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A solvent-free HPLC method for the simultaneous determination of Favipiravir and its hydrolytic degradation product

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During COVID-19 pandemic, Favipiravir (FPV) showed a great efficacy against COVID-19 virus, it produced noticeable improvements in recovery of the patients. The aim of this study was to develop a new, green and simple method for the simultaneous determination of FPV and its acid-induced degradation product (ADP) in its pure and pharmaceutical dosage forms. This method will be key for the inevitable development of FPV solution and inhaler formulations. A green micellar RP-HPLC method was developed using an RP-VDSPPHRE PUR 100 column (5 μm , 250 \times 4.6 mm) and an isocratic mixed micellar mobile phase composed of 0.02 M Brij-35, 0.1 M SDS and 0.01 M potassium dihydrogen orthophosphate anhydrous and adjusted to pH 3.0 with 1.0 mL min⁻¹ flow rate. The detection was performed at 280 nm with a run time of less than six min. Under the optimized chromatographic conditions, linear relationship has been established between peak area and concentration of FPV and its ADP in the range of 5–100 and 10–100 $\mu\text{g mL}^{-1}$ with elution time of 3.8 and 5.7 min, respectively. The developed method was validated according to the ICH guidelines and applied successfully for determination of FPV in its pharmaceutical dosage form.

In December 2019, COVID-19 was discovered in China and caused a global pandemic. About six different strains of Coronavirus were being reported to affect humans, even though, the seventh one which was identified as SARS-CoV-2 was similar to the SARS coronavirus genetically¹. As soon as this pandemic has appeared, scientists and drug developers in all companies tried to develop new drugs and vaccinations to control this crisis and save peoples' life. Novelty of virus's strain made the old treatments and vaccinations ineffective against it, so there was an urgent need to develop new medications. Favipiravir (FPV) was one of the most effective drugs and made a great influence in the treatment.

Favipiravir (6-Fluoro-3-hydroxypyrazine-2-carboxamide) is a purine nucleic acid analog developed in Japan by Toyama Chemical for the treatment of viral infections. It is a pro-drug that is ribosylated and phosphorylated intracellularly to form its active metabolite Favipiravir ribofuranosyl-5'-triphosphate (FPV-RTP) that works by inhibiting RNA dependent RNA polymerase (RdRp) enzyme². It is currently formulated as tablet dosage form.

Good results were achieved by the FPV treatment in patients with mild to moderate COVID-19 infections which was indicated by increased discharge rate and less mechanical ventilation^{3,4}. It was also reported that patients who were treated with FPV showed a considerably higher viral clearance rate, after about 2 weeks from initiating FPV treatment⁵.

However, recent study showed a lack of positive effect of FPV on mortality, that could be attributed to the low plasma concentrations of FPV following oral administration⁶. Some adverse effects were noted with FPV such as diarrhea, changes in liver function parameters and hyperuricemia⁷. Accordingly, new dosage forms are being formulated for administration of FPV through lung inhalation⁸. Furthermore, there is an increasing need to formulate oral solution dosage forms suitable for children use⁹. Although being just preliminary studies without actual dosage forms in the market till now, these new formulations increased the importance of stability study of FPV especially in solutions. These studies will help optimize the formulation bioavailability and stability¹⁰. It's well known that hydrolysis is the most common pathway for degradation in solutions; therefore,

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studying the hydrolytic degradation pathways of FPV is of utmost importance for these formulations to succeed and reach markets.

Green chemistry recently represents a universal trend, as a lot of studies are done in which many toxic chemicals and reagents are used, generating toxic waste products. Scientists and researchers should pay attention that these all-hazardous chemicals and wastes be replaced with more green, less toxic and eco-friendly ones. Paul Anastas and John Warner¹¹ announced “The Twelve Principles of Green Chemistry” which are summarized PRO-DUCTIVELY. Simply, green chemistry is the usage of chemistry for contamination and toxic waste hindrance. Recently, green chemistry approach was extending to different analytical techniques such as electrochemistry, spectrophotometry, spectrofluorimetry and chromatography^{12–17}.

HPLC technique is the optimum choice among analytical techniques for many pharmaceutical applications such as stability-indicating and assay of pharmaceutical dosage forms. The common presence of organic solvents is always a challenge to develop a green HPLC method. A simple and important mode of HPLC is Micellar liquid chromatography (MLC) which can be used for determination of drugs in pharmaceutical dosage forms and physiological fluids. In MLC separation of charged and uncharged solutes can be achieved using surfactants at a concentration exceeding their critical micelle concentration (CMC)¹⁸. MLC offers unique separation selectivity, high reproducibility, robustness, and safety; all these properties grabbed the attention of analysts for MLC¹⁹. Selection of surfactant in MLC is based mainly on their CMC. Surfactants with low CMC values are preferred as high CMC surfactants usually lead to a sticky mobile phase, which need higher pressure and may cause noise in the detector response²⁰. Common surfactants used in MLC are sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB), and polyoxyethylene 23 lauryl ether (Brij-35) which are known to be safe and biodegradable. In MLC, monomers of surfactants are adsorbed on the stationary phases thus altering their properties. In addition, they form micelles in the mobile phase that solubilizes the analytes. SDS is a commonly used anionic surfactant for retention of positively charged analytes, while Brij-35 is a non-ionic one. Usually, hybrid mobile phases are used in MLC where a small portion of organic modifiers (3–15%) is used with the surfactants to solve the problem of slow mass transfer from the stationary phase¹⁹. This problem can also be solved by increasing temperature in the chromatography column to increase the mass transfer kinetics²¹, or using the non-ionic Brij-35 in combination with SDS²². The presence of Brij-35 can eliminate the need for presence of organic modifiers by decreasing stationary phase polarity. These facts make MLC a green procedure compared to traditional MLC and RP-HPLC.

According to the literature, some methods have been reported for determination of FPV including spectrophotometry^{23,24}, spectrofluorimetry^{25–27}, electrochemical^{28–31} and chromatographic methods^{32–49}. Although stability studies have been reported for FPV^{36,37,49}, they either lack the eco-friendly advantage, or use expensive instruments that are uncommon in QC laboratories (LC–MS).

