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All-in-One Approach Enabling Simultaneous Derivatization and Preconcentration of Favipiravir for Sensitive Determination by GC-MS

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Abstract

In this research, a simultaneous derivatization and preconcentration method followed by gas chromatography-mass spectrometry (GC-MS) was established for the determination of favipiravir in synthetic serum, synthetic urine and domestic/synthetic wastewater samples. Acetic anhydride derivatization reaction was performed to obtain volatile and less polar derivatives of favipiravir. The derivatization reaction was simultaneously carried out with spraying based-liquid phase microextraction (SB-LPME) method. Eight different parameters such as acetic anhydride concentration, spraying cycle, pH/volume of buffer solution, extraction solvent type, initial sample volume, mixing type and mixing period were meticulously optimized by univariate optimization approach. Under the optimum SB-LPME-GC-MS method, limit of detection (LOD), limit of quantification (LOQ), dynamic range and coefficient of determination (R^2) were determined to be 16.8 µg/kg, 56.0 µg/kg, 0.063-5.32 mg/kg and 0.9998, respectively. Recovery experiments were carried out using spiked synthetic serum, synthetic urine and domestic/synthetic wastewater samples. The SB-LPME-GC-MS method exhibited satisfactory recovery results ranged from 75.6% to 134.9% for the samples. Based on these results, the developed analytical strategy has great potential to be used for the qualitative and quantitative determination of favipiravir in human serum, urine and wastewater samples.

Keywords: Favipiravir, spraying-based liquid-phase microextraction, GC-MS, serum, urine, wastewater

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1. Introduction

Favipiravir is a chemical compound with IUPAC name of 5-fluoro-2-oxo-1H-pyrazine-3-carboxamide. It shows broad spectrum antiviral effect, especially against RNA viruses like influenza and COVID-19 [1]. Not only coronaviruses but also H5N1 influenza, Ebola and seasonal respiratory diseases can be treated due to its efficacy against highly virulent virus strains [2]. Favipiravir depresses the action of RNA polymerase enzyme; thus, the replication of RNA virus is blocked [3]. Its bioavailability has been reported as 97.6% and C_{max} for the recommended dosage has been determined as 51.5 μg/mL. The recommended dosage of this drug is 3200 mg/day orally for 1 day and 1200 mg/day orally for 10 days [4]. In addition, the unmetabolized part of the antiviral drugs can generally enter into sewage system via the urine and feces of human bodies. According to scientific studies, more than 60% of antiviral drug dosages are excreted by patients [5]. Hence, an accurate and reliable analytical method for the determination of favipiravir is an important task in order to support the investigations of this drug in viral treatment [6].

In the literature, favipiravir has been quantified by several analytical techniques such as high performance liquid chromatography-diode array detector (HPLC-DAD) [7], liquid chromatography-mass spectrometry (LC-MS) [8], molecular fluorescence spectrometry [9] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [10]. Gas chromatography-mass spectrometry (GC-MS) is a powerful and popular analytical system due to efficient chromatographic separation by the GC system and molecular identification and confirmation by the MS system. It has excellent mass spectra libraries used to identify unknown structures, as well as good selectivity and sensitivity [11]. In addition to these, GC-MS is generally more accessible and lower-cost compared to LC-MS/MS, owing to its mature technology, simpler instrumentation and lower operational expenses [12]. Most of the drugs are frequently derivatized before their GC-MS determination due to several reasons such as transformation of analyte's chemical form to be compatible with the instrument, and improvement of resolution, detection and structural elucidation efficiency [13]. In one study, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [11] was used to derivatize favipiravir. However, this derivatization agent needs aprotic solvent medium to achieve high silylation efficiency [14]. In this study, acetic anhydride was employed as a derivatization agent for the determination of favipiravir by the GC-MS system. This agent can be used in both aqueous and nonaqueous media [15]. The other advantages of acetic anhydride are

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low molecular weight and low boiling point, and the fact that it can be easily separated from the analyte containing solution [16].

Although chromatographic methods are one of the most powerful separation techniques, they cannot overcome the matrix effects arising from the biological and environmental samples [17,18]. Sample preparation methods can isolate and enrich the target analyte(s) from the complex matrices [19]. Sample preparation methods are based on conventional techniques such as liquid-liquid extraction (LLE) and solid phase extraction (SPE). However, these methods requires huge amounts of organic solvents and labor intensive steps [20]. Microextraction means sample preparation methods which employ low volume of extraction solvent for effective sampling process [21]. Microextraction methods used for the sample preparation started with solid phase microextraction in 1987-1990 and continued with liquid phase microextraction (LPME) methods such as single drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME), hollow-fiber microextraction (HF-LPME) and electro membrane extraction (EME) [22]. Among them, DLLME has become an indispensable preconcentration method due to its high enrichment factors, good recoveries, and easy, low-cost procedure [23]. It depends on the formation of microemulsion via the injection of extraction and dispersive solvents into aqueous sample solution [24]. However, this method needs high volume of halogenated organic solvents in routine analyses of hundreds of samples [25]. In addition, the selection of proper dispersive solvent [26], the increment of analyte solubility in aqueous sample solution [27] and the endangerment of environmental health [28] are other negative aspects of this method. Spraying-based liquid-phase microextraction (SB-LPME) has been introduced to the literature in order to eliminate the usage of dispersive solvent from DLLME method [29]. In the SB-LPME method, the selected extraction solvent is transferred into the tank of a spraying system and then sprayed into the aqueous sample solution after the connection of the centrifuge tube to the cap located at the top of the spray system [30]. Hence, the extraction solvent is finely and homogenously dispersed throughout the sample solution by means of the spraying system [29].

In this work, an analytical method for the qualification/quantification of favipiravir in synthetic human, synthetic urine and domestic/synthetic wastewater samples by the GC-MS system was developed. Acetic anhydride was utilized as a derivatization agent to convert the analyte into its volatile and less polar derivatives. The SB-LPME method was also performed to preconcentrate

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the analyte derivatives from aqueous sample matrices. The derivatization agent was added to the sample solution with the help of spraying system consisting of both extraction solvent and acetic anhydride solution. Under the optimum derivatization and microextraction conditions, the parameters of system analytical performance were determined. The accuracy and applicability of the developed SB-LPME method were evaluated by recovery experiments for synthetic serum, synthetic urine, and domestic/synthetic wastewater samples.

