

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

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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 $\mu\text{g/L}$; recovery = 95.4 – 103.7%), pig serum (LOD = 11.5 $\mu\text{g/L}$; recovery = 103.2 – 106.2%), and synthetic urine (LOD = 11.2 $\mu\text{g/L}$; 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

1 **Introduction**

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

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

24 facility. As a consequence, PS-MS has been applied in the detection of many compound classes
25 such as herbicides,²⁰ dyes,²¹⁻²² therapeutic drugs,^{16,23} and antibiotics²⁴ in various biological
26 matrices such as blood.^{16,23,25} Food testing applications can undoubtedly benefit from this
27 technique, as has been reported for the determination of resveratrol in red wine,²⁶ the analysis of
28 bisphenol analogues in food packaging materials,²⁷ and the analysis of the toxin microcystin in
29 water as a result of cyanobacterial blooms.²⁸

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

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

47 whilst reducing the cost and time per analysis further, particularly for complex biological matrices,
48 as are encountered in practice. Many previous studies have relied on liquid chromatography
49 coupled with MS,^{10,35} UV-vis³⁶⁻³⁹ or fluorescence detection.^{5,40-41} Despite providing high
50 sensitivity and selectivity, these chromatography-based methods are still considered to be too time-
51 consuming, especially when a large number of samples are to be analyzed. In addition, extensive
52 sample pretreatments such as solid phase microextraction,^{39,41} the use of ionic liquids,^{36,38} or
53 chemical derivatization⁴⁰ are required in almost all cases for more complex matrices such as blood.
54 Other methods based on enzyme-linked immunosorbent assays (ELISA)⁴²⁻⁴⁵ have also been
55 developed for the detection of sulfonamides. Whilst offering high sensitivity, these methods are
56 not universal and suffer from issues of cross-reactivity.

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

65 **Materials and Methods**

66 **General Information**

67 Chemical reagents and solvents were purchased from Sigma-Aldrich, Carlo Erba, Merck, TCI
68 Chemicals, Carbosynth, RCI Labscan and PanReac. Sulfamethazine-*d*₄ was purchased from

69 Toronto Research Chemicals. Papers used in this study were Whatman 1Chr chromatography
70 papers. Pig blood and serum samples were generously provided by Charoen Pokphand Foods PCL
71 (Thailand). Synthetic urine matrix was prepared based on an adaptation from a previous study.⁵⁰
72 This consisted of 0.33 M urea, 0.12 M NaCl, 0.016 M KH₂PO₄, 0.004 M Na₂HPO₄·2H₂O, and
73 0.007 M creatinine in deionized water.

74 **Calibration Plots and Spiking Experiments**

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

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

88 **Sample Preparations for Paper Spray Mass Spectrometry**

89 Final solutions for PS-MS analysis were prepared by combining a 50- μ L sample with 5 μ L of
90 4-mg/L sulfamethazine- d_4 (the internal standard). After brief vortexing, 100 μ L of ethyl acetate
91 (or other solvents for the optimization experiment) was added and the resulting mixture was
92 vortexed for 1 min, followed by 30-s centrifugation (5000 \times g) by a minicentrifuge. Three μ Ls of
93 the organic layer was removed and dropped on a triangular paper (see details below). This paper
94 was dried at ambient condition for at least 30 min before performing the PS-MS analysis.

95 **Paper Spray Mass Spectrometry Experiments**

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

100 Spraying condition was achieved using a DC power supply (3B scientific model U33010) that
101 was set at 5 kV. The apex of the paper piece was positioned at a distance of about 5 mm from the
102 inlet of the mass spectrometer. The mass spectrometer in this experiment was a Thermo Scientific
103 TSQ Quantum EMR triple quadrupole mass spectrometer. Selected reaction monitoring (SRM)
104 was used for all quantification experiments with the following parameters, capillary temperature:
105 300 $^{\circ}$ C; scan width: 1.000; scan time: 0.100 s; Q1 peak width: 0.70; Q3 peak width: 0.50; collision
106 cell (Q2); Ar pressure: 1.5 mTorr. Each compound (direct infusion by a syringe) was optimized
107 for collision energy and tube lens voltage using the TSQ tune software, which was also used for
108 all instrument controls. The optimized collision energy and tube lens voltage, along with the parent
109 and the product m/z values for each compound, are shown in Table 1 below. All data processing
110 was performed with the Xcalibur software.