The target of this manuscript is to progress a new simple, specific, and eco-friendly method for the simultaneous determination of FPV and its hydrolytic degradation product in its pure and pharmaceutical dosage form. The method can be further used in the development phase of FPV solution and inhaler formulations.

Experimental

Materials and reagents

All chemicals and reagents utilized throughout the experiments were all of analytical grade. FPV powder ($100.03 \pm 0.61\%$) was supplied from Marcyrl company. Brij35, SDS and Methanol HPLC grade were purchased from Sigma Aldrich (Germany), potassium dihydrogen orthophosphate anhydrous (98%) from Loba Chemie Pvt Ltd (India), sodium hydroxide was obtained from AVANTOR VWR Chemicals (USA) and hydrochloric acid from PIOCHEM (Egypt).

PIRAVAFI tablets, batch no. 2132642, labelled to contain 200 mg of FPV was purchased from local market (Marcyrl Pharmaceutical Industries, Egypt).

Instruments

Chromatographic analysis was performed using Waters 2695 Alliance HPLC System with photodiode array (PDA), a quaternary, low-pressure mixing pump, autosampler and vacuum degassing. The detector is a PDA (model 2996) with a wavelength range of 190–800 nm and sensitivity settings from 0.0001 to 2.0000 absorbance units.

Mass spectrum was obtained using UPLC-MS/MS Waters 3100 (USA) with TQ detector, binary solvent manager pump, autosampler and MASS LYNX V4.1 software. IR Spectrophotometer: SHIMADZU 435 (Kyoto, Japan), sampling was undertaken as potassium bromide disks. pH meter (JENWAY) was used for pH adjustment.

Chromatographic conditions

RP-C18 was used as stationary phase using VDSPHER PUR 100[®] column (5 μ m, 250 \times 4.6 mm). A solvent-free chromatographic separation was achieved using a mixed micellar mobile phase composed of 0.02 M Brij35 and 0.1 M SDS in presence of 0.01 M potassium dihydrogen orthophosphate anhydrous (pH 3 ± 0.01). pH was adjusted with orthophosphoric acid, and the elution was isocratic for 8.0 min. The flow rate was 1.0 mL min⁻¹ while detection was done at 280.0 nm for FPV and its acid-induced degradation product (ADP). All Chromatographic separations were carried out at 40 ± 0.5 °C. Column was saturated with mobile phase at least 15 min before starting injection of samples, while at the end, column was washed with methanol then water for about 15 min each to confirm removing any residuals.

Stock standard solution preparation

A stock standard solution of FPV (1 mg mL^{-1}) was prepared by dissolving 25.0 mg of FPV in 10 mL deionized water, sonicated for 10 min then the volume was completed in 25.0 mL-volumetric flask with deionized water to obtain the desired concentration.

Acid-induced degradation solution preparation

A certain weight of FPV (25 mg) was dissolved in 25.0 mL of 1.0 N HCl, refluxed for 2 h in water bath at 100°C . The solution was neutralized after cooling with 1.0 N NaOH, the volume was completed with distilled water in a 100-mL volumetric flask to obtain ADP equivalent to $250.0 \mu\text{g mL}^{-1}$.

Construction of calibration curve

From prepared stocks, different concentrations of FPV and ADP were prepared in the mobile phase and injected in triplicates. The procedure under chromatographic conditions was monitored. The peak areas were plotted against the corresponding concentrations and the regression equations were estimated. Regression equations for the developed methodology were established for FPV and ADP using 7 and 6 concentrations for FPV and ADP, respectively by plotting AUC obtained versus the corresponding concentration. For FPV, the concentrations used to build the calibration curve were 5, 10, 20, 40, 60, 80 and $100 \mu\text{g mL}^{-1}$, while for ADP 10, 20, 40, 60, 80 and $100 \mu\text{g mL}^{-1}$ were used.

Application of the proposed HPLC method to PIRAVAFI tablets

Ten tablets were grinded into fine powder after removing their coats, an amount equivalent to 10.0 mg were accurately weighed, transferred into a 100-mL volumetric flask after that 50 mL methanol was added. The prepared solution was sonicated for 15 min, then it was completed to volume with distilled water. The solution was then filtered with $0.45 \mu\text{m}$ filter, and dilutions were done with the mobile phase to obtain concentrations within the linearity range. The procedure under chromatographic conditions was applied.

Results and discussion

Preparation of degradation product of FPV

According to previous studies, FPV is more liable to acidic degradation than other conditions^{36,37,49}, so in this experiment acidic degradation was applied to obtain the hydrolytic degradation product of FPV. HCl (1.0 N) was added to FPV and refluxed in water bath at 100°C , a sample was taken after different time intervals (1, 2 and 3 h), neutralized with NaOH, evaporated, and dissolved in methanol. To monitor the progress of degradation, solutions were spotted on TLC plates and developed using mobile phase of ethyl acetate: methanol: ammonia (2: 4: 0.1, by volume). Two spots were noticed after 1 h for FPV and the possible hydrolytic degradation product, but after 2 and 3 h one spot only for degradation was observed. For further confirmation, degradation solutions were injected into HPLC after 1 h showed a small peak at the same retention time of FPV but after 2 and 3 h, this peak completely disappeared. Therefore, the time for complete degradation of FPV was set at 2 h.

FPV structure contains an amide group which may be subjected to hydrolysis in acidic media. To confirm this hypothesis FPV and ADP were subjected to IR and LC/MS. The IR spectrum of FPV showed forked peak at 3400 cm^{-1} which represents NH_2 group of the amide (Fig. 1a). On the other hand, spectrum of ADP shows no forked peak of NH_2 besides the appearance of broad peak of OH stretching at 3440 cm^{-1} characteristic to COOH group (Fig. 1b). Furthermore, the mass spectrum of ADP was obtained; it showed a peak at 157 m/z corresponding to ADP molecular weight. The structure of the degradation product is not the main objective of our study. We focus on the stability indicating nature of the proposed method to help develop new solution dosage forms for FPV that will help treating COVID-19. However, our suggested structure is augmented with IR and mass spectra and the fact that it was already reported in literature. However, we will explain the findings that can be drawn from the mass spectrum in this reply but will not be added to the manuscript.