2. Materials and methods

2.1. Chemicals and reagents

Solid standard of favipiravir (purity ≥ 99.65%), which was dissolved in acetonitrile (ACN), was supplied from a pharmaceutical company in Türkiye, İstanbul. Acetic anhydride (purity ≥ 99%) was purchased from Sigma Aldrich (Darmstadt, Germany). Dichloromethane (≥ 99.5%) and 1,2-dichloroethane (≥ 99.5%) were supplied from Riedel-de-Haën (Seelze, Germany). Chloroform (≥ 99.0%), NH_4OH (25%), NaOH (≥ 99%), HCl (35%) and acetic acid (≥ 99%) were bought from Merck (Darmstadt, Germany).

2.2. Instrumentation

Chromatographic separation and mass spectrometric detection were achieved using a coupled 6890N gas chromatograph (GC) and 5973N mass spectrometer (MS, Agilent Technologies, Santa Clara, United States). The GC system was equipped with an Agilent HP-5MS capillary column (30 m length x 0.25 mm i.d., 0.25 µm film thickness) for the optimization experiments while a Trajan ACR5MS capillary column with identical dimensions was utilized for the evaluation of analytical performance and recovery experiments. A single ramp oven temperature program was applied, in which the temperature was raised from an initial 100 ˚C to 300 ˚C at a rate of 35 ˚C/min and then maintained at 300 ˚C for 0.36 min. Inlet temperature, interface heater temperature, ionization source temperature/energy, quadrupole temperature, and injection volume/mode were set at 280 ˚C, 280 ˚C, 230 ˚C/70 eV, 150 ˚C, and 1.0 µL/splitless, respectively.

2.3 SB-LPME-GC-MS procedure

The spray system used for efficient dispersion of extraction solvent into the standard/sample solutions was detailed in our previous studies [29,31]. In this study, the solvent container of spray

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system was filled with acetic anhydride (79.2% (*g/g*)) dissolved in chloroform to perform the derivatization and preconcentration steps simultaneously. In the first of the SB-LPME procedure, a centrifuge tube containing 6.0 mL of the standard or sample solution and 1.0 mL of pH 6 buffer solution was tightened to the placed cap on the spray system. After, the spray system was overturned, and five spray cycles were implemented to disperse the extraction solvent. To promote the derivatization and extraction yield, 60 s of vortex mixing was applied to the resulting solution and then the application of centrifugation was carried out for 2.0 min at 1800 *g* to accelerate the separation of organic and aqueous phase separation. In the final step of this procedure, the lower phase (approximately 150 µL) was pipetted into an insert vial to be introduced to the GC-MS system.

2.4 Samples

Two different SeraFlx synthetic serum samples (possessing two different product dates) were obtained from Mensis Pharma (Lenexa, USA). Synthetic urine samples I and II were purchased from Mensis Pharma (Lenexa, USA) and Biochemazone (Alberta, Canada), respectively. A domestic wastewater sample was obtained from the Department of Environmental Engineering at Yıldız Technical University and a synthetic wastewater sample was prepared according to our previous study [32]. During the preparation of synthetic wastewater sample, 0.440 g of glucose, 0.015 of yeast extract, 0.116 g of bactopeptone, 0.106 g of $(NH_4)_2SO_4$, 0.025 g of KH_2PO_4, 0.042 g of $MgSO_4$, 0.011 g of $FeCl_3$, 0.063 g of $CaCl_2$, 0.002 g of $MnSO_4$ and 0.257 g of $NaHCO_3$ were dissolved in ultrapure water and then filled up to 1000 mL with ultrapure water.

2.4.1 Preparation of wastewater samples

Each wastewater sample (1.40 g) was weighed into separate centrifuge tubes and spiked with a proper amount of favipiravir standard solution. Next, an appropriate amount of acetonitrile was added into the solution to provide equal amounts of acetonitrile for all sample solutions. In the final step, total amount of the solutions was filled up to 14.0 g with ultrapure water. The acetonitrile content and matrix dilution were fixed to 2.50% (*g/g*) and 10-fold, respectively.

2.4.2 Preparation of synthetic urine samples

During the preparation of synthetic urine samples, the sample preparation procedure described in our previous study [33] was followed in order to reflect the real sample preparation steps. Hereby,

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0.50 g of a urine sample was added to the centrifuge tube and then spiked at different concentrations. After the addition of 0.70 g of acetonitrile and 0.20 g of ammonia solution (25%), the solutions were made up to 1.50 g with ultrapure water. Afterwards, the solutions were exposed to the centrifugation for 10 min at 1800 *g*. After the centrifugation, 0.583 g of the supernatant were transferred to a clean centrifuge tube and then completed to 14.0 g with ultrapure water. The contents of ammonia solution and acetonitrile were fixed to 0.56% (*g/g*) and 1.97% (*g/g*) in the final stage.

2.4.3 Preparation of synthetic serum samples

In the preparation of synthetic serum samples, the protocol outlined in our earlier study [33] was applied to closely simulate the procedures used for handling real serum samples. Hence, 0.50 g of the synthetic serum samples were weighed into separate centrifuge tubes. After, the spiking procedures were performed at different concentrations and then 0.90 g of acetonitrile was pipetted into the solution before filling up to 1.50 g with ultrapure water. Then, the centrifugation for 10 min at 1800 *g* was implemented to remove the suspended solid particles. Next, 0.583 g of the supernatant was weighed to a clean centrifuge tube and then made up to 14.0 g with ultrapure water. The content of acetonitrile was set to 2.50% (*g/g*) in the final sample solution.

2.5 Synthesis of favipiravir acetate derivative and its characterization

To synthesize favipiravir acetate derivative, 8.0 mL of favipiravir standard solution (126.4 mg/kg) was pipetted into a clean centrifuge tube. Then, the centrifuge tube was connected to the spray system and 1,2-dichloroethane containing 49.2% (*g/g*) acetic anhydride was sprayed into the standard solution three times. After the spraying process, 45 s vortex and 2.0 min centrifugation (at 1800 *g*) were sequentially applied and then the lower analyte derivative-rich phase was analyzed by the GC-MS system. The derivatization process with acetic anhydride proceeds via nucleophilic acyl substitution at the enolic oxygen of favipiravir, yielding an O-acetylated derivative. This derivatization increases the volatility and decreases the water solubility of the analyte, facilitating its extraction into the organic phase and subsequent GC-MS analysis. After the analysis, it was observed that the acetate derivative of favipiravir was successfully synthesized and extracted from the aqueous phase. Based on the obtained selected ion monitoring (SIM) chromatograms and mass spectrum (Fig. 1), the most intense molecular and fragment ions were observed at mass to charge (*m/z*) ratios of 199 and 157, respectively. The ion at *m/z* 199 is

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consistent with the molecular ion of the O-acetylated favipiravir derivative, corresponding to the addition of an acetyl group to the parent favipiravir molecule. While the molecular ion (*m/z*=199) of favipiravir acetate derivative was employed during the quantitative determination, other fragment ions (*m/z*=114, 142, 157, 158 and 201) were utilized for the qualitative determination. Under electron ionization, the elimination of ketene (CH_2=C=O, Δ*m/z*=42) cause the formation of *m/z* 157, a well-established fragmentation pathway for aromatic/enolic acetate esters [34,35] while the fragment ion at *m/z* 142 is generated via cleavage on the ester side of the molecule [36]. Additionally, the low-intensity ions at m/z 158 and 201 are attributed to isotope ions of m/z 157 and 199, respectively [37].

