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CONTENTS*

Organic Chemistry

- M. Koravović, M. Kovačević, M. Jovanović, G. Tasić, A. Mayasundari, G. Nishiguchi, B. Marković, Z. Ranković and V. Savić: Comparative *in silico/in vitro* analysis of pharmacokinetic profiles of BET inhibitors 837
- B. Anić Marković, I. Cvijetić, M. Milošević, A. Marinković, Lj. Tolić Stojadinović, S. Drmanić and J. Nikolić: *In vitro* antioxidant activity of nicotinic acid hydrazides: Experimental and theoretical study 857

Biochemistry and Bioengineering

- İ. Özgen, A. Gül, C. Baltacı, İ. Öner and E. Aydoğmuş: Determination of antibacterial properties and chemical composition of pyrolysis liquid from feeding wastes of mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) adults and larvae 869

Theoretical Chemistry

- S. Kumar, N. Kumar, R. Kumar and K. K. Mishra: The role of non-covalent interactions in the solvation dynamics of metronidazole in water: A theoretical study 883
- M. M. Petković-Benazzouz, I. Radović, D. V. Miloškov and G. V. Janjić: Molecular docking's pioneering role in materials science. Metal ion site-preference in fluorapatite 899

Physical Chemistry

- M. Ristić, N. Krstevski, M. Marković, A. Šajić and M. Kuzmanović: Influence of pressure and mixing with air on the equilibrium composition of laser-induced plasma on ODS steel 915
- M. Febriansyah, D. K. Putri, A. T. Nabila, I. Radiansyah, M. Nurrahman and I N. Candra: Innovation in printer ink based on carbon quantum dots (CQDs) derived from styrofoam waste for anti-counterfeiting document technology 929

Analytical Chemistry

- C. Son Yue, C. Fung Loke, S. Hong Teo, H. Hong Teo, S. Iiangovan and K. Niang Chee: Acetic acid liquid–liquid extraction and UHPLC-DAD detection of polycyclic aromatic hydrocarbons in toasted and fried foods 943

Chemical Engineering

- S. Mansouri and S. Cheknoun: Ruthenium(III)-monosubstituted Keggin-type polyoxotungstate: Synthesis, characterization and application in the catalytic methanation of carbon dioxide 957

Environmental Chemistry

- T. Marjanović, M. Bogunović Koljaja, J. Prodanović, B. Barta Holló and I. Ivančević-Tumbas: Influence of dissolved organic carbon nature on adsorption of ibuprofen, caffeine and diclofenac by powdered activated carbon from water 973
- D. Tahirović M. Balaban, T. Muhić Šarac, E. Članjak-Kudra, M. Smajlović, F. Čaklović and V. Antić: Arsenic in Bosnia and Herzegovina market seafood: Effects of matrix modifiers on measured concentration 987

History of and Education in Chemistry

- A. Kargopoulos, C. Skordoulis, P. Giannakoudakis and A. Mavropoulos: Teaching chemical reactions to upper secondary school students: A laboratory approach 1001

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Comparative *in silico/in vitro* analysis of pharmacokinetic profiles of BET inhibitors

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Abstract: Pharmacokinetic limitations are a common cause of drug development failure, making early-stage profiling essential to mitigate financial risks and guide compound optimization. The computational tools capable of predicting pharmacokinetic properties offer a valuable resource for the prioritizing of candidates during early discovery phases. This study provides a comparative analysis of *in silico* and *in vitro* pharmacokinetic profiles of bromodomain and extraterminal domain (BET) inhibitors (BIs), focusing on sixteen (+)-JQ1-derived compounds we previously synthesized. Using ADMETlab 2.0 software, we predicted key absorption, distribution, metabolism, and excretion (ADME) parameters and compared them with experimentally determined data. Strong correlations were observed for plasma protein binding, whereas notable discrepancies were identified in permeability and clearance values. Additionally, the analysis underscores the role of CYP3A4 as a critical enzyme in the metabolism of several BIs. These findings demonstrate the utility of computational tools like ADMETlab 2.0 for early pharmacokinetic profiling while highlighting the need for validation and refinement of predictive models. This work provides valuable insights into the pharmacokinetics of BIs and supports the integration of computational and experimental approaches in drug discovery.

Keywords: (+)-JQ1; ADME analysis; ADMETlab 2.0; drug development.

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INTRODUCTION

The challenges related to pharmacokinetics frequently contribute to the failure of drug development, emphasizing the importance of early evaluation and refinement of these properties during drug discovery. In the context of drug development, particularly for orally administered agents like bromodomain and extraterminal domain (BET) inhibitors, the desirable pharmacokinetic properties include: high intestinal absorption and permeability to ensure sufficient bioavailability; moderate to high metabolic stability to avoid rapid degradation; balanced plasma protein binding to ensure the adequate free (unbound) drug concentration and avoid the potential variability related to the albumin concentration; optimal volume of distribution, indicating the ability to reach the target tissues, including those with poor blood supply; reasonable half-life to maintain the therapeutic plasma levels without frequent dosing; and the low potential for drug-drug interactions, especially via cytochrome P450 (CYP450) enzymes like CYP3A4. For BET inhibitors (BIs) targeting systemic or central nervous system (CNS) malignancies, additional desirable features include blood–brain barrier (BBB) penetration and resistance to P-glycoprotein (P-gp)-mediated efflux, which can hinder CNS drug delivery. These criteria guide early-stage screening to prioritize candidates likely to exhibit effective and safe pharmacological profiles *in vivo*.

The computational tools, such as ADMETlab 2.0, enable the prediction of absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties, offering valuable insights for drug design.¹ Building on our previous research,² this study evaluates the pharmacokinetics of (+)-JQ1-derived, BIs using a combination of *in silico* and *in vitro* approaches.

The BET protein family, comprising BRD2, BRD3, BRD4 and BRDT, plays a crucial role in transcriptional regulation by recognizing the acetylated lysine residues on chromatin.^{3–5} These proteins are implicated in various diseases, making them attractive therapeutic targets. BIs have shown efficacy in hematological malignancies such as acute myeloid leukemia,⁷ multiple myeloma⁸ and diffuse large B-cell lymphoma,⁹ as well as in solid tumors including triple-negative breast cancer,¹⁰ glioblastoma¹¹ and NUT midline carcinoma.¹² Beyond oncology, BET inhibition is being explored in inflammatory and autoimmune disorders such as systemic lupus erythematosus¹³ and rheumatoid arthritis¹⁴ and even in fibrotic diseases¹⁵ and cardiovascular conditions.¹⁶ The wide range of potential indications highlights the clinical relevance of pharmacokinetically optimizing BIs for both systemic and tissue-targeted therapies. Among the BIs, (+)-JQ1 has shown remarkable preclinical efficacy across numerous disease models.¹⁷ However, its limited pharmacokinetic properties have posed challenges for clinical application.¹⁸

To address these limitations, our group has developed a series of potent (+)-JQ1-derived amides.² Expanding on this work, we utilized ADMETlab 2.0 to predict the pharmacokinetic profiles of these novel compounds and compared the

computational predictions with experimental data to identify correlations. These findings enhance the understanding of Bis' pharmacokinetics, facilitating the development of next-generation BIs and enabling the selection of promising candidates for further evaluation. This study systematically correlates *in silico* and *in vitro* ADME results for BIs, offering a comprehensive pharmacokinetic discussion that integrates computational predictions with experimental findings.

The chemical structures of the studied BIs are presented in Fig. 1.

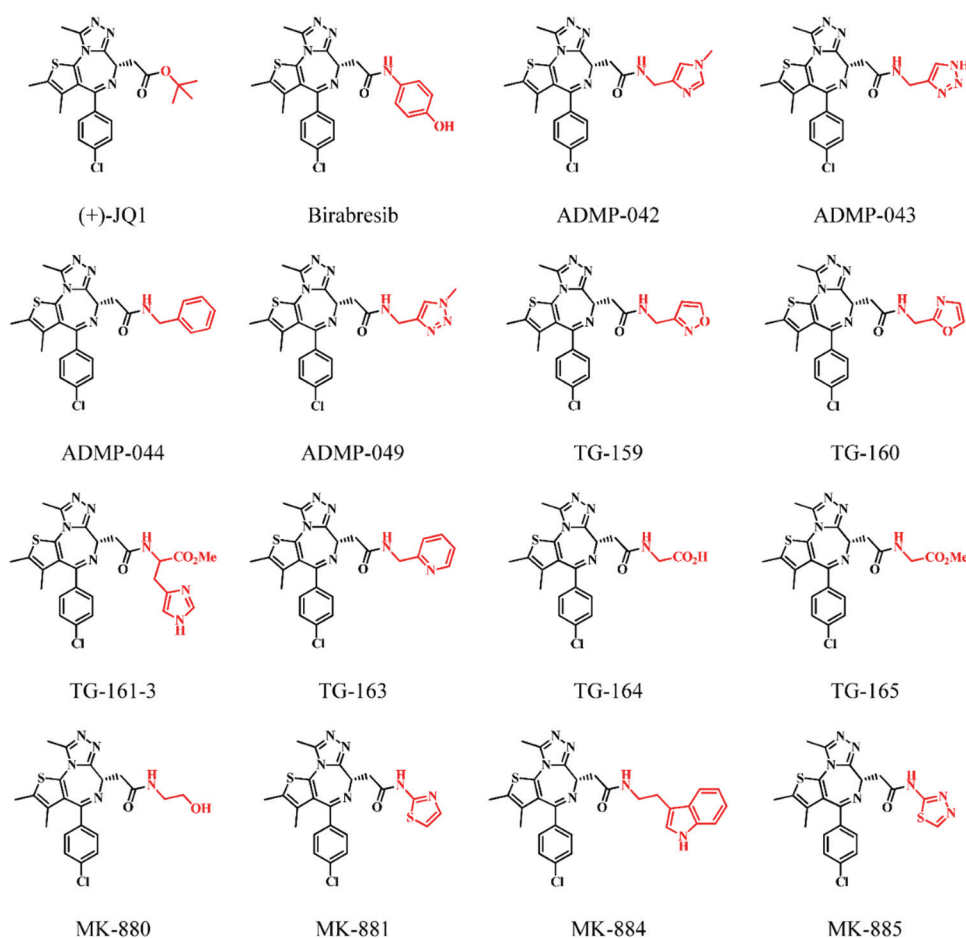


Fig. 1. Chemical structures of the studied (+)-JQ1-derived BIs (parts of molecules marked in red indicate structural differences among the studied BIs).

MATERIALS AND METHODS

In this study, ADMETlab 2.0 was used to predict the ADMET properties of previously characterized BIs *in vitro*.² This platform utilizes a comprehensive ADMET database for predicting various pharmacokinetic and toxicological parameters using QSAR models built from

288,967 chemicals.^{1,19} The predictions are based on six modelling algorithms: random forests (RF), support vector machine (SVM), recursive partitioning regression (RP), partial least square (PLS), naive Bayes (NB) and decision tree (DT).¹ ADMETlab 2.0 includes 17 physicochemical properties, 13 medicinal chemistry properties, 23 ADME properties, 27 toxicity endpoints and 8 toxicophore rules (751 substructures).²⁰ The structures of BIs were drawn using ChemDraw Ultra 12.0, with SMILES input and output retrieved as .csv files. ADMETlab 2.0 was chosen for its accuracy and large database,^{1,20} providing the comprehensive pharmacokinetic predictions that align with the study's needs.

Additionally, MarvinSketch 4.1.13 was used to predict ionization and logD values. Statistical analysis was performed using IBM SPSS Statistics 28.0. The strength and the direction of association between ADMETlab 2.0 output and experimental data were evaluated using bivariate Spearman's correlation, with Spearman's rank correlation coefficients (ρ) and p values reported. Correlation interpretation followed these criteria: $\rho \geq 0.700$ (strong), $0.400 \leq \rho < 0.700$ (moderate) and $\rho < 0.400$ (weak).²¹ The correlations were also assessed using the Wilcoxon signed-rank test, and the results were visualized with box-and-whisker plots. Pharmacokinetic parameters were represented by median values and interquartile ranges (*IQRs*).

A sample of sixteen compounds was selected to balance dataset robustness with detailed analysis. This structurally diverse set allows evaluation of pharmacokinetic properties and their relationship to structural variation. The focused nature of the study provides detailed data for further research.

To ensure the clarity and the consistency throughout this study, we introduce a specific notation: results obtained through computational predictions, referred to as *in silico*, are denoted with the superscript "IS" (e.g., predicted pharmacokinetic parameter^{IS}). Similarly, the results derived from experimental assays, referred to as *in vitro*, are denoted with the superscript "IV" (e.g., measured pharmacokinetic parameter^{IV}). This notation is applied consistently throughout the text to provide a clear and immediate distinction between the two types of datasets. However, in tables where the data are already explicitly classified into *in silico* and *in vitro* groups, the superscripts "IS" and "IV" are omitted to maintain visual simplicity.

RESULTS AND DISCUSSION

Absorption

A total of seven pharmacokinetic parameters relevant for absorption were predicted using ADMETlab 2.0: three parameters predicting the extent of absorption – *HIA prob.*^{IS}, *F(20%)*^{IS} and *F(30%)*^{IS}, two parameters predicting the permeability – *Caco-2 perm.*^{IS} and *MDCK perm.*^{IS}, and two parameters predicting whether the studied BIs act as P-gp substrates or inhibitors – *P-gp sub.*^{IS} and *P-gp inh.*^{IS}, respectively. The obtained *in silico* and *in vitro* results regarding absorption are presented in Tables I and II.

Heatmap is based on an *in silico* empirical decision. The cells marked in green, yellow or red denote excellent, medium or poor results, respectively.²²

The extent of the human intestinal absorption (*HIA*) was reported through the probabilities (*HIA prob.*^{IS} values) of the tested compounds to have *HIA* below 30 %. Hence, the lower the *HIA prob.*^{IS}, the higher the intestinal absorption. These probabilities ranged from 0.004 to 0.047. Thirteen out of sixteen tested candidates had *HIA prob.*^{IS} below 0.010, appropriate for orally administered drugs. The

compound MK-881 showed the best *HIA prob.*^{IS} of 0.004, similar to birabresib as the reference drug. Interestingly, the lowest value of intestinal absorption was predicted for (+)-JQ1, whose *HIA prob.*^{IS} was as high as 0.047.

TABLE I. Absorption-related *in silico* results

Compound	<i>HIA prob.</i> ^a	<i>F</i> (20%) ^b	<i>F</i> (30%) ^c	<i>Caco-2 perm.</i> ^d	<i>Caco-2 perm. calc.</i> , nm s ⁻¹	<i>MDCK perm.</i> ^e , nm s ⁻¹	<i>P-gp sub.</i> ^f	<i>P-gp inh.</i> ^g
(+)-JQ1	0.047	0.002	0.022	-4.475	334.97	215.99	0.006	0.001
Birabresib	0.004	0.001	0.001	-4.787	163.31	187.98	0.714	0.001
ADMP-042	0.007	0.001	0.218	-4.656	220.80	268.83	0.812	0
ADMP-043	0.007	0.001	0.002	-4.826	149.28	171.63	0.992	0
ADMP-044	0.005	0.001	0.001	-4.703	198.15	182.99	0.033	0.002
ADMP-049	0.018	0.002	0.009	-4.590	257.04	263.26	0.860	0
TG-159	0.007	0.001	0.001	-4.616	242.10	195.39	0.243	0
TG-160	0.006	0.001	0.001	-4.615	242.66	259.52	0.129	0
TG-161-3	0.006	0.001	0.003	-4.718	191.43	171.08	0.989	0
TG-163	0.005	0.001	0.001	-4.672	212.81	235.96	0.043	0.001
TG-164	0.008	0.001	0.001	-4.793	161.06	246.16	0.685	0.001
TG-165	0.014	0.002	0.004	-4.587	258.82	255.60	0.606	0.001
MK-880	0.005	0.002	0.002	-4.512	307.61	276.85	0.730	0
MK-881	0.004	0.001	0.002	-4.696	201.37	253.39	0.285	0
MK-884	0.006	0.001	0.009	-5.026	94.19	164.93	0.430	0.034
MK-885	0.005	0.001	0.002	-4.712	194.09	277.64	0.936	0

^aThe output values, ranging between 0 and 1, represent the probability that the compound has human intestinal absorption < 30 %. ^bThe output values, ranging between 0 and 1, represent the probability that the compound has bioavailability < 20 %. ^cThe output values, ranging between 0 and 1, represent the probability that the compound has bioavailability < 30 %. ^dOptimal is > -5.15. ^eLow permeability: < 20 nm s⁻¹; medium permeability: 20–200 nm s⁻¹; high permeability: > 200 nm s⁻¹. ^fThe output values, ranging between 0 and 1, represent the probability that the compound is a P-glycoprotein substrate. ^gThe output values, ranging between 0 and 1, represent the probability that the compound is a P-glycoprotein inhibitor

The last two parameters used to predict the extent of absorption were *F*(20%)^{IS} – the probability of a drug having oral bioavailability below 20 % and *F*(30%)^{IS} – the probability of a drug having oral bioavailability below 30 %. As for the previous parameter, lower probability values denote higher oral bioavailability. The range of *F*(20%)^{IS} values (0.001–0.002) was narrow, thus not particularly informative for comparing the compounds, although it did suggest that the studied compounds have very low probabilities of exhibiting bioavailabilities below 20 %. In contrast to *F*(20%)^{IS}, *F*(30%)^{IS} highlighted ADMP-042 as the compound with the highest predicted value, indicating the greatest probability of having bioavailability below 30 % among the studied BIs. The extent of the human intestinal absorption (HIA) was reported through the probabilities (*HIA prob.*^{IS} values) of the tested compounds to have HIA below 30 %. Hence, the lower the *HIA prob.*^{IS}, the higher the intestinal absorption. These probabilities ranged from 0.004 to 0.047. Thirteen out of sixteen tested candidates had *HIA prob.*^{IS} below 0.010,

appropriate for orally administered drugs. The compound MK-881 showed the best *HIA prob.*^{IS} of 0.004, similar to birabresib as the reference drug. Interestingly, the lowest value of intestinal absorption was predicted for (+)-JQ1, whose *HIA prob.*^{IS} was as high as 0.047.

TABLE II. *In vitro* permeability data from PAMPA and Caco-2 assay; Avg. *P* – average permeability; Avg. *P_{app} A–B* – average apparent permeability coefficient from apical to basolateral side; Avg. *P_{app} B–A* – average apparent permeability coefficient from basolateral to apical side; *ER* – efflux ratio; *R* – recovery; *SD* – standard deviation

Compound	PAMPA		Caco-2 permeability assay		
	Avg. <i>P</i> ± <i>SD</i> , 10 ^{−6} cm s ^{−1}	<i>R</i> ± <i>SD</i> / %	Avg. <i>P_{app} A–B</i> ± <i>SD</i> nm s ^{−1}	Avg. <i>P_{app} B–A</i> ± <i>SD</i> , nm s ^{−1}	<i>ER</i>
(+)-JQ1	> 1800	59.52±16.79	63.97±11.63	46.67±12.91	0.73
Birabresib	315.13±23.20	95.20±1.42	221.60±67.35	485.39±142.68	2.19
ADMP-042	0	44.20±6.33	9.16±0.39	311.21±92.63	33.96
ADMP-043	2.09±0.68	23.68±1.17	2.50±0.41	28.03±8.54	11.20
ADMP-044	1797.07±277.74	71.66±11.23	42.20±14.58	44.51±13.17	1.05
ADMP-049	14.04±3.37	32.25±2.47	6.17±3.15	246.45±133.88	39.93
TG-159	274.52±41.45	65.06±1.82	150.34±40.42	777.89±185.65	5.17
TG-160	97.17±7.28	61.69±1.68	30.39±7.46	297.37±55.89	9.79
TG-161-3	0	33.64±0.78	7.74±10.38	14.18±5.75	1.83
TG-163	177.45±20.63	74.32±3.59	98.37±9.55	568.43±106.65	5.78
TG-164	0.54±0.93	8.52±0.26	4.51±0.95	7.81±5.20	1.73
TG-165	231.31±53.54	47.45±5.58	63.76±8.53	307.31±35.59	4.82
MK-880	20.80±1.19	31.11±1.22	6.98±0.95	83.32±15.08	11.94
MK-881	544.83±34.50	80.49±1.47	19.10±12.38	41.09±8.55	2.15
MK-884	>1800	90.51±N/D	5.69±1.43	13.79±7.81	2.42
MK-885	533.96±237.40	91.83±4.43	66.46±26.53	180.11±54.68	2.71

The predicted Caco-2 permeability was reported through *Caco-2 perm.*^{IS} with a subsequent calculation to derive *Caco-2 perm. calc.*^{IS}. All candidates showed an acceptable *Caco-2 perm.*^{IS} since all values were greater than −5.15. The compound MK-884 showed the poorest *Caco-2 perm.*^{IS}, followed by ADMP-043 and TG-164, whereas (+)-JQ1 showed the best *Caco-2 perm.*^{IS}. The set of *in silico*- (*Caco-2 perm. calc.*^{IS}, Table I) and *in vitro*-obtained results (Avg. *P_{app} A–B*^{IV}, Table II) regarding Caco-2 permeability were compared through the Wilcoxon signed-ranked test (Fig. S-1A of the Supplementary material to this paper). The median value regarding predicted Caco-2 permeability notably surpassed that determined through *in vitro* assessment with the respective values of 207.09 nm s^{−1} (*IQR* 170.34–253.44 nm s^{−1}) and 24.74 nm s^{−1} (*IQR* 6.37–65.84 nm s^{−1}). Thus, the direct comparison between *in silico*- and *in vitro*-obtained Caco-2 permeability results demonstrated a statistically significant difference (*p* < 0.001). A bar plot with paired data (Fig. S-1B of the Supplementary material) provides a visual representation that more effectively highlights the relationship between *in silico* and *in*

vitro results, offering a clearer perspective on their degree of alignment. In general, all BIs exhibited higher predicted Caco-2 permeabilities compared to those determined *in vitro*.

MDCK permeability was predicted as well and reported through *MDCK perm.^{IS}*. Based on this parameter, the studied compounds were classified as medium (0.2×10^{-5} – 2×10^{-5} cm s⁻¹) or high permeable (above 2×10^{-5} cm s⁻¹). Ten candidates, including (+)-JQ1, had high, whereas six candidates, including birabresib, had medium predicted MDCK permeabilities.

The final two predicted parameters, *P-gp sub.^{IS}* and *P-gp inh.^{IS}*, keep to absorption and predict whether the tested BIs perform as substrates or inhibitors of P-gp, respectively. Tested compounds differed significantly in terms of being P-gp substrates, having *P-gp sub.^{IS}* values from 0.006 to 0.989. This variability might affect drug distribution and therefore requires attention. The highest probability of being a P-gp inhibitor was predicted for MK-884, with *P-gp inh.^{IS}* of 0.034. In contrast, other candidates showed substantially lower *P-gp inh.^{IS}* comparable to birabresib or (+)-JQ1. Interestingly, nine candidates had *P-gp inh.^{IS}* equal to zero.

In vitro assessment of absorption of the studied BIs was performed *via* PAMPA and Caco-2 permeability assay.² A notable dispersion of results was observed, signifying substantial variability in permeabilities of the tested compounds. PAMPA reported the highest permeability for ADMP-044, approximately six times higher than for birabresib. Also, high permeabilities were observed for MK-881 and MK-885, followed by birabresib. Only four compounds (MK-881, MK-884, MK-885 and birabresib) had recovery (*R*) above 80 %.

Table S-I of the Supplementary material provides a detailed overview of the statistical correlations between *in silico*- and *in vitro*-assessed pharmacokinetic parameters relevant to absorption. Moderate negative correlations were noted between *HIA prob.^{IS}* and PAMPA results, *P-gp sub.^{IS}* and Avg. *P^{IV}*, and *P-gp inh.^{IS}* and *ER^{IV}*.

Distribution

The pharmacokinetic parameters relevant for distribution include BBB penetrability (*BBB pen.^{IS}*), plasma protein binding (*PPB^{IS}* and *PPB^{IV}*), volume of distribution (*V_d^{IS}*) and unbound fraction (*F_u^{IS}*). The results obtained from ADMETlab 2.0 and *in vitro* assessments are presented in Table III.

The predicted BBB penetrability showed high values ranging from 0.808 to 0.992, with birabresib having a probability of 0.923. The lowest *BBB pen.^{IS}* was estimated for compound TG-164, likely due to its higher hydrophilicity resulting from the presence of a free carboxylic acid. Despite the high values being marked in red according to the ADMETlab 2.0's empirical decision, BBB penetrable BIs are generally desirable for certain CNS diseases.²³

TABLE III. Representation of *in silico*- and *in vitro*-obtained results regarding distribution; Heatmap is based on an *in silico* empirical decision. Cells marked in green or red denote excellent or poor results, respectively;²² V_d – volume of distribution; F_u – unbound fraction; SD – standard deviation

Compound	<i>In silico</i> -obtained results				<i>In vitro</i> -obtained results
	<i>BBB pen.</i> ^a	<i>PPB</i> ^b , %	V_d^c / L kg ⁻¹	F_u^d / %	<i>PPB</i> ± <i>SD</i> , %
(+)-JQ1	0.987	93.97	1.419	9.76	99.15±0.03
Birabresib	0.923	96.10	1.17	5.97	97.27±0.15
ADMP-042	0.904	82.36	1.674	26.74	98.53±0.40
ADMP-043	0.940	91.87	1.683	13.73	94.77±0.32
ADMP-044	0.991	94.89	0.933	11.07	98.68±0.14
ADMP-049	0.931	91.08	1.686	20.18	97.54±0.04
TG-159	0.971	93.59	1.239	11.02	93.45±0.45
TG-160	0.981	86.44	1.030	23.21	94.08±0.57
TG-161-3	0.937	77.41	1.105	28.23	92.68±0.71
TG-163	0.984	90.26	1.238	17.14	97.21±0.24
TG-164	0.808	85.37	0.876	15.73	85.57±2.50
TG-165	0.992	86.02	1.478	22.58	91.03±1.46
MK-880	0.985	79.77	1.607	24.88	86.90±0.67
MK-881	0.985	91.96	1.136	11.16	98.24±0.14
MK-884	0.983	95.93	1.210	6.92	99.59±0.03
MK-885	0.983	93.57	1.248	7.87	97.22±0.37

^aThe output values, ranging between 0 and 1, represent the probability that the compound is blood-brain barrier penetrable. ^bOptimal is ≤90 %. ^cOptimal is from 0.04 to 20 L kg⁻¹. ^dLow is <5 %, medium is 5–20 % and high is >20 %

The *in vitro* experiments revealed higher affinities for 14 out of 16 tested compounds compared to *in silico* predictions. The Wilcoxon signed-rank test (Fig. S-2A of the Supplementary material) showed a statistically significant difference ($p < 0.001$) between PPB^{IS} and PPB^{IV} , with median values of 91.47 % (*IQR* 85.69–93.78 %) and 97.21 % (*IQR* 92.87–98.46 %), respectively. A bar plot with paired data (Fig. S-2B of the Supplementary material) visually depicts the relationship between *in silico* and *in vitro* results, providing a more intuitive understanding of their degree of alignment.

The V_d^{IS} ranged between 0.876 and 1.686 L kg⁻¹, with TG-164 predicted to have the lowest V_d^{IS} . ADMP-049 demonstrated the highest V_d^{IS} , despite not being classified among the most lipophilic compounds.

Table S-I provides a comprehensive representation of the statistical correlations between *in silico*- and *in vitro*-assessed pharmacokinetic parameters relevant to distribution. Moderate positive correlation was observed between $BBB pen.^{IS}$ and Avg. P^{IV} . Additionally, PPB^{IS} exhibited strong positive correlations with both Avg. P^{IV} and R^{IV} . Moderate positive correlations were also found between *in silico*- and *in vitro*-assessed plasma protein binding values, as well as

between V_d^{IS} and ER^{IV} . Notably, a strong negative correlation was identified between F_u^{IS} and $Avg. P^{IV}$, while F_u^{IS} and R^{IV} demonstrated a moderate negative correlation.

Metabolism and excretion

In silico evaluation of metabolism included predicting the probabilities of the BIs being substrates or inhibitors of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Results are shown in Table IV.

TABLE IV. Probabilities of the studied bis being cyp450 substrates and/or inhibitors; the output values, ranging between 0 and 1, represent the probability that the compound is cytochrome P450 substrate and/or inhibitor

Compound	CYP1A2		CYP2C9		CYP2C19		CYP2D6		CYP3A4	
	Sub.	Inh.	Sub.	Inh.	Sub.	Inh.	Sub.	Inh.	Sub.	Inh.
(+)-JQ1	0.943	0.098	0.115	0.837	0.909	0.635	0.019	0.040	0.959	0.458
Birabresib	0.952	0.173	0.728	0.890	0.744	0.495	0.078	0.399	0.960	0.665
ADMP-042	0.933	0.170	0.057	0.371	0.748	0.251	0.055	0.008	0.956	0.863
ADMP-043	0.889	0.186	0.085	0.430	0.708	0.209	0.032	0.010	0.952	0.662
ADMP-044	0.864	0.143	0.177	0.900	0.856	0.643	0.077	0.069	0.958	0.861
ADMP-049	0.916	0.136	0.066	0.327	0.912	0.228	0.060	0.007	0.956	0.646
TG-159	0.668	0.144	0.096	0.791	0.858	0.391	0.068	0.014	0.956	0.862
TG-160	0.671	0.126	0.067	0.746	0.852	0.382	0.071	0.011	0.957	0.768
TG-161-3	0.893	0.094	0.072	0.669	0.637	0.252	0.014	0.177	0.960	0.932
TG-163	0.626	0.144	0.121	0.639	0.873	0.311	0.070	0.010	0.956	0.863
TG-164	0.377	0.113	0.762	0.095	0.829	0.114	0.073	0.013	0.949	0.079
TG-165	0.644	0.129	0.084	0.448	0.876	0.262	0.071	0.008	0.959	0.750
MK-880	0.778	0.164	0.115	0.232	0.856	0.156	0.057	0.007	0.957	0.450
MK-881	0.979	0.378	0.648	0.821	0.844	0.590	0.043	0.104	0.961	0.799
MK-884	0.961	0.197	0.740	0.942	0.773	0.847	0.076	0.649	0.960	0.956
MK-885	0.979	0.581	0.442	0.686	0.690	0.459	0.021	0.125	0.963	0.833

The predicted metabolic pathway predominantly involves CYP3A4, as all compounds appear to be good substrates for this enzyme. In addition, CYP1A2 was expected to have a significant contribution to metabolic transformations, with most compounds showing high probabilities of being CYP1A2 substrates. However, TG-164 exhibited distinct behaviour, with a lower probability of being a CYP1A2 substrate (0.377) and a much higher probability of being a CYP2C9 substrate (0.762) compared to other BIs. Interestingly, apart from TG-164, all studied BIs seem to be potential inhibitors of CYP3A4. This is an important information for considering potential drug-drug interactions.

Table V presents the pharmacokinetic parameters relevant to elimination: clearances (CL^{IS} and CL_{int}^{IV}) and half-lives ($t_{1/2}^{prob.IS}$, $t_{1/2}^{calc.IS}$ and $t_{1/2}^{IV}$).

CL^{IS} values ranged from 0.851 to 3.344 mL min⁻¹ kg⁻¹. The highest values were reported for (+)-JQ1 (3.291 mL min⁻¹ kg⁻¹) and TG-161-3 (3.344 mL min⁻¹ kg⁻¹), whereas CL^{IS} for birabresib was predicted to be 1.365 mL min⁻¹ kg⁻¹.

