverpool, L69 3GJ, United Kingdom.

[†]These authors contributed equally in this study.

***Corresponding Author**

Email: Thanit.P@chula.ac.th. Tel: +66-2-218-7638

Abstract

We report herein a practical method for non-lethal detection of the antibiotic sulfamethazine in pig body fluids via the combination of simple extraction and paper spray mass spectrometry (PS-MS). This method requires minimal sample preparation, while still providing high sensitivities and accuracies in complex matrices including pig whole blood (LOD = 7.9 μg/L; recovery = 95.4 – 103.7%), pig serum (LOD = 11.5 μg/L; recovery = 103.2 – 106.2%), and synthetic urine (LOD = 11.2 μg/L; recovery = 99.1 – 103.2%). Given a known correlation between the level of sulfamethazine in body fluids and edible tissues, this method shows great promise as a practical and non-lethal solution for rapid testing of the drug, which can substantially aid managerial decision in the livestock industry.

Keywords

Paper Spray; Mass Spectrometry; Sulfamethazine; Extraction; Ambient Ionization

Introduction

Veterinary drugs are an essential part of livestock industries in many regions across the world due to their wide range of benefits including disease treatment, disease prevention and growth acceleration.[1] However, the unwanted accumulation of several drug residues in various kinds of products from livestock can lead to serious public health and environmental effects.[1] Therefore, the detection and quantification of veterinary drug residues has been an important topic in food safety; one of which may critically affect the acceptance of foods by importing countries. In this regard, a variety of analytical techniques are currently available including gas chromatography – mass spectrometry (GC-MS),[2] high-performance liquid chromatography with photodiode array detector (HPLC-PDA),[3] HPLC with fluorescence detection (HPLC-FLD),[4-5] liquid chromatography – mass spectrometry (LC-MS),[6-7] and liquid chromatography – tandem mass spectrometry (LC-MS/MS).[8-10] Among these, LC-MS/MS has been widely accepted as a gold standard for sample inspections in most regions due to its superior sensitivity and selectivity.[11] Techniques that allow for simpler sample preparations, are more rapid and/or are amenable for centralized testing, offer significant advantages that are sought after by both industry and inspection agencies, particularly in resource-limited settings.

Ambient ionization techniques for MS have been developed to fulfill the aforementioned requirements.[12-15] In particular, paper spray (PS) ionization, a technique whereby a high electrical voltage is applied to a paper piece to create ion sprays for MS analysis,[16-19] has emerged as a promising technique as it offers several advantages. In particular, the use of paper as a medium offers a relatively economical solution, which also creates a logistical advantage due to the ease of transporting large numbers of samples in the form of dry sheets of paper. This approach can facilitate the analysis of samples from various rural locations to take place in a centralized lab

facility. As a consequence, PS-MS has been applied in the detection of many compound classes such as herbicides,[20] dyes,[21-22] therapeutic drugs,[16,23] and antibiotics[24] in various biological matrices such as blood.[16,23,25] Food testing applications can undoubtedly benefit from this technique, as has been reported for the determination of resveratrol in red wine,[26] the analysis of bisphenol analogues in food packaging materials,[27] and the analysis of the toxin microcystin in water as a result of cyanobacterial blooms.[28]

In this study, for the first time, we explore the possibility of detecting sulfamethazine (Figure 1) in whole blood and serum from pigs using PS-MS. Furthermore, a synthetic urine was also used to test the applicability for analyzing the drug in real pig urine samples. Sulfamethazine is a sulfonamide antibiotic that is widely used to prevent bacterial infection in agricultural and livestock industries.[29-30] Over- and prolonged usages of sulfamethazine can cause undesirable consequences such as bacterial resistance.[31] These potential threats to human health exist, although this particular compound is not used as human medicine. This is because sulfonamides released from animal sources like feces or urine are usually quite stable in the environment. Polluted in soil, natural water, or even drinking water, sulfamethazine can eventually accumulate in the human body and cause the aforementioned harmful effects.[32] As a consequence, a maximum limit of the amount of sulfamethazine in imported meats has been established by many regulatory organizations around the world, *e.g.,* EU regulation at 100 μg/kg in all kinds of tissues.[33] Thailand, which has a relatively mature swine industry and is a growing global exporter of pork particularly in the ASEAN market, also set a maximum residue limit of 100 μg/kg as imposed by the Thai Food and Drug Administration.[34]