3. Results and discussion

3.1 Optimization of chromatographic conditions

The preconcentrated standard solution of favipiravir detailed in *Section 2.5* was employed to find an efficient temperature program in terms of signal intensity. For this purpose, the preconcentrated standard solution was introduced to the GC-MS system using three different programs (Table S1). As can be seen in Fig. S1, the highest signal to noise ratio for the analyte was recorded from program 3 which was determined as the optimum GC program. Temperature ramps above 35 ˚C were not evaluated to prevent potential damage to temperature-related components of the GC system. In addition, the solvent peak was observed between 0.70 and 2.75 min. To hinder possible interference arising from the solvent peak region, higher temperature ramps and helium flow rates exceeding 35 ˚C and 2.0 mL/min, respectively, were not employed.

3.2 Optimization of derivatization and microextraction parameters

A one-variable-at-a-time approach, in which all other variables were kept constant, was used throughout the optimization experiments to determine the optimum conditions for each parameter. Furthermore, Tukey's post hoc test was used to assess the statistical differences among the tested variables. The mean value and error bars of each variable found in the optimization figures were produced via triplicate measurements.

Acetic anhydride was used as a derivatization reagent to form favipiravir acetate derivative; therefore, the concentration of acetic anhydride is a substantial parameter to increase the signal to noise ratio of the analyte. In the first step of this optimization, five different concentrations of

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acetic anhydride (39.4%, 49.2%, 59.5%, 69.3% and 79.1% (*g/g*)) were prepared in 1,2-dichloroethane were investigated to find more efficient one. As can be seen in Fig. S2 (A), the analytical signals enhanced while increasing the concentration of acetic anhydride and the analyte-rich phase obtained from 79.1% of acetic anhydride solution was not introduced to the GC-MS system due to obtaining low phase separation. Following the increase in spraying cycles from 3 to 5, the acetic anhydride concentration was re-optimized since higher concentration levels of the derivatization agent led to improved analytical responses. The signal intensities increased from 68.4% (*g/g*) to 88.7% (*g/g*) of acetic anhydride (Fig. S2 (B)). Although the response at 88.7% (*g/g*) was 5.6% higher than that at 79.2% (*g/g*), the difference between these two concentrations was not statistically significant. Therefore, 79.2% of acetic anhydride was selected as the optimum concentration to reduce the reagent consumption.

In the SB-LPME method, the extraction solvent volume is directly proportional to the number of spraying cycles. Therefore, the spraying cycle is a critical parameter affecting the preconcentration factor, as an inverse relationship exists between the extraction solvent volume and preconcentration factor [33,38]. Furthermore, the spraying cycle influences derivatization efficiency because the derivatization reagent is present in the extraction solvent. For this purpose, the effect of spraying cycle on both the preconcentration factor and derivatization efficiency was examined by evaluating two, three, four, five and six spraying cycles. Contrary to expectations, the analytical signals obtained with three spraying cycles were lower than those recorded for four, five and six spraying cycles while no significant difference in peak areas was observed among the latter three conditions. This behavior might be attributed to the preparation of acetic anhydride in the extraction solvent since the volume derivatization reagent sprayed rises as the number of spraying cycles increases. As shown in Fig. S3, the increment in the reagent volume with increasing spraying cycle could improve the derivatization yield and could help prevent a decrease in the preconcentration factor. Considering that collection of the analyte-rich phase was more straightforward at five spraying cycles which were selected for subsequent experiments.

During the preliminary experiments, the standard solutions with and without the addition of NaOH (2.0 mol/L) were compared to evaluate the effect of pH on the derivatization yield. The addition of NaOH resulted in lower analytical signals compared to the solutions without NaOH. Therefore, buffer solutions at pH 7 and above were excluded from a further experiment, and pH 3, 4, 5 and 6

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were investigated to improve the signal intensity. The most stable analytical responses were obtained at pH 6 (Fig. S4 (A)), which was thus selected as the optimum condition. Additionally, the buffer solution volumes (0.50, 1.0 and 1.5 mL) were evaluated to find one of the most effective. The highest analytical signals were attained from 1.0 mL of pH 6 buffer solution (Fig. S4 (B)) which was chosen as the optimum volume. The decrease in the analytical signals at 1.5 mL might be attributed to excessive dilution of the solution, and the increased ionic strength might have negatively affected the derivatization reaction.

In the LPME methods, the extraction solvent should exhibit low solubility in the aqueous phase, higher density than water and strong affinity toward the target analyte(s) [39,40]. Accordingly, solutions containing 80% (*g/g*) of acetic anhydride were individually prepared in three different chlorinated organic solvents (1,2-dichloroethane, dichloromethane and chloroform) and both the preconcentration and derivatization steps were carried out using these mixtures. No phase separation was observed for the solution prepared with dichloromethane, likely due to its relatively high solubility in water [41,42]. As shown in Fig. S5, the highest analytical responses were recorded from chloroform containing 80% (*g/g*) of acetic anhydride, which was therefore determined to be the optimum extraction solvent.

In a microextraction method, sample volume optimization plays a critical role in obtaining low limits of detection and enhanced preconcentration factors [42]. For this reason, initial sample volumes of 4.0, 6.0, 8.0 and 10 mL were tested to enhance the signal intensity. The lowest analytical signals were recorded at an initial sample volume of 4.0 mL while 6.0, 8.0 and 10 mL produced nearly identical responses (Fig. S6). Since 6.0 mL provided a sufficiently high signal to noise ratio, it was selected as the optimum initial sample volume.