TABLE V. Clearances and half-lives of the studied compounds; Heatmap is based on an *in silico* empirical decision. Cells marked in green, yellow, or red denote excellent, medium, or poor results, respectively;²² CL – total clearance; $t_{1/2}$ – half-life; CL_{int} – intrinsic clearance; SD – standard deviation

Compound	<i>In silico</i> -obtained results			<i>In vitro</i> -obtained results	
	CL^a mL min ⁻¹ kg ⁻¹	$t_{1/2} prob.^b$	$t_{1/2} calc. / h$	$CL_{int} /$ mL min ⁻¹ kg ⁻¹	$t_{1/2} \pm SD / h$
(+)-JQ1	3.291	0.117	4.98	51.21	0.41 ± 0.01
Birabresib	1.365	0.172	9.90	12.33	1.69 ± 0.08
ADMP-042	2.352	0.442	8.22	42.52	0.49 ± 0.03
ADMP-043	1.419	0.359	13.70	35.87	0.58 ± 0.02
ADMP-044	1.687	0.105	6.39	329.76	0.06 ± 0.00
ADMP-049	2.708	0.199	7.20	38.79	0.54 ± 0.02
TG-159	1.406	0.184	10.18	60.09	0.35 ± 0.01
TG-160	1.589	0.219	7.49	3.60	5.78 ± 0.47
TG-161-3	3.344	0.434	3.82	4.40	4.72 ± 0.59
TG-163	1.538	0.191	9.30	229.14	0.09 ± 0.01
TG-164	0.851	0.465	11.89	1.84	11.29 ± 2.41
TG-165	1.620	0.307	10.54	19.23	1.08 ± 0.03
MK-880	1.851	0.300	10.03	6.52	3.19 ± 0.13
MK-881	1.562	0.120	8.40	18.86	1.10 ± 0.11
MK-884	2.644	0.108	5.29	79.26	0.26 ± 0.03
MK-885	1.246	0.103	11.57	6.52	3.19 ± 0.13

^aHigh is >15 mL min⁻¹ kg⁻¹, moderate is 5–15 mL min⁻¹ kg⁻¹, and low is <5 mL min⁻¹ kg⁻¹. ^bThe output values, ranging between 0 and 1, represent the probability that the compound has half-life >3 h

The calculated values ranged from 3.82 h (TG-161-3) to 11.89 h (TG-164). Birabresib had $t_{1/2} calc.^{IS}$ of 9.90 h, while it was 4.98 h for (+)-JQ1. In general, the calculated half-lives were greater than those experimentally assessed. In line with this, the Wilcoxon signed-rank test (Fig. S-3A of the Supplementary material) showed a statistically significant difference ($p < 0.001$) between half-lives obtained *in silico* and *in vitro*. The corresponding median values are 8.85 h (*IQR* 6.59–10.45 h) and 0.83 h (*IQR* 0.36–3.19 h). A bar plot with paired data (Fig. S-3B) provides a visual representation that simplifies and makes the understanding of calculated and *in vitro*-assessed half-lives more straightforward. The best agreement was observed for TG-164 (11.89 and 11.29 h, respectively), and very good agreement was noted for TG-160 and TG-161-3.

As already described, a metabolic stability assay using human liver microsomes was employed to assess intrinsic clearances (CL_{int}^{IV}) of the studied BIs.² The obtained results were as follows: ten compounds showed high CL_{int}^{IV} , while three compounds had moderate CL_{int}^{IV} (birabresib, MK-880, MK-885). These

molecules might be considered to have appropriate metabolic stability. By contrast, compounds showing low metabolic stability (*i.e.*, having very high CL_{int}^{IV}) had quite short $t_{1/2}^{IV}$, which ultimately makes them unsuitable for further investigation (ADMP-044 and TG-163).

Table S-I provides a detailed representation of the statistical correlations between pharmacokinetic parameters relevant to metabolism and excretion, obtained from both *in silico* and *in vitro* studies. Summarily, $t_{1/2}^{prob.IS}$ showed strong negative correlations with both Avg. P^{IV} and R^{IV} and a moderate negative correlation with PPB^{IV} . On the other hand, $t_{1/2}^{calc.IS}$ showed a moderate negative correlation with PPB^{IV} .

This study assessed the pharmacokinetic properties of BIs derived from (+)-JQ1 using *in silico* predictions from ADMETlab 2.0, which were subsequently compared with *in vitro* data. An effective *in silico* model would streamline the optimization of Bis' structures and facilitate the selection of candidates for further development, reducing the need for extensive experimental efforts. ADMETlab 2.0 was chosen for its comprehensive database and proven accuracy in predicting ADME properties.^{1,20} While some discrepancies were noted between the predictions and experimental results, the tool still offered valuable insights. These differences highlight the challenges in accurately modelling pharmacokinetics computationally and emphasize the need to validate predictions with experimental data. Rather than detracting from ADMETlab 2.0's utility, the findings suggest areas for improvement in predictive models. The study focused on sixteen structurally diverse compounds, providing a detailed comparison and forming the basis for future research with larger datasets.

Absorption

Newly synthesized BIs are intended for oral administration in contemporary anticancer therapy, requiring sufficient intestinal permeability. A moderate negative correlation was observed between $HIA^{prob.IS}$ (indicating the likelihood of human intestinal absorption being below 30 %) and PAMPA-obtained *in vivo* results (Avg. P^{IV} and R^{IV}). This relationship reflects the shared focus of both methods on gut wall permeability: $HIA^{prob.IS}$ predicts the likelihood of absorption through the gut wall using a computational model, while PAMPA experimentally assesses passive permeability across a lipid membrane in an *in vivo* setting. The observed correlation aligns with the expectation that compounds predicted to have poor absorption by $HIA^{prob.IS}$ are also less likely to exhibit high passive permeability in the PAMPA assay.

In contrast, no significant correlation was found between $F(20\%)^{IS}$ or $F(30\%)^{IS}$ and PAMPA-obtained *in vivo* results (Avg. P^{IV} and R^{IV} , Table S-I). This discrepancy arises from the distinct scope of these methods. Unlike $HIA^{prob.IS}$,

which exclusively evaluates gut wall absorption, $F(20\%)^{IS}$ and $F(30\%)^{IS}$ incorporate presystemic metabolic processes, such as metabolism occurring in the gut wall or liver, to provide a broader estimate of a compound's efficiency in reaching systemic circulation. The absence of correlation with PAMPA-obtained *in vivo* results, which do not account for metabolism, highlights the differences in these methods' focus. Consequently, the moderate negative correlation between $HIA_{prob.}^{IS}$ and Avg. P^{IV} and R^{IV} reflects their mutual emphasis on gut wall permeability. In contrast, the lack of correlation between $F(20\%)^{IS}$ or $F(30\%)^{IS}$ and Avg. P^{IV} and R^{IV} underscores the influence of presystemic metabolism on these broader pharmacokinetic predictions.

No significant correlation was found between *in silico*-predicted and *in vitro*-assessed Caco-2 permeability (Fig. S-1A, Table S-I). This suggests that the *in silico* model may not fully capture the complexity of the *in vitro* conditions, which involve multiple biological processes, such as transporter interactions and efflux mechanisms, that are difficult to simulate accurately. High $P_{app} A-B^{IV}$ values may suggest good permeability, but many BIs exhibited undesirable ER^{IV} s over 3 (Table II), indicating potential active efflux involvement that could significantly impact bioavailability. These findings highlight the limitations of relying solely on *in silico* predictions for permeability assessment, as they may overlook critical biological factors influencing drug absorption. The further refinement of predictive models, considering transporter activity and efflux mechanisms, could help improve their alignment with experimental results and enhance their use in early drug development phases.

No correlation was found between Caco-2 permeability assay obtained Avg. $P_{app} A-B^{IV}$ and MDCK $perm.^{IS}$. The Caco-2 assay assesses intestinal absorption using cells derived from human colon adenocarcinoma, forming tight junctions and mimicking intestinal epithelium characteristics.²⁴ The alternative cell-based permeability assay employs MDCK cells. These exhibit rapid growth and differentiation, significantly shortening the duration of *in vitro* transport studies. Using MDCK cells in permeability-based assays offers an appealing substitute to Caco-2 cells, enabling the enhanced throughput and the expedited completion of transport studies for novel compounds.²⁵ The lack of correlation between *in vitro* assessment of Caco-2 permeability and *in silico* prediction of MDCK permeability could be due to differences in their origins, membrane composition, metabolic activity, and transporter expression.²⁶

A negative correlation between $P-gp_{sub.}^{IS}$ and Avg. P^{IV} (Table S-I) is due to the physicochemical properties of P-gp substrates, which are typically larger, more polar, and have multiple hydrogen bond donors and acceptors.²⁷ These properties hinder passive diffusion across lipid membranes, which PAMPA measures. Thus, PAMPA assesses passive permeability, which is inversely related to P-gp substrate recognition traits.

PAMPA showed variability in Avg. P^{IV} and R^{IV} values among BIs (Table II). The highest absorption was observed for (+)-JQ1, birabresib, ADMP-044, MK-881, MK-884 and MK-885, likely due to their high log D values (Table S-II of the Supplementary material). Combining PAMPA and Caco-2 permeability assay results (Table II) suggests these BIs exhibit favorable permeabilities, with minimal ionization at physiological pH, except for TG-164 (Table S-II).

A negative correlation was observed between $P\text{-gp inh.}^{IS}$ and ER^{IV} (Table S-I), suggesting that compounds more likely to inhibit P-gp tend to have lower ER^{IV} s. In other words, substances that inhibit P-gp-mediated efflux may lead to reduced ER^{IV} s in the Caco-2 permeability assay. This could be due to P-gp inhibitors blocking efflux activity, thereby decreasing the active drug efflux in the Caco-2 assay. Thus, higher $P\text{-gp inh.}^{IS}$ values may result in lower ER^{IV} s. It can be hypothesized that the studied BIs reduce their efflux by inhibiting P-gp.

While no statistically significant correlation between $P\text{-gp sub.}^{IS}$ and ER^{IV} was observed (Table S-I), a trend was noted wherein some compounds with higher $P\text{-gp sub.}^{IS}$ values also exerted higher ER^{IV} values. For instance, as shown in Tables I and II, the compounds such as ADMP-042, ADMP-043, ADMP-049 and MK-880, which exhibited higher $P\text{-gp sub.}^{IS}$ values (0.812, 0.992, 0.860 and 0.730, respectively), were also associated with elevated, double-digit ER^{IV} values (33.96, 11.20, 39.93 and 11.94, respectively). This observation aligns with the hypothesis that the substrates of P-gp may exhibit increased efflux, as reflected by higher ER^{IV} s. However, this relationship was not strong enough to be conclusive. Notably, phase I/II clinical trials have confirmed that new BIs are P-gp substrates but still show favourable absorption rates after oral administration.^{28–32} This discordance suggests that additional factors, beyond P-gp substrate status, influence BIs' absorption.

Distribution

The distribution phase is responsible for delivering a drug to its site of action. For BIs, BBB penetration is crucial, particularly for CNS tumor treatments. Based on $BBB pen.^{IS}$ values (Table III), all studied BIs, including TG-164 (despite its ionized carboxylic acid at physiological pH; Table S-II), are likely to cross the BBB, which in some cases might be desirable as birabresib has been investigated for recurrent glioblastoma multiforme.²³ A moderate positive correlation was observed between $BBB pen.^{IS}$ and Avg. P^{IV} (Table S-I), suggesting that higher passive permeability, as assessed by PAMPA, is associated with an increased probability of BBB penetration. This correlation likely reflects the overlapping physico-chemical properties required for both processes, such as optimal lipophilicity and molecular size. While passive permeability is an important determinant, other mechanisms such as active transport may also influence BBB penetration. For instance, TG-164's predicted BBB penetration, despite its ionized carboxylic acid

moiety at physiological pH, could be attributed to its amide-bound glycine group, which might facilitate LAT-1-mediated transport, similar to what is observed with levodopa.³³

The plasma protein binding plays a critical role in drug distribution. Based on PPB^{IS} values (Table III), ten out of sixteen BIs exhibited high plasma protein binding (more than 90 %), with the highest PPB^{IS} (96.10 %) predicted for birabresib. Generally, greater lipophilicity corresponds to higher plasma protein binding, as hydrophobic compounds preferentially bind to plasma proteins for transport. This relationship was supported by strong positive correlations between PPB^{IS} and both Avg. P^{IV} and R^{IV} (Table S-I), indicating that lipophilic compounds tend to exhibit higher permeability and recovery in the PAMPA assay as well as greater protein binding. Conversely, strong and moderate negative correlations were observed between F_u^{IS} and Avg. P^{IV} , and between F_u^{IS} and R^{IV} , respectively, suggesting that lower unbound fractions are associated with higher permeability and recovery. This highlights the interplay between lipophilicity, protein binding, and permeability. Additional factors, such as acid-base properties and specific protein interactions, should also be considered.

A moderate positive correlation was observed between *in silico*-predicted and *in vitro*-assessed plasma protein binding values using bivariate Spearman's correlation, indicating that the *in silico* model effectively captures relative trends in plasma protein binding among the studied compounds. However, the significant differences between the two datasets, as identified by the Wilcoxon signed-rank test (Fig. S-2A), suggest some systematic discrepancies in their central tendencies. This implies that while the *in silico* model is useful for ranking compounds based on the relative plasma protein binding values, further refinement is needed to enhance its predictive accuracy for absolute values.

The volume of distribution describes the apparent distribution extent of drugs within the body.³⁴ ADMETlab 2.0 predictions place V_d^{IS} values within the optimal range (0.04–20 L kg⁻¹),²² specifically from 0.876 to 1.686 L kg⁻¹. Predicted V_d^{IS} values exceeded the total body water (0.5–0.6 L kg⁻¹),³⁵ suggesting high tissue affinity. Multiplying V_d^{IS} values by 70 kg indicates distribution between 61 and 118 L. A moderate positive correlation was observed between V_d^{IS} and ER^{IV} , suggesting that higher efflux activity, often associated with tissue partitioning, may contribute to greater apparent distribution volumes. For example, TG-164 had a V_d^{IS} of 0.876 L kg⁻¹, which is linked to its low log D , likely due to the free carboxylic acid moiety (Fig. 1). However, its high probability of BBB penetration may be attributed to active transport mechanisms, as discussed earlier, potentially involving its amide-bound glycine group facilitating LAT-1-mediated transport. ADMP-049 had the highest V_d^{IS} (1.686 L kg⁻¹), despite its low log D (1.72; Table III and Table S-II). Overall, predicted V_d^{IS} values align with those from first-in-human BI studies, which report steady-state V_d^{IS} values of approximately 70 L.^{36,37}

Metabolism and excretion

ADMETlab 2.0 predicts the probabilities of compounds being substrates or inhibitors of CYP450 isoenzymes, including CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Table IV). It was predicted that CYP3A4, CYP1A2 and CYP2C19 are dominant in the metabolism of the tested BIs, and that these compounds are also likely CYP3A4 and CYP2C9 inhibitors. These insights are valuable for guiding the future research into (+)-JQ1-derived BIs. The genetic polymorphism of CYP450, particularly CYP2C19,³⁸ can significantly affect drug exposure and clinical outcomes, which is important as CYP2C19 is predicted to play a major role in BI metabolism (Table IV).

Selecting the optimal BI is challenging due to *in silico* results' inability to predict competition among CYP450 isoenzymes for a given compound. Nonetheless, these results align with findings that CYP3A4 is key in producing (+)-JQ1 metabolites *in vitro*.¹⁸ Apart from its role in hepatic clearance, CYP3A4 also participates in first-pass metabolism in the gut, potentially reducing bioavailability, which could apply to the studied BIs.³⁹ The detailed metabolic profiling of the studied BIs might identify active metabolites; for example, molibresib forms two active metabolites via CYP3A4.⁴⁰ Evaluating BIs for CYP450 inhibition or induction is crucial for assessing drug-drug interactions. All BIs, except TG-164, were predicted to inhibit CYP2C9, CYP2C19 and CYP3A4.

Elimination of the compounds studied was evaluated through CL^{IS} , $t_{1/2}^{prob.IS}$ and $t_{1/2}^{calc.IS}$ (Table V). No correlation was found between CL^{IS} and CL_{int}^{IV} , as intrinsic clearance reflects intrinsic organ clearance independent of blood flow or binding.⁴¹ Similarly, there was no correlation between $t_{1/2}^{prob.IS}$ or $t_{1/2}^{calc.IS}$ and $t_{1/2}^{IV}$, potentially due to a non-hepatic component of clearance, such as renal elimination. These discrepancies highlight that calculated half-lives might not fully capture *in vivo* dynamics. Despite these limitations, *in silico* predictions provide a basis for designing new (+)-JQ1-derived BIs.

Based on the comparative analysis of *in silico* and *in vitro* pharmacokinetic profiles, metabolic stability appears to be the most limiting factor for many of the studied BIs. Several compounds exhibited high CL_{int}^{IV} and short $t_{1/2}^{IV}$ values (Table V), suggesting the rapid hepatic metabolism that could compromise systemic exposure and therapeutic efficacy. Notably, metabolism appears to be mediated by multiple CYP450 isoenzymes, especially CYP3A4, which is involved in both hepatic and intestinal first-pass metabolism, as well as CYP1A2, CYP2C9 and CYP2C19 (Table IV). This broad CYP450 involvement raises concerns not only about fast clearance but also about potential drug-drug interactions, especially in polypharmacy contexts like oncology. While some BIs demonstrated promising absorption and distribution profiles, their advancement into preclinical development may be hindered unless metabolic pathways are optimized through structural modifications or formulation strategies. Thus, metabolism – and the complexity of

CYP450-mediated biotransformation – is currently the most critical pharmacokinetic barrier for further development of these compounds.

Among the tested BIs, (+)-JQ1, ADMP-042, ADMP-049, TG-161-3 and MK-884 were predicted to have significantly higher CL^{IS} than birabresib, indicating rapid elimination (Table V). Other BIs, excluding TG-164, had CL^{IS} values comparable to birabresib. MK-880 and MK-885, alongside birabresib, showed moderate CL_{int}^{IV} values, making them suitable for further development. Compounds with low metabolic stability, such as ADMP-044 and TG-163, exhibited high CL_{int}^{IV} and short half-lives (3–6 min), limiting their potential for further research.⁴² Conversely, highly stable compounds like TG-160, TG-161-3 and TG-164 demonstrated relatively long *in vivo* half-lives.

Taking into account the balance of absorption, distribution, metabolism, and excretion parameters, our data suggest that MK-880 and MK-885, along with the reference compound birabresib, are the most promising candidates for further investigation. These compounds exhibited moderate CL_{int}^{IV} values (Table V), indicating acceptable metabolic stability, and had favorable plasma protein binding (Table III), adequate permeability (Tables I and II), and appropriate V_d^{IS} values (Table III). MK-885, in particular, showed a favorable balance between *in silico* and *in vitro* data, including high passive permeability (Table II), moderate $t_{1/2}^{IV}$ values (Table V) and a manageable CYP450 interaction profile (Table IV). Although CYP3A4 involvement was predicted, the overall interaction profile does not suggest an unmanageable risk of drug-drug interactions. While the compounds such as TG-160 and TG-164 demonstrated prolonged $t_{1/2}^{IV}$ (Table V), other limitations – such as high *ER* observed for TG-160 or low permeability (Table II) and high ionization (Table S-II) of TG-164 – may reduce their immediate suitability. Therefore, from a pharmacokinetic standpoint, MK-880 and MK-885 offer the most balanced profiles, supporting their prioritization in further preclinical development.

CONCLUSIONS

This study provides a comprehensive analysis of the pharmacokinetic profiles of (+)-JQ1-derived BIs, expanding on prior work. By comparing *in silico* and *in vitro* data, we evaluated the efficacy of ADMETlab 2.0 as a screening tool for these compounds. This is the first study to explore *in silico/in vitro* correlations for the pharmacokinetics of newly synthesized (+)-JQ1-derived BIs. Our findings revealed a moderate negative correlation between $HIA\ prob.^{IS}$ and PAMPA-obtained *in vivo* results (Avg. P^{IV} and R^{IV}) (Table S1), while a moderate positive correlation was observed for plasma protein binding despite statistically significant differences (Fig. S-2A). Both approaches indicated the plasma protein binding values above 90 % for most compounds (Table III). The predictions of interactions with CYP450 isoenzymes were instrumental in understanding the potential of

drug-drug interactions (Table IV). While discrepancies between CL^{IS} and CL_{int}^{IV} (Table V) were observed, these were reasonable, given the insight CL_{int}^{IV} offers into hepatic clearance. Overall, most compounds exhibited favorable pharmacokinetic characteristics, with MK-880 and MK-885 standing out alongside birabresib for their superior CL_{int}^{IV} values.

We emphasized the importance of integrating *in silico* predictions with experimental data to achieve a more complete understanding of pharmacokinetic profiles. The combination of these approaches enables researchers to validate and refine predictive models, ensuring reliable data applicable to real-world drug development. Early-stage ADME screening, as demonstrated through ADMETlab 2.0, is essential for identifying promising candidates and accelerating their progression through the drug development process. However, the discrepancies between *in silico* and *in vitro* results highlight the need for further refinement of computational predictions.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13160>, or from the corresponding author on request.

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ИЗВОД

КОМПАРАТИВНА АНАЛИЗА ФАРМАКОКИНЕТИЧКИХ ПРОФИЛА ВЕТ ИНХИБИТОРА: УВИД ИЗ *IN SILICO* И *IN VITRO* ПОДАТАКА

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Фармакокинетичка ограничења представљају чест узрок неуспеха у развоју лекова, што чини рану процену фармакокинетичког профила кључном за смањење финансијског ризика и усмеравање оптимизације једињења. Рачунарски алати способни да предвиђају фармакокинетичке особине представљају драгоцен ресурс за одређивање приоритета

кандидата у раним фазама откривања лекова. Ова студија пружа компаративну анализу *in silico* и *in vitro* фармакокинетичких профила ВЕТ (енг. *bromodomain and extraterminal domain*) инхибитора, са посебним освртом на шеснаест једињења изведених из (+)-JQ1 које смо претходно синтетисали. Коришћењем софтверске платформе ADMETLab 2.0, предвиђени су кључни параметри апсорпције, дистрибуције, метаболизма и екскреције (АДМЕ), који су затим упоређени са експериментално добијеним подацима. Уочена је јака корелација у вези са везивањем за плазматске протеине, док су значајна одступања примећена код вредности пермеабилности и клиренса. Анализа, такође, наглашава улогу ензима CYP3A4 као кључног фактора у метаболизму неколико ВЕТ инхибитора. Добијени резултати потврђују корисност рачунарских алата попут ADMETLab 2.0 у раној фармакокинетичкој процени, али и указују на потребу за валидацијом и додатним унапређењем предиктивних модела. Ово истраживање пружа драгоцене увиде у фармакокинетику ВЕТ инхибитора и подржава интеграцију рачунарских и експерименталних приступа у процесу откривања лекова.

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SUPPLEMENTARY MATERIAL TO
**Comparative *in silico/in vitro* analysis of pharmacokinetic
profiles of BET inhibitors**

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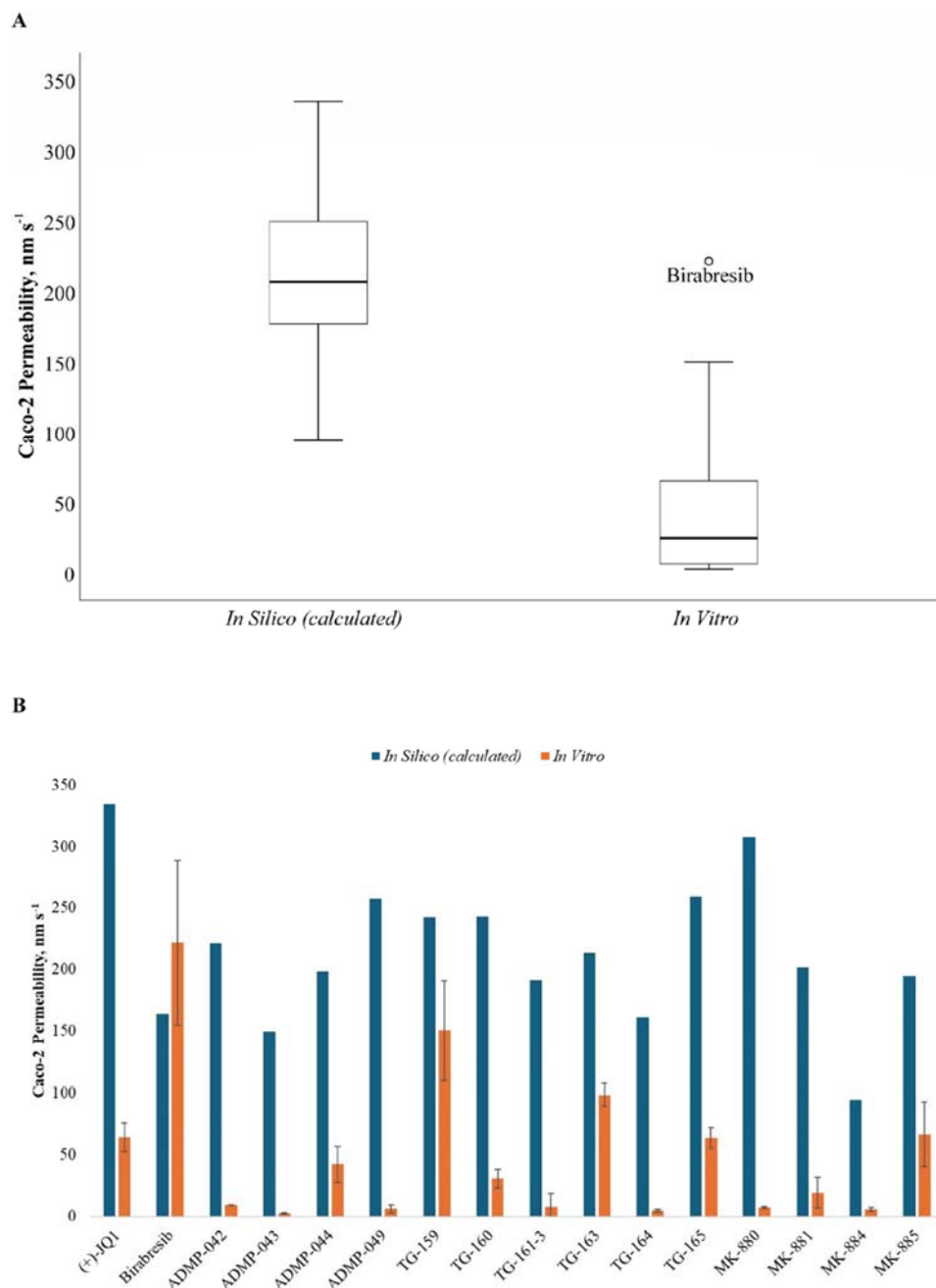


Fig. S-1. (A) Results of the Wilcoxon signed-rank test comparing *in silico* (*Caco-2 perm. calc.*) and *in vitro* (*Avg. P_{app} A-B*) *Caco-2* permeabilities, demonstrating a statistically significant difference. (B) Bar plot with paired data emphasizing the differences between *in silico* and *in vitro* *Caco-2* permeability results.

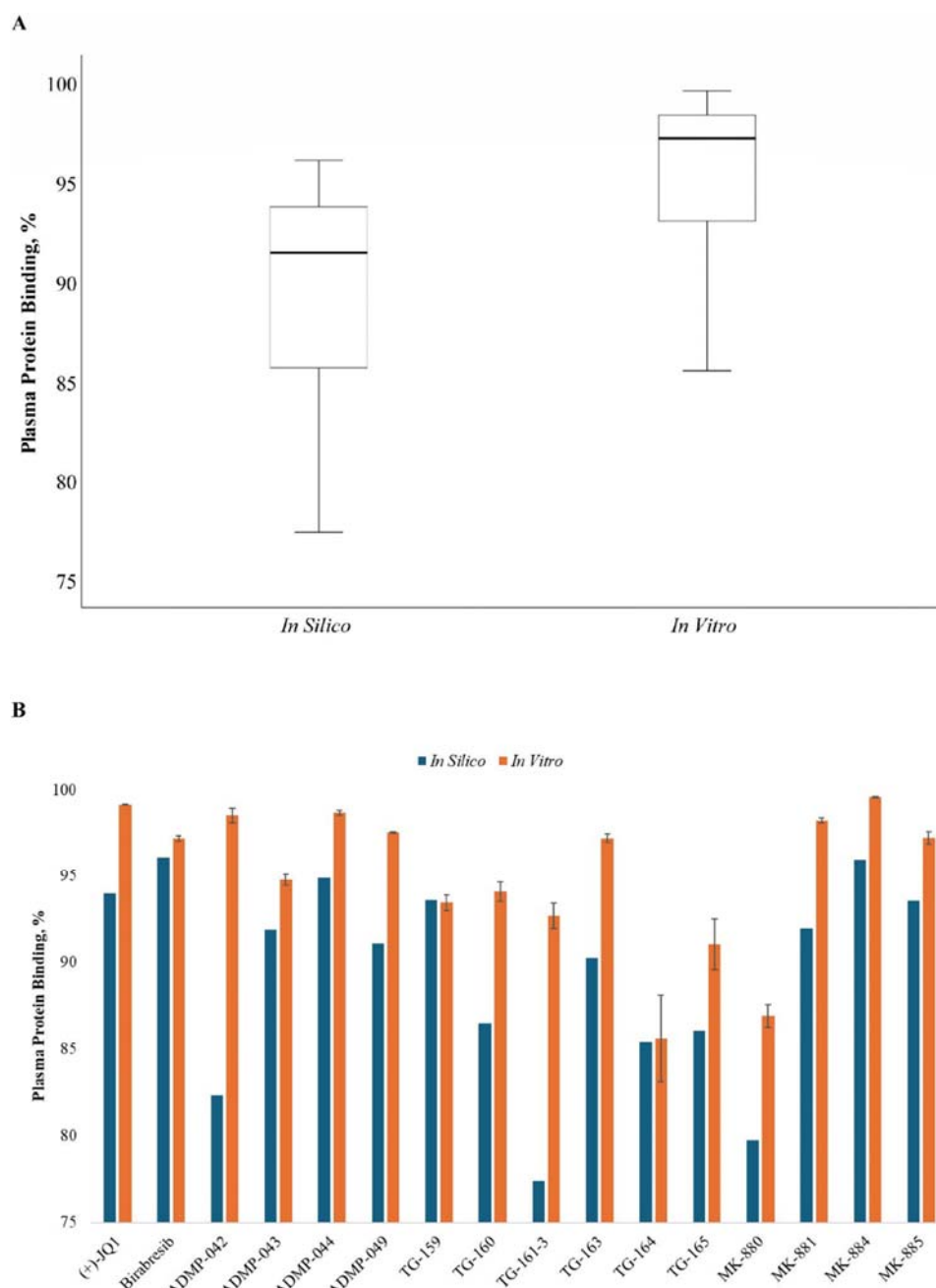


Fig. S-2. (A) Results of the Wilcoxon signed-rank test indicating a statistically significant difference between *in silico*-predicted and *in vitro*-assessed plasma protein binding values. (B) Bar plot with paired data comparing *in silico* and *in vitro* plasma protein binding values, illustrating the relation between the two data sets.