Various detection methods for sulfonamides have been developed, yet there remains a desire from the swine industry for new analytical innovations that provide adequate performance metrics,

whilst reducing the cost and time per analysis further, particularly for complex biological matrices, as are encountered in practice. Many previous studies have relied on liquid chromatography coupled with MS,[10,35] UV-vis[36-39] or fluorescence detection.[5,40-41] Despite providing high sensitivity and selectivity, these chromatography-based methods are still considered to be too time-consuming, especially when a large number of samples are to be analyzed. In addition, extensive sample pretreatments such as solid phase microextraction,[39,41] the use of ionic liquids,[36,38] or chemical derivatization[40] are required in almost all cases for more complex matrices such as blood. Other methods based on enzyme-linked immunosorbent assays (ELISA)[42-45] have also been developed for the detection of sulfonamides. Whilst offering high sensitivity, these methods are not universal and suffer from issues of cross-reactivity.

In this study, we developed a method that offers a good balance between the aforementioned factors for the detection and quantification of sulfamethazine in pig body fluids by combining a simple extraction method with PS-MS detection. Our selection of sample types is critical as there is a clear correlation between sulfamethazine levels in body fluids and edible tissues.[46-49] This is of critical importance for the swine industry as it allows effective and efficient management decisions to be carried out in a timely fashion. This ultimately prevents the unnecessary loss of any yet-to-be-qualified pigs. Method development, optimizations, and key analytical figures of merit are reported and discussed herein.

Materials and Methods

General Information

Chemical reagents and solvents were purchased from Sigma-Aldrich, Carlo Erba, Merck, TCI Chemicals, Carbosynth, RCI Labscan and PanReac. Sulfamethazine-d_4 was purchased from

Toronto Research Chemicals. Papers used in this study were Whatman 1Chr chromatography papers. Pig blood and serum samples were generously provided by Charoen Pokphand Foods PCL (Thailand). Synthetic urine matrix was prepared based on an adaptation from a previous study.[50] This consisted of 0.33 M urea, 0.12 M NaCl, 0.016 M KH_2PO_4, 0.004 M $Na_2HPO_4 \cdot 2H_2O$, and 0.007 M creatinine in deionized water.

Calibration Plots and Spiking Experiments

Calibration plots for sulfamethazine were created by first preparing solutions of the compound at 1 g/L in MeOH. Thereafter, diluted solutions of sulfamethazine at 20 mg/L were prepared, whereby 5 μL was added into a 195-μL blank matrix solution (blank serum, blank whole blood, or blank synthetic urine). The resulting concentrations at this stage were 500 μg/L (500 ppb). Serial dilutions using blank matrix solution of choice were then followed, resulting in the concentrations of 1, 5, 10, 25, 50, 100, and 200 μg/L. Aliquots of 50 μL were then taken for sample preparation described below. Regression analysis was then performed to determine the limit of detection (LOD) and the limit of quantification (LOQ) using the formula $3.3S_b/m$ and $10S_b/m$, respectively (where S_b is the standard deviation of the blank, and m is the slope in the calibration plot).

Spiking experiments were performed by preparing sulfamethazine solutions at 4, 8, 16 mg/L. Each solution (5 μL) was then added to a blank solution of choice (195 μL) to create three sample sets (100 μg/L, 200 μg/L, and 400 μg/L of sulfamethazine). Aliquots of 50 μL were then taken from each solution for sample preparations described below.