A mixing process can improve the contact between the analyte and the extraction solvent [43]. Therefore, the solutions subjected to vortex mixing, ultrasonic treatment and no mixing were compared to evaluate the effect of mixing process on the preconcentration and derivation steps. As illustrated in Fig. S7 (A), vortex mixing produced the highest signal to noise ratio; thus, it was selected for subsequent experiments to achieve lower detection and quantitation limits. In addition, five different vortex mixing periods ranging from 15 s to 75 s were investigated to further improve the signal to noise ratio. The analytical signals increased gradually from 15 s to 60 s, while 60 s

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and 75 s yielded nearly identical responses based on mean values and statistical analysis (Fig. S7 (B)). In conclusion, vortex mixing of 60 s was chosen as the optimum period.

Normally, the increments in spraying cycles and sample volumes should lead to the decrement and enhancement in analytical signals, respectively [33]. However, the optimization of both spraying cycle and initial sample volume produced unexpected outcomes. Therefore, a series of experiments was conducted as described below:

- 1st condition: An 8.0 mL of favipiravir standard solution (10.74 mg/kg) was pipetted into a centrifuge tube and 1.0 mL of pH 6 buffer solution was added onto the solution. After, chloroform containing 80% (*g*/*g*) acetic anhydride was sprayed five times. In the final step, the solution was centrifuged for 2.0 min at 1800 *g* following 60 s of vortex mixing.
- 2nd condition: A volume of 8.0 mL of favipiravir standard solution (10.74 mg/kg) was transferred into a centrifuge tube, followed by the addition of 1.0 mL of pH 6 buffer solution. Next, 100 µL of acetic anhydride (99%) was directly added into the mixture and vortexed for 30 s. Subsequently, chloroform was sprayed one time, and the mixture was vortexed again for 60 s. Finally, the solution was centrifuged for 2.0 min to facilitate phase separation.
- 3rd condition: An 8.0 mL aliquot of favipiravir standard solution (10.74 mg/kg) was added into a centrifuge tube, and 1.0 mL of pH 6 buffer solution was pipetted. Following this step, 800 µL of acetic anhydride (99%) was added directly, and the mixture was vortexed for 30 s. Afterward, chloroform was sprayed once, followed by an additional 60 s of vortex mixing. The mixture was then centrifuged for 2.0 min.
- 4th condition: A centrifuge tube was loaded with 8.0 mL of favipiravir standard solution (10.74 mg/kg), and 1.0 mL of pH 6 buffer solution was subsequently added. Then, 800 µL of acetic anhydride (99%) was introduced directly into the solution and vortexed for 30 s. After this, chloroform was sprayed twice, and the mixture was vortexed again for 60 s. In the final step, the centrifugation process was applied to the solution for 2.0 min.

It should be noted that the selected optimum condition of five spraying cycles was equivalent to delivering approximately 1.0 mL of chloroform-based solution composed of 80% (*g*/*g*) acetic anhydride. Within this volume, approximately 800 µL corresponded to acetic anhydride

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and about 200 µL to chloroform. Moreover, each individual spraying cycle of chloroform represented an approximate volume of 200 µL.

As shown in Fig. S8, the lowest analytical responses were obtained at the smallest acetic anhydride volume (2nd condition), as expected. When 2nd and 3rd conditions were compared, 3rd condition produced approximately 94% higher analytical signals based on peak areas. In contrast, no statistically significant difference in analytical responses was observed between the 1st and 3rd conditions. Additionally, a comparison between the 3rd and 4th conditions revealed a pronounced decrease in analytical signals in the 4th condition. This outcome is consistent with expectations, as increasing the volume of extraction solvent leads to a reduction in the preconcentration factor. Based on these findings, the absence of a decrease in analytical signals with increasing extraction solvent volume (for spraying cycles of four, five and six times) during spraying cycle optimization can be attributed to the simultaneous increase in derivatization efficiency with higher spraying cycles, which compensates for the decline in the preconcentration factor. Furthermore, the observation of similar analytical signals for initial sample volumes of 6, 8 and 10 mL in the optimization step indicates that the amount of acetic anhydride present in the sprayed extraction solvent becomes a limiting factor in terms of derivatization yield for the analyte. Additionally, the observed results might be explained by the tautomeric nature of favipiravir [44–46] which might adversely affect the derivatization reaction.

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3.3 Evaluation of LOD, LOQ and dynamic range

To assess limit of detection (LOD), limit of quantitation (LOQ) and dynamic range of the developed SB-LPME-GC-MS method for the analyte, the standard solutions ranging from 0.063 mg/kg to 101.98 mg/kg were subjected to the developed SB-LPME method. The presented SB-LPME-GC-MS method demonstrated the dynamic range between 63.04 µg/kg and 5.32 mg/kg, with the LOD of 16.80 µg/kg and the LOQ of 56.02 µg/kg. After the concentration of 5.32 mg/kg, the GC-MS system gave non-linear responses; therefore, it was determined as limit of linearity (LOL). During the estimation of LOD and LOQ, the relationship between standard deviation of the analytical signals at the lowest concentration ($n= 6$) and the slope of external standard calibration plot (Fig. 2 (A)) [33,47] was used. The experimental LOQ signal, calibration plot and calibration curve are illustrated in Fig. 2.

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3.4 Recovery experiments

In the first step of recovery experiments, non-spiked sample solutions were subjected to the developed simultaneous derivatization and microextraction method and then the analyte-rich phases were injected to the GC-MS system. The non-spiked sample solutions did not generate analytical signals belonging to the analyte. Therefore, the recovery experiments were carried out using the spiked sample solutions to assess the accuracy and applicability of the developed method. For this purpose, domestic and synthetic wastewater, synthetic urine, and synthetic serum samples were spiked with suitable amounts of a favipiravir standard solution (sample preparation procedures are described in *Section 2.4*). For the calculation of percent recoveries, both external standard and matrix-matching calibration approaches were implemented to identify the most appropriate calibration method in order to obtain more reliable analytical results. The external standard calibration plot shown in Fig. 2 (A) was used to calculate the recovery values. The matrix-matching calibration plots were also employed to figure out the recovery results across different but comparable sample types. For instance, the calibration plot generated from the spiked domestic wastewater samples (Fig. 3 (A)) was used to determine the recoveries of the spiked synthetic wastewater samples, and vice versa. The same procedure was applied to all spiked samples groups to simulate similar matrix conditions in order to improve the developed method's accuracy. Based on the results presented in Table 1, positive matrix effects were observed for all sample matrices when the recovery results were calculated using the external standard calibration method. In contrast, the matrix-matching calibration approach yielded acceptable recovery values overall; however, comparatively higher recoveries were noted at the lowest spiked concentration levels in synthetic urine I and II, and synthetic serum II samples. For the middle and highest spiked concentration levels, satisfactory recovery results were obtained across all samples. These findings indicate that the matrix-matching calibration method provides more reliable and accurate analytical results than the external standard calibration approach. Therefore, the use of matrix-matching calibration or standard addition calibration methods (particularly for samples containing favipiravir) is recommended to compensate the matrix-induced deviations and improve the accuracy. Additionally, matrix effects were quantified for each sample matrix using the expression "$\%Matrix\ effect = \frac{Slope\ of\ matrix-matching\ calibration\ plot}{Slope\ of\ external\ standard\ calibration\ plot} - 1$". The calculated values were 166% for domestic wastewater, 192% for synthetic wastewater, 99% for synthetic urine sample I,

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112% for synthetic urine sample II, 172% for synthetic serum sample I and 188% for synthetic serum sample II. Representative SIM chromatograms obtained from the standard and spiked sample solutions can be seen in Fig. 4.