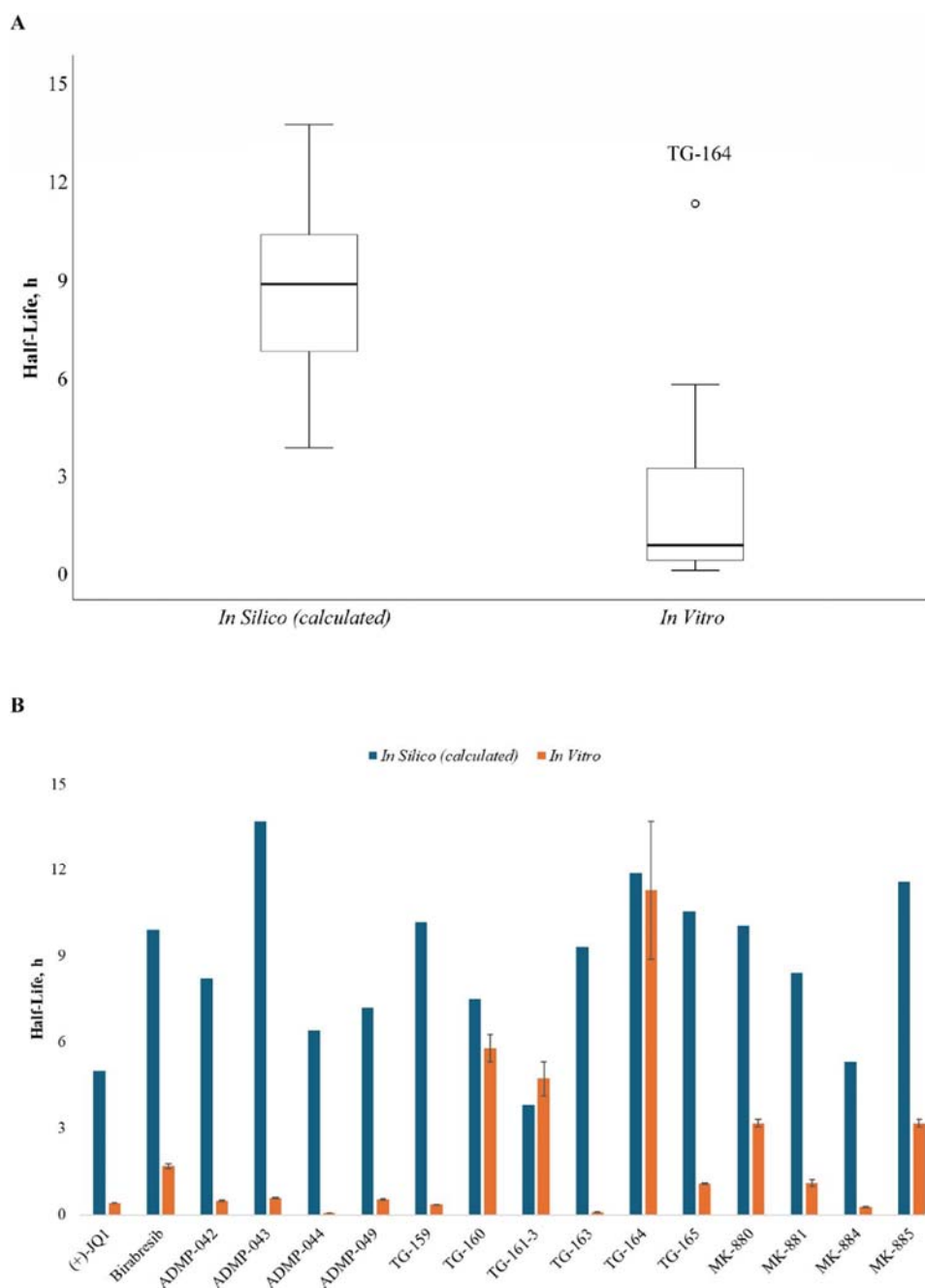


Fig. S-3. (A) Results of the Wilcoxon signed-rank test indicating a statistically significant difference between calculated and *in vitro*-assessed half-lives. (B) Bar plot with paired data emphasizing the differences between calculated and *in vitro*-assessed half-lives.

TABLE S-I. Statistical Correlations Between *In Silico*- and *In Vitro*-Obtained Results

		<i>In Vitro</i> -Obtained Results							
		ABSORPTION				DISTRIB.	METABOLISM & EXCRETION		
		<i>Avg. P</i>	<i>R</i>	<i>Avg. P_{app} A-B</i>	<i>Avg. P_{app} B-A</i>	<i>ER</i>	<i>PPB</i>	<i>CL_{int}</i>	<i>t_{1/2}</i>
		<i>P-gp</i>	<i>MDC</i>	<i>aco-F</i>	<i>(20% HI)</i>	<i>prob.</i>			
<i>In Silico</i> -Obtained Results	ABSORPTION	$\rho = -0.581$	$\rho = -0.618$	$\rho = -0.304$	$\rho = -0.067$	$\rho = \mathbf{0.122}$	$\rho = \mathbf{-0.094}$	$\rho = 0.076$	$\rho = -0.076$
		$\rho = 0.042$	$\rho = 0.011$	$\rho = 0.252$	$\rho = 0.805$	$\rho = \mathbf{0.652}$	$\rho = \mathbf{0.729}$	$\rho = 0.779$	$\rho = 0.779$
		$\rho = -0.108$	$\rho = -0.376$	$\rho = -0.063$	$\rho = 0.094$	$\rho = \mathbf{0.188}$	$\rho = \mathbf{-0.094}$	$\rho = 0.031$	$\rho = -0.031$
		$\rho = 0.713$	$\rho = 0.152$	$\rho = 0.818$	$\rho = 0.729$	$\rho = \mathbf{0.486}$	$\rho = \mathbf{0.729}$	$\rho = 0.908$	$\rho = 0.908$
		$\rho = -0.436$	$\rho = -0.216$	$\rho = -0.338$	$\rho = -0.190$	$\rho = 0.169$	$\rho = 0.398$	$\rho = 0.155$	$\rho = -0.155$
		$\rho = 0.119$	$\rho = 0.421$	$\rho = 0.200$	$\rho = 0.480$	$\rho = 0.531$	$\rho = 0.127$	$\rho = 0.566$	$\rho = 0.566$
		$\rho = 0.053$	$\rho = -0.194$	$\rho = 0.303$	$\rho = 0.491$	$\rho = 0.279$	$\rho = -0.091$	$\rho = 0.091$	$\rho = -0.091$
		$\rho = 0.658$	$\rho = 0.471$	$\rho = 0.254$	$\rho = 0.053$	$\rho = 0.295$	$\rho = 0.737$	$\rho = 0.737$	$\rho = 0.737$
		$\rho = 0.053$	$\rho = -0.194$	$\rho = 0.303$	$\rho = 0.491$	$\rho = 0.279$	$\rho = -0.091$	$\rho = 0.091$	$\rho = -0.091$
		$\rho = 0.658$	$\rho = 0.471$	$\rho = 0.254$	$\rho = 0.053$	$\rho = 0.295$	$\rho = 0.737$	$\rho = 0.737$	$\rho = 0.737$
		$\rho = 0.004$	$\rho = -0.132$	$\rho = 0.082$	$\rho = 0.359$	$\rho = 0.491$	$\rho = -0.215$	$\rho = -0.350$	$\rho = 0.350$
		$\rho = 0.988$	$\rho = 0.625$	$\rho = 0.762$	$\rho = 0.172$	$\rho = 0.053$	$\rho = 0.425$	$\rho = 0.184$	$\rho = 0.184$
	DISTRIBUTION	$\rho = -0.550$	$\rho = -0.379$	$\rho = -0.462$	$\rho = -0.206$	$\rho = 0.412$	$\rho = -0.308$	$\rho = -0.468$	$\rho = 0.468$
		$\rho = 0.042$	$\rho = 0.147$	$\rho = 0.072$	$\rho = 0.444$	$\rho = 0.113$	$\rho = 0.249$	$\rho = 0.068$	$\rho = 0.068$
		$\rho = 0.338$	$\rho = 0.317$	$\rho = 0.139$	$\rho = -0.168$	$\rho = -0.558$	$\rho = 0.327$	$\rho = 0.436$	$\rho = -0.436$
		$\rho = 0.237$	$\rho = 0.232$	$\rho = 0.609$	$\rho = 0.533$	$\rho = 0.025$	$\rho = 0.217$	$\rho = 0.092$	$\rho = 0.092$
		$\rho = 0.632$	$\rho = 0.284$	$\rho = 0.293$	$\rho = 0.012$	$\rho = -0.259$	$\rho = 0.172$	$\rho = 0.362$	$\rho = -0.362$
		$\rho = 0.015$	$\rho = 0.286$	$\rho = 0.271$	$\rho = 0.965$	$\rho = 0.332$	$\rho = 0.523$	$\rho = 0.168$	$\rho = 0.168$
		$\rho = 0.790$	$\rho = 0.703$	$\rho = 0.403$	$\rho = 0.068$	$\rho = -0.376$	$\rho = 0.656$	$\rho = 0.494$	$\rho = -0.494$
		$\rho < 0.001$	$\rho = 0.002$	$\rho = 0.122$	$\rho = 0.803$	$\rho = 0.151$	$\rho = 0.006$	$\rho = 0.052$	$\rho = 0.052$
		$\rho = -0.257$	$\rho = -0.300$	$\rho = -0.126$	$\rho = 0.312$	$\rho = 0.685$	$\rho = 0.082$	$\rho = 0.244$	$\rho = -0.244$
		$\rho = 0.374$	$\rho = 0.259$	$\rho = 0.641$	$\rho = 0.240$	$\rho = 0.003$	$\rho = 0.762$	$\rho = 0.362$	$\rho = 0.362$
		$\rho = -0.739$	$\rho = -0.665$	$\rho = -0.371$	$\rho = 0.032$	$\rho = 0.456$	$\rho = -0.491$	$\rho = -0.312$	$\rho = 0.312$
		$\rho = 0.003$	$\rho = 0.005$	$\rho = 0.158$	$\rho = 0.905$	$\rho = 0.076$	$\rho = 0.053$	$\rho = 0.240$	$\rho = 0.240$
	METABOLISM & EXCRETION	$\rho = -0.359$	$\rho = -0.224$	$\rho = -0.276$	$\rho = -0.197$	$\rho = 0.035$	$\rho = 0.365$	$\rho = 0.271$	$\rho = -0.271$
		$\rho = 0.208$	$\rho = 0.405$	$\rho = 0.300$	$\rho = 0.464$	$\rho = 0.897$	$\rho = 0.165$	$\rho = 0.311$	$\rho = 0.311$
		$\rho = -0.915$	$\rho = -0.803$	$\rho = -0.488$	$\rho = -0.068$	$\rho = 0.365$	$\rho = -0.638$	$\rho = -0.432$	$\rho = 0.432$
		$\rho < 0.001$	$\rho < 0.001$	$\rho = 0.055$	$\rho = 0.803$	$\rho = 0.165$	$\rho = 0.008$	$\rho = 0.094$	$\rho = 0.094$
		$\rho = 0.029$	$\rho = -0.176$	$\rho = 0.006$	$\rho = 0.159$	$\rho = 0.268$	$\rho = -0.571$	$\rho = -0.303$	$\rho = 0.303$
		$\rho = 0.923$	$\rho = 0.513$	$\rho = 0.983$	$\rho = 0.557$	$\rho = 0.316$	$\rho = 0.021$	$\rho = 0.254$	$\rho = 0.254$

Cells in green, yellow, and red denote strong ($\rho \geq 0.700$ and $\rho \leq -0.700$), moderate ($0.400 \leq \rho < 0.700$ and $-0.400 \geq \rho > -0.700$), or weak correlation ($-0.400 < \rho < 0.400$), respectively, for $p < 0.050$.

TABLE S-II. Predicted Degrees of Ionization and $\log D$ Values

Compound	Degree of Ionization (pH = 7,4)	$\log D$ (pH = 7,4)
(+)-JQ1	99.90% non-ionized	3.64
Birabresib	99.06% non-ionized	4.03
ADMP-042	95.09% non-ionized	2.01
ADMP-043	96.25% non-ionized	1.76
ADMP-044	99.88% non-ionized	3.93
ADMP-049	99.93% non-ionized	1.72
TG-159	99.96% non-ionized	2.59
TG-160	99.96% non-ionized	2.08
TG-161-3	87.96% non-ionized	1.68
TG-163	99.87% non-ionized	2.77
TG-164	99.92% ionized	-1.94
TG-165	99.92% non-ionized	1.73
MK-880	99.91% non-ionized	1.41
MK-881	99.65% non-ionized	3.57
MK-884	99.86% non-ionized	4.28
MK-885	99.13% non-ionized	2.23



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In vitro* antioxidant activity of nicotinic acid hydrazides: Experimental and theoretical study

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Abstract: The formation of reactive oxygen species (ROS) in the human body can lead to cell damage. Despite the body's natural defences, including the enzyme superoxide dismutase, novel antioxidant small molecules are needed. In this work, *in vitro* antioxidant activity of seven nicotinic acid amides (NcAs) derived from nicotinic acid and *mono*-thiocarbohydrazones was investigated using DPPH, ABTS, CUPRAC and TAC assays. The compounds exhibited IC_{50} values between 0.202 to 1.297 mM in the DPPH assay, which improved to 0.114–0.638 mM upon the addition of water in the system. In the ABTS assay, IC_{50} values ranged from 0.107 to 0.365 mM. CUPRAC and FRAP assays indicated reducing antioxidant power of 1.973–4.650 and 1.564–3.472 mM L⁻¹, respectively. Moderate antioxidant activity was also observed in the phosphomolybdenum assay for total antioxidant capacity. The density functional theory calculations revealed that the S–H bond of thioenol 1 tautomer, with a low bond dissociation enthalpy (*BDE*) of around 270 kJ mol⁻¹, is the favourable site for hydrogen atom transfer (HAT) to reactive free radicals. Additionally, all compounds exhibited high stability constants with Fe²⁺ and Fe³⁺ ($K_s \approx 10^8$), forming complexes with ML stoichiometry.

Keywords: biological activity; ROS; DFT; metal complexes; imino derivatives; pyridinecarboxylic acid derivatives.

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*This article is dedicated to the memory of our esteemed colleague Jasmina Nikolić whose scholarly contributions and dedication greatly enriched this work.
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INTRODUCTION

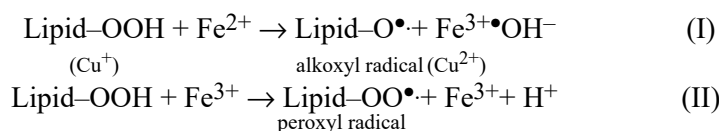
The human body is a complex system involving numerous processes, including metabolism, cellular repair and the transport of vital substances through the bloodstream.¹ Oxygen plays the main part in these processes, particularly in the production of adenosine triphosphate (ATP), which is essential for energy transfer within cells. ATP facilitates oxygen transport by haemoglobin and supports respiration.² While oxygen is crucial for cellular metabolism, its metabolic transformation into reactive oxygen species (ROS) presents potential downsides. ROS can lead to cell inflammation, cell death³ and oxidative stress, which have both harmful and beneficial effects on the body. At regulated levels, ROS can inhibit tumour growth and neutralize pathogens. However, excessive ROS levels contribute to oxidative stress, which is linked to numerous diseases including cancer, cardiovascular disease and neurodegenerative disorders. Alongside ROS, reactive nitrogen species (RNS)⁴ also play significant role in the oxidative stress. The most abundant ROS and RNS species include superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and nitric oxide ($NO^{\bullet}$) radicals.

An example of free radical formation is the interaction of gamma radiation with water in tissue cells. The energy absorbed splits the O–H bonds homolytically, producing hydrogen atom (H) and OH-radical.⁵ ROS and RNS can be generated both enzymatically and non-enzymatically. Enzymatic production involves enzymes such as peroxidase, NADPH oxidase, xanthine oxidase and others. Non-enzymatic formation occurs during oxidative phosphorylation in the mitochondria, where ATP is produced.⁴ When mitochondria are dysfunctional, there is an increased production of $O_2^{\bullet-}$, leading to the elevated levels of ROS and an increased risk of DNA mutations, which accelerate aging.⁶

Superoxide dismutase (SOD) is a key enzyme that reduces the harmful effects of free oxygen radicals. It neutralizes superoxide radicals by converting them to H_2O_2 and oxygen. However, H_2O_2 can be toxic to cells, as it can damage DNA through the reactions involving Fe^{2+}/Fe^{3+} .¹ SOD plays an important role in preventing diseases such as cancer, inflammatory conditions and rheumatoid arthritis, and current research focuses on its potential use in medical treatments.⁷

The human body system defends against ROS through three main mechanisms: 1) the use of antioxidants, 2) the action of SOD and 3) preventing the formation of free radicals with metal-ion binding proteins. Antioxidants can be either lipophilic or hydrophilic. Many fruits and vegetables contain hydrophilic antioxidants, such as vitamins and minerals, which are easily absorbed and eliminated from the body. In contrast, lipophilic antioxidants require fat for absorption and are not as rapidly eliminated.

Transition metals such as Fe^{2+}/Fe^{3+} and Cu^+/Cu^{2+} can react with O_2^- and H_2O_2 to produce $HO^{\bullet}$. When these metals interact with lipids, they degrade peroxides into peroxy and alkoxy radicals, further contributing to oxidative damage.⁵



The redox activity of iron ions contributes to accumulation of ROS, and excessive iron can lead to cell death.⁸ As a result, many studies have focused on iron chelation using various natural products. For instance, ginger contains curcumin, which chelates Fe^{2+} and prevents the formation of Fe^{2+} -ferrozine complex. This process help mitigate iron overload.⁹ Moreover, the complexation of metal ions such as Al(III), Zn(II), Cu(II) and Fe(II) with natural antioxidants like flavonoids has proven to be an effective strategy for developing novel compounds with enhanced anti-inflammatory, anticancer and antioxidative properties compared to their uncomplexed forms. In particular, superior antioxidative activity of these metal complexes is often attributed to their ability to activate SOD.¹

Thiosemicarbazones are well-known metal chelators due to the presence of sulphur and nitrogen atoms, which enable strong coordination with metal ions. These metal complexes exhibit potent anticancer activity¹⁰ along with other valuable biological properties, such as antimicrobial effects.¹¹ Among various thiosemicarbazones, phenyl-thiosemicarbazones have been extensively studied.¹²

In this study, we report the antioxidant activity of novel amides synthesized *via* condensation between nicotinic acid and mono-thiocarbohydrazones. The antioxidant activity was assessed using several assays, including DPPH, ABTS, CUPRAC, FRAP and TAC methods. Moreover, we determined the stoichiometry and stability constants of complexes formed between Fe^{2+} and Fe^{3+} and the synthesized ligands. Finally, quantum chemical calculations were conducted to investigate the structure-antioxidant relationship of these compounds.

EXPERIMENTAL

Chemicals

All chemicals and solvents for synthesis were obtained from Sigma Aldrich and Merck and used without additional purification. Sodium phosphate, ammonium molybdate tetrahydrate, dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazil (DPPH $\cdot$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Aldrich. Neocuproine was obtained from Acros Organics. Copper(II) chloride dihydrate and ammonium acetate were purchased from Merck. All chemicals and reagents for antioxidant analysis were of commercial quality and used without further purification.

Structure of nicotinic acid amides with monothiocarbohydrazones (NcA)

Synthesis and structural characterization of compounds 1–7 (Fig. 1) were described in our previous work.¹³ The analytical and spectral data for all synthesized compounds are given in the Supplementary material to this paper.

Computational methods

Bond dissociation enthalpy (BDE) is calculated by optimizing the geometries of a molecule, corresponding radical and H atom at the B3LYP/6-311++g(d,p) level of theory. The unrestricted DFT calculations are performed for radicals, with multiplicity set to 2. The frequency calculations confirmed that there are no negative vibrational frequencies, so each geometry obtained corresponds to the minimum. The zero point energy correction is applied to the molecule and corresponding radical. All calculations were done in Gaussian 09, version B.01.¹⁴

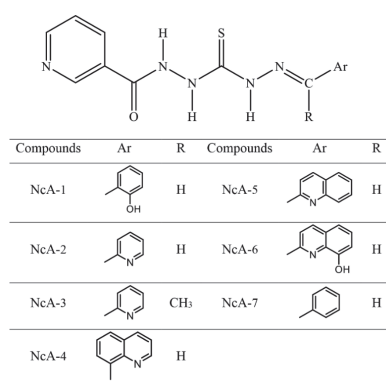


Fig. 1. Structures of synthesized NcA derivatives.

BDE was calculated by the following equation:

$$BDE = H_{\text{radical}} + H_{\text{H_atom}} - H_{\text{molecule}} \quad (1)$$

Herein, H_{radical} , $H_{\text{H_atom}}$, H_{molecule} represent enthalpies of radical species, H atom and ground state, respectively.

Antioxidant methods

DPPH• and ABTS free radical scavenging assays were conducted by measuring the changes in the absorbance of DPPH• at 517 nm and ABTS•⁺ at 734 nm, as described in the literature.¹⁵ Ascorbic acid was used as a control and the scavenging activity was calculated as:

$$\text{Scavenging activity} = 100 \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \quad (2)$$

where A_{sample} and A_{control} refer to the absorbances at 517 and 734 nm of DPPH• and ABTS•⁺ in the samples and control solutions, respectively.

The cupric ion reducing antioxidant capacity (CUPRAC) assay method was prepared according to the method¹⁶ by measuring the changes in the absorbance at 450 nm. Trolox was used as a control. The results were reported as Trolox equivalent antioxidant capacities (TEAC) and calculated as ratio of molar absorptivity of each tested compounds and molar absorptivity of Trolox using the following equation:

$$TEAC = \frac{\epsilon_{\text{sample}}}{\epsilon_{\text{Trolox}}} \quad (3)$$

where ϵ_{sample} and ϵ_{Trolox} are molar absorptivities of sample and Trolox solutions, respectively.

Ferric reducing antioxidant power assay (FRAP) method was prepared according to the methods given in reference¹⁷ by measuring the changes in the absorbance at 593 nm. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was used as a control to construct a calibration curve (0.1, 0.4, 0.8, 1.0, 1.12, 1.5 mM) and the results are expressed as mM Fe^{2+} /L of the compounds.

Phosphomolybdenum assay, which defines the total antioxidant capacity (TAC), was performed according to the method described elsewhere.¹⁷ The absorbance at 695 nm was recorded on Shimadzu 1800 UV/Vis spectrophotometer. The antioxidant capacity was expressed as the mass equivalents of ascorbic acid.

Mole ratio method – determination of stability constant

The stoichiometry and stability constants of the metal complexes formed between Fe^{2+} and Fe^{3+} and nicotinic acid amides (NcAs) were determined according to the procedure reported in the literature.¹⁸ Stock solutions of metal ions (1.0×10^{-2} M) were prepared using Fe^{2+} ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and Fe^{3+} ($(\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O})$). Stock solutions of compounds NcA1–NcA7 were prepared in a mixture of deionized water (10 ml) and DMSO (2 ml). Working solutions of compounds were prepared within a concentration range that ensured the absorbance of 0.5–0.6 at λ_{max} . Following the procedure, 3 ml of the compound solution was mixed with 2 μl of the stock metal solution, and the reaction mixture was mixed using the magnetic stirrer for 10 min at 200 rpm. After the equilibration, the UV spectra were recorded. The compounds were titrated with the stock metal solution until the UV spectra stabilised, indicating the endpoint of the reaction.

RESULTS AND DISCUSSION

Antioxidant capacity of nicotinic acid amides (NcAs)

The antioxidant activity of nicotinic acid amide derivatives was investigated *in vitro* using various assays, including DPPH, ABTS, CUPRAC, FRAP and TAC. Ascorbic acid and Trolox were used as standard antioxidants. The tested derivatives exhibited varying degrees of antioxidant activity, ranging from weak to strong, with the results summarized in Table I.

TABLE I. IC_{50} values (mM) determined by DPPH and ABTS methods, and the results of CUPRAC, TAC and FRAP assays

Compound	DPPH	DPPH DMSO+H ₂ O	ABTS	CUPRAC TEAC	TAC Phosphomolybdenum test	FRAP mM/L
NcA-1	0.232	0.138	0.365	3.791	0.490	3.321
NcA-2	0.202	0.114	0.107	4.650	0.484	3.472
NcA-3	0.186	0.241	0.279	1.973	0.754	1.564
NcA-4	0.215	0.283	0.147	4.373	0.670	3.460
NcA-5	0.952	0.638	0.152	3.894	0.697	2.278
NcA-6	1.297	0.281	0.112	4.516	0.897	3.470
NcA-7	1.584	0.135	0.142	4.138	0.727	3.454
Ascorbic acid	0.094	0.211	0.150	—	—	—
Trolox	—	—	—	1.000	—	—

In the DPPH assay, compound NcA-3 demonstrated the highest activity among the derivatives, with an IC_{50} value of 0.183 mM (Table I), although this activity was still lower than that of ascorbic acid ($IC_{50} = 0.0939$). While all derivatives showed lower antioxidant activity compared to the standards, the derivatives displayed notable antioxidant potential in the DMSO/water, ranging from 0.114 to 0.638 mM at the same concentration. Specifically, compounds NcA-1, NcA-2 and NcA-7 were more potent toward DPPH• than ascorbic acid. As reported by Assaleh *et al.*¹⁹ the addition of water to DMSO led to increased antioxidative activity of thiocarbohydrazones in the DPPH assay. This effect was ascribed to the *E/Z* isomerism around the imine bond, thione/thiol tautomerism, and hydrogen bonding interactions between the studied compounds and solvent.

The antioxidant activity of NcAs varied across the different assays used (Table I). In the ABTS assay, all compounds effectively scavenged ABTS•⁺, with NcA-1 and NcA-3 showing comparatively lower activities. On the other hand, higher activities – *i.e.*, lower IC_{50} values – were observed for the ABTS assay compared to the DPPH and DPPH (DMSO + H₂O) assays. Similar activities of NcA-2 and NcA-4–NcA-7 derivatives were identified by ABTS method (Table I).

The antioxidant activity is influenced by the presence and position of substituents, as well as the nature of electron-accepting and electron-donating groups and the inclusion of heteroatoms in the aromatic core. In particular, the presence of a hydroxyl group in the ortho position on the quinoline ring significantly increased activity, as evidenced by the literature.^{15,20} The results suggest that extended π -delocalization in the most potent derivatives, along with the electron-donating effect of the hydroxyl group in NcA-6, play a crucial role in the resonance stabilization of the radical, contributing to the higher activity than NcA-5 in the ABTS assay. Additionally, intramolecular hydrogen bonding (IHB) between the phenolic –OH group and imino nitrogen in NcA-1 may hinder efficient hydrogen atom transfer, which likely contributes to its reduced antioxidant activity across all free radical assays.

The CUPRAC assay, which evaluates antioxidative potential based on reduction reactions, is particularly suitable for compounds with chelating groups. Trolox was used as the standard, and the results were expressed as *TEAC*, based on the molar absorptivity ratios of the tested compounds compared to Trolox (Table I and Fig. S-36 of the Supplementary material). Among the derivatives, NcA-2, NcA-6 and NcA-4 exhibited the higher activities, while NcA-3 showed the lower.

The FRAP assay, which measures the ability of compounds to reduce Fe(III) to Fe(II), indicated that all tested derivatives displayed good reducing power, with values ranging from 1.564 and 3.472 mM/L. NcA-2 was identified as the most potent derivative in this assay (Table I).

In the TAC, which evaluates the total antioxidant capacity, the tested nicotinamides showed promising antioxidative activity relative to ascorbic acid. The results, expressed as ascorbic acid equivalents, are presented in Table I. NcA-6 demonstrated the higher antioxidant potential, with 1 mg of the compound equivalent to 0.897 mg of ascorbic acid.

Mechanism of antioxidative activity – bond dissociation enthalpy and spin density distribution of NcA compounds

The molecular structure plays a crucial role in determining the radical scavenging properties of antioxidants. To correctly ascribe the solution structure of thiocarbohydrazones, it is essential to consider both their *E/Z* isomerism and tautomerism. In the solid-state, bis-thiocarbohydrazones predominantly exist as the thione (thioketo) form.^{21,22} However, the presence of the thioenol tautomers cannot be completely ruled out, as the experimentally determined bond C–S length (1.66–1.67 Å) falls between the typical values for a C–S single bond (1.82 Å) and a C=S double bond (1.56 Å).²³

In a solution, solvent-solute interactions may alter the isomeric or tautomeric preferences of a compound. While the thioenol tautomers are less prevalent than the thioketo form, it may play a more significant role in the interaction between the studied compounds and free radicals. Our previous experimental and theoretical studies confirmed the presence of thioenol tautomer of bis-thiocarbohydrazones in aqueous solution.¹⁹

Accordingly, we hypothesize that the S–H bond in the thioenol tautomer is a favourable site for attack by reactive free radicals such as DPPH• and ABTS•+. This hypothesis was tested by calculating the *BDE* of several bonds in thioketo and two thioenol tautomeric forms of NcA-2 (Fig. 2), where the bond with the lowest *BDE* would indicate the most reactive site for the interaction with free radicals. As shown in Fig. 2, the S–H bond in the thioenol 1 form is more susceptible to free radical attack compared to the N–H bonds. Due to significant differences in *BDE*s, we focused exclusively on the thioenol 1 form for all other compounds. Table II lists the *BDE* values of the S–H bonds in the thioenol 1 form for compounds NcA1–NcA7, along with the *BDE*s for phenolic O–H bonds in compounds that contain this functional group.

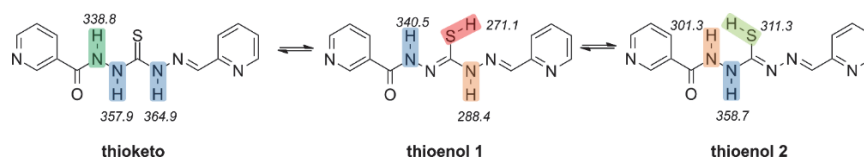


Fig. 2. Tautomeric equilibrium of NcA-2 and bond dissociation enthalpies (*BDE* in kJ mol⁻¹) of the three most reactive heteroatom-hydrogen (X–H) bonds in each tautomer.

TABLE II. *BDE* values (kJ mol⁻¹) for S–H bond of thioenol 1 tautomer of all compounds, and O–H group for NcA-1 and NcA-6

Compound	S–H	O–H
NcA-1	269.43	326.02
NcA-2	271.11	
NcA-3	272.24	
NcA-4	289.95	
NcA-5	291.03	
NcA-6	290.25	318.18
NcA-7	269.08	

According to Eq. (1), the resonance stabilization of radical formed by H atom abstraction decreases the enthalpy of the radical, which results in lower *BDE* values. The extent of this resonance stabilization can be elucidated by visualizing the electron spin density (*ESD*) distribution of the radical. The 2D representation of the *ESD* distribution of S radical of thioenol 1 tautomer (Fig. 3a) shows that the spin is delocalized over the entire thiocarbazono moiety. Only 17.6 % of the spin remains at the S atom, while most of the *ESD* is delocalized over the neighbouring N atom. Accordingly, the extent of O radical delocalization for NcA-1 is lower (Fig. 3b), where 34 % of the initial spin density is retained on the O atom, and the largest part of α and β spin densities is delocalized over the corresponding aromatic ring. The 3D representation of *ESD* distribution provides better visualization of this effect (Fig. 3c and d).