The molecular ion peak corresponding to the degradation product itself showed a weak peak at $m/z = 157$ (M-1) despite having M.Wt. of 158, as ESI was operated in negative ion mode, Fig. 2.

This may suggest that FPV hydrolytic degradation is feasible through cleavage of the amide bond by protonation-addition-deprotonation-protonation-elimination-deprotonation (PADPED) mechanism, resulting in carboxylic acid and ammonium chloride salt⁵⁰. Figure 3 shows the suggested degradation pathway of FPV.

Optimization of chromatographic condition

To obtain high resolution in a short run time for the analyzed components with sharp symmetric peaks, there was an urgent need to study the effect of different factors affecting sensitivity, selectivity, and separation efficiency.

Different columns were tried to obtain the best resolution and separation. Symmetry C18 column ($5 \mu\text{m}$, $75 \times 4.6 \text{ mm}$) and VDSPHERE PUR 100 C18-E ($5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$) column were used. The optimum peak resolution was obtained using the second column, so it was used for analysis of FPV and its ADP. On the other hand, different concentrations of mobile phase components were tried, Brij35 (0.01, 0.02, 0.03 M) and SDS (0.1, 0.12, 0.8 M) in 0.01 M potassium dihydrogen orthophosphate anhydrous were tried. A mobile phase composed of Brij35, SDS, and potassium dihydrogen orthophosphate anhydrous in the concentrations of 0.02: 0.1: 0.01 M gave the best results. As pH is an important parameter in HPLC and has a great effect on results including resolution and retention time, different pH were tested from (3–5) and the best results were obtained at pH 3. At pH below 3, peak of FPV was forked which may be due to protonation of amide group at high acidic medium. so no study was done below pH 3.

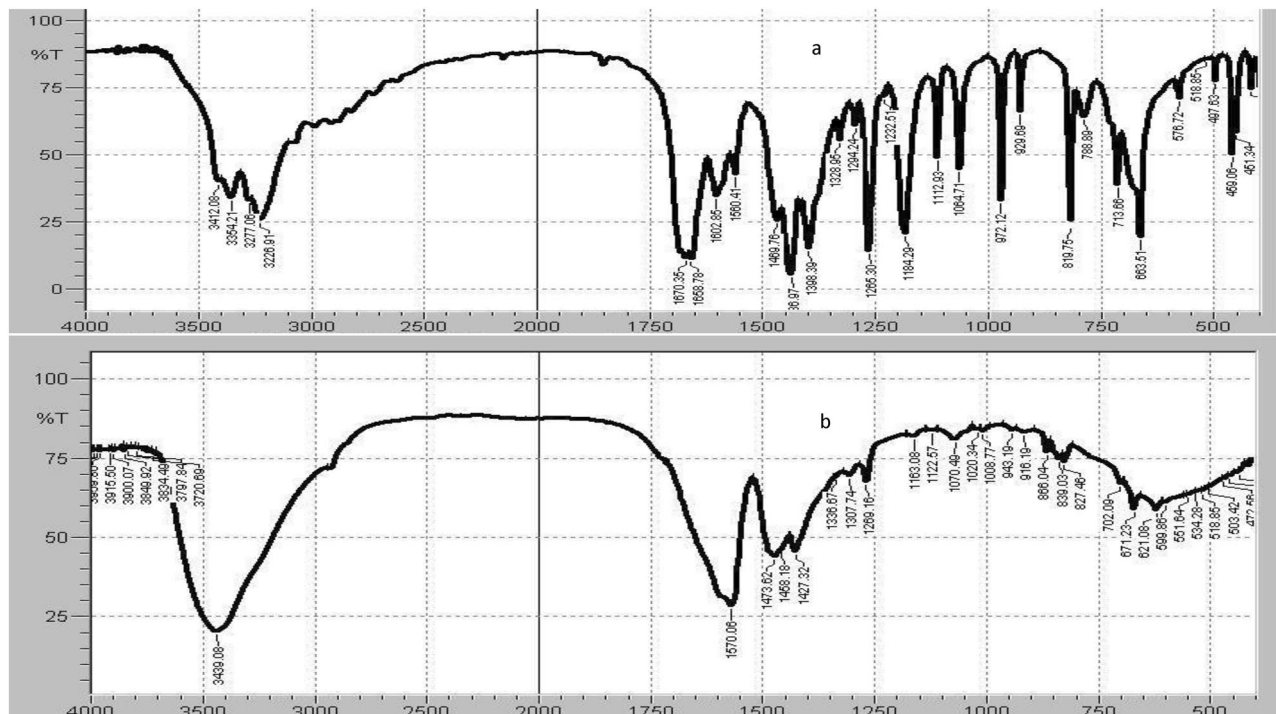


Figure 1. IR spectra of (a) Favipiravir (FPV) and (b) acidic degradation product (ADP).