3.5 Literature comparison

The developed SB-LPME-GC-MS method was compared to some literature findings in terms of dynamic range, LOD, LOQ and recovery. As can be seen in Table 2, the developed SB-LPME-GC-MS method provided a relatively wide dynamic range (0.063–5.32 mg/kg) and achieved LOD and LOQ values of 16.80 µg/kg and 56.02 µg/kg, respectively. Although these detection and quantification limits are higher than those reported for some methods in the literature, this should be considered together with the objectives of the proposed analytical approach. The method was developed to provide a single extraction procedure applicable to chemically different matrices without matrix-specific optimization. In addition, the sensitivity of the developed method might be influenced by several factors, including the efficiency of the derivatization step and the extraction capability of the SB-LPME method. Furthermore, the proposed SB-LPME method could potentially be coupled with GC-MS/MS instrumentation to achieve improved detection/quantification capability. Nevertheless, the recovery values ranged from 75.6% to 134.9%, which are generally consistent with previously reported studies. Overall, the results indicate that the developed method is reliable and versatile, particularly for analyses of complex samples.

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3.6 Greenness and practicability evaluation of the developed method

The developed SB-LPME-GC-MS method was evaluated in terms of greenness and practicability using the AGREEprep [48,49] and BAGI [50] tools. The proposed method obtained scores of 0.29 and 60 according to the AGREEprep and BAGI evaluations, respectively. Although chloroform, which was selected as the extraction solvent, is recognized as a hazardous halogenated solvent, its consumption in the proposed SB-LPME procedure was limited to a very small volume (approximately 200 µL). Therefore, the consumption of this hazardous solvent was minimized compared with traditional liquid-liquid extraction methods. In light of these results, the proposed analytical method can be considered reasonably green and practical. Additionally, the replacement of chloroform with less hazardous extraction solvents such as switchable solvents [51],

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supramolecular solvents [52], ionic liquids [53] and deep eutectic solvents [54] can be considered in future studies to further improve the environmental sustainability of the method.

4. Conclusion

This study presents a method for the simultaneous derivatization and preconcentration of favipiravir from domestic/synthetic wastewater, synthetic urine and synthetic serum samples prior to the GC-MS quantification. To enable both steps in a single procedure, the derivatization reagent (acetic anhydride) was prepared in chloroform (the extraction solvent) and then introduced into the standard/sample solutions using a spray system. Key experimental parameters affecting derivatization and preconcentration efficiency were univariately optimized to improve the detection/quantification performance of the GC-MS system. Under the optimum conditions, a series of favipiravir standard solutions preconcentrated using the developed SB-LPME method was analyzed by the GC-MS system to establish the method's analytical figures of merit. The proposed SB-LPME-GC-MS method exhibited a dynamic range of 0.063–5.32 mg/kg, with the LOD of 16.80 µg/kg and the LOQ of 56.02 µg/kg. The accuracy and applicability of the SB-LPME-GC-MS method was checked using the spiked domestic/synthetic wastewater, synthetic urine and synthetic serum samples. All tested matrices produced signal enhancement effects, indicating the presence of matrix-induced variations. To address this, the matrix-matching calibration strategy was employed, resulting in improved analytical accuracy. Based on the recoveries between 75.6% and 134.9% calculated via the matrix-matching calibration approach, the proposed method can be reliably employed for the determination of favipiravir in diverse environmental and biological samples. However, it should be noted that the observed matrix effects varied considerably among the different matrices tested (ranging from 99% to 192% signal enhancement), reflecting the inherently variable composition of real environmental and biological samples. Consequently, the matrix-matching calibration strategy prepared with one batch or source of sample may not fully represent the matrix compositions of another unknown real samples, which can introduce quantification bias in routine analysis. Therefore, the use of standard addition method or the preparation of strictly matrix-matching calibration plots using the same batch/source as the sample under analysis, is recommended to provide accurate and reliable quantification of favipiravir.

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Declarations

Conflict of interest: The authors declare that they have no competing interests.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material: Data will be available upon reasonable request from corresponding author.

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CRediT authorship contribution statement

Sezin Erarpat Bodur: Conceptualization, Investigation, Methodology, Validation, Writing–original draft.

Süleyman Bodur: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing–original draft.

Bedrihan Kartoğlu: Investigation, Validation.

Ömer Tahir Günkara: Conceptualization, investigation, validation.

Sezgin Bakırdere: Conceptualization, Investigation, Methodology, Validation, Writing–review & editing.

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Tables

Table 1. Recoveries obtained from external standard and matrix-matching calibration methods.

	External standard calibration method	**Matrix-matching calibration method**

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Sample	Spiked concentration, mg/kg	%Recovery	±SD*	%RSD**	%Recovery	±SD*	%RSD**
Domestic Wastewater	0.25	142.1	8.7	6.2	75.6	3.0	4.0
	0.55	238.0	15.0	6.3	93.9	5.1	5.5
	1.01	272.3	11.6	4.3	100.0	4.0	4.0
	2.56	256.9	5.7	2.2	90.6	2.0	2.2
Synthetic Wastewater	0.27	153.8	5.6	3.6	79.8	2.1	2.6
	0.52	287.8	2.4	0.8	119.7	0.9	0.7
	1.01	275.6	9.3	3.4	109.6	3.5	3.2
	2.33	282.2	21.3	7.5	108.7	8.0	7.4
Synthetic Urine I	0.27	194.5	30.2	15.5	134.9	14.3	10.6
	0.50	158.1	9.1	5.7	97.4	4.3	4.4
	1.00	149.0	6.3	4.2	81.8	3.0	3.6
	2.84	185.4	10.1	5.5	91.6	4.8	5.2
	5.38	196.5	19.7	10.0	95.0	9.3	9.8
Synthetic Urine II	0.29	172.0	8.2	4.8	129.6	4.1	3.2
	0.52	168.4	5.4	3.2	108.8	2.7	2.5
	1.04	176.9	12.3	7.0	101.1	6.2	6.1
	2.87	200.2	25.9	13.0	104.9	13.0	12.4
	5.24	208.1	10.4	5.0	106.9	5.2	4.9
Synthetic Serum I	0.28	173.4	21.8	12.6	107.7	7.6	7.0
	0.39	207.5	19.8	9.5	106.3	6.9	6.5
	1.00	191.8	2.9	1.5	80.2	1.0	1.2
	2.72	270.7	15.6	5.7	99.1	5.4	5.5
	4.98	262.8	8.2	3.1	94.1	2.9	3.0
Synthetic Serum II	0.26	202.5	17.3	8.5	127.4	6.3	5.0
	0.50	197.0	19.4	9.8	100.0	7.1	7.1
	0.99	227.1	15.1	6.7	97.3	5.6	5.7
	2.62	277.9	37.4	13.4	107.2	13.7	12.8
	5.19	279.6	4.7	1.7	105.3	1.7	1.7