Sample Preparations for Paper Spray Mass Spectrometry

Final solutions for PS-MS analysis were prepared by combining a 50-μL sample with 5 μL of 4-mg/L sulfamethazine-d_4 (the internal standard). After brief vortexing, 100 μL of ethyl acetate (or other solvents for the optimization experiment) was added and the resulting mixture was vortexed for 1 min, followed by 30-s centrifugation (5000 ×g) by a minicentrifuge. Three μLs of the organic layer was removed and dropped on a triangular paper (see details below). This paper was dried at ambient condition for at least 30 min before performing the PS-MS analysis.

Paper Spray Mass Spectrometry Experiments

Paper sheets were cut into isosceles triangles with dimensions of 0.6 (base) × 1.3 cm (height) by a commercial laser cutter. The resulting paper pieces were briefly cleansed by being rinsed and immersed in deionized water with shaking for 5 min (2×), followed by 5-min shaking in MeOH. After drying at ambient condition, the paper pieces were ready to be used.

Spraying condition was achieved using a DC power supply (3B scientific model U33010) that was set at 5 kV. The apex of the paper piece was positioned at a distance of about 5 mm from the inlet of the mass spectrometer. The mass spectrometer in this experiment was a Thermo Scientific TSQ Quantum EMR triple quadrupole mass spectrometer. Selected reaction monitoring (SRM) was used for all quantification experiments with the following parameters, capillary temperature: 300 °C; scan width: 1.000; scan time: 0.100 s; Q1 peak width: 0.70; Q3 peak width: 0.50; collision cell (Q2); Ar pressure: 1.5 mTorr. Each compound (direct infusion by a syringe) was optimized for collision energy and tube lens voltage using the TSQ tune software, which was also used for all instrument controls. The optimized collision energy and tube lens voltage, along with the parent and the product *m/z* values for each compound, are shown in Table 1 below. All data processing was performed with the Xcalibur software.

Results and Discussion

Our experiment commenced with a confirmation of mass fragmentation patterns from a sulfamethazine standard in PS-MS format. It was found that sulfamethazine in MeOH gave similar fragmentation patterns to typical electrospray ionization MS experiments (Figure S1). Nevertheless, our control experiment on blank paper confirmed a previous finding[16] that dibutyl phthalate, a common plasticizer in the paper industry, could also be found at the same molecular mass as that of sulfamethazine (Figure S2A), *i.e.*, *m/z* 279 for $[M+H]^+$. MS/MS experiments also confirmed this, whereby a daughter ion at *m/z* 149 was the most abundant peak, as suspected (Figure S2B). We found that more extensive washing sequences including other solvents did not substantially solve this problem. Nevertheless, this did not affect the quantification by SRM where the most abundant daughter ion at *m/z* 186 was eventually selected (after optimizing for collision energy and tube lens voltage, as shown in Table 1). This condition provided sufficient selectivity in the analysis of real samples, thus making the use of any further qualifier ions unnecessary in this study. Thereafter, the effects of different matrices were tested by dropping pig whole blood, pig serum, and synthetic urine solution spiked with sulfamethazine on paper and letting the liquid dry out. This was then followed by adding a fresh solvent for spraying from the paper substrate to enable mass spectrometric analysis. However, it was found that direct spraying was not efficient enough for detecting the compound below its required maximum limit (100 μg/kg)[33] likely due to ion suppression from other species in the matrix. In fact, this phenomenon was not surprising as it is generally accepted that compounds with less basic functional groups in the structure are not ionized well in positive mode, thus providing relatively high LOD values.[23] Thus, some research groups have developed pre-treatment methods to help boost the sensitivity, such as techniques based on solid-phase microextractions,[51] or those based on separated extraction stages. The latter

is of interest since it is still amenable to standard PS-MS without the need for special instrumental/materials setups. For instance, the analysis of resveratrol in red wine could be accomplished after a cleanup by solid phase extraction with hydrophobic C18 materials to eliminate interferences like salts and sugars.[26] Another work from Fang and coworkers reported the use of liquid phase microextraction in a syringe to analyze malachite green and crystal violet from real lake water samples.[52] Also, a study from Yang and coworkers utilized the slug-flow microextraction to extract amphetamine from blood and urine for further analysis by PS-MS.[25]