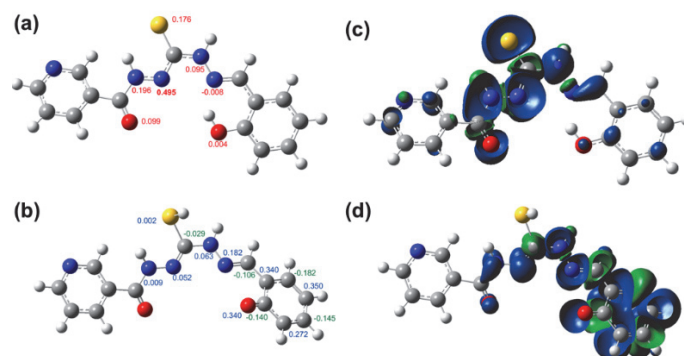


Fig. 3. Mulliken atomic spin densities for compound NcA-1 calculated on B3LYP/6-311++g(d,p) level of theory. a) 2D representation of electron spin density (*ESD*) for S radical of thioenol 1 form; b) 2D representation of *ESD* for O radical of thioenol 1 form; c) 3D *ESD* distribution of S radical; d) 3D *ESD* distribution of O radical.

In the case of thienol 2 form, the most favourable site for H-atom abstraction is the amidic N₁–H bond (*BDE* = 301.3 kJ mol⁻¹), while the S–H and N₂–H are less reactive, with *BDE*s of 311.3 and 358.7 kJ mol⁻¹, respectively. The exceptional reactivity of the amidic N–H bond can be attributed to pronounced spin

delocalization in the resulting radical, where only 45.9 % of spin is retained on N₁ atom and the rest is distributed, mostly over thiocarbohydrazide moiety. In contrast, the reactivity of the amidic N–H bond toward H-atom transfer is significantly reduced in the thioketo and thioenol 1 tautomers, with corresponding BDEs of 338.8 and 340.5 kJ mol^{−1}, respectively.

Given the low *BDE* value of NcA-1 in its thioenol 1 form, it would be expected to exhibit stronger antioxidative activity. Indeed, the *IC*₅₀ value decreases from 0.232 to 0.138 mM with the addition of water to the solvent system. However, despite the fact that the *BDE* of the NcA-2 derivative is 1.5 kJ mol^{−1} higher than that of NcA-1, NcA-2 demonstrates greater antioxidant activity. This can be attributed to the formation of an intramolecular, six-membered hydrogen-bonded ring between the phenolic –OH group and the imino-N atom, which stabilizes the ground state and consequently reduces its reactivity toward free radicals.

Iron chelating ability

We next investigated the iron-chelating capabilities of the synthesized nicotinamides toward biologically relevant Fe²⁺ and Fe³⁺. Compounds containing functional groups such as C=O, C=S, C–OH and C–SH demonstrate high affinity for metal ion binding, allowing them to uptake iron ions from tissues and promote their excretion through urine and faeces. These compounds act as antioxidants by reducing the availability of iron ions available for •OH generation *via* the Fenton reaction.²⁴

Nicotinamides show strong affinity for Fe²⁺ and Fe³⁺, with stability constants (*K*_s) ranging from 10⁷ to 10⁹ M^{−1} (Table III and Fig. S-37 of the Supplementary material). The stoichiometry of the resulting complexes is 1:1, indicating that all complexes are of the ML-type. As discussed, the compounds with C=O and C=S groups form stable complexes with iron ions, and the nicotinamides studied here contain both moieties, resulting in high chelating abilities toward Fe²⁺ and Fe³⁺.

TABLE III. Stability constants of nicotinamide complexes with Fe²⁺ and Fe³⁺

Compound	<i>K</i> _s (Fe ²⁺)×10 ^{−8} /M ^{−1}	<i>K</i> _s (Fe ³⁺)×10 ^{−8} /M ^{−1}
NcA-1	3.89	0.211
NcA-2	5.32	77.3
NcA-3	12.1	9.5
NcA-4	13.2	2.97
NcA-5	7.76	9.69
NcA-6	37.9	47.8
NcA-7	8.76	33.2

Among the compounds, the NcA-6 formed the most stable complexes with iron ions, likely due to the presence of 8-hydroxy group on the 2-quinoline moiety, which may serve as an additional ligand for metal ions. NcA-1 exhibited the lowest iron-chelating ability, probably due to a strong intramolecular hydrogen bond and

the reduced coordination potential of its imino nitrogen atom and phenolic OH group.¹⁹

In a previous study by Assaleh *et al.*¹⁸ cinnamic acid hydrazides in complexes with iron ions demonstrated high antimicrobial activity, especially against *Acinetobacter baumannii*. Trends in the observed biological activity correlated with the stability constants of the complexes, influenced by electron density distribution across the thiocarbohydrazone bridge and significant atomic charges on carbonyl oxygen, sulphur and nitrogen atoms. Given the structural similarity between our nicotinamides and cinnamic acid amides, iron complexation could play an important role in the antimicrobial mechanism of NcAs.¹³ This aspect will be further explored in future research.

CONCLUSIONS

In this study the structure-antioxidant activity relationship of novel nicotinamides (NcAs) using DPPH, ABTS, CUPRAC, *TAC* and iron chelating assays was investigated. Quantum-chemical calculations of bond dissociation enthalpies (*BDEs*) provided further insights into their antioxidant mechanisms. Our findings revealed that the addition of water promotes thione/thiol isomerism and favours the formation of the S–H bond, a key site for hydrogen atom transfer due to its exceptionally low *BDE* value. Additionally, NcAs demonstrated strong iron-chelating properties, with the 8-hydroxy-2-quinoline derivative NcA-6 forming the most stable 1:1 complexes. In contrast, the intramolecular hydrogen bonding (IHB) in NcA-1 was found to reduce both its antioxidant capacity and iron-chelating ability. The future studies will focus on optimizing the antioxidant properties of NcAs, particularly by investigating their interaction with metal ions and how this influences their radical-scavenging activity.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13117>, or from the corresponding author on request.

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ИЗВОД

IN VITRO АНТИОКСИДАТИВНА АКТИВНОСТ ХИДРАЗИДА НИКОТИНСКИХ
КИСЕЛИНА: ЕКСПЕРИМЕНТАЛНА И ТЕОРЕТСКА СТУДИЈА

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Формирање реактивних кисеоничних врста у организму може да оштети ћелије. Поред природних одбрамбених механизма као што је ензим супероксид-дизмутаза, потребни су и нови мали молекули као антиоксиданси. У овом раду испитивана је анти-оксидативна активност више амида никотинске киселине, синтетисаних у реакцији са топо-тиокарбо-хидразонима, помоћу DPPH, ABTS, CUPRAC и TAC метода. IC₅₀ вредности су биле између 0,202 и 1,297 mM за DPPH (0,114–0,638 mM уз додатак воде) и 0,107–0,365 mM за ABTS. Дистрибуција електронског спина је показала да је S–H веза тио-енолног облика испитиваних никотинамида погодна места за трансфер атома водоника на реактивне слободне радикале. Сва једињења су показала високе константе стабилности комплекса типа ML са Fe²⁺ и Fe³⁺.

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SUPPLEMENTARY MATERIAL TO

***In vitro* antioxidant activity of nicotinic acid hydrazides: Experimental and theoretical study**

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1. STRUCTURAL CHARACTERIZATION OF COMPOUNDS NCA-1- NCA-7

1.1. (NcA-1) 1-[(2-Hydroxyphenyl) methyl]-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a slightly yellow powder was obtained with a yield of 72% and a melting point of 48.9 °C.

Elem. anal.: Measured on C₁₄H₁₃N₅O₂S (M_w = 315.35 g mol⁻¹): C, 53.32; H, 4.16; N, 22.21; O, 10.15; S, 10.17%. Found: C, 53.36; H, 4.14; N, 22.28; O, 10.08; S, 10.17 %. IR(KBr, cm⁻¹) ν_{max}: 3054.48 (N-H stretching vibration of amide group), 1686.08 (C=O stretching vibration of amide group), 1489.52 (C=S vibration), 1583.23 (C=N vibration). ¹H NMR (400 MHz, DMSO-*d*₆, δ(ppm)): 6.84–6.91 (m, 2H, H-C13, H-C11); 7.25 (t, 1H, J = 8.0 Hz, H-C12); 7.58 (s, 1H, H-C3); 8.08 (d, 1H, J = 8.0 Hz, H-C14); 8.26 (d, 1H, J = 8.0 Hz, H-C4), 8.45 (s, 1H, H-C2); 8.78 (d, 1H, J = 4.0 Hz, H-C8); 9.08 (s, 1H, H-C1), 9.99 (brs, 1H, H-N3); 10.35 (brs, 1H, H-N2); 10.78 (s, 1H, H-C10(OH)), 11.84 (s, 1H, H-N4). ¹³C NMR (100 MHz, DMSO-*d*₆) δ ppm, TMS): 116.58 (C11); 119.69 (C9); 120.66 (C13); 124.11 (C3); 127.49 (C14); 129.16 (C5); 131.81 (C12); 135.74 (C4); 141.03 (C8); 148.97 (C2); 152.85 (C1); 157.07 (C10); 164.78 (C=O); 179.43 (C=S), [M–H]⁺, m/z 314.28.

1.2. (NcA-2) 1-(2-Pyridinylmethylene)-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a slightly yellow powder was obtained with a yield of 52% and a melting point of 207.6 °C.

Elem. anal.: Measured on C₁₃H₁₂N₆OS (M_w = 300.34 g mol⁻¹): C, 51.99; H, 4.03; N, 27.98; O, 5.33; S, 10.67%. Found: C, 52.00; H, 4.02; N, 27.91; O, 5.43; S, 10.64%. IR (KBr, cm⁻¹) ν_{max}: 3145.36 (N-H stretching vibration of amide group), 1669.80 (C=O stretching vibration of amide group), 1470.97 (C=S vibration), 1590.27 (C=N vibration). ¹H NMR (400 MHz, DMSO-

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d_6) δ (ppm): 7.38–7.42 (m, 1H, H-C8); 7.58–7.59 (m, 1H, H-C12); 7.86–7.90 (m, 1H, H-C3), 8.08–8.44 (m, 3H, H-C11, C10, H-C4); 8.56–8.60 (m, 1H, H-C13); 8.74–8.78 (m, 1H, H-C2); 9.10 (s, 1H, H-C1), 10.86 (s, 1H, H-N3), 11.80 (s, 1H, H-N2), 12.14 (s, 1H, H-N4). ^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 121.04 (C10); 124.19 (C3); 124.81 (C12); 129.10 (C5); 135.80 (C4); 137.15 (C11); 144.00 (C1); 148.93 (C2); 149.80 (C13); 152.90 (C8); 153.59 (C9); 164.80 (C=O); 179.94 (C=S), $[\text{M}-\text{H}]^-$, m/z 299.17.

1.3. (NcA-3) 1-[1-(2-Pyridinyl)ethylidene]-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a yellow powder was obtained with a yield of 84% and a melting point of 200.5 °C.

Elem. anal.: Measured on $\text{C}_{14}\text{H}_{14}\text{N}_6\text{OS}$ ($M_w = 314.37 \text{ g mol}^{-1}$): C, 53.49; H, 4.49; N, 26.73; O, 5.09; S, 10.20%. Found: C, 53.45; H, 4.54; N, 26.74; O, 5.08; S, 10.20%. IR (KBr, cm^{-1}) ν_{max} : 3181.03 (N-H stretching vibration of amide group), 1636.07 (C=O stretching vibration of amide group), 1466.72 (C=S vibration), 1596.43 (C=N vibration). ^1H NMR (400 MHz, DMSO- d_6 , δ (ppm)): 2.45 (d, 3H, $J = 24.0 \text{ Hz}$, H-C9); 7.42 (t, 1H, $J = 4.0 \text{ Hz}$, H-C3), 7.58 (t, 1H, $J = 4.0 \text{ Hz}$, H-C13); 7.83 (t, 1H, $J = 8.0 \text{ Hz}$, H-C12); 8.28 (d, 1H, $J = 8.0 \text{ Hz}$, H-C11); 8.59–8.61 (m, 2H, H-C4, H-C14); 8.79 (d, 1H, $J = 4.0 \text{ Hz}$, H-C2); 9.10 (s, 1H, H-C1); 10.48 (s, 1H, H-N4); 10.86 (s, 1H, H-N3); 10.88 (s, 1H, H-N2). ^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 12.71 (C9); 121.70 (C12); 124.14 (C3); 124.61 (C10); 129.15 (C5); 135.76 (C4); 136.78 (C11); 148.96 (C1, C2); 150.11 (C14); 152.87 (C13); 154.93 (C8); 164.71 (C=O); 180.62 (C=S), $[\text{M}-\text{H}]^-$, m/z 313.28.

1.4. (NcA-4) 1-(8-Quinolylmethylene)-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a brown powder was obtained with a yield of 73% and a melting point of 191.8 °C.

Elem. anal.: Measured on $\text{C}_{17}\text{H}_{14}\text{N}_6\text{OS}$ ($M_w = 350.40 \text{ g mol}^{-1}$): C, 58.27; H, 4.03; N, 23.98; O, 4.57; S, 9.15%. Found: C, 58.21; H, 4.11; N, 23.96; O, 4.60; S, 9.12%.

IR (KBr, cm^{-1}) ν_{max} : 3167.12 (N-H stretching vibration of amide group), 1667.82 (C=O stretching vibration of amide group), 1473.60 (C=S vibration), 1592.34 (C=N vibration). ^1H NMR (400 MHz, DMSO- d_6 , δ (ppm)): 7.54–7.63 (m, 2H, H-C3, H-C14), 7.70 (t, 1H, $J = 8.0 \text{ Hz}$, H-C11); 8.08 (d, 1H, $J = 4.0 \text{ Hz}$, H-C4); 8.27 (d, 1H, $J = 8.0 \text{ Hz}$, H-N10); 8.44 (d, 1H, $J = 8.0 \text{ Hz}$, H-C12); 8.70–8.81 (m, 2H, H-C8, H-C13); 8.99 (s, 1H, H-C2); 9.06 (s, 1H, H-C15); 9.42 (s, 1H, H-C1); 10.56 (s, 1H, H-N3); 10.83 (s, 1H, H-N2); 12.15 (s, 1H, H-N4).

^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 122.31 (C11); 124.15 (C14); 126.89 (C3, C12a); 128.46 (C10); 129.16 (C12); 130.53 (C5); 131.38 (C9); 135.76 (C4); 137.12 (C13); 140.74 (C8); 145.89 (C15a); 148.97 (C2); 150.92 (C1); 152.87 (C15); 164.82 (C=O); 179.76 (C=S), $[\text{M}-\text{H}]^-$, m/z 349.17.

1.5. (NcA-5) 1-(2-Quinolylmethylene)-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a slightly yellow powder was obtained with a yield of 53% and a melting point of 174.7 °C.

Elem. anal.: Measure on $\text{C}_{17}\text{H}_{14}\text{N}_6\text{OS}$ ($M_w = 350.40 \text{ g mol}^{-1}$): C, 58.27; H, 4.03; N, 23.98; O, 4.57; S, 9.15%. Found: C, 58.20; H, 4.10; N, 23.93; O, 4.65; S, 9.12%. IR (KBr, cm^{-1}) ν_{max} : 3191.56 (N-H stretching vibration of amide group), 1668.0 (C=O stretching vibration of amide group), 1501.50 (C=S vibration), 1596.50 (C=N vibration). ^1H NMR (400 MHz, DMSO- d_6 , δ (ppm)): 7.44–7.66 (m, 2H, H-C3, H-C13); 7.80 (t, 1H, $J = 8.0 \text{ Hz}$, H-C8); 8.02–8.06 (m, 2H,

H-C10, H-C14); 8.30–8.31 (m, 2H, H-C12, H-C15); 8.42 (d, 1H, $J = 6.0$ Hz, H-C4), 8.61 (dd, 1H, H-C11); 8.79 (d, 1H, $J = 4.0$ Hz, H-C2), 9.12 (s, 1H, H-C1); 10.75 (s, 1H, H-N3), 10.91 (s, 1H, H-N2); 12.30 (s, 1H, H-N4). ^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 118.77 (C10); 124.18 (C3); 127.74 (C13); 128.39 (C12, C15); 129.31 (C5, C14); 130.45 (C11a); 135.75 (C4); 136.79 (C11); 144.14 (C8); 147.84 (C2); 148.96 (C1); 152.94 (C9, C15a); 164.81 (C=O), 179.95 (C=S), $[\text{M}-\text{H}]^-$, m/z 349.32.

1.6. (NcA-6) 1-[(8-Hydroxy)-2-quinolylmethylene]-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a brown powder was obtained with a yield of 81% and a melting point of 210 °C.

Elem. anal.: Measured on $\text{C}_{17}\text{H}_{14}\text{N}_6\text{O}_2\text{S}$ ($M_w = 366.40$ g mol^{-1}): C, 55.73; H, 3.85; N, 22.94; O, 8.73; S, 8.75%. Found: C, 55.80; H, 3.78; N, 22.99; O, 8.68; S, 8.75%. IR (KBr, cm^{-1}) ν_{max} : 3200.65 (N-H stretching vibration of amide group), 1658.31 (C=O stretching vibration of amide group), 1455.72 (C=S vibration), 1590.32 (C=N vibration).

^1H NMR (400 MHz, DMSO- d_6 , δ (ppm)): 7.13 (d, 1H, $J = 8.0$ Hz, H-C14); 7.42–7.46 (m, 2H, H-C3, H-C8); 7.58–7.62 (m, 1H, H-C13); 8.33–8.41 (m, 3H, H-C4, H-C10, H-C12); 8.57 (d, 1H, $J = 8.0$ Hz, H-C11); 8.80 (d, 1H, $J = 4.0$ Hz, H-C2); 9.12 (s, 1H, H-C1) 9.90 (s, 1H, H-C15(OH)), 10.72 (s, 1H, H-N2), 10.91 (s, 1H, H-N3); 12.35 (s, 1H, H-N4).

^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 112.62 (C14); 118.23 (C12); 119.09 (C10); 124.18 (C3); 128.70 (C13); 129.09 (C5); 129.34 (C11a); 135.76 (C4); 136.64 (C11); 138.68 (C15a); 144.00 (C8); 148.96 (C2); 152.09 (C1); 152.93 (C9), 153.93 (C15); 164.83 (C=O); 179.95 (C=S), $[\text{M}-\text{H}]^-$, m/z 365.37, $[\text{M}-\text{OH}]^-$, m/z 349.30.

1.7. (NcA-7) 1-(Phenyl-methylen)-5-(pyridine-3-carbonyl) Dihydrazide Thiocarbonyl Acid

After synthesis, a white powder was obtained with a yield of 64% and a melting point of 191 °C.

Elem. anal.: Measured on $\text{C}_{14}\text{H}_{13}\text{N}_5\text{OS}$ ($M_w = 299.35$ g mol^{-1}): C, 56.17; H, 4.38; N, 23.40; O, 5.34; S, 10.71%. Found: C, 56.20; H, 4.47; N, 23.63; O, 5.35; S, 10.35%. IR (KBr, cm^{-1}) ν_{max} : 3211.24 (N-H stretching vibration of amide group), 1660.20 (C=O stretching vibration of amide group), 1449.19 (C=S vibration), 1600.27 (C=N vibration).

^1H NMR (400 MHz, DMSO- d_6 , δ (ppm)): 7.44 (s, 3H, H-C11, H-C12, H-C13); 7.58 (t, 1H, $J = 4.0$ Hz, H-C3); 7.91 (s, 1H, H-C10); 7.92 (s, 1H, H-C14); 8.13 (s, 1H, H-C4); 8.27 (d, 1H, $J = 8.0$ Hz, H-C8); 8.78 (s, 1H, H-C2); 9.09 (s, 1H, H-C1); 10.44 (s, 1H, H-N3); 10.81 (s, 1H, H-N2); 11.93 (s, 1H, H-N4).

^{13}C NMR (100 MHz, DMSO- d_6 , δ ppm, TMS): 124.15 (C3); 128.07 (C11, C13); 129.14 (C10, C14, C5); 130.54 (C12); 134.49 (C9); 135.76 (C4); 143.88 (C8); 148.96 (C2); 152.86 (C1); 164.80 (C=O); 179.69 (C=S), $[\text{M}-\text{H}]^-$, m/z 298.17.

IR spectrometry showed characteristic peaks of amide bond, thione form, and carbonyl group. Stretching vibrations of the amide group are presented around 3054–3211 cm^{-1} . Characteristic strong absorption bands between 1583 and 1600 cm^{-1} are attributed to the azomethine $\nu(\text{C}=\text{N})$ stretching vibration. The stretching band of amide group $\nu(\text{C}=\text{O})$ shows absorption between 1636 and 1686 cm^{-1} . Group characteristics for thiocarbohydrazide are shown between 1449 and 1501 cm^{-1} as the form $\nu(\text{C}=\text{S})$.

^1H NMR spectroscopy showed characteristic peaks of nicotinic amides for N-H hydrogens according to δ 10.48–12.30 for N4 atom, δ 9.99–10.87 for N3 atom and δ 10.35–11.8 for N2 atom. Near them is a singlet for O-H atom on δ 9.88–10.78 for NcA-1 and NcA-6. Also, one of the characteristic peaks is azomethine ($-\text{CH}=\text{N}$), which could be seen between δ 7.40 and 8.79

with the exception of NcA-3. NcA-3 has a methyl group ($-\text{CH}_3$) connected to azomethine moiety, giving a singlet on δ 2.45. Aromatic rings from nicotinic acid, phenol, and quinoline nuclei are shown between δ 6.87 and 9.42.

^{13}C NMR spectrum showed characteristic peaks for all seven compounds. Thione carbon ($\text{C}=\text{S}$) is present between 179.43 and 180.62 ppm, carbonyl carbon ($\text{C}=\text{O}$) is shown at 164.71–164.83 ppm, and azomethine carbon ($\text{C}=\text{N}$) between 140.74 and 154.93 ppm. NcA-3 has a characteristic methyl group at 12.71 ppm. The carbon atom attached to the O-H group at NcA-1 and NcA-6 is from 127.49 to 153.93 ppm. Also, the carbon atoms resulting from phenyl and quinoline groups are shown between 112.62 and 157.07 ppm.

The MS analysis was used for the identification of analytes present in the synthesized nicotinamides. A mass spectrum was obtained for each sample. Mass spectra have shown that masses of the obtained analytes correspond to deprotonated $[\text{M}-\text{H}]^-$ ions of nicotinamide derivatives, and those ions were the most intense in the obtained spectra.

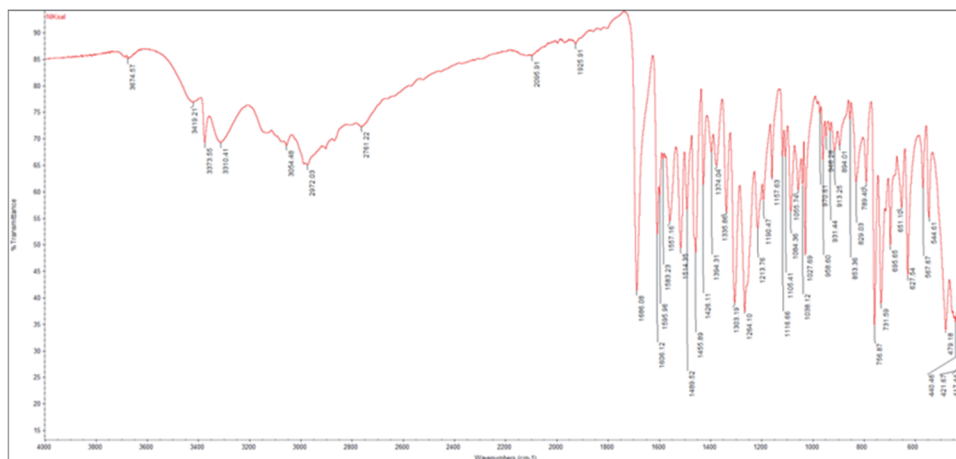
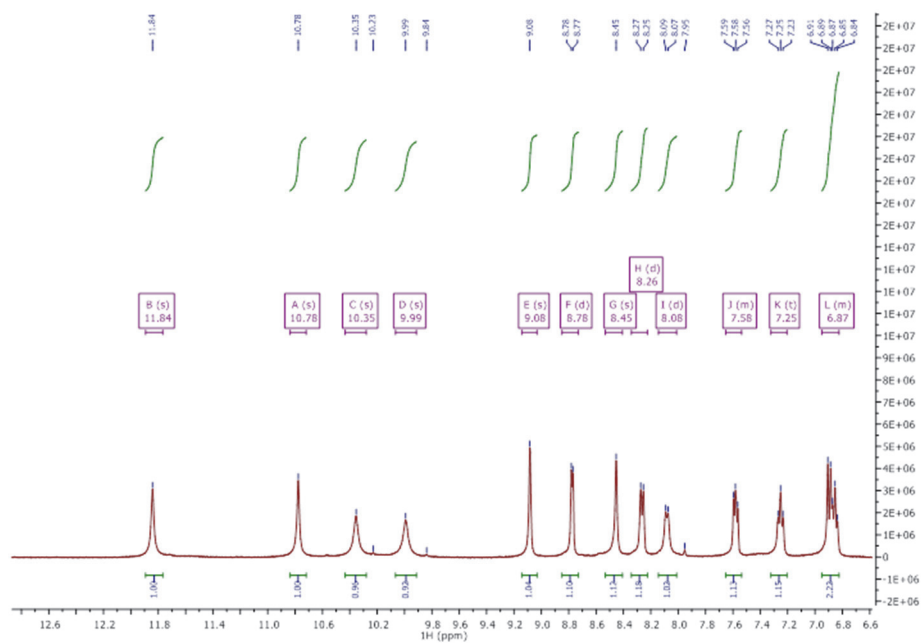


Fig. S1. FTIR spectrum of NcA-1

Fig. S2. ^1H spectrum of NcA-1

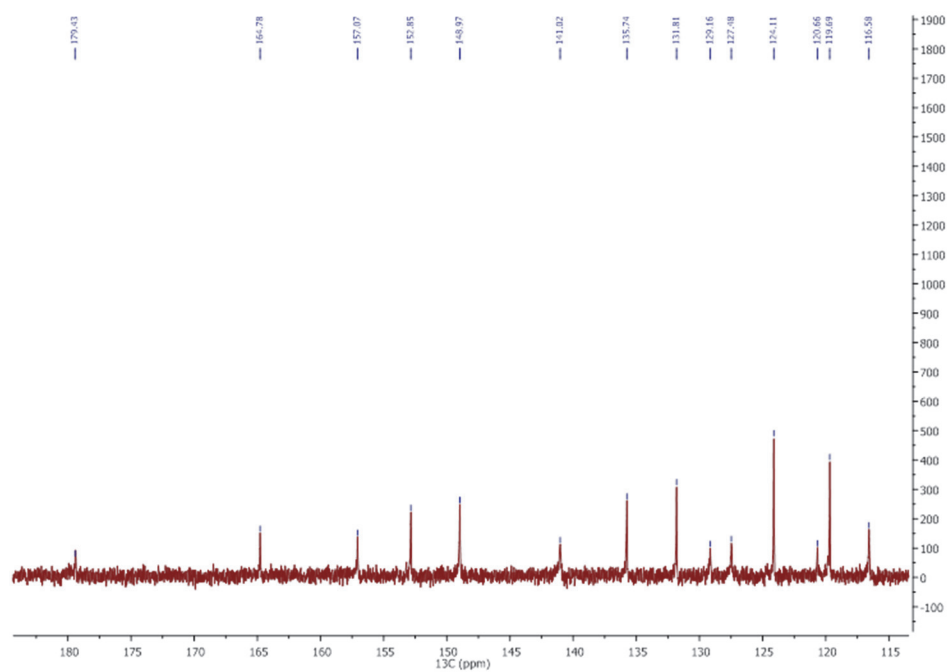
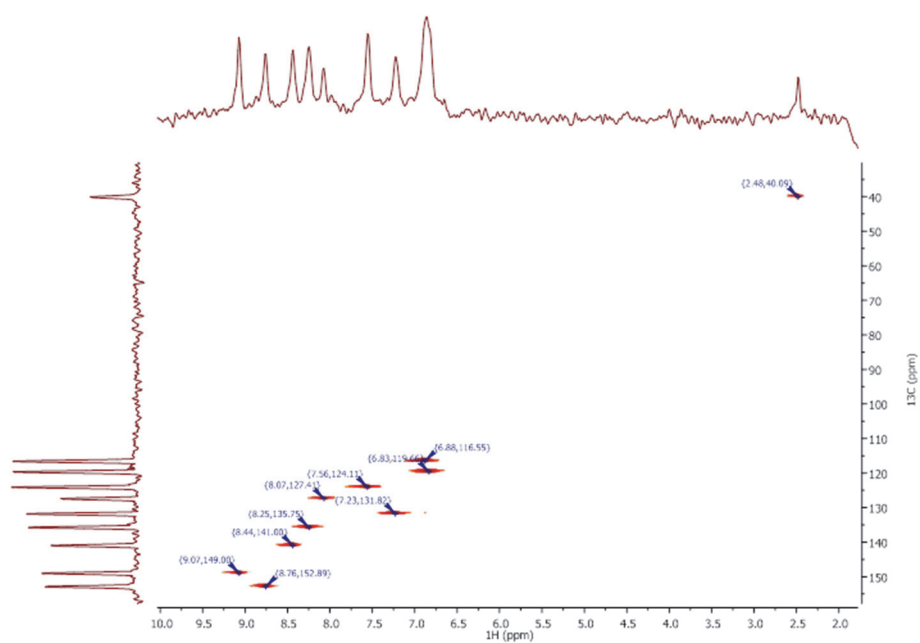
Fig. S3. ^{13}C spectrum of NcA-1