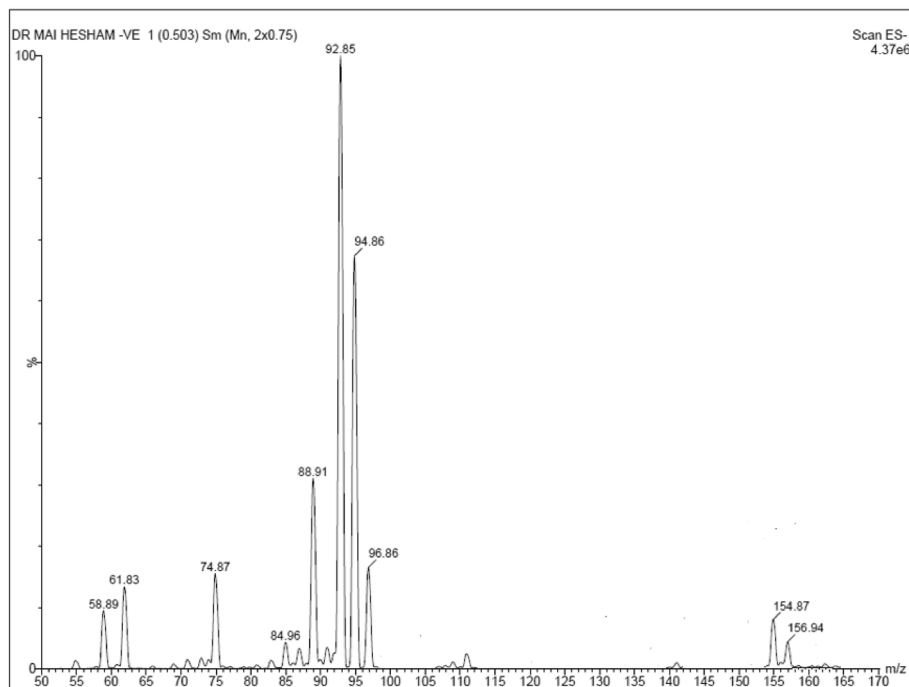


Figure 2. Mass spectrum of acidic degradation product (ADP) of Favipiravir.

UV detection was done at different wavelengths 323, 227 and 280 nm, the best sensitivity was obtained at 280 nm at which measurements were done. Comparing between various flow rates 0.8, 1.0 and 1.2 the best results obtained at flow rate 1.0 ml min⁻¹.

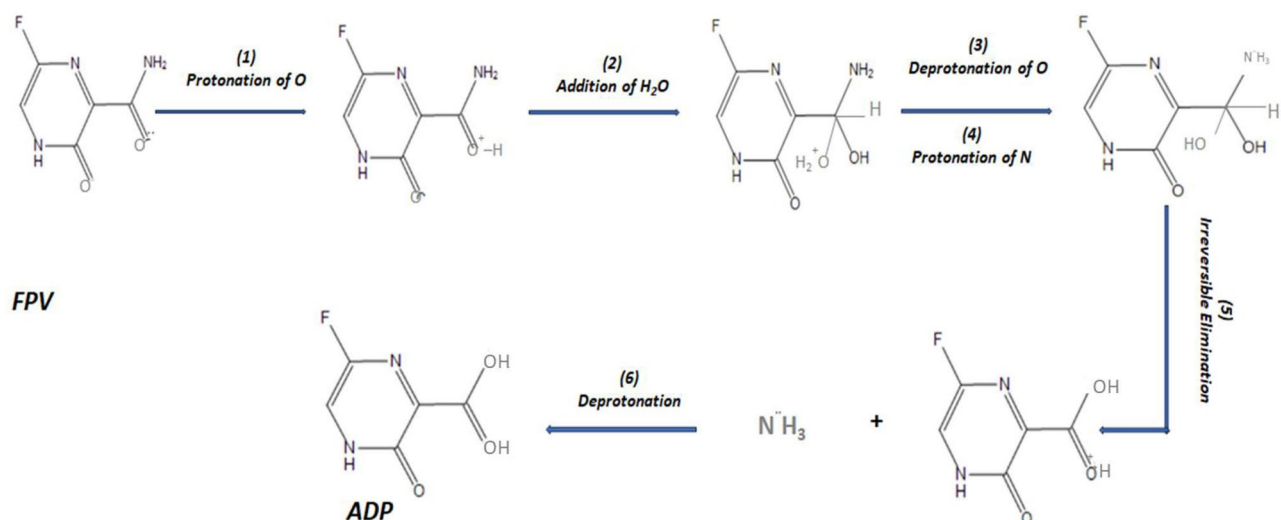


Figure 3. Suggested hydrolytic degradation pathway of Favipiravir.

Method validation

The developed methodology were validated according to ICH guidelines⁵¹. Specificity of the developed method was confirmed by analysis of a mixture of FPV, and its ADP and comparing the results of pure FPV and its dosage form (Fig. 4). Regression equation for the developed methodology was established for FPV and ADP after plotting the area under curve (AUC) obtained versus the concentration of FPV and ADP. Linearity was obtained in the range of 5–100 and 10–100 $\mu\text{g mL}^{-1}$ with correlation coefficients of 0.9999 and 0.9993 for FPV and ADP, respectively (Table 1). The correction factor was calculated for ADP and found to be 25.7⁵².

Accuracy of the developed method was tested and confirmed by calculating percentage recoveries (actual conc./claimed conc * 100) for five different concentrations in triplicate for each compound. Concentrations used for both FPV and ADP were 15, 30, 50, 70 and 90 $\mu\text{g mL}^{-1}$. The closeness of the calculated percentage recovery results to the true values confirmed the accuracy of the method. Accuracy obtained was 100.51 and 99.61% for FPV and its ADP, respectively (Table 1). Repeatability (intra-day) and intermediate precision (inter-days) were established at three different measurements (40, 60 and 80 $\mu\text{g mL}^{-1}$) within the same day and on three different days respectively, and acceptable values of RSD% were obtained that didn't exceed 1.6% as presented in (Table 1).

Limits of detection [LOD] and quantification [LOQ] were calculated as $3.3 \sigma/S$, and $10 \sigma/S$, respectively, where (σ) is the residuals standard deviation and (S) is the slope of calibration curve, Table 1. There is urgent need to confirm and ensure that developed method can withstand small changes in the experimental conditions, thus robustness was assessed. Trials were performed to assess the impact of minor changes in concentrations of SDS