111 **Results and Discussion**

112 Our experiment commenced with a confirmation of mass fragmentation patterns from a
113 sulfamethazine standard in PS-MS format. It was found that sulfamethazine in MeOH gave similar
114 fragmentation patterns to typical electrospray ionization MS experiments (Figure S1).
115 Nevertheless, our control experiment on blank paper confirmed a previous finding¹⁶ that dibutyl
116 phthalate, a common plasticizer in the paper industry, could also be found at the same molecular
117 mass as that of sulfamethazine (Figure S2A), *i.e.*, m/z 279 for $[M+H]^+$. MS/MS experiments also
118 confirmed this, whereby a daughter ion at m/z 149 was the most abundant peak, as suspected
119 (Figure S2B). We found that more extensive washing sequences including other solvents did not
120 substantially solve this problem. Nevertheless, this did not affect the quantification by SRM where
121 the most abundant daughter ion at m/z 186 was eventually selected (after optimizing for collision
122 energy and tube lens voltage, as shown in Table 1). This condition provided sufficient selectivity
123 in the analysis of real samples, thus making the use of any further qualifier ions unnecessary in
124 this study. Thereafter, the effects of different matrices were tested by dropping pig whole blood,
125 pig serum, and synthetic urine solution spiked with sulfamethazine on paper and letting the liquid
126 dry out. This was then followed by adding a fresh solvent for spraying from the paper substrate to
127 enable mass spectrometric analysis. However, it was found that direct spraying was not efficient
128 enough for detecting the compound below its required maximum limit ($100 \mu\text{g}/\text{kg}$)³³ likely due to
129 ion suppression from other species in the matrix. In fact, this phenomenon was not surprising as it
130 is generally accepted that compounds with less basic functional groups in the structure are not
131 ionized well in positive mode, thus providing relatively high LOD values.²³ Thus, some research
132 groups have developed pre-treatment methods to help boost the sensitivity, such as techniques
133 based on solid-phase microextractions,⁵¹ or those based on separated extraction stages. The latter

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

141 In our study, we simplified the extraction process by using a standard microcentrifuge tube as
142 a reservoir, and micropipettor as a transferring device. Excluding chlorinated solvents,
143 hydrocarbons, and polar solvents that are substantially miscible with water, three solvents
144 including ethyl acetate (EtOAc), diethyl ether (Et₂O), and acetonitrile (ACN) were tested for their
145 performances as an organic layer in the extraction process. It was found that EtOAc provided the
146 highest extraction efficiency (Figure 2A). While Et₂O provided acceptable performance with about
147 less than one half of that obtained from EtOAc, this solvent was not eventually considered due to
148 its high volatility, which may lead to increased error and inconsistency. The performance of ACN
149 was significantly inferior to EtOAc, likely due to its partial miscibility with water. As a result,
150 EtOAc was selected as the extraction solvent. Also, we surmised that the pH of the aqueous
151 solution to be extracted may have some influence on extraction efficiency due to the fact that
152 sulfamethazine (Figure 1) has some ionizable functional groups, including an amino group of the
153 aniline moiety, and the sulfonamido -NH- group. Therefore, an additional set of experiments was
154 performed by adding 20 μ L of 100-mM phosphate buffer solution at a fixed pH (ranging from pH
155 5 to 9) into a whole blood sample before extraction with EtOAc. The results (Figure S3) revealed
156 that the pH seemed to exhibit no obvious effect to the extraction efficiency. Given that the pH

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

161 Encouraged by these results, further optimization of spraying solvents was conducted to
162 increase the sensitivity even further (Figure 2B). It was found that 80:20 MeOH: H₂O provided
163 the best result, surpassing other pure solvents including MeOH and ACN. Interestingly, the
164 addition of formic acid, over the range of 0.01 – 1 % (v/v) did not increase the ion intensity (Figure
165 S4). Therefore, formic acid was not added to the spray solvent. Combined together, these
166 optimizations resulted in significant increases of the intensities of sulfamethazine fragment ions in
167 all matrices tested, which can be clearly seen in the MS/MS, Figure 3.

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

175 Notably, there have been previous studies showing definite correlation between the amount of
176 the drug in body fluids and edible tissues.⁴⁶⁻⁴⁹ For example, it was found that the sulfamethazine
177 content in kidney, liver, and muscle tissue will exceed the maximum allowance limit of 100
178 µg/kg³³ when the compound can be detected in pig serum at the level above 190, 110, and 420