Fig. S4. HSQC spectrum of NcA-1

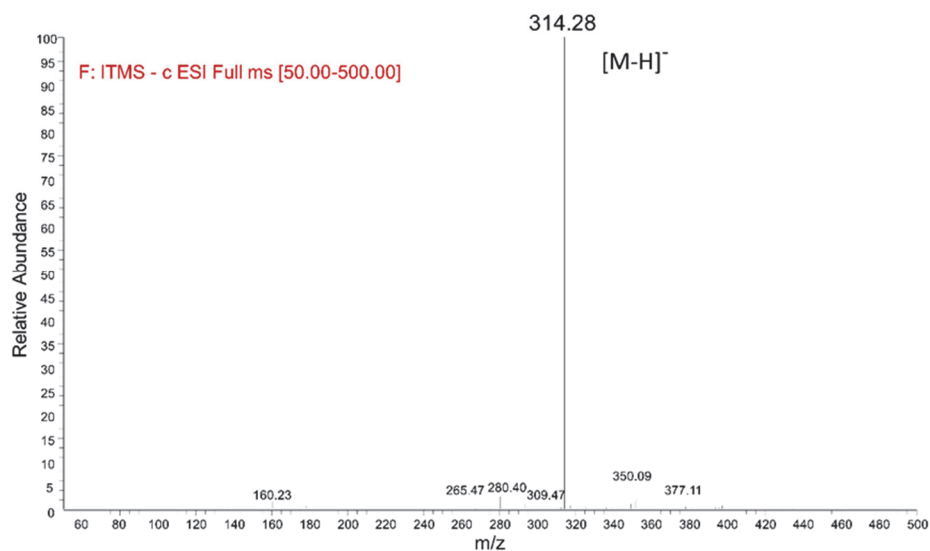
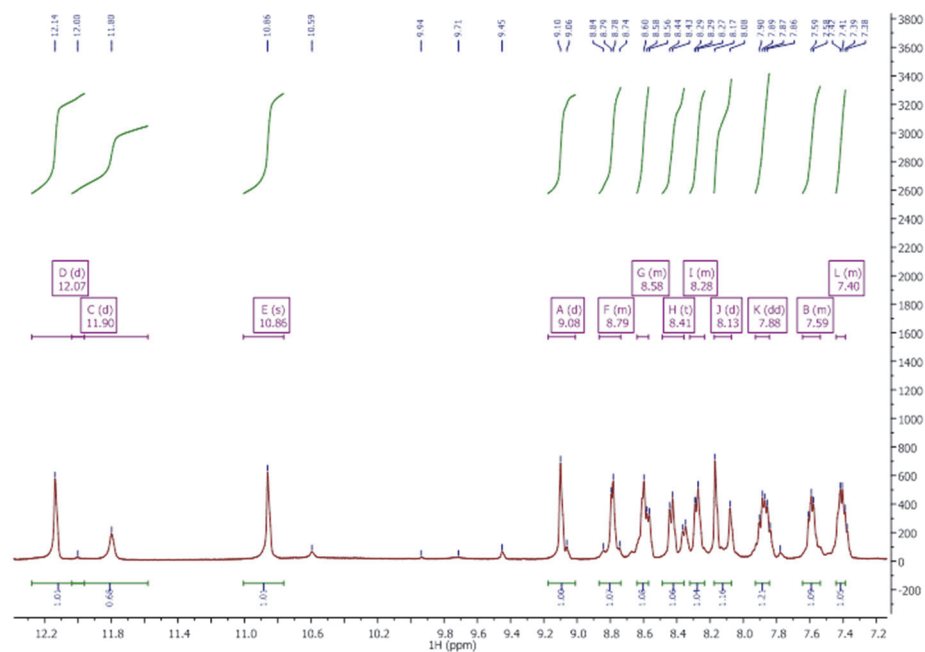
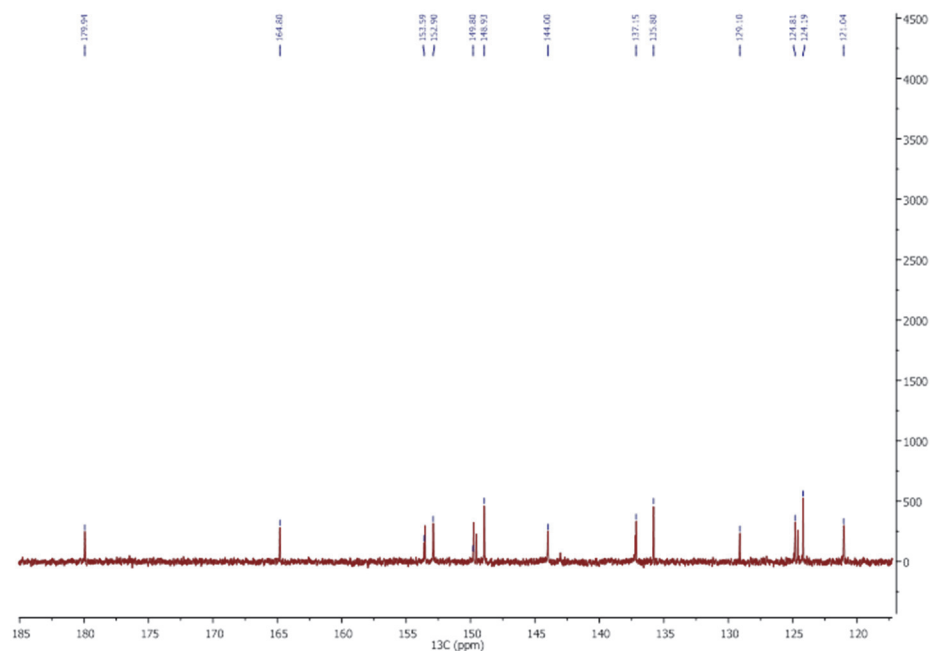


Fig. S5. MS spectrum of compound NcA-1



Fig. S6. FTIR spectrum of NcA-2

Fig. S7. ¹H spectrum of NcA-2Fig. S8. ¹³C spectrum of NcA-2

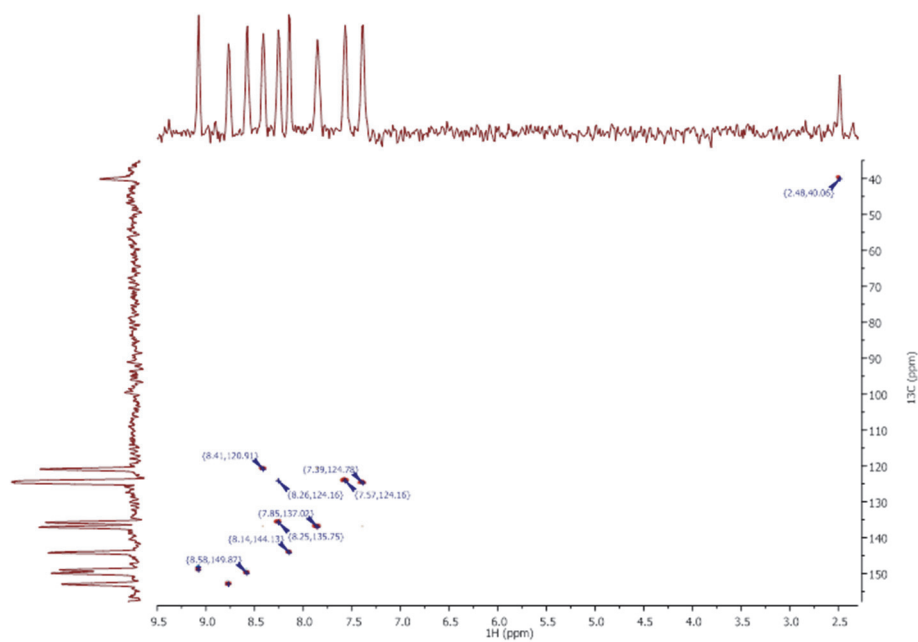


Fig. S9. HSQC spectrum of NcA-2

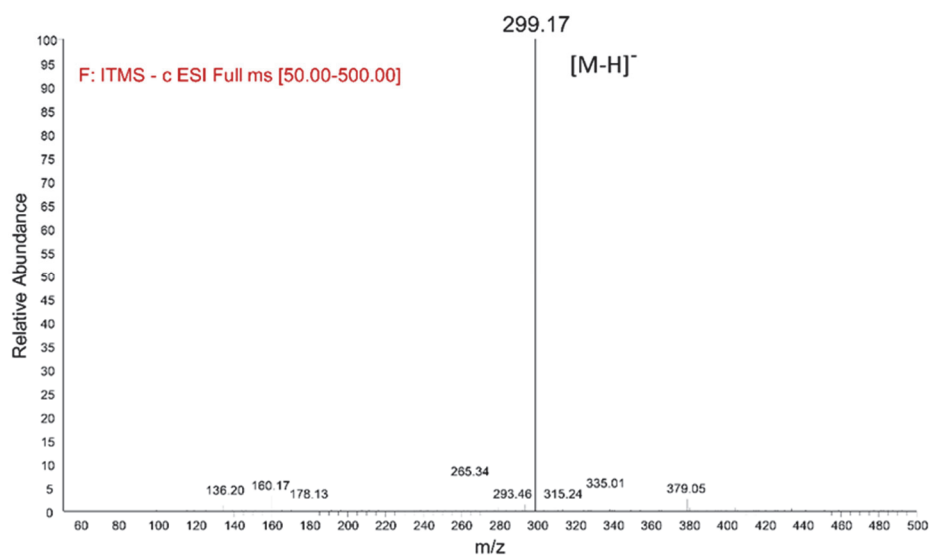


Fig. S10. MS spectrum of compound NcA-2

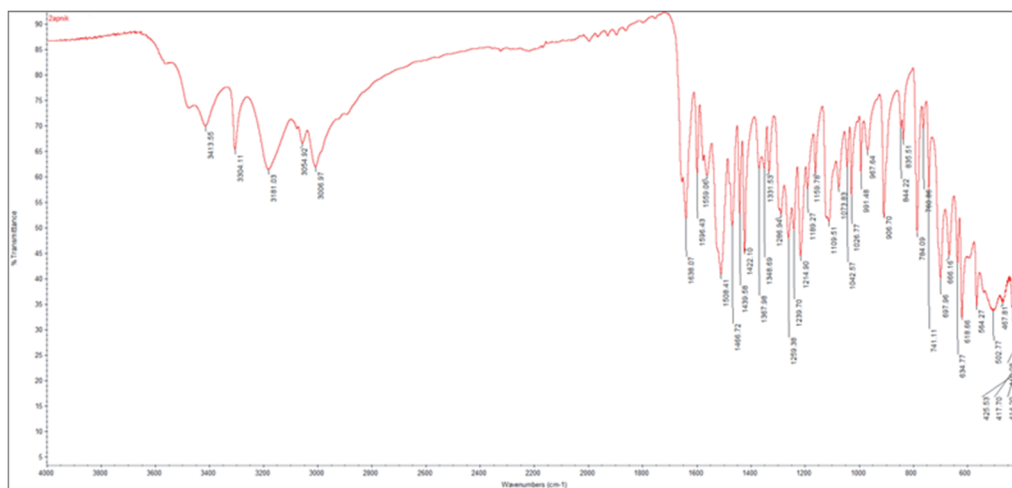
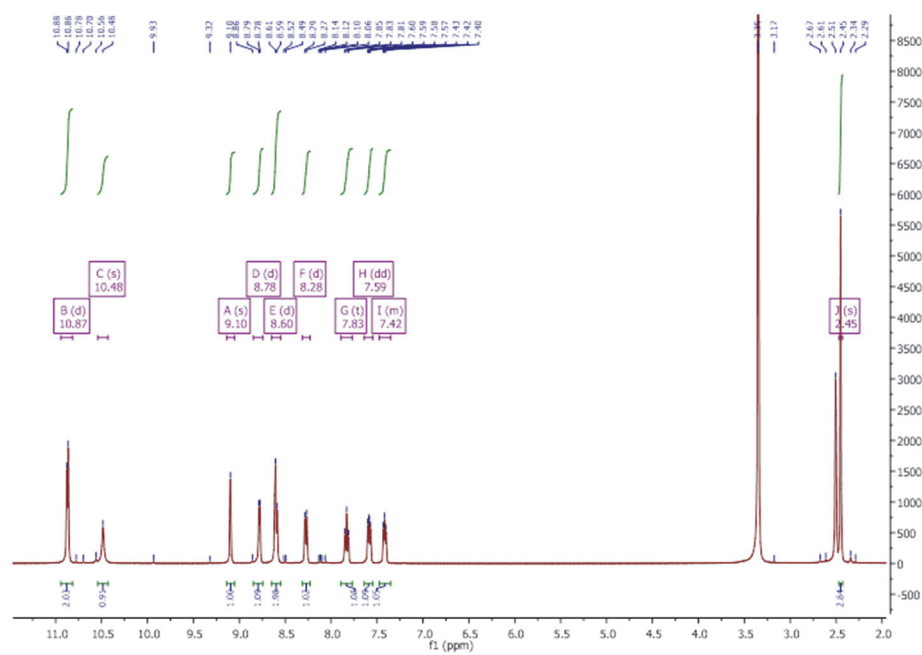


Fig. S11. FTIR spectrum of NcA-3

Fig. S12. ^1H spectrum of NcA-3

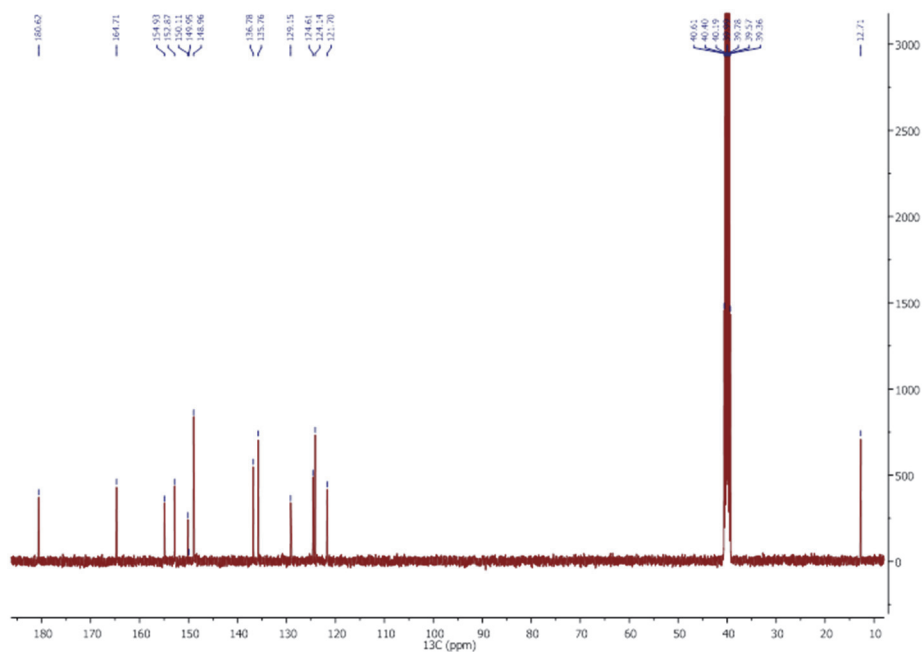
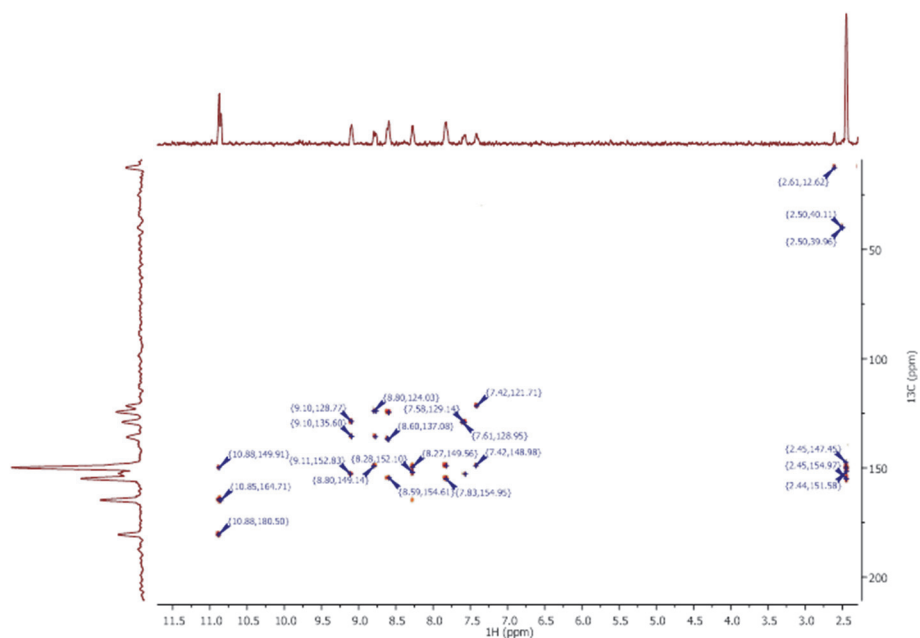
Fig. S13. ^{13}C spectrum of NcA-3

Fig. S14. HSQC spectrum of NcA-3

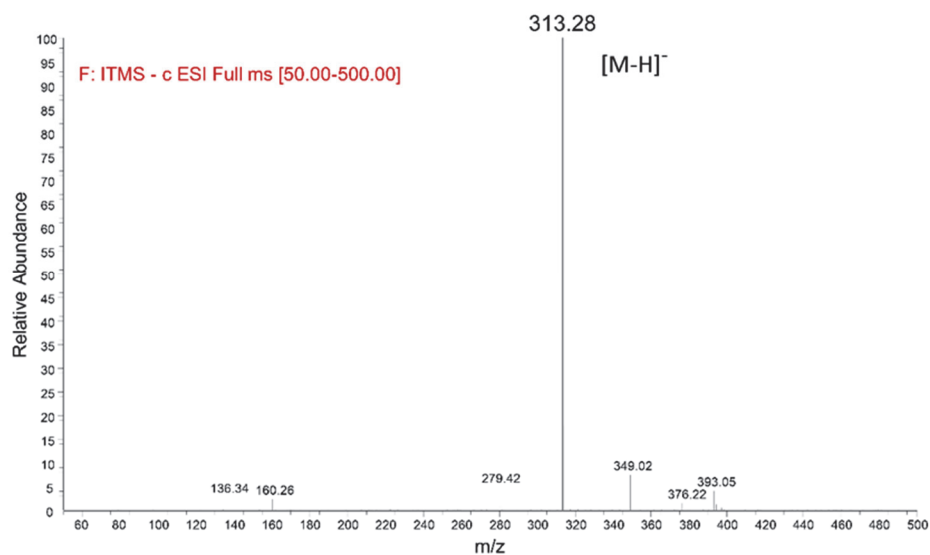


Fig. S15. MS spectrum of compound NcA-3

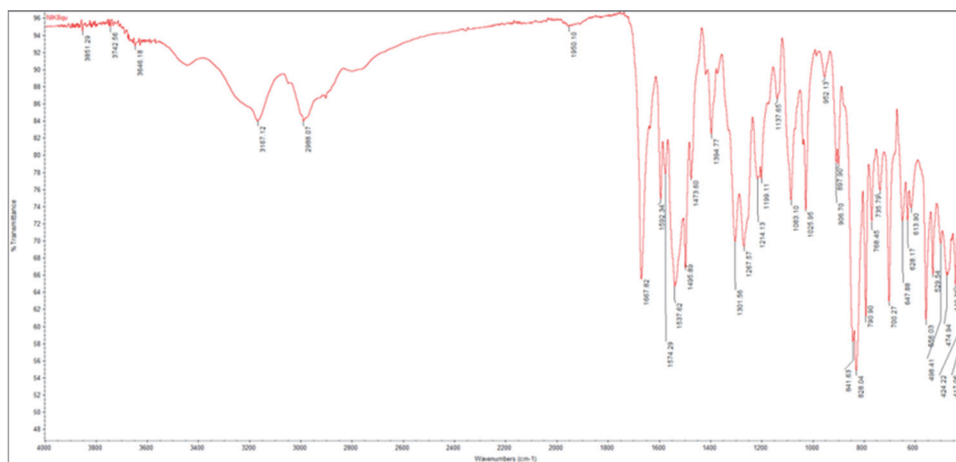
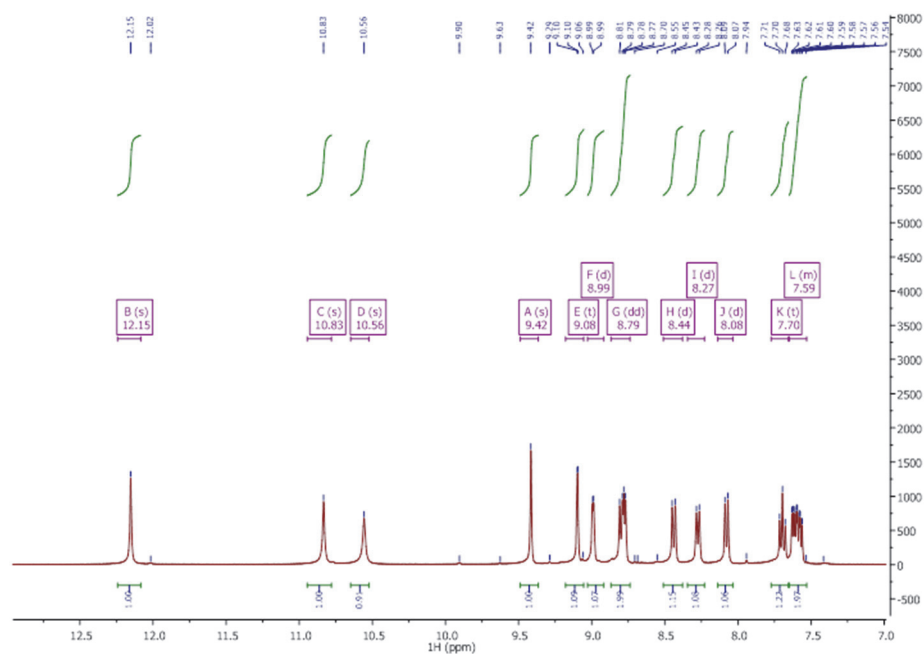
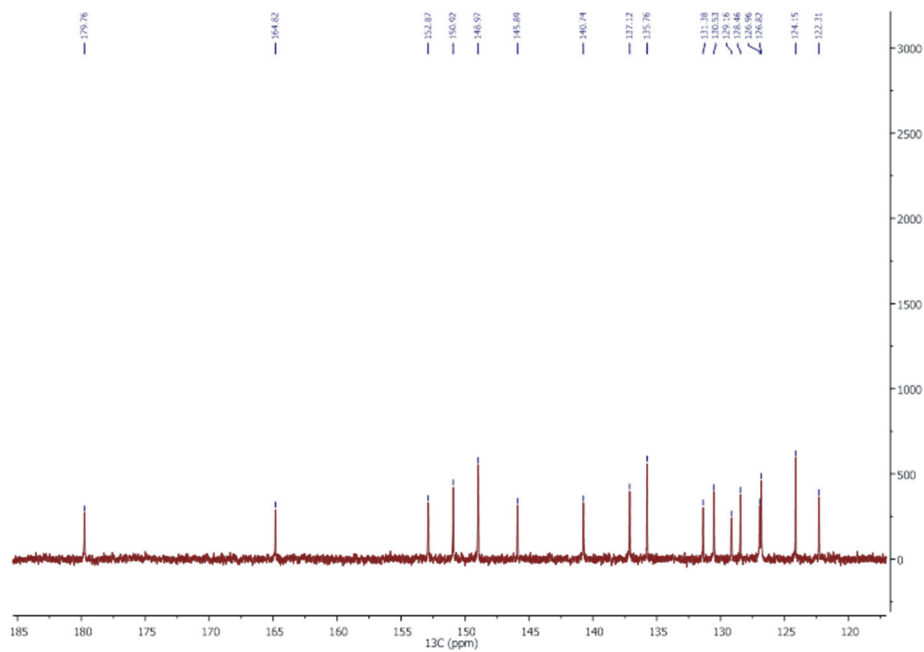


Fig. S16. FTIR spectrum of NcA-4

Fig. S17. ¹H spectrum of NcA-4Fig. S18. ¹³C spectrum of NcA-4

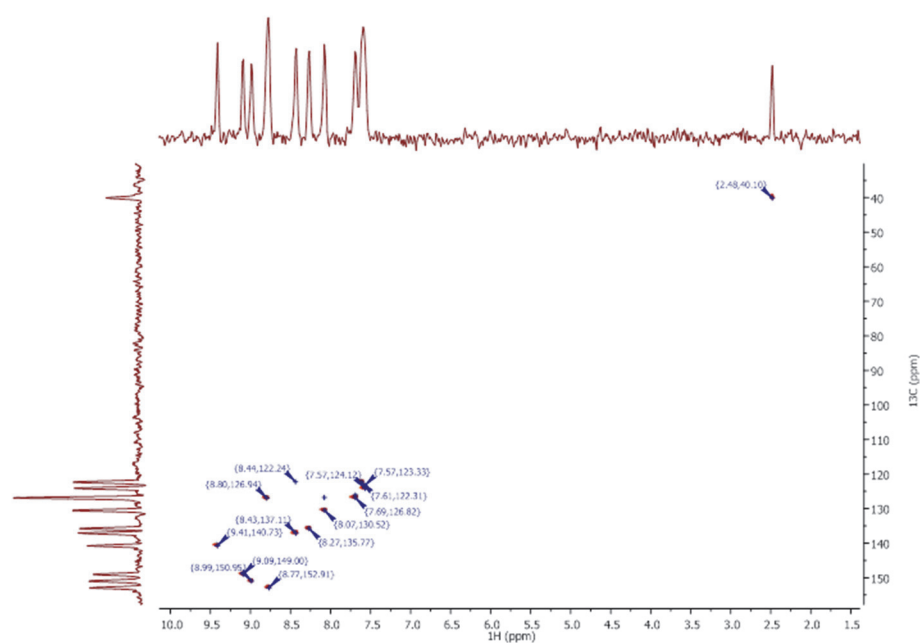


Fig. S19. HSQC spectrum of NcA-4

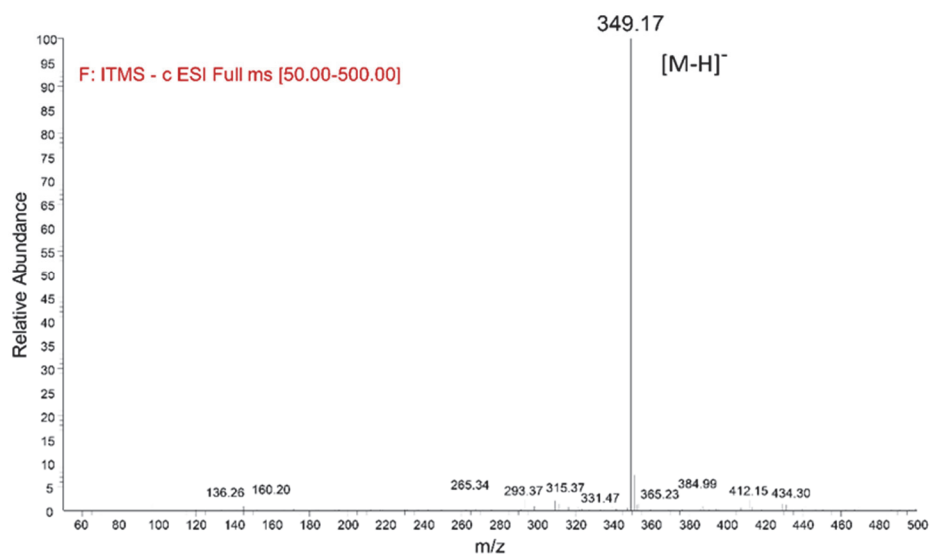


Fig. S20. MS spectrum of compound NcA-4

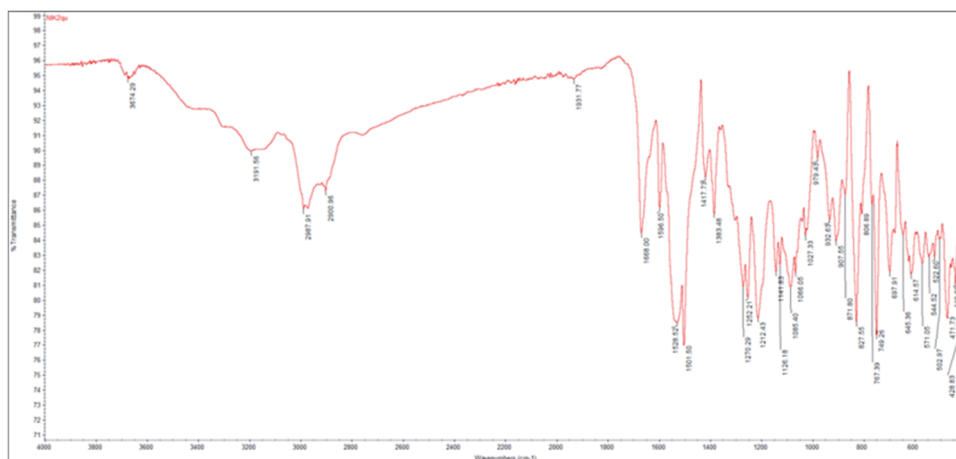
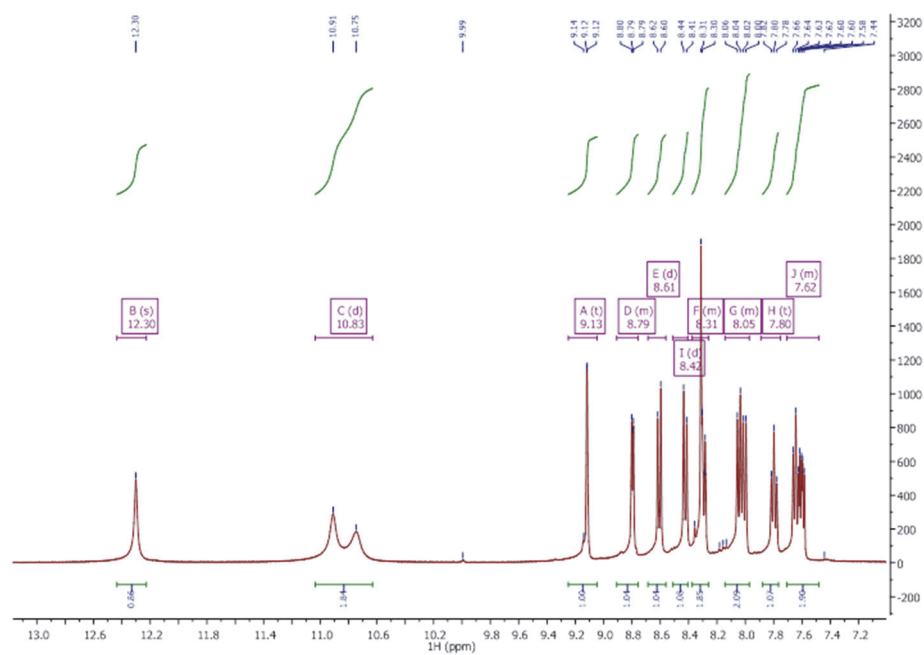


Fig. S21. FTIR spectrum of NcA-5

Fig. S22. ^1H spectrum of compound NcA-5

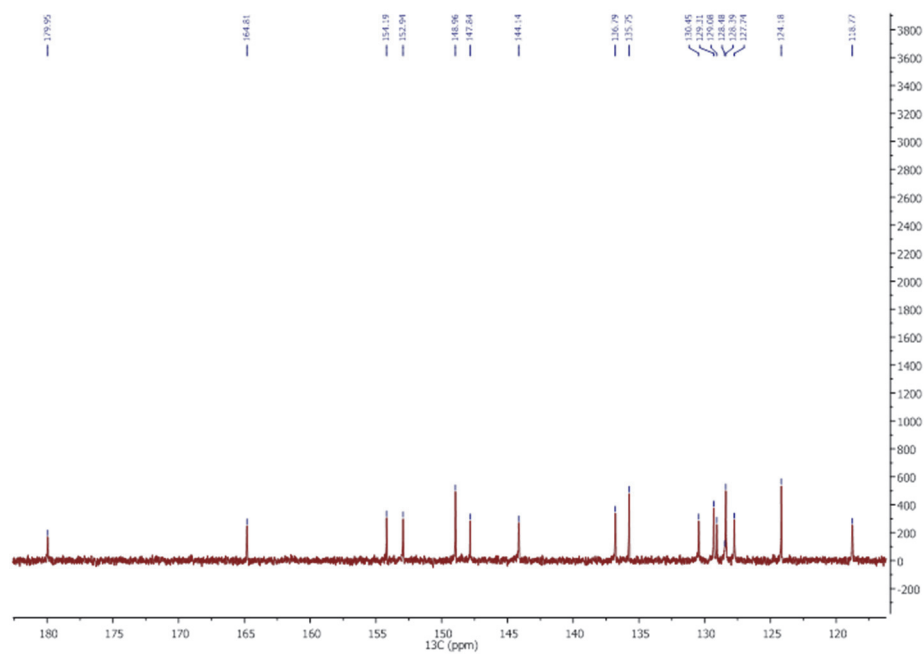
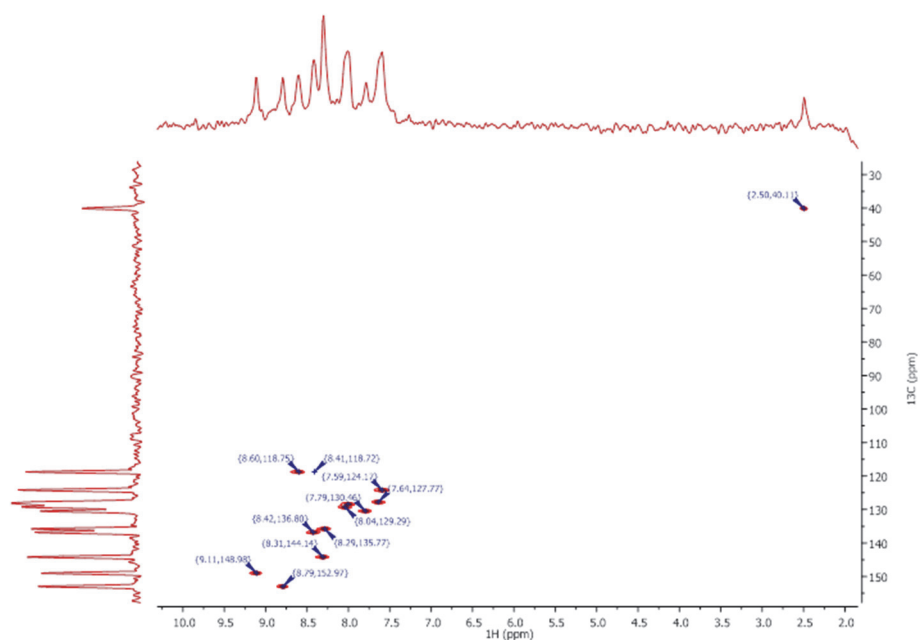
Fig. S23. ^{13}C spectrum of NcA-5