±SD: Standard deviation for n=4.

***%RSD: Percent relative standard deviation for n=4.*

Table 2. Comparison of the developed method with existing studies.

Method	Sample	Dynamic range	LOD	LOQ	%Recovery	Reference

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Molecular fluorescence spectrometry	Human plasma	40–280 µg/L	9.44 µg/L	28.60 µg/L	97.88%–102.02%	[9]
High performance liquid chromatography–diode array detector	Pharmaceutical formulation	6.25–250.00 mg/L	1.02 mg/L	3.10 mg/L	101.06%–101.55%	[55]
Molecular fluorescence spectrometry	Human plasma	0.02–0.35 mg/L	0.004 mg/L	0.011 mg/L	94.2%–101.9%	[56]
High performance liquid chromatography–UV detection		10–100 mg/L	0.985 mg/L	2.986 mg/L	–	
Menthol-assisted homogeneous liquid-liquid microextraction–high performance liquid chromatography–UV detection	Human plasma	100-100000 µg/L	–	100 µg/L	96.20%–102.66%	[57]
Fabric phase sorptive extraction-gas chromatography-mass spectrometry	Pharmaceutical formulation, urine, plasma and whole blood	0.01–10 mg/L	0.001–0.0026 mg/L	0.003–0.0086 mg/L	86.3%–100.2%	[11]
Fabric phase sorptive extraction–high performance liquid chromatography–UV detection	Human plasma and breast milk	0.2–50 mg/L (for plasma) 0.5–25 mg/L (for breast milk)	0.06 mg/L (for plasma) 0.15 mg/L (for breast milk)	0.2 mg/L (for plasma) 0.5 mg/L (for breast milk)	86.0%–94.7%	[58]
Spraying based-liquid phase	Domestic/synthetic wastewater,	0.063 – 5.32 mg/kg	16.80 µg/kg	56.02 µg/kg	75.6%–134.9%	This study

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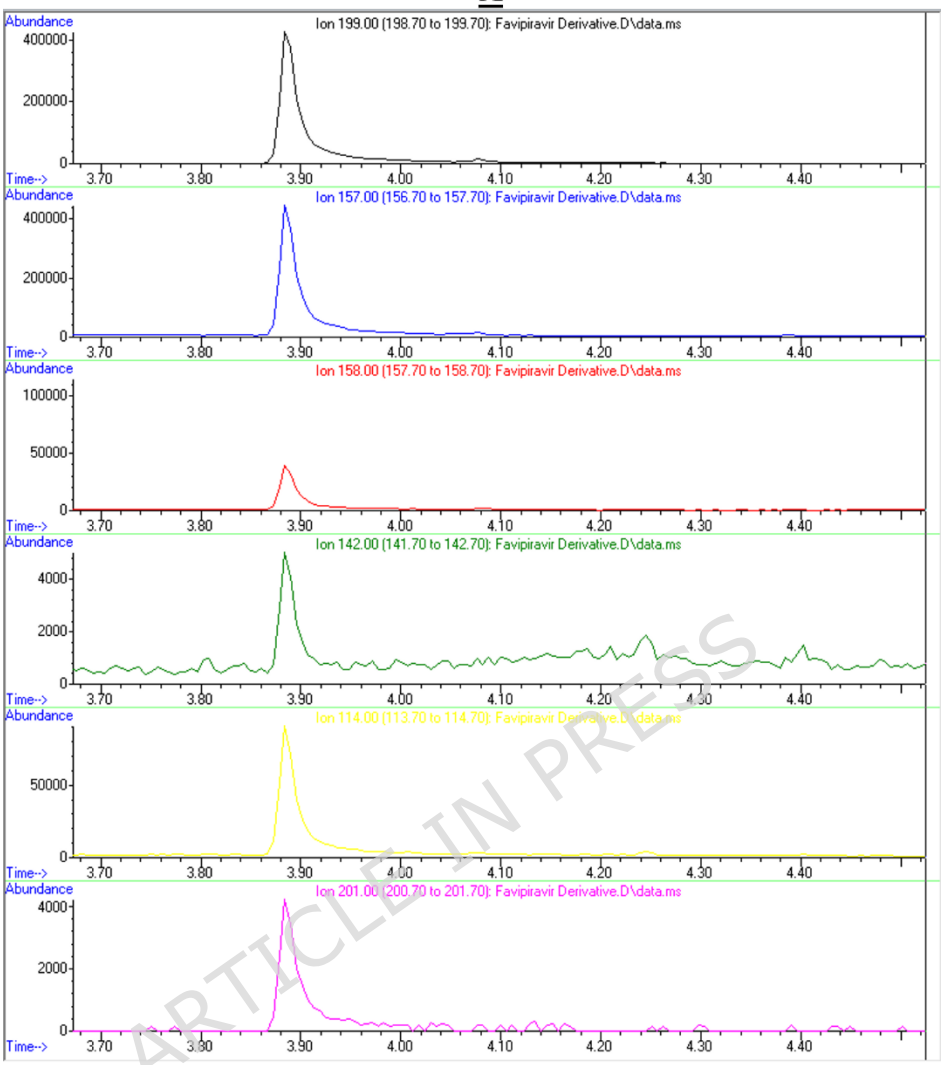
microextraction–gas chromatography–mass spectrometry	synthetic urine and synthetic serum