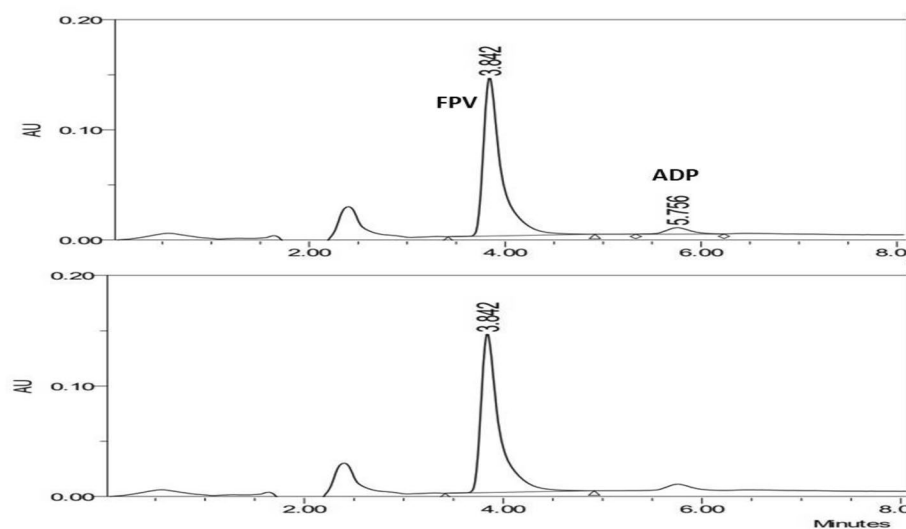


Figure 4. HPLC chromatograms of (a) synthetic mixture of Favipiravir (FPV) and its acid-induced degradation product (ADP) and (b) PIRAVAFI tablet dosage form ($100 \mu\text{g mL}^{-1}$) at 280.0 nm.

Parameter	FPV	ADP
Linearity range ($\mu\text{g mL}^{-1}$)	5–100	10–100
Slope	18,474	718.42
Intercept	17,771	6374.7
Regression coefficient (r)	0.9999	0.9993
Accuracy (mean \pm SD) ^a	100.51 \pm 1.20	99.61 \pm 1.57
SD of residuals	7721.034	711.6004
LOD ($\mu\text{g mL}^{-1}$) ^b	1.379	3.268
LOQ ($\mu\text{g mL}^{-1}$) ^b	4.179	9.905
Intra-day precision ^c	0.428	1.075
Inter-day precision ^d	1.101	1.561

Table 1. Validation parameters data of the proposed HPLC method for Favipiravir (FPV) determination and its acid-induced degradation product (ADP). ^aMean ($n=3$) recovery % of five concentrations. ^bLimits of detection “LOD” and quantitation “LOQ” are determined via calculations, LOD = 3.3 (SD of the residuals/slope), LOQ = 10 (SD of the residuals/slope). ^cIntra-day precision ($n=3$), three concentrations (40, 60 and 80 $\mu\text{g mL}^{-1}$) repeated three times within the same day. ^dInter-day precision ($n=3$), three concentrations (40, 60 and 80 $\mu\text{g mL}^{-1}$) repeated three times in three successive days.

and Brij-35 of the mobile phase. Changes in surfactant concentrations were (0.1 ± 0.02 M) and (0.02 ± 0.005 M) for SDS and Brij-35, respectively. By detecting changes in the AUC and retention times in terms of RSD%, it was within the acceptable limits as shown in Table 2. Short term stability was studied and results showed that FPV is stable for 24 h as shown in Table 2.

System suitability parameters

Checking system suitability parameters of the proposed HPLC method was done to confirm that the system was working accurately during the experiment. Number of theoretical plates (N), heights equivalent to theoretical plates (HETP), tailing factor (T), capacity factor (K'), resolution and selectivity (α) were examined and were within the acceptable levels as shown in Table 3.

Application to pharmaceutical dosage form

The developed analytical method was used for determination of FPV in PIRAVAFI tablets. Calculated percentage recovery of FPV was statistically comparison to the reported method⁴⁹. Student's t-test and F-test showed no significant difference among the results (Table 4), proving the proposed method can be successfully applied for analysis of FPV dosage forms in QC laboratories.

Parameters	RSD%	
	AUC	Retention time
SDS concentration (0.1 ± 0.02 M)	1.132	1.357
Brij-35 concentration (0.02 ± 0.005 M)	1.838	1.040
Short-term stability (24 h)	0.308	0.256

Table 2. Robustness and short-term stability of Favipiravir for the proposed HPLC method.

Parameters	FPV	ADP	Reference values ⁵³
Retention time	3.842	5.756	
Number of theoretical plates(N)	2745	3583	>2000
Tailing factor (T)	2.016	1.245	≤ 2
Capacity factor (K')	0.921	1.878	>2.0
Height equivalent to theoretical plates (HETP)	0.0655 mm	0.0502 mm	
Resolution ^a	5.75		>2
selectivity factor (α) ^a	2.086		>1

Table 3. System suitability parameters of the proposed HPLC methods for the determination of Favipiravir (FPV) and its acid degradation product (ADP). ^aResolution and selectivity factor are determined between the peaks of FPV and ADP.

Parameters	Reported method ^{44a}	Proposed method
Mean recovery % ^b	99.405	98.890
Variance	0.338	0.429
n	4	4
Students t-test ^c	–	1.173 (2.44)
F-test ^c	–	1.26 (9.276)

Table 4. Statistical analysis of the proposed HPLC method and the reported method for the analysis of PIRAVAFI tablets. ^aInertsil ODS-3V C18 maintained at 30 °C, using Phosphate buffer (pH 3.5) and acetonitrile, (90:10, v/v) as mobile phase with flow rate of 1.0 mL min⁻¹ at 358.0 nm. ^bAverage of three determinations. ^cValues between parenthesis are corresponding to the theoretical values of *t* and *F* (*P* = 0.05).

Comparison with other reported methods

Regarding greenness assessment, developing environmentally friendly analytical methods has become a universal issue, greenness of the developed method and five reported methods was evaluated using two greenness assessment techniques: the green analytical procedure index (GAPI)⁵⁵ and the AGREE metric⁵⁶. GAPI assessments includes three parts firstly, sample preparation which involves the steps, transportation, and organic solvents. Secondly, the quantity of reagents and their National Fire Protection Association score. The third part takes instrument and waste into consideration.

As shown in Table 5, GAPI pictogram of the proposed method has only two red zones that stand for the offline sampling and no waste treatment which represents no problem as all reagents used were eco-friendly and totally green. Assessment of the proposed method was the same as the two reported green methods^{42,47} which offered no stability study. On the other hand, the reported methods^{48,49,54} are less eco-friendly as shown in the upper left part with red colors and upper right part with yellow colors related to using organic solvents, so their safety and toxicity is more compared with the green color in both parts of the proposed method pictogram.