In our study, we simplified the extraction process by using a standard microcentrifuge tube as a reservoir, and micropipettor as a transferring device. Excluding chlorinated solvents, hydrocarbons, and polar solvents that are substantially miscible with water, three solvents including ethyl acetate (EtOAc), diethyl ether (Et_2O), and acetonitrile (ACN) were tested for their performances as an organic layer in the extraction process. It was found that EtOAc provided the highest extraction efficiency (Figure 2A). While Et_2O provided acceptable performance with about less than one half of that obtained from EtOAc, this solvent was not eventually considered due to its high volatility, which may lead to increased error and inconsistency. The performance of ACN was significantly inferior to EtOAc, likely due to its partial miscibility with water. As a result, EtOAc was selected as the extraction solvent. Also, we surmised that the pH of the aqueous solution to be extracted may have some influence on extraction efficiency due to the fact that sulfamethazine (Figure 1) has some ionizable functional groups, including an amino group of the aniline moiety, and the sulfonamido -NH- group. Therefore, an additional set of experiments was performed by adding 20 μL of 100-mM phosphate buffer solution at a fixed pH (ranging from pH 5 to 9) into a whole blood sample before extraction with EtOAc. The results (Figure S3) revealed that the pH seemed to exhibit no obvious effect to the extraction efficiency. Given that the pH

values of all matrices (7.28, 8.49, and 6.26 for pig whole blood, pig serum, and synthetic urine) used in this study were in this pH range, that bloods generally have some buffer capacity, and that adding a buffer before extraction means an extra sample preparation step, it was determined that no buffer was needed before the extraction process.

Encouraged by these results, further optimization of spraying solvents was conducted to increase the sensitivity even further (Figure 2B). It was found that 80:20 MeOH: H_2O provided the best result, surpassing other pure solvents including MeOH and ACN. Interestingly, the addition of formic acid, over the range of 0.01 – 1 % (v/v) did not increase the ion intensity (Figure S4). Therefore, formic acid was not added to the spray solvent. Combined together, these optimizations resulted in significant increases of the intensities of sulfamethazine fragment ions in all matrices tested, which can be clearly seen in the MS/MS, Figure 3.

After the optimal conditions that allow sufficiently sensitive analysis of sulfamethazine were established, calibration plots that accounted for each matrix (pig blood, pig serum, synthetic urine) were created (Figure 4). Using regression analysis, LOD and LOQ values for each matrix were calculated. Importantly, it was found that the developed extraction technique boosted the signal intensity in all matrices, resulting in LOD values of 7.9 μg/L (pig whole blood), 11.5 μg/L (pig serum), and 11.2 μg/L (synthetic urine), and LOQ values of 23.9 μg/L (pig whole blood), 34.9 μg/L (pig serum), and 34.0 μg/L (synthetic urine).

Notably, there have been previous studies showing definite correlation between the amount of the drug in body fluids and edible tissues.[46-49] For example, it was found that the sulfamethazine content in kidney, liver, and muscle tissue will exceed the maximum allowance limit of 100 μg/kg[33] when the compound can be detected in pig serum at the level above 190, 110, and 420

μg/L, respectively.[48] The corresponding numbers for urine were found to be even higher at the level of 630, 370, and 1,300 μg/L. Clearly, this means that the developed method as proposed herein (all LOQ values being below 50 μg/L) is capable of detecting sulfamethazine at the level significantly below the required limits using the aforementioned correlation data. Therefore, it can be adopted for practical uses where the developed method is used for non-lethal analyses of blood or urine sample to provide crucial data before slaughtering. Interestingly, the performance of this pre-extraction in combination with PS-MS, is comparable with other previous studies[36-38] that analyzed sulfamethazine in blood samples (LODs around 1.4-12.3 μg/L). As mentioned above, these related studies required more sophisticated sample preparation including the use of ionic liquids for extraction, multiple steps of pre-treatments and/or the need for HPLC separation – all of which significantly increases the complexity and operation time for sample preparation, thus limiting the turnaround time. Hence, our developed technique provides a promising alternative for the rapid analysis of sulfamethazine in complex biological samples with minimal sample preparations.