179 $\mu\text{g/L}$, respectively.⁴⁸ The corresponding numbers for urine were found to be even higher at the
180 level of 630, 370, and 1,300 $\mu\text{g/L}$. Clearly, this means that the developed method as proposed
181 herein (all LOQ values being below 50 $\mu\text{g/L}$) is capable of detecting sulfamethazine at the level
182 significantly below the required limits using the aforementioned correlation data. Therefore, it can
183 be adopted for practical uses where the developed method is used for non-lethal analyses of blood
184 or urine sample to provide crucial data before slaughtering. Interestingly, the performance of this
185 pre-extraction in combination with PS-MS, is comparable with other previous studies³⁶⁻³⁸ that
186 analyzed sulfamethazine in blood samples (LODs around 1.4-12.3 $\mu\text{g/L}$). As mentioned above,
187 these related studies required more sophisticated sample preparation including the use of ionic
188 liquids for extraction, multiple steps of pre-treatments and/or the need for HPLC separation – all
189 of which significantly increases the complexity and operation time for sample preparation, thus
190 limiting the turnaround time. Hence, our developed technique provides a promising alternative for
191 the rapid analysis of sulfamethazine in complex biological samples with minimal sample
192 preparations.

193 After calibration, we proceeded to evaluate the performance of this new approach to
194 sulfamethazine detection by spiking experiments at three concentrations including 100, 200, and
195 400 $\mu\text{g/L}$ (Table 2). The results showed that the obtained percentage recoveries were generally
196 good, ranging from 95.4 to 106.2 %, with acceptable to good precision in all matrices (RSD < 20
197 %, except for one case). Importantly, while an internal standard is required for the proposed
198 method (as generally known in PS-MS analysis),¹⁴ the internal standard does not have to be the
199 isotopically labeled version of the analyte of interest.⁵³ Any compound having similar chemical
200 and physical properties may be used as a substitute – this thus creates more opportunity and
201 flexibility of the method. Overall, the obtained performance underscores the viability of the

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

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

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212 whole blood samples. Scispec Co., Ltd. is acknowledged for its support on the MS instrument used
213 in this study.

214 **Associated Content**

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

216 Comparison of MS/MS spectra between direct infusion and PS-MS, MS spectra of MeOH solution
217 sprayed from blank paper, the effect of pH to extraction efficiency, and the effect of formic acid
218 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- $\mu\text{g/L}$ 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:H₂O 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 $\mu\text{g/L}$ 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 <i>m/z</i>	Product <i>m/z</i>	Collision Energy (V)	Tube Lens Voltage (V)
sulfamethazine	279.0	186.0	17	91
sulfamethazine- <i>d</i> 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 (%)
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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