Figures

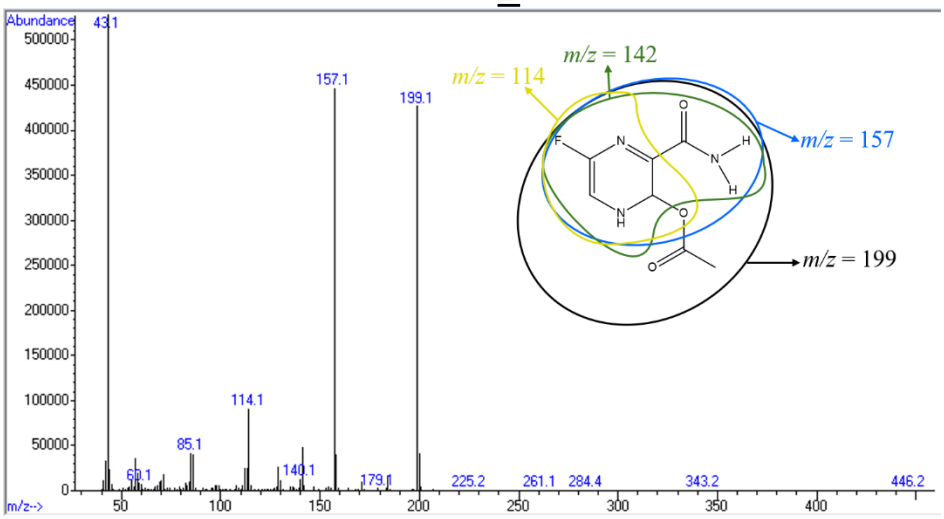
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A



B



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Fig. 1. SIM chromatograms (A) and mass spectrum (B) belonging to the fragments of favipiravir acetate derivative.

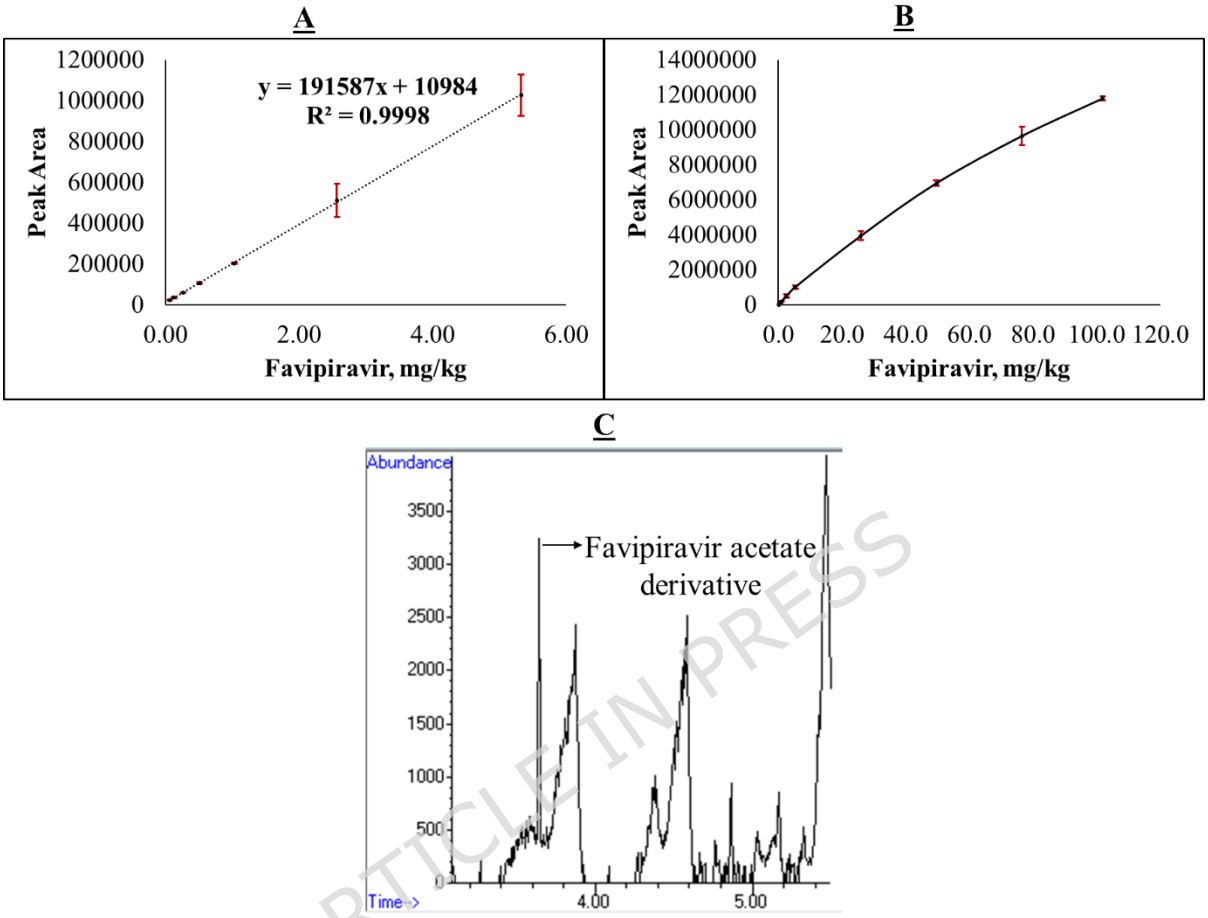


Fig. 2. The constructed calibration plot (A), calibration curve (B) and experimental LOQ signal (63.04 µg/kg).