After calibration, we proceeded to evaluate the performance of this new approach to sulfamethazine detection by spiking experiments at three concentrations including 100, 200, and 400 μg/L (Table 2). The results showed that the obtained percentage recoveries were generally good, ranging from 95.4 to 106.2 %, with acceptable to good precision in all matrices (RSD < 20 %, except for one case). Importantly, while an internal standard is required for the proposed method (as generally known in PS-MS analysis),[14] the internal standard does not have to be the isotopically labeled version of the analyte of interest.[53] Any compound having similar chemical and physical properties may be used as a substitute – this thus creates more opportunity and flexibility of the method. Overall, the obtained performance underscores the viability of the

developed extraction/PS-MS method for rapid and efficient analysis of sulfamethazine in samples from pigs.

In conclusion, we have developed a simple extraction protocol that readily allows the detection and quantification of sulfamethazine, a class of sulfonamide drugs, in body fluids from pigs by PS-MS. The whole operation is very simple, fast, and practical, while still delivering suitable performance in terms of sensitivity, accuracy, and precision. This method should also be amenable to other compounds having similar chemical structures with great potential usage for analyses where the non-lethal inspection of drug levels in pigs is desired.

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Associated Content

The Supporting Information is available free of charge at DOI:.

Comparison of MS/MS spectra between direct infusion and PS-MS, MS spectra of MeOH solution sprayed from blank paper, the effect of pH to extraction efficiency, and the effect of formic acid in the spraying solvent.

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FIGURE CAPTIONS

Figure 1. The chemical structure of sulfamethazine (*m/z* 279 for $[M+H]^+$) and its prominent fragmentation patterns.

Figure 2. A) Extraction efficiencies (as represented by the ion intensity of the daughter ion at *m/z* 186) of various organic solvents, with MeOH as the spraying solvent; B) Evaluation of various spraying solvents with EtOAc being used as the extraction solvent. The errors were fairly high due to the lack of an internal standard, but the conclusion from these data sets was still considered to be valid. Each data point was from at least five replicates. FA = Formic acid.

Figure 3. Comparison of MS/MS spectra of 500-μg/L sulfamethazine from PS-MS in various matrices including A&B) pig whole blood, C&D) pig serum, and E&F) synthetic urine. Extractions with EtOAc were performed on experiments in B), D), and F). In all experiments, 80:20 MeOH:H_2O was used as the spraying solvent.

Figure 4. Calibration plots between the ratio of the intensity of *m/z* 186 daughter ion from sulfamethazine (*m/z* 279) over the intensity of the corresponding daughter ion from sulfamethazine-d_4 for concentrations 1-500 μg/L in: A) pig whole blood, B) pig serum, and C) synthetic urine.

TABLES

Table 1. Parent and product *m/z* values, collision energies, and tube lens voltages for all compounds in this study.

Compound	Parent *m/z*	Product *m/z*	Collision Energy (V)	Tube Lens Voltage (V)
sulfamethazine	279.0	186.0	17	91
sulfamethazine-d_4	283.0	186.0	17	92

Table 2. Recovery percentages of the detections of sulfamethazine in three matrices by PS-MS. Each analysis was repeated for at least 8 independent measurements.

Matrix	Spiked Concentration (μg/L)	Found (μg/L) ± SD	%RSD	Recovery (%)

Pig whole blood	100	103.7 ± 17.1	16.4 %	103.7
	200	194.7 ± 16.8	8.6 %	97.4
	400	381.6 ± 43.0	11.3 %	95.4
Pig serum	100	106.2 ± 13.3	12.6 %	106.2
	200	208.3 ± 17.3	8.3 %	104.1
	400	412.7 ± 34.3	8.3 %	103.2
Synthetic urine	100	102.5 ± 19.3	18.8 %	102.5
	200	206.4 ± 21.3	10.3 %	103.2
	400	396.2 ± 89.0	22.5 %	99.1