Fig. S24. HSQC spectrum of NcA-5

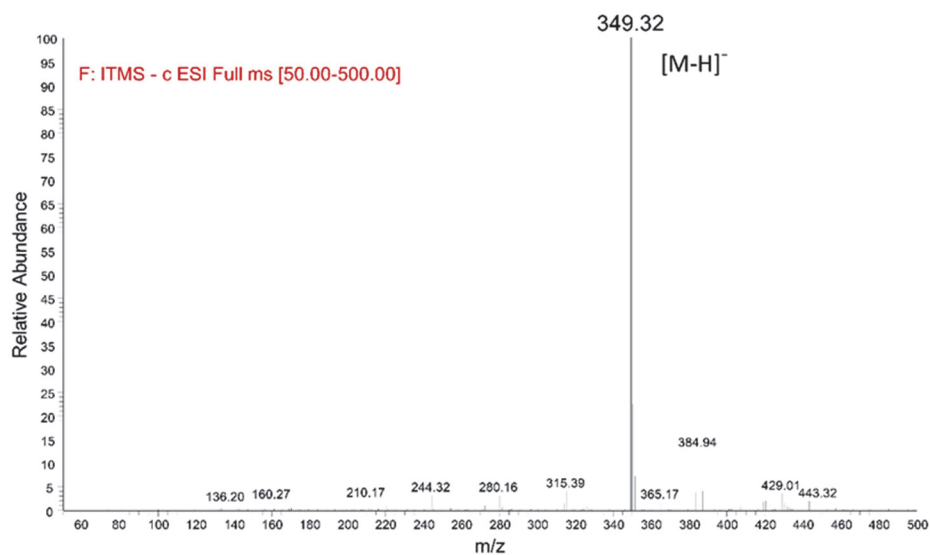


Fig. S25. MS spectrum of compound NcA-5

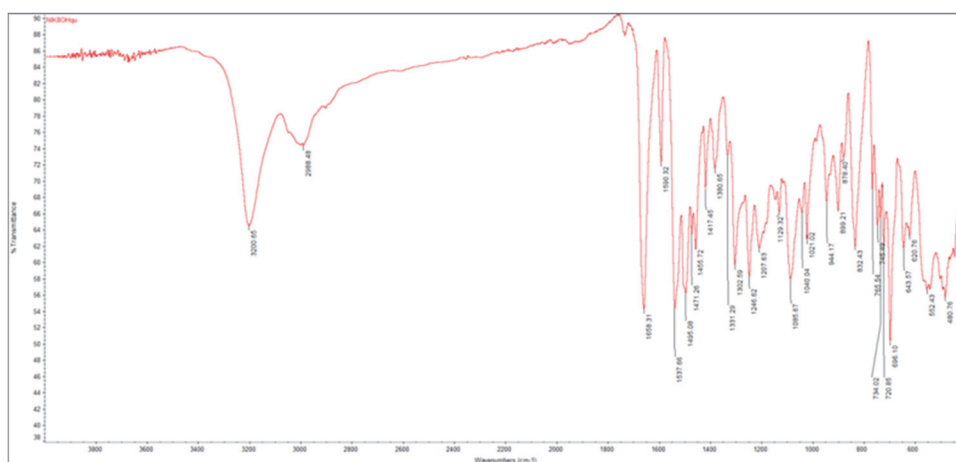
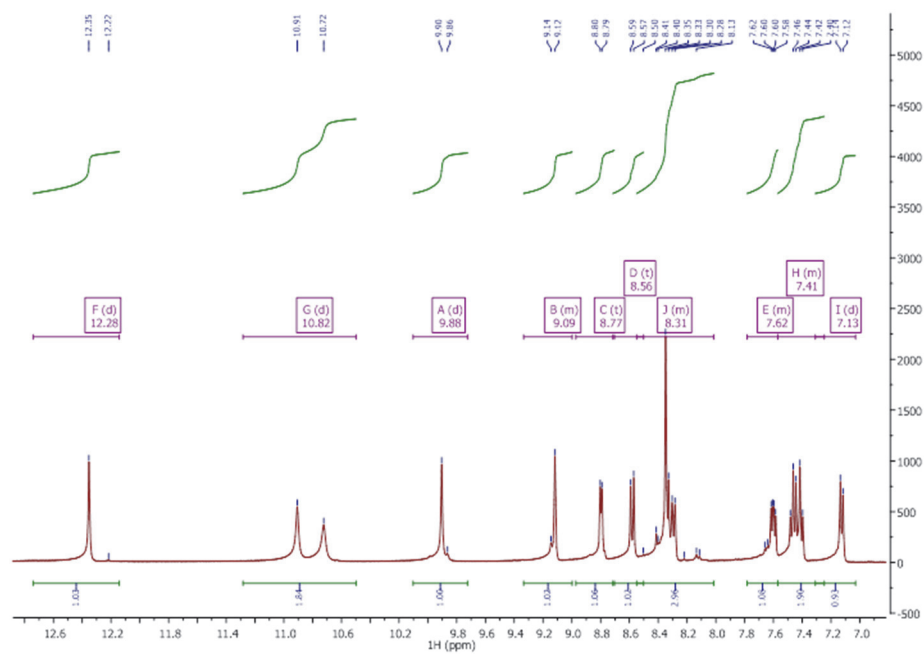
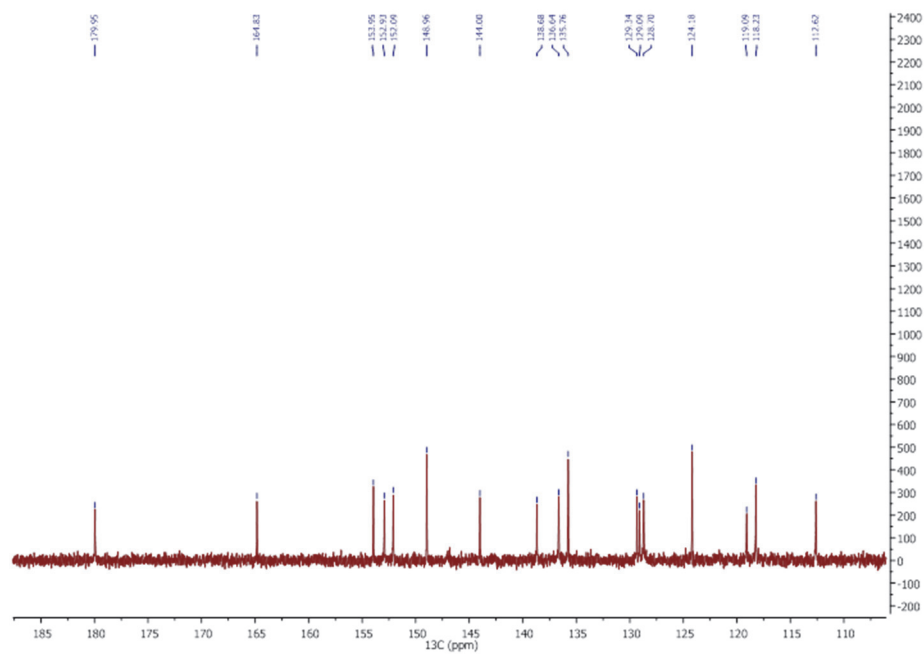


Fig. S26. FTIR spectrum of NcA-6

Fig. S27. ^1H spectrum of NcA-6Fig. S28. ^{13}C spectrum of NcA-6

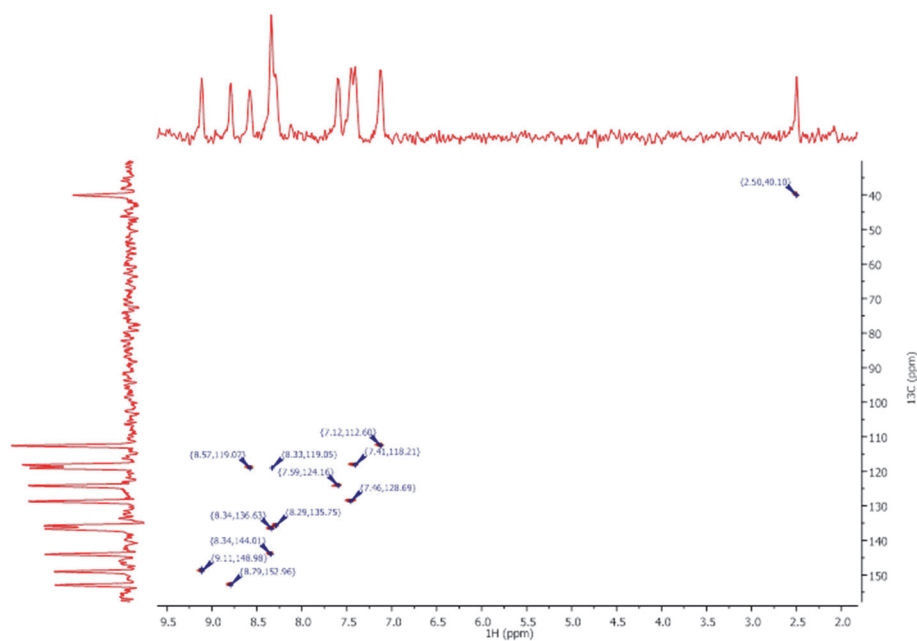


Fig. S29. HSQC spectrum of NcA-6

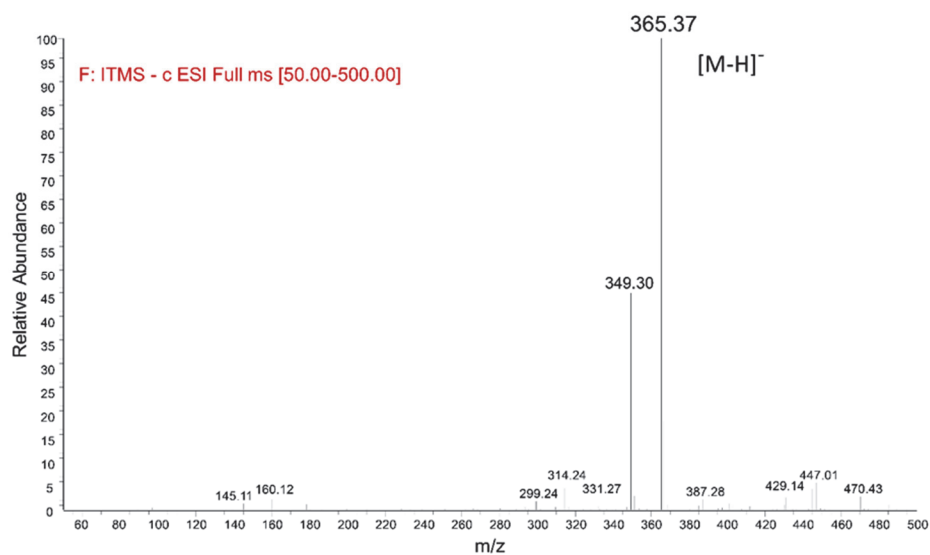


Fig. S30. MS spectrum of compound NcA-6

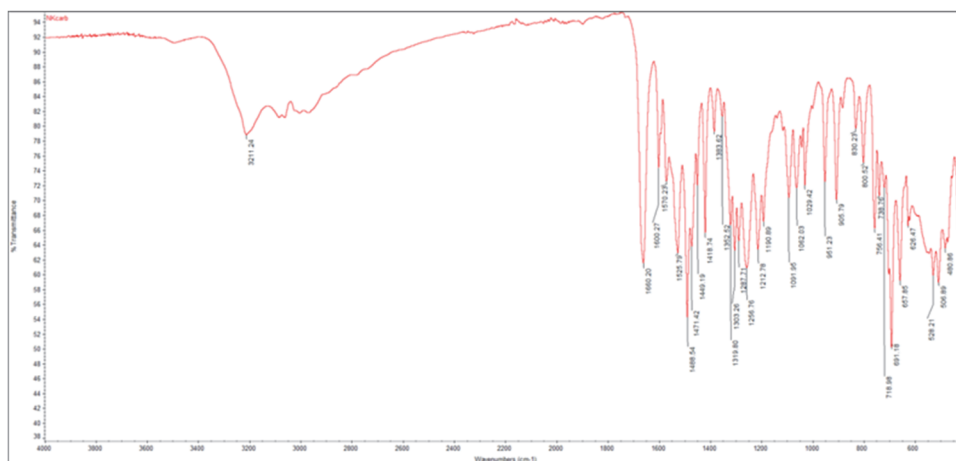
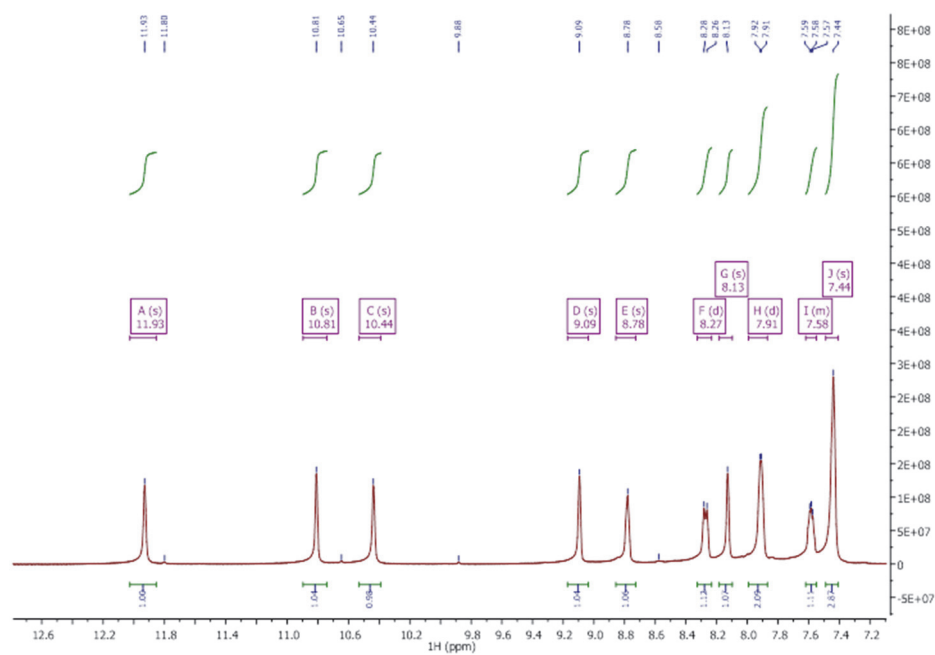


Fig. S31. FTIR spectrum of NcA-7

Fig. S32. ¹H spectrum of NcA-7

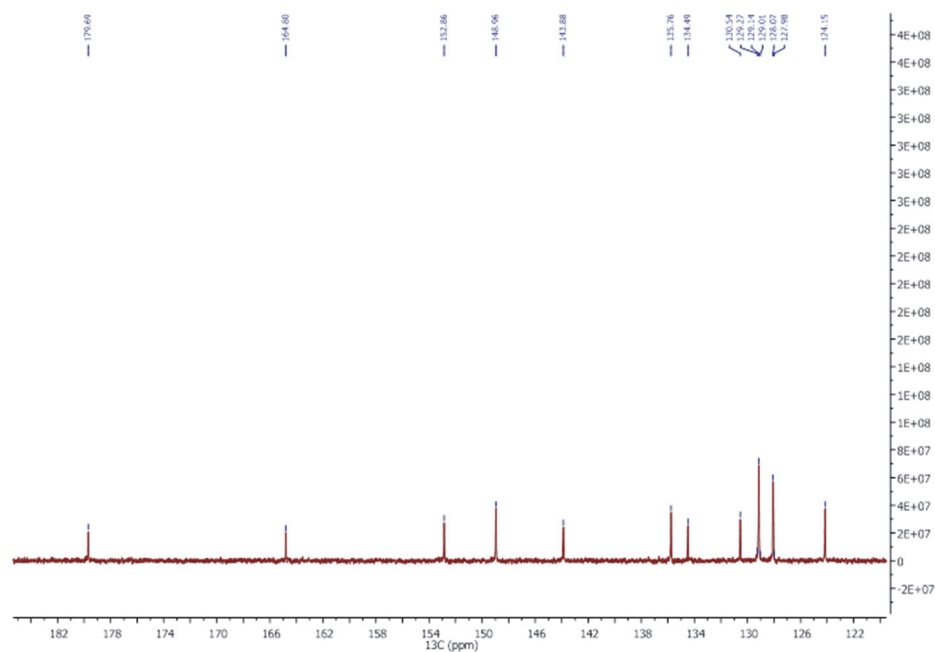
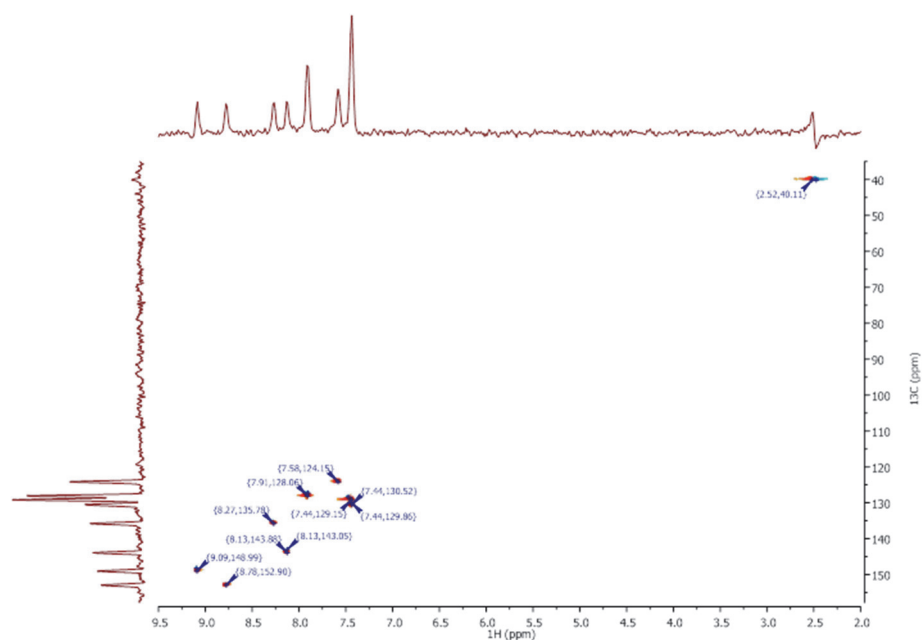
Fig. S33. ^{13}C spectrum of NcA-7

Fig. S34. HSQC spectrum of NcA-7

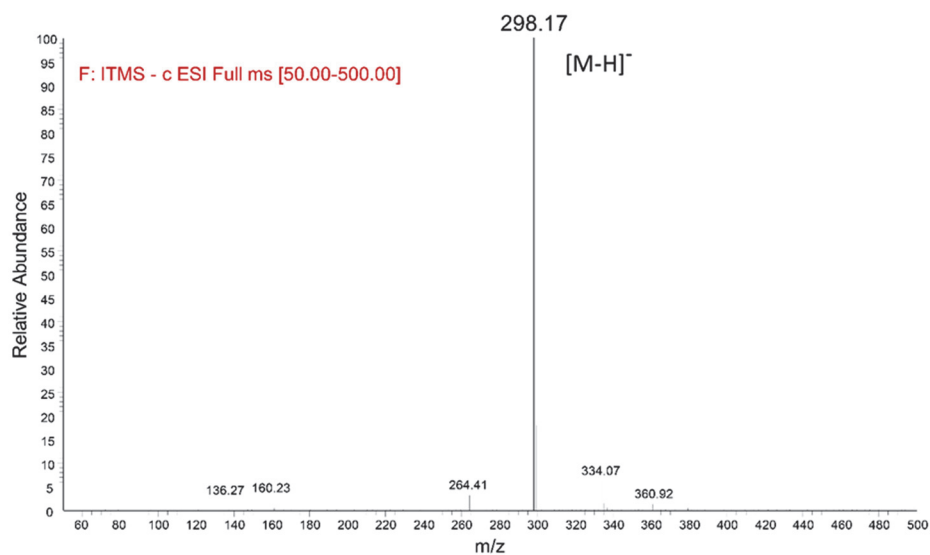


Fig. S35. MS spectrum of compound NcA-7

2. ANTIOXIDANT CAPACITY OF NCAS

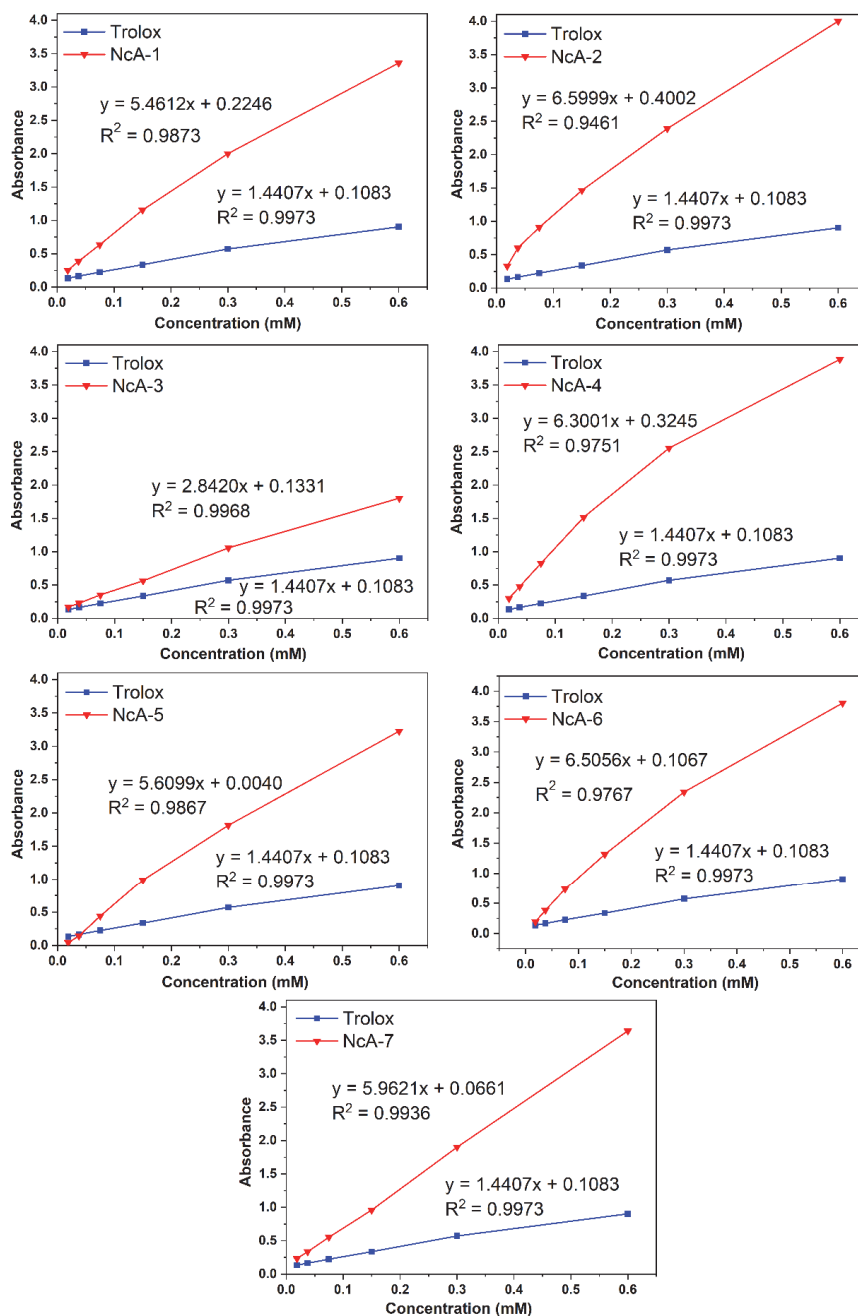


Fig. S36. Calibration curves of 7 NcAs and a standard (Trolox) for the determination of molar absorptivity and TEAC

3. IRON CHELATING ABILITY OF NCA-6

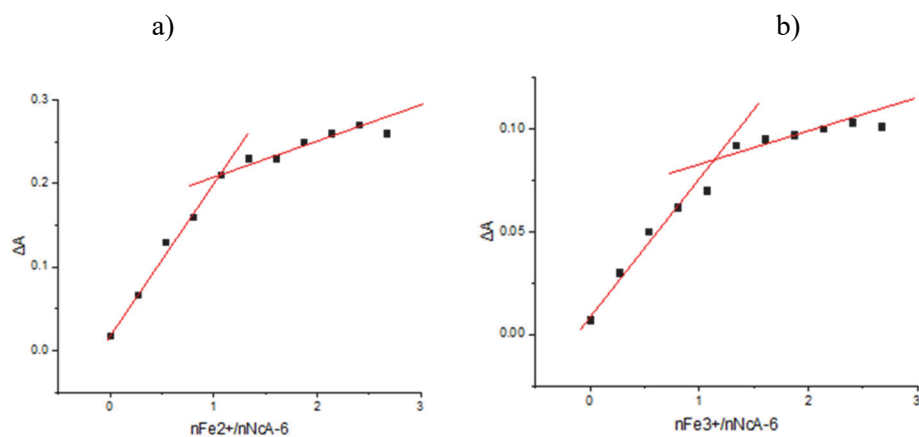


Fig. S37. The mole ratio plot for the formation of complexes between the NcA-6 and a) Fe^{2+} ; b) Fe^{3+}



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Determination of antibacterial properties and chemical composition of pyrolysis liquid from feeding wastes of mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) adults and larvae

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Abstract: This study aimed to evaluate the antibacterial properties and phytochemical composition of pyrolysis liquids obtained from the biowaste of the mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) adults and larvae. Biowaste was subjected to pyrolysis at 295 ± 5 °C for 7 h under a nitrogen atmosphere using a laboratory-scale system equipped with a dual condensation unit to separate light and heavy fractions. The biowaste pyrolysis liquid (BPL) were purified and analysed for their chemical content using LC–MS/MS, which identified seven key phenolic compounds, with cinnamic acid being the most abundant ($358.05 \mu\text{g/g}$), followed by salicylic acid, 4-hydroxybenzoic acid and naringenin. Notably, the most of flavonoid derivatives were either absent or below detection limits, indicating the extract's phenolic acid-dominated profile. The antibacterial activity of BPL was assessed against two gram-negative (*E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853) and two gram-positive (*E. faecalis* ATCC-23212, *S. aureus* ATCC-25923) bacterial strains using the agar well diffusion method. The results demonstrated significant antibacterial effects, particularly against *S. aureus* (29.5 ± 0.3 mm) and *P. aeruginosa* (23.5 ± 0.2 mm), followed by *E. coli* (21.7 ± 0.1 mm). These findings highlight the potential of insect-derived pyrolysis liquids as natural antibacterial agents, driven largely by their phenolic acid content. This study underscores the value of biowaste valorisation for developing bioactive compounds with potential applications in antimicrobial therapies.

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<https://doi.org/10.2298/JSC250512044O>

Keywords: *Tenebrio molitor*; biowaste pyrolysis; antibacterial activity; phytochemical composition; LC–MS/MS analysis.

INTRODUCTION

The growing demand for sustainable and eco-friendly solutions to combat microbial infections has intensified the search for novel antibacterial agents from unconventional sources.¹ In this context, insect biowaste, particularly from species such as mealworm *Tenebrio molitor*, has emerged as a promising substrate due to its rich organic content.² Through pyrolysis, biowaste can be thermochemically converted into valuable bio-oils containing a diverse range of phytochemicals.³ These bio-oils offer potential applications not only as fuels but also as bioactive materials with antioxidant and antimicrobial properties. Understanding the chemical composition and biological activities of these pyrolysis-derived liquids is crucial for unlocking their potential in pharmaceutical and environmental applications.⁴

The growing global concern over antibiotic resistance has prompted extensive research into alternative sources of antimicrobial agents. Natural and waste-derived compounds have emerged as promising candidates due to their bioactive properties and potential to combat resistant bacterial strains. One such source is the insect-derived biowaste, which has received increasing attention for its rich chemical profile and potential biological activities.⁵

Pyrolysis, a thermochemical decomposition process performed under an inert atmosphere, is widely used to convert organic waste materials into valuable products, including pyrolysis liquids rich in bioactive compounds.⁶ Pyrolysis liquids derived from plant biomass, algae, and animal waste have been shown to possess antimicrobial, antioxidant, and anti-inflammatory properties, making them suitable for pharmaceutical and agricultural applications.⁷ However, the use of insect-derived biowaste as a substrate for pyrolysis remains relatively unexplored, presenting a unique opportunity to valorize such materials.

Phenolic compounds, particularly phenolic acids and flavonoids, are key contributors to the biological activities of plant and waste-derived pyrolysis liquids.⁸ These compounds are well known for their antioxidant capacities, free radical scavenging activities, and ability to modulate inflammatory responses. Their antibacterial mechanisms include disrupting bacterial membranes, chelating metal ions and inhibiting nucleic acid synthesis.⁹ Identifying these compounds using advanced analytical techniques, such as liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS), allows for a detailed characterization of their potential therapeutic applications.

Several studies have demonstrated the antibacterial activity of pyrolysis products from different biomass sources. For example, Kim *et al.*¹⁰ reported that the pyrolysis liquids derived from pine and oak wood exhibited significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Similarly,

Demirbas¹¹ highlighted that bio-oils produced from agricultural residues showed antimicrobial effects against various Gram-positive and Gram-negative bacteria.