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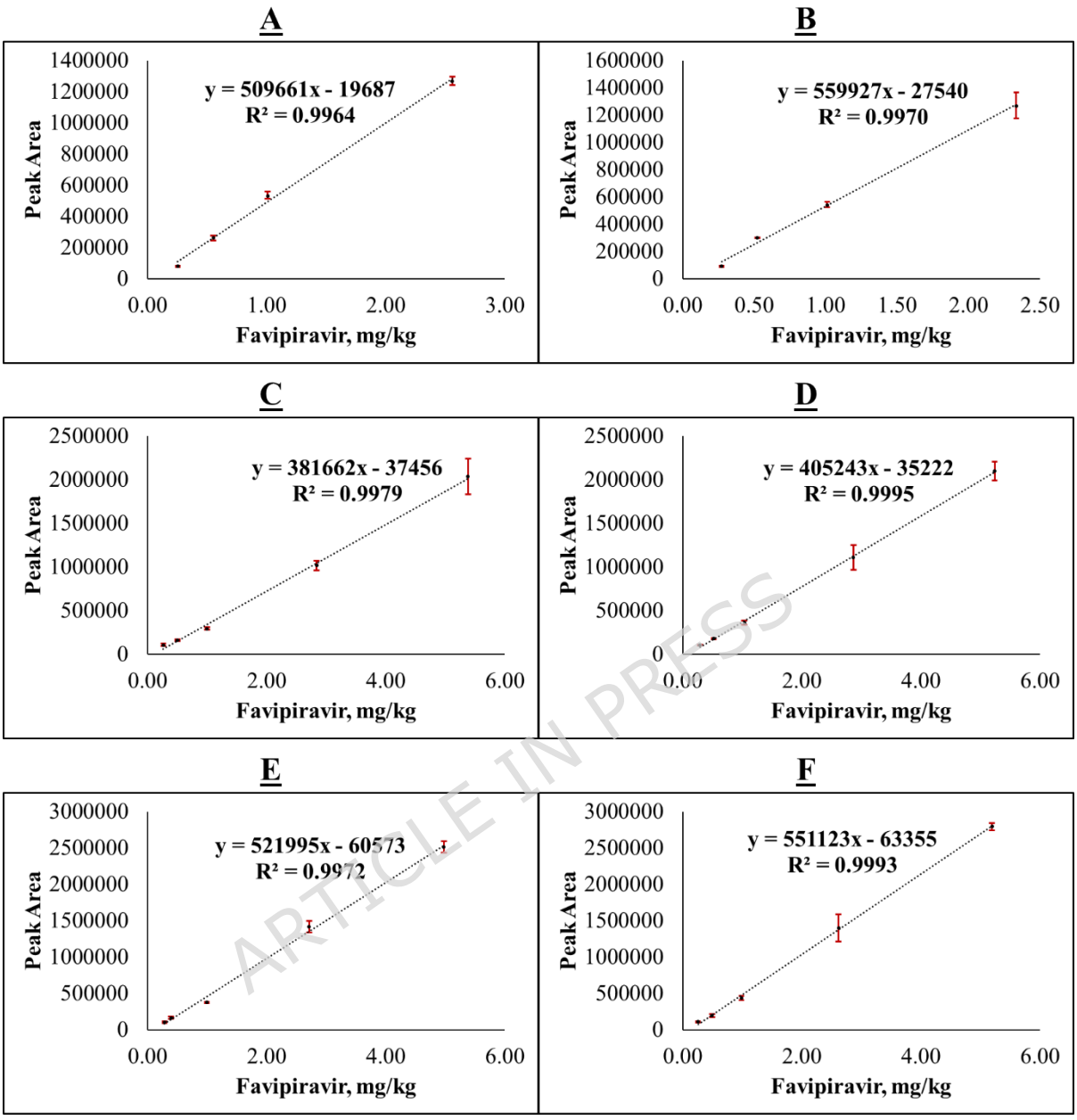
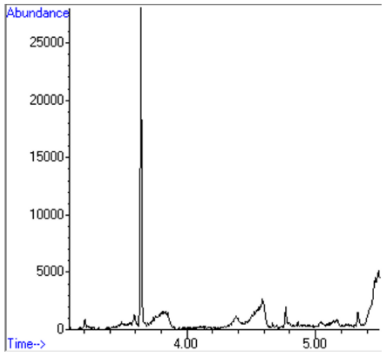


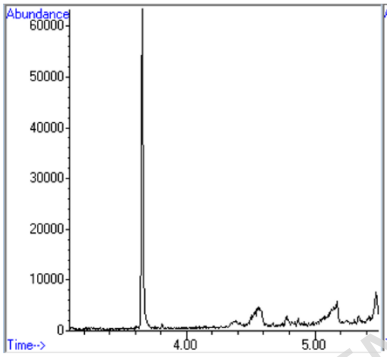
Fig. 3. Matrix-matching calibration plots constructed from the spiked domestic wastewater (A), synthetic wastewater (B), synthetic urine I (C), synthetic urine II (D), synthetic serum I (E) and synthetic serum II (F).

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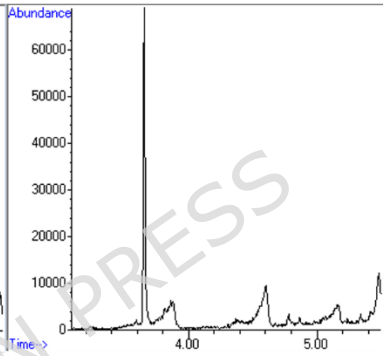
Favipiravir Standard Solution (1.03 mg/kg)



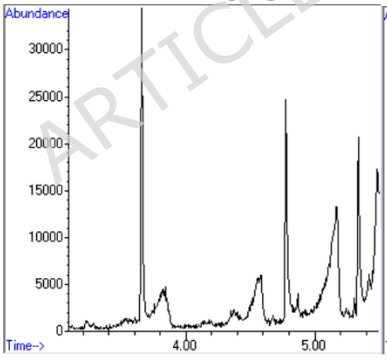
Spiked Domestic Wastewater (1.01 mg/kg)



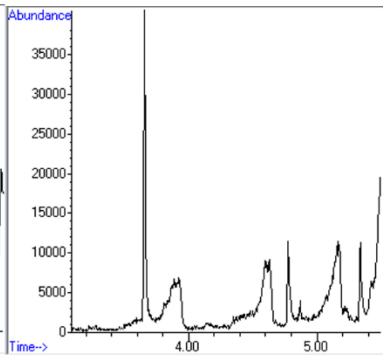
Spiked Synthetic Wastewater (1.01 mg/kg)



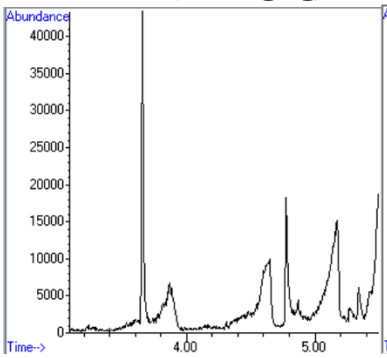
Spiked Synthetic Urine I (1.00 mg/kg)



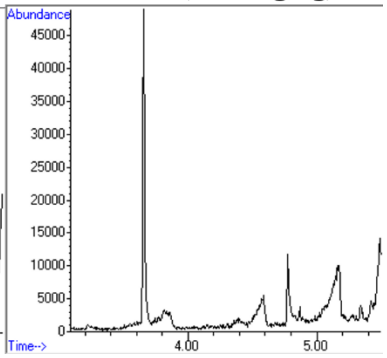
Spiked Synthetic Urine II (1.04 mg/kg)



Spiked Synthetic Serum I (1.00 mg/kg)



Spiked Synthetic Serum II (0.99 mg/kg)



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Fig. 4. Representative SIM chromatograms obtained from the standard and spiked sample solutions.

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