AGREE is represented as a clock-shaped chart with a perimeter divided into 12 sections, each corresponding to one of the Green Analytical Chemistry principles with color scale (red-yellow-green). The AGREE pictogram has an overall evaluation color indicating the final score, performance of the analytical procedure in each criterion as well as an overall assessment score on a scale from 0 to 1. As shown in Table 5, the high score of

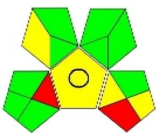
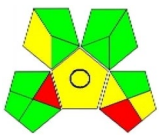
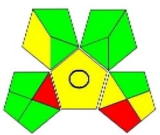
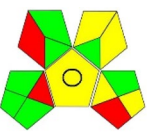
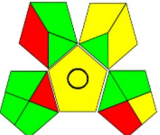
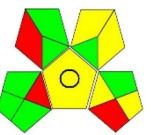






	Proposed method	Reported method ⁴²	Reported method ⁴⁷	Reported method ⁴⁸	Reported method ⁴⁹	Reported method ⁵⁴
Technique	Micellar HPLC–UV	Micellar HPLC–UV	Micellar HPLC–UV	HPLC–UV	HPLC–DAD	RP-HPLC
Linearity range (µg ml ⁻¹)	FPV: (5–100) ADP: (10–100)	FPV: (0.5–50.0)	FPV: (10–100)	FPV:(10–100)	FPV:(6.25–250.00)	FPV:(50–250)
Organic solvent	Totally free	Totally free	Totally free	Phosphate buffer (pH 3.2) and acetonitrile, (90:10, v/v)	Phosphate buffer (pH 3.5) containing 0.1% heptane, sulphonic acid sodium salt, methanol and acetonitrile, (62:28:10, v/v)	Phosphate buffer (pH 3.5) and acetonitrile, (90:10, v/v)
Run time (min)	8.0	5.0	4.0	8.0	10.0	15.0
Column	RP-VDSpher PUR 100	RP-C18 core-shell	VDSpher-150 C18-E	Inertsil ODS-3V C18	Zorbax C18	Inertsil ODS-3V C18
LOD (µg ml ⁻¹)	FPV: 1.379 ADP: 3.268	0.040	0.985	1.200	1.020	2.186
LOQ (µg ml ⁻¹)	FPV: 4.179 ADP: 9.905	0.120	2.986	3.600	3.100	6.626
Application	Stability assessment of FPV and ADP in pharmaceutical dosage form	Molnupiravir and FPV in their dosage forms	Bulk powder	Bulk powder	Stability assessment of FPV in its tablet dosage form	Stability assessment of FPV in its tablet dosage form
GAPI						
AGREE						

Table 5. Comparison between the proposed micellar HPLC and the reported methods for analysis of Favipiravir (FPV) and its acid degradation product (ADP).

the proposed method (0.84) confirms high greenness of method. The two reported methods^{42,47} have the same greenness score of the proposed procedure, but lacking quantification and separation of ADP mentioned in this study. Comparing with the three other reported methods^{48,49,54}, they have low AGREE score as presented by the red color at Sect. (12) corresponding to hazardness, corrosiveness, toxicity and flammability of reagents used. In addition, yellow color at Sect. (10) which represents the type of reagents used compared with the a green section of the proposed method.

Considering other method characteristics, all the previously reported methods used either organic solvents as mobile phase^{48,49,54} or green solvents^{42,47}, but none of them included offered quantitation of the degradation product.

Conclusions

Simple, accurate and eco-friendly HPLC method was developed and validated for the simultaneous determination of FPV and its hydrolytic degradation product. The developed method was successfully applied to marketed pharmaceutical dosage forms. The method can be used for the development of solutions or inhalers dosage forms for FPV. These dosage forms are urgently needed to increase its efficacy in treatment of SARS-CoV-2 viral infection. The greenness of the proposed method was estimated using GAPI and AGREE metrics and proved its low ecological impact and made it preferred to other reported methods to be applied in quality control laboratories.

Data availability

The data analyzed during the current study are available from the corresponding author on reasonable request.