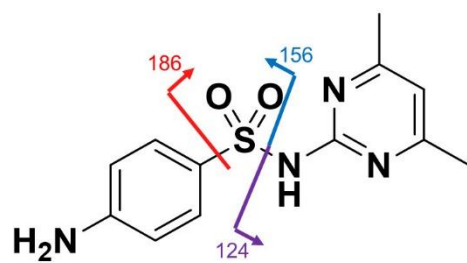


Figure 1

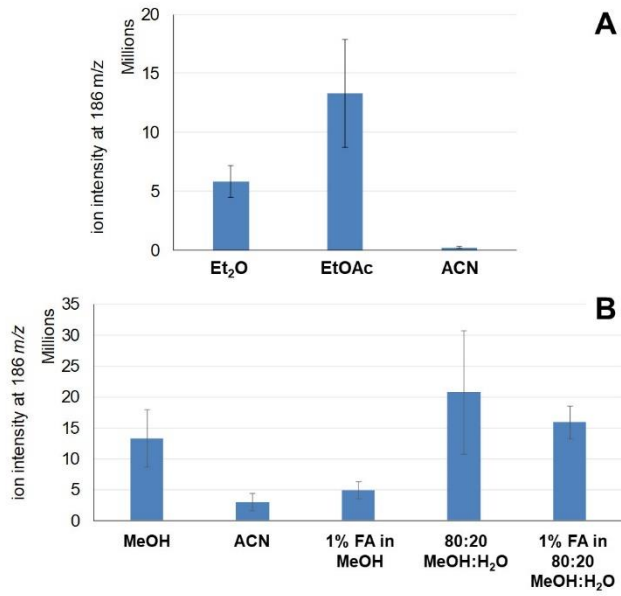


Figure 2

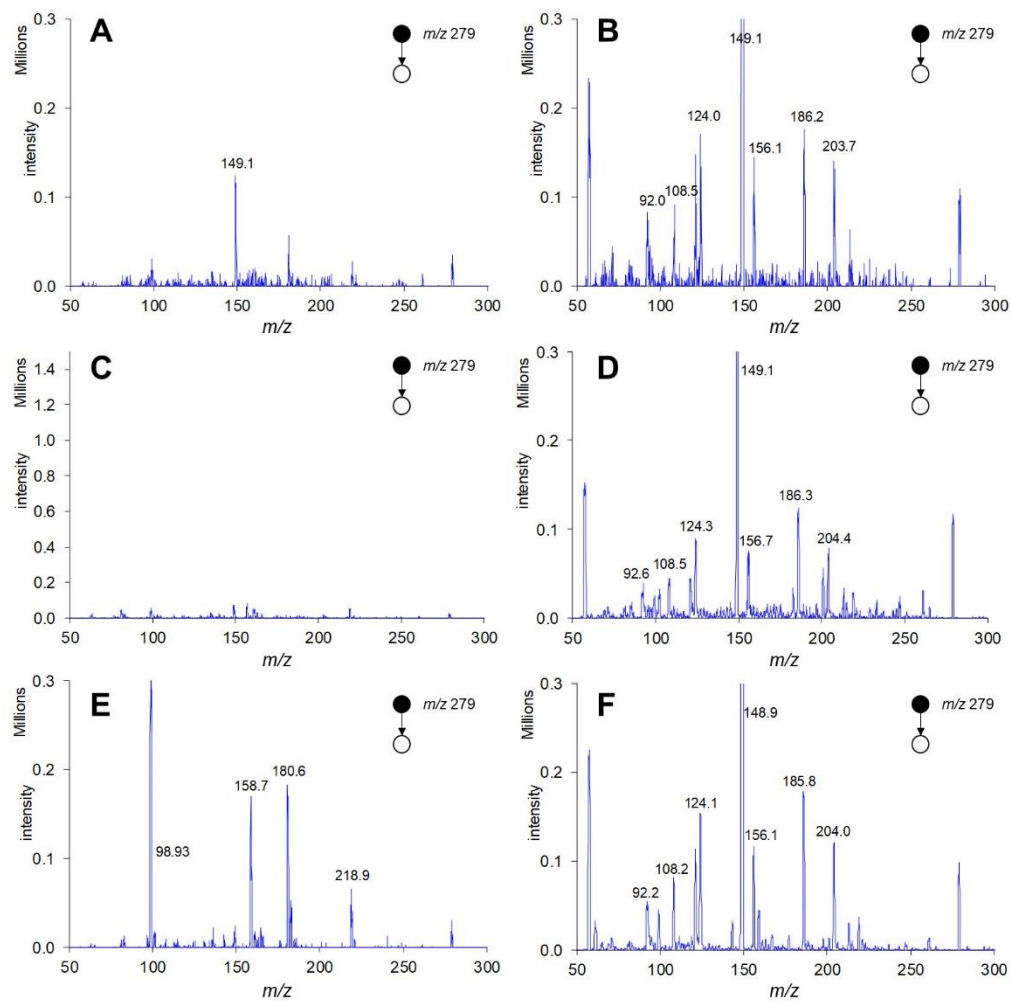


Figure 3

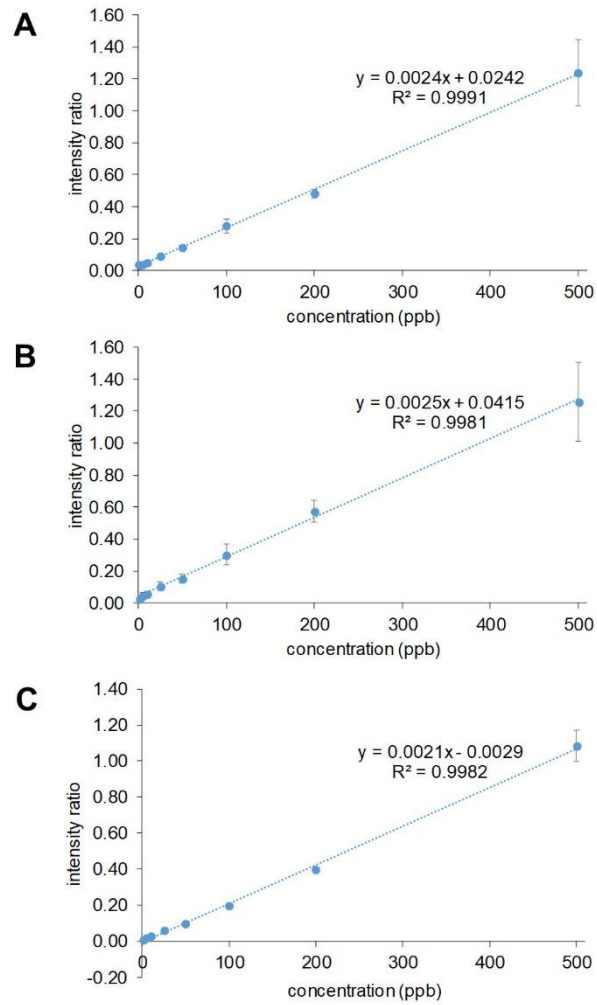


Figure 4

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