Insects such as *T. molitor* (mealworms) and *Hermetia illucens* (black soldier fly) have been increasingly studied for waste management and biomass production, but few studies have investigated the chemical analysis and antibacterial potential of their waste by-products.¹² Exploring the biowaste of *T. molitor* through pyrolysis offers a novel approach to both waste valorisation and bioactive compound discovery. The anticipated presence of phenolic acids such as cinnamic acid, salicylic acid, and ferulic acid could contribute significantly to antibacterial and antioxidant activities.¹³

The antimicrobial resistance among common pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* poses a severe threat to public health worldwide.¹⁴ The search for novel antibacterial agents, particularly from natural and waste-derived sources, is therefore a critical research priority. Investigating the antibacterial activity of pyrolysis liquids from insect waste not only addresses this need but also aligns with sustainable waste management practices and circular bioeconomy principles.

The present study aims to determine the antibacterial properties of extracts obtained from the pyrolysis of feeding waste of *T. molitor* adults and larvae and to analyse their chemical composition using LC–MS/MS. By evaluating their effects against both gram-positive and gram-negative bacteria, this research contributes to the expanding field of bioactive compound discovery from unconventional sources, the potential of insect-derived biowaste as an alternative antimicrobial reservoir.

By combining chemical profiling with biological evaluation, this study provides a comprehensive assessment of BPL derived from *T. molitor* biowaste, highlighting its potential as a natural antibacterial agent. The integration of waste management, chemical valorisation and antimicrobial exploration presents an exciting frontier in the development of sustainable solutions for public health challenges.

EXPERIMENTAL

Mealworm T. molitor production and waste handling

T. molitor adults and larvae were reared in controlled laboratory conditions at 70 % relative humidity and 28±2 °C under appropriate conditions. The insects were monitored weekly by keeping their food in plastic containers and fresh medium was added as needed in order to maintain optimum rearing conditions. The mixtures of the food given to the insects were adjusted as 30 wt. % wheat bran, 60 wt. % wheat flour and 10 wt. % corn flour (Fig. 1). The Fig. 1 shows the initial state of the mixture given as food to *T. molitor* individuals (left) and the waste state of the mixture (right). The darker coloured waste was used in the studies. The waste material consisting of feces, exuvia and leftover feed was collected after a period of four weeks by carefully sieving the contents of the containers to separate the insects from the accumulated waste. The collected feed waste was then air dried at room temperature for 48 h to remove

moisture and then ground into fine powder using a laboratory mill. This powdered biowastes were stored in airtight containers until further use in pyrolysis experiments.



Fig. 1. Mixture used in the study (dark coloured waste was used).

Obtaining pyrolysis liquid from biowastes

In this study, the biowaste pyrolysis liquid (BPL) derived from mealworms was used as a primary feedstock for the pyrolysis experiments (Fig. 2). The biowaste materials were first collected and subjected to a drying process at 60 °C for 24 h to remove excess moisture. Once dried, the biowaste was ground using a mechanical grinder to achieve a uniform particle size, enhancing thermal homogeneity and improving reaction efficiency during pyrolysis. Prior to the thermal degradation process, all materials were stored in airtight containers to prevent contamination and moisture absorption.



Fig. 2. PID controlled system producing BPL from biomass produced by mealworms.

The pyrolysis experiments were carried out using a custom-built laboratory-scale pyrolysis system equipped with the proportional-integral-derivative (PID) temperature controller for precise thermal management. The reactor chamber, fabricated from stainless steel, was designed to operate under oxygen-free conditions, ensuring the process occurred under a strictly inert nitrogen atmosphere. Nitrogen gas with a purity of 99 % was continuously supplied at a controlled flow rate of 60 mL min⁻¹ to maintain the inert environment and minimize oxidation of the volatile compounds. The entire pyrolysis run was conducted at a constant temperature of 295±5 °C, maintained for 7 h to ensure complete thermal conversion of the biomass material.

The reactor was equipped with a dual-stage condensation system consisting of two water-cooled condensers arranged in series. This configuration enabled the selective separation of heavy and light condensable vapors. During pyrolysis, the vapours generated from thermal decomposition were directed through these condensers, where cooling led to the formation of two distinct liquid fractions based on their boiling points. The heavy fractions were collected in the first condensation unit, while the lighter fractions were captured in the second condenser and stored in separate glass collection flasks.

After the pyrolysis process was completed, the collected liquid fractions were carefully transferred to a separation funnel to undergo phase separation and purification. The funnel was allowed to stand undisturbed for several hours to ensure complete settling of the immiscible layers. Following the separation, the upper and lower liquid phases – representing light and heavy pyrolysis oils respectively – were individually isolated and filtered to remove any particulate residues. These purified fractions were then stored in amber glass bottles at 4 °C prior to subsequent chemical characterization and analysis. Consistent preparation methods, standardized operating conditions and controlled environments were maintained throughout the study.

Identification of phenolic compounds by LC–MS/MS

A Thermo Scientific brand LC–MS/MS device was used to measure the amounts of phenolic components in the biowaste pyrolysis liquid (BPL) studied in this research. After the samples were dissolved in methanol to be 10 mg mL⁻¹, they were filtered through a 0.45 µm syringe filter and transferred to the vial. The chromatographic separation was carried out on an ODS HYPERSIL model (250 mm×4.6 mm, 5 µm) analytical column. The analysis test conditions used in the study are given in Table I.

TABLE I. LC–MS/MS analysis conditions of biowaste pyrolysis liquid (BPL)

	Time, min	A (0.1 % formic acid in water)	B (% methyl alcohol)
Solvent program	0	100	0
	1	100	0
	22	5	95
	25	5	95
	30	0	100
Solvent flow rate	0.7 mL min ⁻¹		
Column oven temperature	30 °C		
Column properties	ODS HYPERSIL 250 mm×4.6 mm, 5 µm column		
Injection volume	20 µL		
Analysis time	34 min		

Mass spectrometric determination was performed using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI–MS/MS data were processed by LabSolutions software (Shimadzu). Multiple reaction monitoring (MRM) mode was used for quantification of phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on scanning of ion transitions from the specified precursor phytochemical to the fragment. Collision energies (*CE*) were optimized to produce the optimal phytochemical fragmentation and maximum transmission of desired product ions.¹⁵ MS analysis, drying gas flow, and MS/MS operating conditions are expressed in Table II.

TABLE II. LC–MS/MS instrument operating conditions

Capillary temperature	300 °C
Vaporizer temperature	350 °C
Sheath gas pressure	30 Arb
Aux gas pressure	13 Arb
Spray voltage (positive polarity)	4000 V
Spray voltage (negative polarity)	2500 V
Discharge current	4.0 μ A

Determination of antibacterial activity

The antibacterial activity of the samples was investigated against two gram-negative (*E. coli* ATCC-25922 and *P. aeruginosa* ATCC-27853) and two gram-positive (*E. faecalis* ATCC-23212 and *S. aureus* ATCC-25923) bacterial strains. The antimicrobial activities of the samples were assessed using the agar well diffusion method with some modifications, as described by Karpuz *et al.*¹⁶ For this purpose, Petri dishes prepared with nutrient agar (NA) medium were inoculated with overnight cultures of the bacterial strains using the spread plate technique. To allow diffusion into the agar layer, the inoculated Petri dishes were kept in a laminar air flow cabinet (Thermo Fisher scientific laminar air flow, USA) for approximately 1 h. After this period, wells with a diameter of 6 mm were made in the agar plates using a cork borer. Each well was filled with 50 μ L of sample using a micropipette. Ampicillin (working concentration 50 μ g mL⁻¹) was used as a positive control. The prepared Petri dishes were then incubated at 37 °C for 24 h in an incubator (Mettler, Germany). At the end of the incubation period, the diameter of the inhibition zones formed around the wells was measured to determine the antibacterial activity.

Statistical analysis

All experimental measurements were performed in triplicate and the results were reported as mean $\pm$ standard deviation (STD). The data obtained were analysed by one-way analysis of variance (One-way ANOVA) using IBM SPSS Statistics 21 software. Statistically significant differences were accepted at the $p < 0.05$ level.

RESULTS AND DISCUSSION

LC–MS/MS analysis results of phenolic compounds

Phenolic compounds found in plants play a significant role as potent antioxidants capable of neutralizing free radicals in biological systems. These compounds provide various health benefits by reducing cellular damage. Among phenolic compounds, flavonoids are particularly noteworthy due to their broad distribution and diverse biological activities. As key members of the polyphenol family, flavonoids comprise more than 4,000 compound types present in different plant organs, including roots, flowers, leaves and fruits. The biological properties of these compounds form an essential basis for understanding the health effects of plant-based foods.

To accurately identify phenolic and flavonoid compounds, advanced analytical techniques are employed, and in this context, mass spectrometry (MS) is frequently preferred, particularly for analyses requiring high sensitivity and accu-

acy.⁸ Furthermore, the LC–MS/MS technique offers higher selectivity and sensitivity compared to other liquid chromatography methods, making it widely used for quantitative analyses. This method provides effective results in the detailed analysis of flavonoids and other phenolic compounds.¹⁷

In line with this information, LC–MS/MS analysis was employed in the present study to identify the phytochemicals present in the BPL. Among the 32 standard compounds used by LC–MS/MS, seven phytochemical compounds were detected. The distribution of the quantitative findings of the phenolic compounds in the bio-waste is presented in Table III. Additionally, the analytical performance of the LC–MS/MS method is shown in Table IV. The main peak chromatogram rep-

TABLE III. Distribution of quantitative findings of phenolic compounds of BPL; the mean of three replicate samples ($n = 3$) $\pm$ STD ($p < 0.05$); NF – not found

Compound	Content, μg phenolic compound/g pyrolysis liquid
Gallic acid	NF
Protocatechuic acid	NF
Protocatechuic aldehyde	NF
Sesamol	NF
Gentisic acid	24.75 $\pm$ 1.21
Catechin	NF
Chlorogenic acid	NF
Epicatechin	NF
Caffeic acid	4.38 $\pm$ 0.13
Vanillin	NF
Syringic acid	NF
p_Coumaric acid	NF
Taxifolin	NF
Ferulic acid	18.47 $\pm$ 1.34
Salicylic acid	36.26 $\pm$ 1.56
4-Hydroxybenzoic acid	37.35 $\pm$ 1.98
Hesperidin	NF
Rosmarinic acid	NF
Oleuropein	NF
Luteolin-7-glucoside	NF
Rutin	NF
Resveratrol	NF
Ellagic acid	NF
Cinnamic acid	358.05 $\pm$ 11.32
Naringenin	37.11 $\pm$ 2.12
Quercetin	NF
Luteolin	NF
Apigenin	NF
Pinoembrin	NF
Chrysin	NF
Galangin	NF
Flavone	NF

representing the composition of the bio-waste obtained by the LC–MS/MS method is provided in Fig. 3. The quantities of the flavonoid compounds and phenolic acid types, identified in the extracts are reported as μg analyte/g extract.

TABLE IV. Analytical performance of LC–MS/MS method; linear range: 0.125–10 ppm

Phenolic compound	Equation	R^2
Gallic acid	$Y = -1772.12 + 66065.7X$	0.9949
Protocatechuic acid	$Y = 27640.8 + 35692.1X$	0.9926
Protocatechuic aldehyde	$Y = 506291 + 900489X$	0.9963
Sesamol	$Y = 190429 + 294285X$	0.9965
Gentisic acid	$Y = 81035.3X$	0.9979
Catechin	$Y = -46484.8 + 91012.1X$	0.9987
Chlorogenic acid	$Y = 21653.3X$	0.9937
Epicatechin	$Y = -137604 + 217920X$	0.9951
Caffeic acid	$Y = 166126 + 470479X$	0.9981
Vanillin	$Y = 18407.3 + 4851.4X$	0.9937
Syringic acid	$Y = 150673 - 55037.7X$	0.9149
p_Coumaric acid	$Y = 79931.9 + 73318.2X$	0.9927
Taxifolin	$Y = 8374.89 + 42570.1X$	0.9979
Ferulic acid	$Y = 96528.3 + 177434X$	0.9941
Salicylic acid	$Y = 485398 + 972318X$	0.9960
4-Hydroxybenzoic acid	$Y = 537544 + 881649X$	0.9951
Hesperidin	$Y = 1.43184e + 006X$	0.9977
Rosmarinic acid	$Y = -127182 + 253431X$	0.9980
Oleuropein	$Y = 375327 + 1.76005e + 006X$	0.9969
Luteolin-7-Glycoside	$Y = 135610X$	0.9985
Routine	$Y = 2.20056e + 006 + 1.00456e + 007X$	0.9977
Resveratrol	$Y = 97778.9 + 63601.2X$	0.9988
Ellagic Acid	$Y = 745549 + 1.07598e + 006X$	0.9977
Cinnamic Acid	$Y = 536496X$	0.9957
Naringenin	$Y = 2.35935e + 006X$	0.9986
Quercetin	$Y = 111352 + 2.43427e + 006X$	0.9941
Luteolin	$Y = 1.48908e + 007X$	0.9981
Apigenin	$Y = 831953X$	0.9987
Pinocembrin	$Y = 789059X$	0.9979
Krisin	$Y = 1.33937e + 007X$	0.9972
Galangin	$Y = 1.66971e + 006X$	0.9929
Flavone	$Y = 1.57469e + 007 + 3.51252e + 007X$	0.9954

As seen in Table III, cinnamic acid was the phenolic compound detected at the highest concentration (358.05 $\mu\text{g/g}$), making it the predominant phenolic compound in the extract. Salicylic acid (36.27 $\mu\text{g/g}$), 4-hydroxybenzoic acid (37.35 $\mu\text{g/g}$), and naringenin (37.11 $\mu\text{g/g}$) were also found in considerable amounts and are important in terms of biological activity (anti-inflammatory, antioxidant effects). The hydroxycinnamic acid derivatives such as caffeic acid and ferulic acid were also present, contributing to the antioxidant potential of the extract. Most

flavonoid derivatives (e.g., quercetin, apigenin, rutin) were not detected, suggesting that the phenolic composition of the extract is predominantly centred around phenolic acids. The LC–MS/MS analysis revealed that phenolic acid derivatives were the major constituents in the analysed sample. Notably, cinnamic acid, salicylic acid, and 4-hydroxybenzoic acid were detected at high concentrations. In contrast, most flavonoid compounds were below the detection limit or absent in the sample. These findings suggest that the antioxidant capacity of the extract may largely be attributed to its phenolic acid content. Table IV shows the analytical performance of the LC–MS/MS method for the samples.

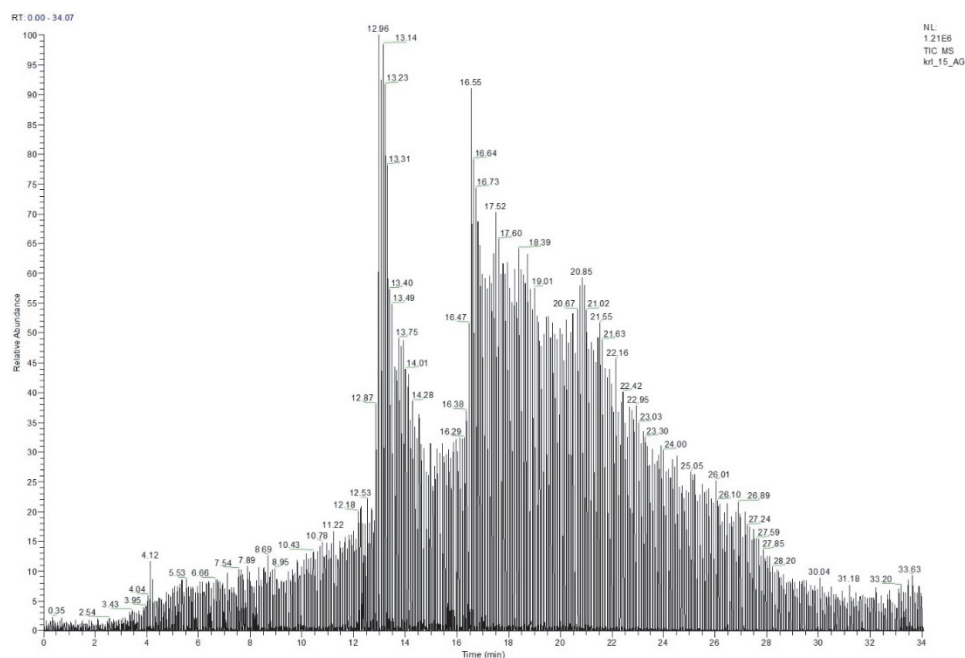


Fig. 3. LC–MS/MS total ion chromatogram (TIC) of biowaste pyrolysis liquid (BPL).

Antibacterial analysis results

The antibacterial properties of BPL were investigated using the agar well diffusion method. Antibacterial activity was evaluated against two gram-negative (*E. coli* and *P. aeruginosa*) and two gram-positive (*E. faecalis* and *S. aureus*) bacterial strains. These strains were selected due to their significant threat to human health and the urgent need for new antibiotics to combat them. Gram-positive and gram-negative bacteria represent major global public health concerns.¹⁴ Ampicillin antibiotic was used as a positive control to determine antibacterial efficacy. Images of the Petri dishes obtained from the experiment are presented in Fig. 4.

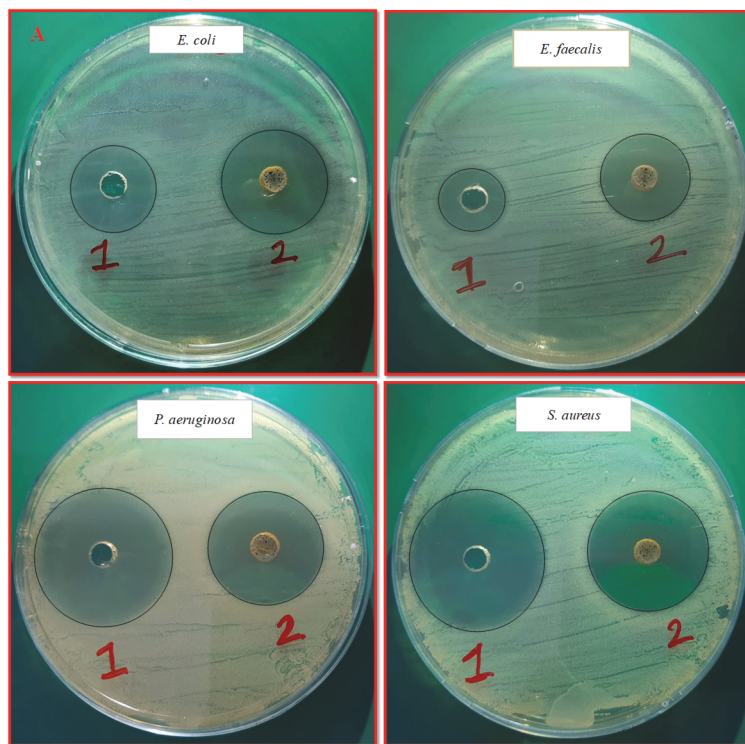


Fig. 4. Petri plates showing the antibacterial activity; 1: ampicillin ($50 \mu\text{g mL}^{-1}$) and 2: BPL sample (20 mg L^{-1}).

It is well known that the antibacterial activity of NPs is dependent on their concentration and size.¹⁸ Therefore, preliminary experiments were first conducted in this study to determine the minimum BPL concentration. Based on this information, the inhibitory zones were assessed to evaluate the effects of the BPL sample on bacterial growth inhibition. The BPL sample exhibited good antibacterial activity against all bacterial strains tested in this study. The highest antibacterial activity of the BPL sample was observed against *S. aureus*, with an inhibition zone of $29.5 \pm 0.3 \text{ mm}$, followed by *P. aeruginosa* ($23.5 \pm 0.2 \text{ mm}$) and *E. coli* ($21.7 \pm 0.1 \text{ mm}$). The ampicillin antibiotic used in the study also demonstrated antibacterial activity against all bacterial strains (Fig. 5). Overall, the BPL sample showed good antibacterial efficacy when compared with the antibiotic ampicillin.

CONCLUSION

The present study successfully demonstrated that biowaste pyrolysis liquids (BPL) obtained from *T. molitor* adults and larvae exhibit promising antibacterial properties against both gram-positive and gram-negative bacterial strains. The agar well diffusion results showed clear inhibitory zones, with the strongest effects obs-

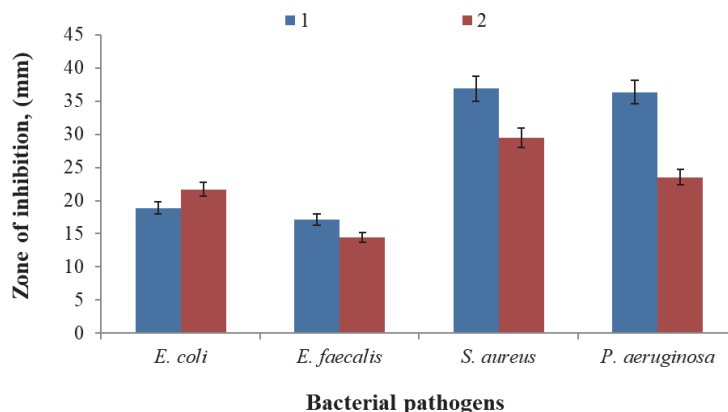


Fig. 5. Antibacterial activity of BPL sample against tested bacterial strains based on inhibition zone diameters; 1: ampicillin (50 µg mL⁻¹) and 2: BPL samples (20 mg L⁻¹). The values were the mean of three replicate samples ($n = 3$) $\pm$ STD. Statistical analysis was performed using one-way ANOVA ($p < 0.05$) in IBM SPSS Statistics 21.

erved against *S. aureus* and *P. aeruginosa*, suggesting the broad-spectrum antibacterial potential of BPL. These findings are particularly noteworthy given the growing threat of antibiotic-resistant bacteria, highlighting the importance of exploring the alternative antimicrobial agents derived from natural and sustainable sources.

The LC–MS/MS phytochemical analysis revealed that the major contributors to the observed biological activities were phenolic acids, especially cinnamic acid, which was present at remarkably high levels. This compound, along with salicylic acid and 4-hydroxybenzoic acid, is known for its antioxidant, anti-inflammatory, and antimicrobial activities, supporting the observed antibacterial effects of the BPL samples. Interestingly, most flavonoid derivatives were absent or below detection limits, suggesting that the antimicrobial activity is largely attributable to phenolic acids rather than flavonoids.

The thermal degradation process during pyrolysis appears to selectively enrich certain bioactive compounds while eliminating others, offering a unique method for the obtaining of antibacterial agents from insect biowastes.¹⁹ This aligns with the current interest in circular bioeconomy approaches that aim to upcycle waste materials into high-value products with therapeutic potential.^{20,21}

While the study provides some valuable insights, further research is needed to understand the mechanism of antibacterial action, optimize the pyrolysis conditions for maximum yield of active compounds and assess the safety and cytotoxicity of these extracts in clinical settings. Moreover, investigating the synergistic effects of BPL with conventional antibiotics, may open new avenues for combating multi-drug-resistant pathogens.

In conclusion, the findings support the potential of insect-derived pyrolysis liquids as an innovative and sustainable source of antibacterial agents. With further

refinement and validation, BPL extracts could contribute to the development of novel antimicrobial formulations, addressing a critical need in global health.

ИЗВОД

ОДРЕЂИВАЊЕ АНТИБАКТЕРИЈСКЕ АКТИВНОСТИ И ХЕМИЈСКОГ САСТАВА
ПИРОЛИТИЧКЕ ТЕЧНОСТИ НАСТАЛЕ ИЗ НУТРИТИВНОГ ОТПАДА ОДРАСЛОГ ЦРВА
И ЛАРВЕ *Tenebrio molitor* L. (Coleoptera: Tenebrionidae)

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У овој студији су испитиване антибактеријске особине и фитохемијски састав пиролитичке течности добијене из биоотпада одраслог црва и ларве *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Биоотпад је подвргнут пиролизи на 295 ± 5 °C током 7 h у атмосфери азота, користећи лабораторијски систем са двоструким кондензатором у циљу раздвајања тешких и лаких фракција. Пиролитичка течност биоотпада (BPL) је пречишћена и анализиран је хемијски састав применом LC–MS/MS. Идентификовано је седам главних фенолних једињења, од којих је цинаминска киселина била најприсутнија (358,05 µg/g), а затим салицилна киселина, 4-хидроксибензојева киселина и нарингенин. Већина флавоноида је била одсутна или испод детекционог лимита, указујући да су фенолне киселине доминантне у екстракту. Антибактеријска активност BPL је тестирана спрам два соја Грам-негативних (*E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853) и два соја Грам-позитивних бактерија (*E. faecalis* ATCC-23212, *S. aureus* ATCC-25923), користећи метод дифузије у агару. Резултати су показали значајан антибактеријски ефекат спрам *S. aureus* ($29,5 \pm 0,3$ mm) и *P. aeruginosa* ($23,5 \pm 0,2$ mm), као и *E. coli* ($21,7 \pm 0,1$ mm). Добијени подаци указују на антибактеријски потенцијал пиролитичких течности пореклом од инсеката, пре свега због присуства фенолних киселина. Ова студија истиче важност испитивања биоотпада у циљу развоја биоактивних једињења која се могу применити у антибактеријској терапији.

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The role of non-covalent interactions in the solvation dynamics of metronidazole in water: A theoretical study

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Abstract: Metronidazole, the medicine with the brand name Flagyl, is used to treat the gastrointestinal infection and the activity against anaerobic bacteria like protozoa. The current work describes the interaction of metronidazole drug with water solvent needed for oral ingestion, which is the most common and convenient pathway for the administration of the drug in the body. Computational calculations are performed to optimize the metronidazole drug having solvent (water) at different positions. NBO and AIM calculations employed to determine the strength of intermolecular hydrogen bonding interactions between metronidazole and solvent (water). The second perturbation energy was calculated and the result was a maximum of 67 kJ mol⁻¹ for O–H...O hydrogen bonding interaction. The solvation energy of the metronidazole drug, determined using the solvation model based on density (SMD) model, is found to be –69.2 kJ mol⁻¹. The bonding parameters of the solvated drug have been analysed through critical point calculations based on the atom-in-molecule (AIM) theory. Furthermore, an *ab initio* molecular dynamics (AIMD) study reveals that the lowest decomposition energies of metronidazole in the presence of water, considering all pores and different pores, are –927.86 and –699.003 a.u., respectively.

Keywords: drug; flagyl; gastrointestinal infection; SMD; AIM; NBO.

INTRODUCTION

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is a well-known drug for gastrointestinal infection and popularly used in Flagyl medicine.^{1–3} The compound show activity against both gram-negative and gram-positive anaerobic bacteria as well as protozoa. It is previously found effective against protozoan such as giardia lamblia, entamoeba histolytica.^{4,5} It has rapid as well as effective abs-

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orption due to its smaller molecular structure. It penetrates all tissues such as bones and blood bone barrier.^{4–6}

Oral ingestion is the most common and preferred route for drug administration due to its cost-effectiveness, patient compliance, and dosage flexibility.⁷ Nearly two-thirds of small-molecule drugs are delivered orally, accounting for about 90 % of the global medicine market, valued at approximately \$35 million.^{8,9} However, poor solubility remains a major challenge, affecting dissolution rate, efflux susceptibility and first-pass metabolism.

Solvation plays a crucial role in determining a drug's ability to achieve the therapeutic concentrations necessary for effective medicinal application. The pharmacological efficacy of a drug is strongly influenced by its aqueous solubility; therefore, poorly soluble drugs often fail to attain the required plasma concentrations for optimal therapeutic action. Limited water solubility adversely affects the drug's formulation, bioavailability and disaggregation in pharmaceutical preparations. Moreover, the low solubility and poor dissolution rate of sparingly soluble drugs can lead to suboptimal absorption in the gastrointestinal tract, potentially causing adverse effects and compromising oral drug delivery. In this context, the solvation dynamics of metronidazole are investigated to obtain molecular-level insights into its solubility behaviour. These dynamics play a pivotal role in determining its oral bioavailability, dissolution rate, and hydrogen bonding interactions with the aqueous environment – factors that are essential for the effective drug absorption and pharmacological performance. Additionally, a detailed analysis of the non-covalent interactions between metronidazole and water at different solvation sites has been conducted to provide the mechanistic insights into its solvation characteristics.

COMPUTATIONAL DETAILS

Structural conformation

Structural confirmation of metronidazole is performed using Vconf software.^{10,11} This software is an important tool for conformational search through the second generation mining minimum algorithm and it utilizes free energy of the molecular system.

Structure optimization

A quantitative study of the interaction between molecules in complexes has been performed. The structural optimization and binding energy calculations along with electrostatic potential have been carried out using Turbomole^{12,13} and G09¹⁴ quantum calculation package and graphical user interface TmoleX¹⁵ and Gview.¹⁶ To evaluate the reliability and consistency of the computed structural and energetic parameters, calculations were performed using a range of electronic structure methods, including the hybrid functional B3LYP,¹⁷ the meta-hybrid functional M05-2X¹⁸ known for its improved treatment of noncovalent interactions, the dispersion-corrected B97-D¹⁹ functional, and the correlated wavefunction-based MP2^{20,21} method. All computations employed the correlation-consistent triple- ζ quality basis set, cc-pVTZ,^{22–24} which offers a balanced description of valence electron correlation. This multi-level approach ensures a robust comparison, with a higher level than the density functional and post-Hartree–

–Fock frameworks, particularly in capturing subtle intermolecular interactions. Harmonic vibrational frequency analyses were carried out for the conformers of metronidazole at several theoretical levels to ensure that the optimized structures represented true local minima. The interaction energies of different conformers were refined by incorporating corrections for both basis set superposition error (BSSE) and zero-point vibrational energy (ZPE). BSSE was accounted for using the counterpoise technique proposed by Boys and Bernardi.²⁵

Natural bond orbital (NBO), non-covalent interactions (NCI), atom in molecule (AIM) and eenergy decomposition analysis (EDA) study

The NBO analysis provides insights into bonding, electron density, and charge transfer, while NCI analysis quantifies non-covalent interactions like hydrogen bonding and van der Waals forces.^{26–28} AIM analysis offers a topological view of electron density, and EDA quantifies intermolecular interactions. Geometry optimizations at the M05-2X/cc-pVTZ^{18,22–24} level were used for NBO,²⁹ NCI,²⁸ AIM³⁰ and EDA analyses.³¹ NCI calculations were performed using NCIPLOT,²⁸ AIM using Multiwfn,^{32–34} and NBO using G09 with NBO7.²⁷ The intermolecular interaction energies in dimer and trimer systems were analysed via EDA using the localized molecular orbital (LMO) approach in the GAMESS (U.S.A.) program.³⁵

Solvation study

A solvation study involves analysing the interactions and effects of a solvent on a solute. One effective approach to incorporating solvent effects is through implicit solvation models such as the SMD model.^{36,37} Developed by the Cramer and Truhlar research groups, the SMD solvation model has been applied to investigate the solvent influence on metronidazole. This advanced continuum solvation method is based on the quantum mechanical charge density of the solute, treating the solvent as a continuous medium. The term “SMD” signifies the direct use of full electron density (“D” for density) without the need to define partial atomic charges. Rather than explicitly modelling solvent molecules, this approach represents the solvent as a dielectric medium with surface tension at the solute–solvent interface, providing a more efficient and accurate depiction of solvation effects.³⁷ The model uses the full solute electron density without explicit charges on solute whereas uses the surface tension over the solute–solvent boundary.

The implicit solvation model is applied for the study of solvation of Metronidazole using at PBE0-D3/def2-TZVP^{38–40} “tightopt” level. The solvated free energy change (ΔG_{solv}) can be calculated using the equation: $\Delta G_{\text{solv}} = E_{\text{solv}} + \Delta G_{\text{el}} + (\Delta G_{\text{cav}} + \Delta G_{\text{disp}})$. It includes liquid phase expectation value, electron-polarization and non-electrostatic contribution. The non-electrostatic contribution (ΔG_{net}) of the solvated system includes cavitation energy (ΔG_{cav}) and the dispersion energy (ΔG_{disp}) during solvation of the solute.