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References

1. Agrahari, R. *et al.* Update vision on COVID-19: Structure, immune pathogenesis, treatment and safety assessment. *Sens. Int.* **2**, 100073 (2021).
2. Vora, A. & Tiwaskar, M. Favipiravir. *J. Assoc. Phys. India* **68**, 91–92 (2020).
3. Alamer, A. *et al.* Effectiveness and safety of favipiravir compared to supportive care in moderately to critically ill COVID-19 patients: A retrospective study with propensity score matching sensitivity analysis. *Curr. Med. Res. Opin.* **37**(7), 1085–1097 (2021).
4. Almoosa, Z. *et al.* Favipiravir versus standard of care in patients with severe COVID-19 infections: A retrospective comparative study. *J. Infect. Public Health* **14**(9), 1247–1253 (2021).
5. Deng, W. *et al.* Evaluation of favipiravir in the treatment of COVID-19 based on the real-world. *Expert Rev. Anti Infect. Ther.* **20**(4), 555–565 (2022).
6. Kow, C. S., Ramachandram, D. S. & Hasan, S. S. Future of antivirals in COVID-19: The case of favipiravir. *Int. Immunopharmacol.* **103**, 108455 (2022).
7. Ghasemnejad-Berenji, M. & Pashapour, S. Favipiravir and COVID-19: A simplified summary. *Drug Res.* **71**(03), 166–170 (2021).
8. Yildiz Pekoz, A. *et al.* Pulmonary delivery of favipiravir inhalation solution for COVID-19 treatment: In vitro characterization, stability, in vitro cytotoxicity, and antiviral activity using real time cell analysis. *Drug Deliv.* **29**(1), 2846–2854 (2022).
9. Tuesuwan, B. *et al.* Basis to aid crisis: Favipiravir oral solution for hospital compounding during COVID-19 drug shortage. *J. Pharm. Sci.* **112**(2), 610–617 (2023).
10. Gad, M. A., Amer, S. M., Zaazaa, H. E. & Hassan, S. A. Strategies for stabilizing formulation and QbD assisted development of robust stability indicating method of azilsartan medoxomil/chlorthalidone. *J. Pharm. Biomed. Anal.* **178**, 112910 (2020).
11. Anastas, P. T. & Warner, J. C. Principles of green chemistry. *Green Chem.* **29** (1998).
12. Hassan, S. A., Mahmoud, A. M., Ahmed, M. K., Abbas, S. S. & Michael, A. M. Design of solid-contact ion-selective electrode with multiwall-carbon nanotubes for assay of sulfacetamide in rabbit aqueous humour. *Curr. Anal. Chem.* **19**(4), 320–329 (2023).
13. ElDin, N. B., Abd El-Rahman, M. K., Zaazaa, H. E., Moustafa, A. A. & Hassan, S. A. Supramolecular green chemistry; an eco-friendly spectrophotometric approach for determination of non-chromophoric methacholine via host-guest interactions with 4-sulfocalix [4] arene. *Microchem. J.* **168**, 106419 (2021).
14. Hassan, S. A., Helmy, A. H., Youssef, N. F., Weshahy, S. A. & El-zeany, B. A. Fluorescence imaging approaches for the eco-friendly determination of perampanel in human plasma and application for therapeutic drug monitoring. *Luminescence* **38**(6), 729–735 (2023).
15. Ibrahim, N., Elzanfaly, E. S., El Gendy, A. E. & Hassan, S. A. Development, optimization, and validation of a green spectrofluorimetric method for the determination of moxifloxacin using an experimental design approach. *Res. J. Pharm. Technol.* **14**(4), 1880–1886 (2021).
16. El-Azab, N. F., Abdelaal, S. H., Hassan, S. A. & El-Kosasy, A. M. Dietary supplement mislabelling: Case study on selected slimming products by developing a green isocratic HPLC method for their quality control. *Sci. Rep.* **12**(1), 22305 (2022).
17. Kelani, K. M., Fayeze, Y. M., Abdel-Raouf, A. M., Fekry, R. A. & Hassan, S. A. Development of an eco-friendly HPLC method for the stability indicating assay of binary mixture of ibuprofen and phenylephrine. *BMC Chem.* In Press (2023)
18. Kalyankar, T., Kulkarni, P., Wadher, S. & Pekamwar, S. J. J. O. A. P. S. Applications of micellar liquid chromatography in bioanalysis: A review. *J. Appl. Pharm. Sci.* **4**(1), 128–134 (2014).
19. El-Shaheny, R. N., El-Maghrabey, M. H. & Belal, F. F. Micellar liquid chromatography from green analysis perspective. *Open Chem.* **13**(1) (2015).
20. Medina Hernandez, M. & Garcia Alvarez-Coque, M. J. A. Solute-mobile phase and solute-stationary phase interactions in micellar liquid chromatography. A review. *Analyst* **117**(5), 831–837 (1992).
21. Ruiz-Angel, M., Carda-Broch, S., Torres-Lapasó, J. R. & Garcia-Alvarez-Coque, M. J. J. O. C. A. Retention mechanisms in micellar liquid chromatography. *J. Chromatogr. A* **1216**(10), 1798–1814 (2009).
22. Ruiz-Angel, M., Peris-García, E. & García-Alvarez-Coque, M. Reversed-phase liquid chromatography with mixed micellar mobile phases of Brij-35 and sodium dodecyl sulphate: A method for the analysis of basic compounds. *Green Chem.* **17**(6), 3561–3570 (2015).
23. Jyothi, B. J. & Kavya, R. V. Ultraviolet spectrophotometric method development for estimation of new antiviral repurposing drug favipiravir. *Asian J. Pharm. Clin. Res.* **14**(7), 67–69 (2021).
24. Megahed, S. M., Habib, A. A., Hammad, S. F. & Kamal, A. H. Experimental design approach for development of spectrofluorimetric method for determination of favipiravir; a potential therapeutic agent against COVID-19 virus: Application to spiked human plasma. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **249**, 119241 (2021).