FIGURES

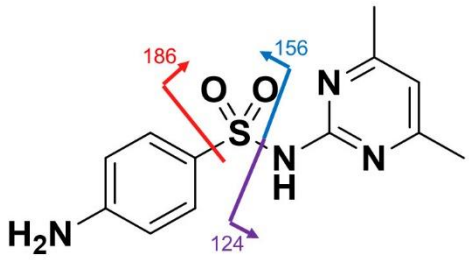

Figure 1

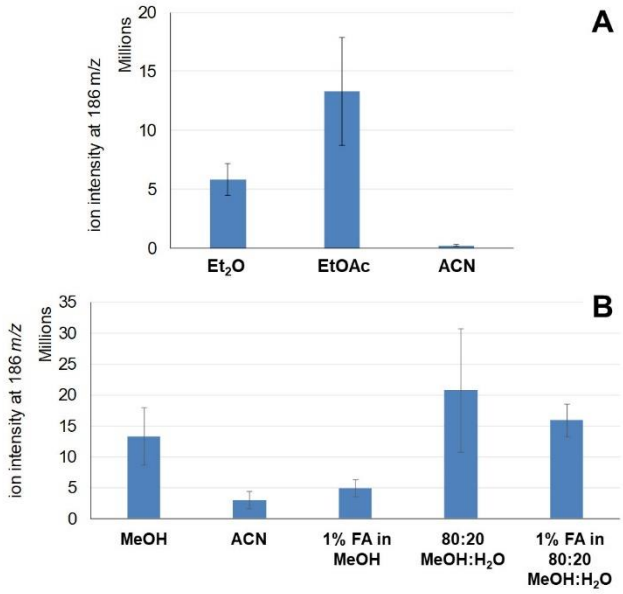
A
ion intensity at 186 m/z
Millions
20
15
10
5
0
Et2O
EtOAc
ACN
B
ion intensity at 186 m/z
Millions
35
30
25
20
15
10
5
0
MeOH
ACN
1% FA in MeOH
80:20 MeOH:H2O
1% FA in 80:20 MeOH:H2O

Figure 2

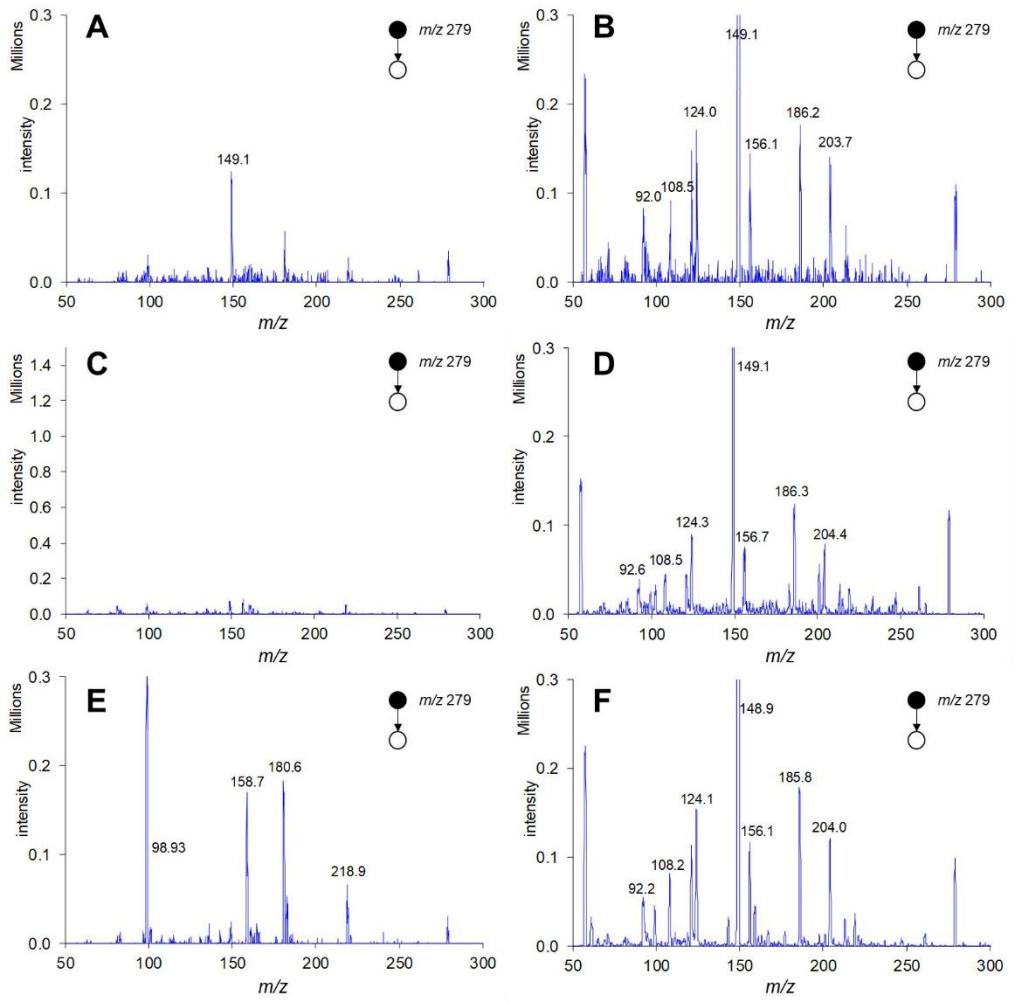
A
m/z 279
Millions
intensity
149.1
m/z
B
149.1
124.0
186.2
156.1
203.7
92.0
108.5
C
1.4
1.2
1.0
0.8
0.6
0.4
0.2
D
186.3
124.3
156.7
204.4
92.6
108.5
E
180.6
158.7
98.93
218.9
F
148.9
185.8
124.1
156.1
204.0
108.2
92.2