25. El Sharkasy, M. E., Tolba, M. M., Belal, F., Walash, M. & Aboshabana, R. Quantitative analysis of favipiravir and hydroxychloroquine as FDA-approved drugs for treatment of COVID-19 using synchronous spectrofluorimetry: Application to pharmaceutical formulations and biological fluids. *Luminescence* **37**(6), 953–964 (2022).
26. El-Awady, M., Elmansi, H. & Belal, F. Insights on the quantitative concurrent fluorescence-based analysis of anti-COVID-19 drugs Remdesivir and Favipiravir. *J. Fluoresc.* **32**(5), 1941–1948 (2022).
27. Ramzy, S., Abdelazim, A. H., Osman, A. & Hasan, M. A. Spectrofluorimetric quantitative analysis of favipiravir, remdesivir and hydroxychloroquine in spiked human plasma. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **281**, 121625 (2022).
28. Galal, A., Ahmed, Y. M., Ahmed, M. H. & Atta, N. F. Electrochemistry and determination of an antiviral drug at ionic liquids crystals-carbon nanotubes modified glassy carbon electrode. *J. Electrochem. Soc.* **168**(11), 116512 (2021).
29. Mehmandoust, M., Khoshnavaz, Y., Tuzen, M. & Erk, N. Voltammetric sensor based on bimetallic nanocomposite for determination of favipiravir as an antiviral drug. *Microchim. Acta* **188**(12), 1–15 (2021).
30. Allahverdiyeva, S., Yunusolu, O., Yardm, Y. & Entrk, Z. H. First electrochemical evaluation of favipiravir used as an antiviral option in the treatment of COVID-19: A study of its enhanced voltammetric determination in cationic surfactant media using a boron-doped diamond electrode. *Anal. Chim. Acta* **1159**, 338418 (2021).
31. Mohamed, M. A. *et al.* Innovative electrochemical sensor for the precise determination of the new antiviral COVID-19 treatment Favipiravir in the presence of coadministered drugs. *J. Electroanal. Chem.* **895**, 115422 (2021).
32. Abdallah, I. A., El-Beairy, M. F., Ahmed, R. M. & Fayed, M. A. The anti-COVID-19 drug favipiravir: Degradation, method development, validation, NMR/LC–MS characterization, and In-vitro safety evaluation. *Chem. Pap.* **76**(10), 1–12 (2022).
33. Aydinoglu, S. & Bozyel, M. Favipiravir determination in pharmaceutical formulation via HPLC chromatographic Approach. *Iran. J. Chem. Chem. Eng* (2022).
34. Balu, P. A. & Pares, M. S. Stability-indicating RP HPLC method development for estimation of favipiravir in bulk and pharmaceutical dosage form. *World J. Pharm. Res.* **10**(14), 1444–1465 (2021).
35. Emam, A. A., Abdelaleem, E. A., Abdelmomen, E. H., Abdelmoety, R. H. & Abdelfatah, R. M. Rapid and ecofriendly UPLC quantification of Remdesivir, Favipiravir and Dexamethasone for accurate therapeutic drug monitoring in Covid-19 Patient's plasma. *Microchem. J.* **179**, 107580 (2022).
36. Gökce, S., Höl, A. & Bulduk, I. Development and validation of UPLC-MS/MS method for obtaining favipiravir tablet dosage form and evaluation of its behavior under forced conditions. *J. Pharm. Res. Int.* **33**(56), 130–140 (2021).
37. Kalshe, M. & Adlinge, S. G. Development and validation of HPLC method for quantification of favipiravir in tablet. *Res. J. Pharm. Technol.* **15**(3), 1319–1322 (2022).
38. Nazifa Sabir Ali, S. *et al.* Analytical method development and validation and forced degradation stability-indicating studies of Favipiravir by RP-HPLC and UV in bulk and pharmaceutical dosage form. *J. Pharm. Res. Int.* **33**(48B), 254–271 (2021).
39. Noureldeen, D. A., Boushra, J. M., Lashien, A. S., Hakiem, A. F. A. & Attia, T. Z. Novel environment friendly TLC-densitometric method for the determination of anti-coronavirus drugs “Remdesivir and Favipiravir”: Green assessment with application to pharmaceutical formulations and human plasma. *Microchem. J.* **174**, 107101 (2022).
40. Onmaz, D. E., Abusoglu, S., Onmaz, M., Yerlikaya, F. H. & Unlu, A. Development and validation of a sensitive, fast and simple LC-MS/MS method for the quantitation of favipiravir in human serum. *J. Chromatogr. B* **1176**, 122768 (2021).
41. Saraya, R. E., Deeb, S. E., Salman, B. I. & Ibrahim, A. E. Highly sensitive high-performance thin-layer chromatography method for the simultaneous determination of Molnupiravir, Favipiravir, and Ritonavir in pure forms and pharmaceutical formulations. *J. Sep. Sci.* **45**(14), 2582–2590 (2022).
42. Sharaf, Y. A., El Deeb, S., Ibrahim, A. E., Al-Harrasi, A. & Sayed, R. A. Two green micellar HPLC and mathematically assisted UV spectroscopic methods for the simultaneous determination of molnupiravir and favipiravir as a novel combined COVID-19 antiviral regimen. *Molecules (Basel, Switzerland)* **27**(7), 2330 (2022).
43. Duse, V. P. & Baheti, G. K. Bioanalytical method development and validation for the determination of Favipiravir in spiked human plasma by using RP-HPLC. *J. Pharm. Res. Int.* **33**(47A), 275–281 (2021).
44. Vemuri, D. K., Gundla, R., Konduru, N., Mallavarapu, R. & Katari, N. K. Favipiravir (SARS-CoV-2) degradation impurities: Identification and route of degradation mechanism in the finished solid dosage form using LC/LC–MS method. *Biomed. Chromatogr.* **36**(6), e5363 (2022).
45. Rezk, M. R., Badr, K. A., Abdel-Naby, N. S. & Ayyad, M. M. A novel, rapid and simple UPLC-MS/MS method for quantification of favipiravir in human plasma: Application to a bioequivalence study. *Biomed. Chromatogr.* **35**(7), e5098 (2021).
46. Eryavuz Onmaz, D., Abusoglu, S., Onmaz, M., Yerlikaya, F. H. & Unlu, A. Development and validation of a sensitive, fast and simple LC-MS/MS method for the quantitation of favipiravir in human serum. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **1176**, 122768 (2021).
47. Mikhail, I. E., Elmansi, H., Belal, F. & Ehab Ibrahim, A. Green micellar solvent-free HPLC and spectrofluorimetric determination of favipiravir as one of COVID-19 antiviral regimens. *Microchem. J.* **165**, 106189 (2021).
48. Bulduk, I. HPLC-UV method for quantification of favipiravir in pharmaceutical formulations. *Acta Chromatogr.* **33**(3), 209–215 (2021).
49. Marzouk, H. M., Rezk, M. R., Gouda, A. S. & Abdel-Megied, A. M. A novel stability-indicating HPLC-DAD method for determination of favipiravir, a potential antiviral drug for COVID-19 treatment; application to degradation kinetic studies and in-vitro dissolution profiling. *Microchem. J.* **172**, 106917 (2022).
50. O'Connor, C. Acidic and basic amide hydrolysis. *Q. Rev. Chem. Soc.* **24**(4), 553–564 (1970).
51. In *ICH harmonised tripartite guideline Q2 (R1): Validation of analytical procedures: text and methodology*. International conference on harmonization (2005).
52. Epshtein, N. J. P. C. J. Correction factors in formulas for calculating impurity contents: Essence and determination methods and their limitations. *Pharm. Chem. J.* **53**(5), 477–482 (2019).
53. Food, Administration, D., Reviewer Guidance: Validation of chromatographic methods. *cited November* (1994).
54. Ali, S. N. S. *et al.* Analytical method development and validation and forced degradation stability-indicating studies of Favipiravir by RP-HPLC and UV in bulk and pharmaceutical dosage form. *J. Pharm. Res. Int.* **33**(48B), 254–271 (2021).
55. Plotka-Wasyłka, J. A new tool for the evaluation of the analytical procedure: Green analytical procedure index. *Talanta* **181**, 204–209 (2018).
56. Pena-Pereira, F., Wojnowski, W. & Tobiszewski, M. AGREE—Analytical GREENness metric approach and software. *Anal. Chem.* **92**(14), 10076–10082 (2020).

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Additional information

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