Figure 3

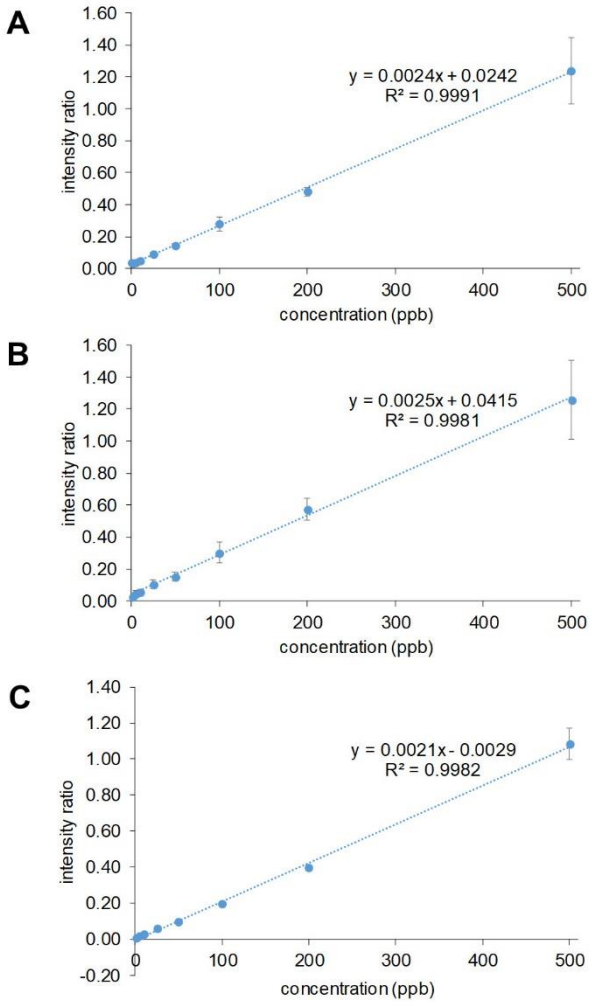
A
y = 0.0024x + 0.0242
R² = 0.9991
intensity ratio
concentration (ppb)
B
y = 0.0025x + 0.0415
R² = 0.9981
intensity ratio
concentration (ppb)
C
y = 0.0021x - 0.0029
R² = 0.9982
intensity ratio
concentration (ppb)

Figure 4

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