Ab initio molecular dynamics assay

Ab initio molecular dynamics (AIMD) study has already shown some significant benefits over traditional molecular dynamics.^{41,42} AIMD is on-the-fly method to obtained electronic structure related information from the recent trajectory. AIMD study is performed for Metronidazole with water using ORCA 5.0.3 software⁴³ at a timestep of 1.0 fs. The temperature was kept constant at 350 K whereas Timecon of 10.0 fs is used. The study was performed at PBE0-D3/def2-SVP level of theory using Orca 5.0.3.⁴³ The interesting fact was that the variation in energy scales between the SMD implicit the solvation model and the AIMD arises from their distinct methodologies. The SMD uses a static, continuum approach that approximates solvation effects without explicit solvent molecules or thermal fluctuations, yielding single-point

energies. In contrast, AIMD includes the explicit solvent interactions and thermal motion, providing time-averaged energies that account for entropic and dynamic effects.

RESULTS AND DISCUSSION

Geometry of metronidazole and its solvated structure

The lowest-energy conformer of Metronidazole, identified using Vconf and binding energy calculations (Fig. 1a), is reported in the Supplementary material to this paper. It comprises 21 atoms: 3 nitrogen, 3 oxygen, 6 carbon and 9 hydrogen. The solvated metronidazole has water at four positions, which are pore1, pore2, pore3 and pore4. These optimized geometries are abbreviated considering the position of water, which are Met-wtr.P1, Met-wtr.P2, Met-wtr.P3 and Met-wtr.P4, respectively (see Fig. 1) at B3LYP/cc-pVTZ level of theory. Optimized coordinates of metronidazole and its complexes (Met-wtr.P1–P4, All) at the M05-2X/cc-pVTZ level are provided in Tables S-XI–S-XVI of the Supplementary material. The corresponding binding energies with BSSE correction (ΔE_e) are calculated -53.09 , -39.83 , -40.12 and -30.38 kJ mol $^{-1}$, respectively, whereas the binding energies with both BSSE and zero-point energy correction (ΔE_0) are calculated -43.76 , -31.25 , -31.97 , and -23.56 kJ mol $^{-1}$, respectively (see Table I). Eventually the completely solvated metronidazole has all pores filled with water molecules, which is abbreviated as Met-wtr.All. Interestingly, the ΔE_e and ΔE_0 values for completely solvated metronidazole at all pores is calculated -164.35 and -131.84 kJ mol $^{-1}$, respectively. The full vibrational frequency analyses at DFT in the Supporting material to this paper-optimized geometries confirmed all stationary points as true local minima, with no imaginary frequencies observed.

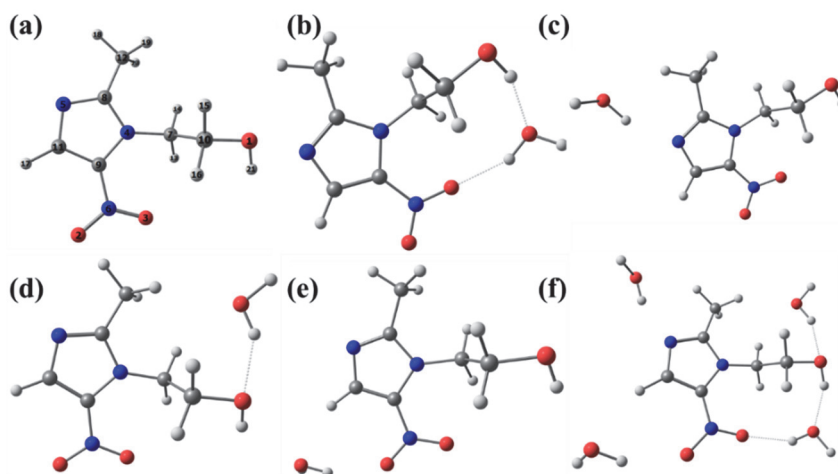


Fig. 1. Optimized structures of: a) Metronidazole, b) Met-wtr.P1, c) Met-wtr.P2, d) Met-wtr.P3, e) Met-wtr.P4 and f) Met-wtr.All at M05-2X/cc-pVTZ level of theory.

TABLE I. Binding energies (in kJ mol⁻¹) for complexes of metronidazole with water solvent molecules

Geometry	B3LYP/cc-pVTZ		M05-2X/cc-pVTZ		B97D/cc-pVTZ		MP2/cc-pVTZ	
	ΔE_e	ΔE_o	ΔE_e	ΔE_o	ΔE_e	ΔE_o	ΔE_e	ΔE_o
Met-wtr.P1	-53.09	-43.76	-134.31	-125.35	-55.98	-47.40	-41.00	-31.97
Met-wtr.P2	-39.83	-31.25	-117.53	-108.53	-40.00	-31.97	-28.70	-20.21
Met-wtr.P3	-40.12	-31.97	-124.06	-115.10	-46.69	-38.28	-30.79	-22.38
Met-wtr.P4	-30.38	-23.56	-107.74	-101.00	-30.00	-23.97	-22.68	-16.15
Met-wtr.All	-164.35	-131.84	-457.39	-424.59	-175.94	-144.01	-84.56	-52.22

In Met-wtr.P1, O1-H21 and O22-H24 bond lengths are 0.96748 and 0.96274 Å, respectively. The corresponding lengths in bare metronidazole and water molecule are 0.95884 and 0.95742 Å, respectively. The increase of bond lengths is attributed to O22...O1-H21 and O3...O22-H24 hydrogen bonding interactions. O22-H24 and C12-H18 bond lengths are 0.96876 and 1.08469 Å, respectively in Met-wtr.P2. The hydrogen bonding interactions such as N5...O22-H24 and O22...C12-H18 are responsible for the increase of bond lengths in Met-wtr.P2. Furthermore O1...O22-H24 and O22...C11-H17 as well as O2...O22-H23 hydrogen bonding interactions are present in Met-wtr.P3 and Met-wtr.P4, respectively.

Charge density analysis of the solvated metronidazole

Mulliken population analysis (MPA) charges of metronidazole-water complexes at four positions (Met-wtr.P1, Met-wtr.P2, Met-wtr.P3, Met-wtr.P4) were calculated at the M05-2X/cc-pVTZ level and are presented in Fig. S-1 of the Supplementary material, with the corresponding data provided in Table S-V of the Supplementary material. The study reveals a decrease in the MPA charge of oxygen O22 from -0.4783 e in bare water to -0.5027, -0.5073, -0.4858 and -0.4877 e in Met-wtr.P1, P2, P3 and P4, respectively, due to hydrogen bonding. MPA charges of O1 and O3 in Met-wtr.P1 increased to -0.3896 and -0.3833 e from -0.3648 and -0.3249 e in bare metronidazole, influenced by O1-H21...O22 and O22-H24...O3 hydrogen bonding. Similarly, the charge of N5 in Met-wtr.P2 rose to -0.3140 from -0.2568 e due to N5...O22-H24 bonding. MPA charges of O1 in Met-wtr.P3 and O2 in Met-wtr.P4 were -0.32804 and -0.27956 e, respectively, reflecting changes due to hydrogen bonding interactions (O1...O22-H24 in Met-wtr.P3 and O2...O22-H23 in Met-wtr.P4).

HOMO-LUMO energy variation of the metronidazole

HOMO and LUMO energies provide crucial chemical information. E_{HOMO} relates to electron donation ability, while E_{LUMO} is related to the electron acceptance. Higher E_{HOMO} increases electron donation tendency, requiring less energy for electrons to jump to unoccupied states. Lower E_{LUMO} enhances electron acceptance. For metronidazole, E_{HOMO} and E_{LUMO} are -7.075 and -2.441 eV,

respectively. On water solvation, E_{LUMO} values for Met-wtr.P1, Met-wtr.P2, Met-wtr.P3, Met-wtr.P4 and Met-wtr.All are -2.716 , -2.664 , -2.351 , -2.699 and -3.045 eV, showing decreased electron acceptance. E_{HOMO} values for these are -7.023 , -7.347 , -6.939 , -7.173 and -7.293 eV, indicating a higher electron donation tendency. Met-wtr.All has a band gap of 4.249 eV, with ionization energy, electron affinity, chemical hardness, electronic chemical potential and electrophilicity index calculated as 7.293 , 3.045 , 2.124 , -5.169 and 12.579 eV, respectively. Various other molecular parameters in order to understand the characteristics features of metronidazole. Various other molecular parameters in order to understand the characteristics features of metronidazole along with water are shown in Table S-I of the Supplementary material.

Solvation of Metronidazole

Solvation model mimics the environment of multiple solvation shells without placing it close to the drug. Metronidazole drug is soluble in water and all the atoms of metronidazole are close to the boundary along with the solvent. Table S-III of the Supplementary material also shows the solvation data with other solvents. The result shows that surface charge (e) indicates the electrostatic contribution to solvation free energy from interactions between the solute's charge distribution and the solvent environment. Water exhibits the highest surface charge (-28.207 e), reflecting its high polarity and strong electrostatic interactions with the solute. Nonpolar solvents like tetrahydrofuran (THF) and chloroform have smaller surface charge values (-14.531 e and -16.932 e, respectively), consistent with weaker electrostatic interactions. Charge correction is a correction term for inaccuracies in the computed electrostatic interactions. The values are very small (0.02 – 0.03), indicating minimal correction is needed, which suggests the computational model accurately represents the solvent environment. The free energy (cavitation + dispersion) represents the combined free-energy contributions from cavity formation (to accommodate the solute) and van der Waals dispersion interactions between solute and solvent. Water shows the largest positive value (18.853 kJ mol $^{-1}$), reflecting a significant cost of cavity formation and weak dispersion interactions. Solvents like pyridine and THF have more negative values (-16.342 and -15.790 kJ mol $^{-1}$), indicating more favourable van der Waals dispersion contributions.

The single point energies of metronidazole and Metronidazole–water system are -623.4575 and -623.4774 a.u., respectively. Therefore, the solvated system of Metronidazole with water has stabilization energy of -0.0199 a.u., *i.e.*, -52.245 kJ mol $^{-1}$.

Using SMD solvation model, CPCM the dielectric energy is calculated -95.980 kJ mol $^{-1}$ and the energy of cavity dispersion solvated system (SMD CDS (Gcgs)) in the water solvent is calculated -0.0264 a.u., *i.e.*, -69.203 kJ mol $^{-1}$.

Non-covalent interactions (NCI) of Metronidazole

NCI analysis provide electron density (s) and reduced density gradient (ρ) for solvated metronidazole in different pores (Fig. S-2 of the Supplementary material). The isosurface extraction of reduced density gradient is depicted in Fig. S-3 of the Supplementary material. Color schemes indicate interaction types: blue for strong attractive, green for weak non-covalent (van der Waals), and red for repulsive forces.^{28,44} Spikes in Fig. S-2 indicate interactions, with those between -0.02 and 0.02 representing hydrogen bonding. Greenish-blue overlaps in Fig. 2 illustrate intermolecular hydrogen bonding and some intramolecular interactions within Metronidazole in vicinity of four water molecules only.

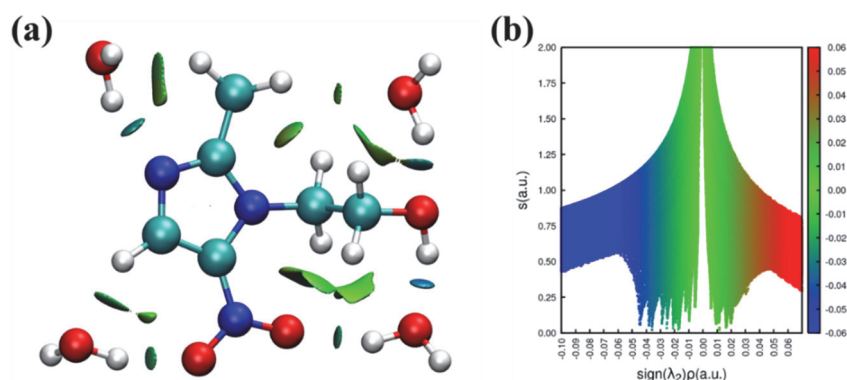


Fig. 2. Isosurface extractions (a) of its RDG plots (b) of NCI analysis of Met-wtr.All.

Energy decomposition analysis (EDA) of Metronidazole with water molecules

The EDA study provides the information regarding the various components of interaction energy for the solvated complexes of metronidazole depending upon the various positions of the water. The total interaction (ΔE_{tot}) is decomposed into exchange energy (ΔE_{ele}), repulsion energy (ΔE_{rep}), polarization energy (ΔE_{pol}) and dispersion energy (ΔE_{disp}). These values are depicted in Table S-II of the Supplementary material. The result assists that the electrostatic component is -70.29 kJ mol^{-1} for Met-wtr.P1, which are -47.95 , -46.69 and -30.96 kJ mol^{-1} for Met-wtr.P2, Met-wtr.P3 and Met-wtr.P4, respectively. The dispersion components are calculated -40.29 , -27.87 , -35.86 and -23.14 kJ mol^{-1} , respectively. Interestingly, the electrostatic component is found nearly double of the dispersion component for Met-wtr.P1 and Met-wtr.P2 whereas the dispersion component is nearly $3/4^{\text{th}}$ of electrostatic component for Met-wtr.P3 and Met-wtr.P4. The high electrostatic component is attributed to the strong hydrogen bonding interaction such as $\text{H-O}\cdots\text{H}$, $\text{O-H}\cdots\text{N}$. The four hydrogen bonding interactions in Met-wtr.All attributed the increase of electrostatic component in comparison to dispersion component.

Natural bond orbital (NBO) study of Metronidazole

NBO analysis was performed for all four pore positions of water near Metronidazole, as well as for metronidazole occupying each pore with surrounding water molecules.⁴⁵ Second order perturbation energy or donor-acceptor interaction energy, $E_{i \rightarrow j}^{(2)}$, the most important parameter, has been evaluated for all interactions between NBO donor (i) and acceptor (j).^{26,46}

The NBO study is performed for metronidazole with water solvent at all four pores or position. At pore1, the water molecule is situated in the pore between nitro- and hydroxyl-groups. In pore2, the water molecule is placed at the pore close to N5 and methyl group (close to H18) of metronidazole while the water molecule is placed closed to methyl and hydroxyl groups (close to O1) of Metronidazole in pore3. In pore4, the water takes the place close to nitro group and H17. In case of Met-wtr.All, all the pores are occupied by water molecules.

Fig. 1 shows the structures of Metronidazole having water at different pores and all pores. Several other interactions are calculated between metronidazole and water molecules (see Table S-IV of the Supplementary material). Fig. S-4 of the Supplementary material shows the NBO overlap for the important non-covalent interactions for Met-wtr.P1, Met-wtr.P2, Met-wtr.P3 and Met-wtr.P4. In Met-wtr.P1, the major interaction of the bonding orbitals LP(2)O22 and LP(1)O3 has been found with BD*(1)O1-H21 and BD*(1)O22-H24 due to H–O...H conventional hydrogen bonding. The $E_{i \rightarrow j}^{(2)}$ value for the corresponding interaction has been calculated 64.48 and 16.32 kJ mol⁻¹, respectively. Similarly, Met-wtr.P2 complexes show the strong hydrogen bonding interaction between metronidazole and water. The interaction has been reported between bonding orbitals of LP(1)N5 and LP(2)O22 with antibonding orbitals BD*(1)O22-H24 and BD*(1)C12-H18 whereas the $E_{i \rightarrow j}^{(2)}$ values are 46.65 and 11.00 kJ mol⁻¹, respectively. Further solvated metronidazole having water in pore3 interacts through its hydroxyl and methyl groups therefore the O1...O22–H24 hydrogen bonding interaction stabilizes the complexes. Both the lone pairs of oxygen interaction with hydrogen bond acceptor O22–H24. Met-wtr.P4 having water in pore 4 of metronidazole interacts through double hydrogen bonding interaction, where the electron delocalization of electron transfer of lone pairs of O22 and O2 to acceptor antibonding orbital BD*(1)C11-H17 and BD*(1)O22-H23, respectively. The second order perturbation energy is calculated 15.61 and 13.51 kJ mol⁻¹, respectively.

It is interesting that in the case of the Metronidazole–water complex in the solvated state, Met-wtr.All, multiple hydrogen bonding interactions form to stabilize the complex utilizing the water molecules sitting at four pores or positions close to metronidazole. The important interactions are highlighted in Fig. 3. Interestingly O22...O1–H21 and O3...O22–H24, N5...O25–H27 and O25...C12–H18, O1...O31–H33 and O28...C11–H17, and O2...O28–H29 hydrogen bonding interactions take place with water sitting at the pore1, pore2, pore3 and pore4, respectively. The

related data has been kept in Table S-IV. The interaction between LP(2)O22 (lone pair on oxygen of metronidazole) and BD(1)O1-H21* (antibonding orbital of a water O–H bond) exhibits the highest stabilization energy of $69.91 \text{ kJ mol}^{-1}$. This indicates a strong hydrogen bond or significant charge transfer from the lone pair of oxygen on metronidazole to the O–H antibonding orbital of water.

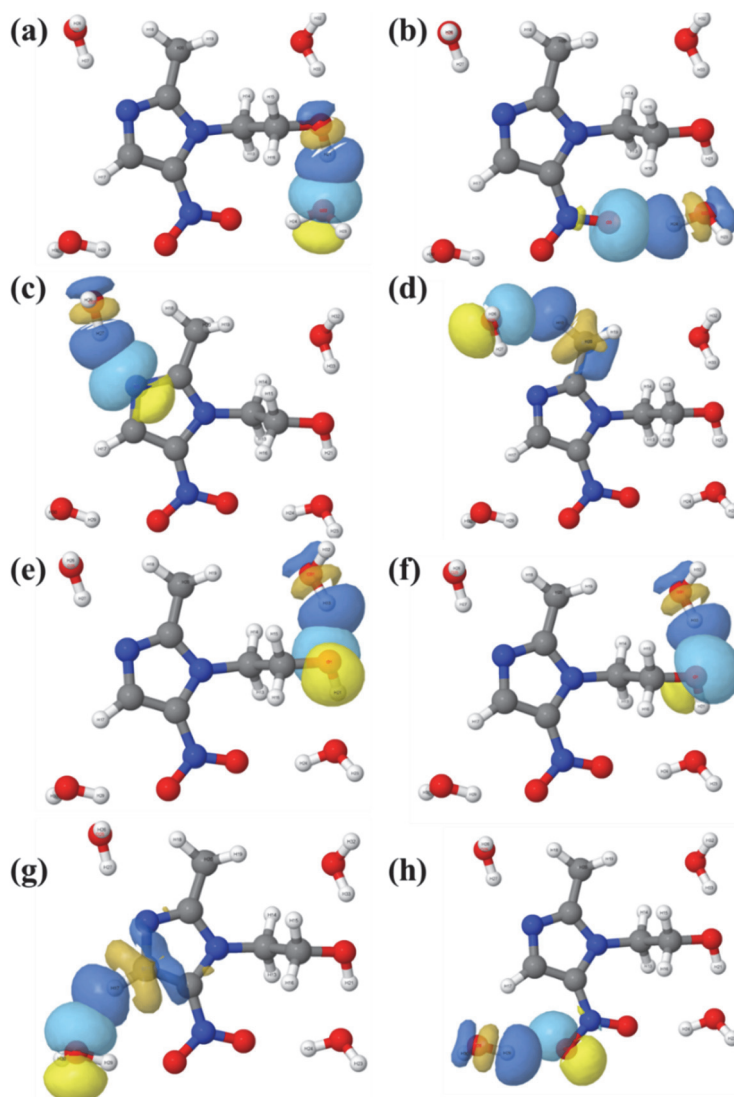


Fig. 3. NBO interaction of: a) LP(2)O22→BD*(1)O1-H21, b) LP(1)O3→BD*(1)O22-H24, c) LP(1)N5→BD*(1)O25-H27, d) LP(2)O25→BD*(1)C12-H18, e) LP(2)O1→BD*(1)O31-H33, f) LP(1)O1→BD*(1)O31-H33, g) LP(2)O28→BD*(1)C11-H17 and h) LP(2)O2→BD*(1)O28-H29 for Met-wtr.All.

The lone pair on the nitrogen atom (N5) interacts with the antibonding orbital of another O–H bond in water, forming another relatively strong hydrogen bond. A lone pair on oxygen (likely a ring oxygen in metronidazole) participates in a hydrogen bond with a water molecule. Interactions involving LP(2)O28, LP(1)O3, LP(2)O2 and LP(2)O25 with various antibonding O–H and C–H orbitals of water or Metronidazole result in lower stabilization energies (ranging from 9.92 to 18.03 kJ mol⁻¹). These are weaker hydrogen bonds or dipolar interactions.

The interactions suggest a robust hydrogen bonding network between Metronidazole and water molecules. This network stabilizes the solvation environment around Metronidazole and facilitates its bioactivity in aqueous systems. The oxygen atoms (O22, O1, O28, O3, O2, O25) are the dominant donors, emphasizing their nucleophilicity and involvement in hydrogen bonding. The nitrogen lone pair (LP(1)N5) also plays a significant role, suggesting its participation in hydrogen bonding or electronic delocalization.

Atom-in-molecule (AIM) analysis for Metronidazole

The AIM study is eligible to provide essential information regarding atom–atom interactions in through either covalent or non-covalent interaction molecular cluster, molecular crystal, *etc.*³⁰ This study provides important tools to measure the electron density, $\rho(r)$ and Laplacian of electron density, $\nabla^2\rho_{\text{bcp}}(r)$ at a bond critical point (BCP).⁴⁷ The yellow lines connecting two closely lying molecules consists small red spheres indicating BCP. AIM calculation measures various topological parameters at these BCPs. The position of the BCP changes depending upon the electronegativity of the connected atoms. The AIM study for Metronidazole with water, *i.e.*, Met-wtr.P1, Met-wtr.P2, Met-wtr.P3, Met-wtr.P4 and Met-wtr, which are all reported in Fig. S-1 of the Supplementary material. The corresponding data has been shown in Tables SVI–SX of the Supplementary material.

Met-wtr.P1 is stabilised by hydrogen bonding interaction between Metronidazole and water. Hydrogen bonding between H21...O22 and O3...H24 mainly stabilize the molecule. The corresponding BCPs between these atoms has $\rho(r)$ of -0.00364 and 0.003184 a.u. whereas $\nabla^2\rho_{\text{bcp}}(r)$ values of 0.111881 and 0.093755 a.u., respectively. In case of Met-wtr.P2, the water molecule is in the pores close to methyl group of metronidazole and interacts through intramolecular O22...H18 and intermolecular H16...O3 hydrogen bonding interactions. The electron density, $\rho(r)$ for these BCPs are 0.001712 and 0.002167 a.u., respectively whereas $\nabla^2\rho_{\text{bcp}}(r)$ for these points are reported 0.035909 and 0.048868 a.u., respectively. In case of metronidazole with water molecule in the pore3, Met-wtr.P3, the water molecule is close to methyl and hydroxy groups therefore linked through O22...H19, O22...H14, O3...H16 and O1...H24 hydrogen bonding interactions. The corresponding $\nabla^2\rho_{\text{bcp}}(r)$ values are reported 0.027317 , 0.034301 , 0.038455 and 0.10487 a.u., respectively. Met-wtr.P4 complex between metronidazole and water stabilize

through intermolecular H16...O3, H17...O22, H23...O2 hydrogen bonding interactions while the intramolecular H13...O3 hydrogen bonding interaction also helps the stability of molecule.

Furthermore, metronidazole occupied with all four pores by water molecules interact with multiple hydrogen bonding interaction as understood through AIM study. The complexes stabilize through H13...O3, H16...O3, H24...O3, H17...O28, H29...O2, H18...O25, H14...O31 and H19...O31 hydrogen bonding interactions.

Ab initio molecular dynamics assay of metronidazole

AIMD study^{41,42} has been performed for metronidazole with water and reported in Fig. 4. The molecular dynamics study was used to understand the velocity of boundary atoms around the metronidazole along with solvated water. Fig. 4 represents the total energy of solvated metronidazole with water versus time (in fs). The total energy is the cumulative of total potential and kinetic energies.

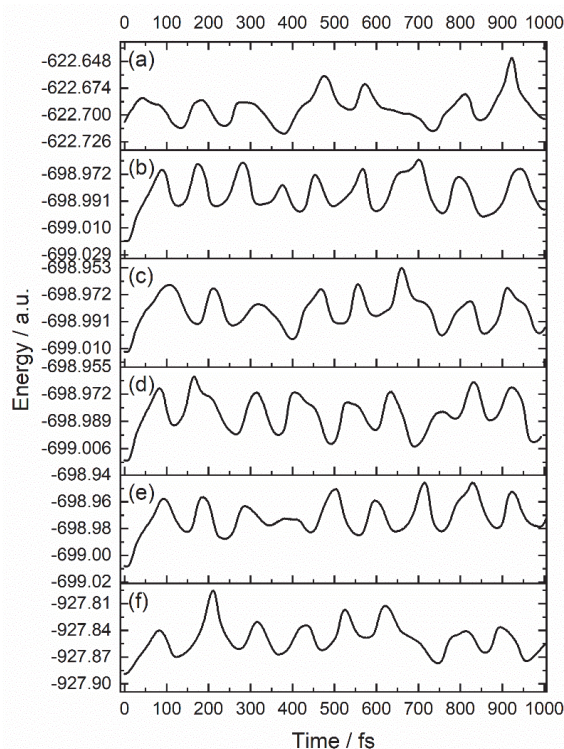


Fig. 4. Simulation of solvated metronidazole with water: a) Metronidazole, b) Met-wtr.P1, c) Met-wtr.P2, d) Met-wtr.P3, e) Met-wtr.P4, f) Met-wtr.All.

The study reveals that the lowest decomposition energy for Metronidazole with water at all pores (Met-wtr.All) is -927.86 a.u. (see Fig. 4f), representing the

most stabilized configuration. This value is significantly lower than the lowest decomposition energies for Metronidazole with water at individual pores (Met-wtr.P1 to Met-wtr.P4), which are approximately -699.003 a.u. The results indicate that the presence of water molecules at all interaction sites around Metronidazole contributes to enhanced stabilisation, as reflected in the much lower decomposition energy. This finding highlights the importance of a fully solvated environment in achieving maximum stability for the system. Furthermore, the lowest decomposition energy for Met-wtr. All was observed between 700–800 fs, consistent with the system reaching its most stable state during this timeframe. The calculated decomposition energies from AIMD simulations align well with the total energy values obtained from quantum chemical calculations, reinforcing the reliability of the results. This correlation underscores the critical role of complete solvation in stabilizing metronidazole, providing valuable insights into its behavior in aqueous environments.

The gradual stabilisation of the Met-wtr system reflects the cumulative impact of hydrogen bonding and electrostatic interactions as water molecules cluster around metronidazole. This supports the notion that metronidazole forms strong solute-solvent interactions, enhancing its solubility and bioavailability in aqueous environments. The oscillatory nature of the energy profiles indicates that solvation dynamics are not static but evolve over time. The system achieves local energy minima due to non-covalent interactions, but the thermal motion causes periodic fluctuations. The trend observed in the data is consistent with solvation theory, where the introduction of polar solvents like water leads to increasing stabilization due to non-covalent interaction networks.

CONCLUSION

Metronidazole is an important drug against gastrointestinal infection, Gram-negative and Gram-positive anaerobic bacteria as well as protozoa. The structures of metronidazole and its solvated structures are studied. The solvation of Metronidazole with solvent (water) is investigated based on SMD model and the free energy is calculated -0.0264 a.u., *i.e.*, -69.20 kJ mol $^{-1}$. AIMD studies has been performed in order to study the velocity of boundary atoms around Metronidazole and the lowest decomposition energies are calculated 699.003 and -927.86 a.u. for metronidazole with water at pore1–pore4 and all pores, respectively. The solvated structure of metronidazole is found stabilized mainly through multiple O–H...O hydrogen bonding interactions. The EDA calculations describes that hydrogen bonding, van der Waals and steric repulsion play a main role in the stability of the solvated metronidazole. The NBO study explains that $E_{i \rightarrow j}^{(2)}$ value for the interaction has been calculated 64.48 and 16.32 kJ mol $^{-1}$, respectively. The results obtained from the solvation dynamics may significantly influence the Metronidazole's oral uptake, the rate of dissolution, and the intermolecular interactions with

the surrounding water molecules key aspects that govern its absorption efficiency and the therapeutic effectiveness.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13011>, or from the corresponding author on request.

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ИЗВОД

УЛОГА НЕКОВАЛЕНТНИХ ИНТЕРАКЦИЈА У ДИНАМИЦИ РАСТВОРА МЕТРОНИДАЗОЛА У ВОДИ: ТЕОРИЈСКА СТУДИЈА

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Метронидазол, под трговачким именом Flagyl, користи се за лечење гастроинтестиналних инфекција и има активност против анаеробних бактерија и протозоа. Овај рад описује интеракцију метронидазола са водом као растварачем, који је неопходан за оралну примену, што је најчешћи и најпогоднији начин за примену лекова у организму. Рачунарски прорачуни су изведени ради оптимизације структуре метронидазола са растварачем (водом) постављеним на различите позиције. NBO (прорачуни засновани на везивним природним орбиталама) и AIM (теорија атома у молекулима) прорачуни су примењени за одређивање јачине интермолекулских водоничних веза између метронидазола и растварача (воде). Пертурбациона енергија другог реда показује максималних 67 kJ mol^{-1} за O—H...O водоничну везу. Енергија солватације метронидазола, одређена коришћењем SMD модела (модел солватације заснован на густини), износи $-69,2 \text{ kJ mol}^{-1}$. Параметри везивања солватисаног лека су анализирани помоћу прорачуна критичних тачака, а на основу AIM теорије. *Ab initio* молекулска динамика (AIMD) је показала да су најниже енергије декомпозиције метронидазола у присуству воде, узимајући у обзир све поре и различите поре, редом $-927,86$ и $-699,003$ а.у.

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SUPPLEMENTARY MATERIAL TO

The role of non-covalent interactions in the solvation dynamics of metronidazole in water: A theoretical study

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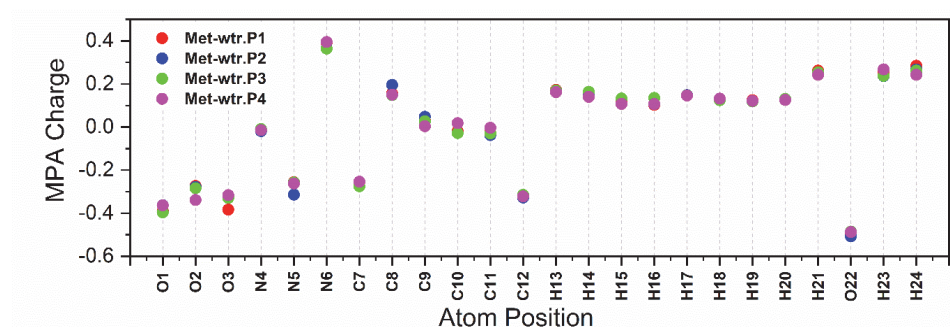


Fig. S-1. Mulliken population analysis (MPA) charge for (a) Met-wtr.P1, (b) Met-wtr.P2, (c) Met-wtr.P3, and (d) Met-wtr.P4.

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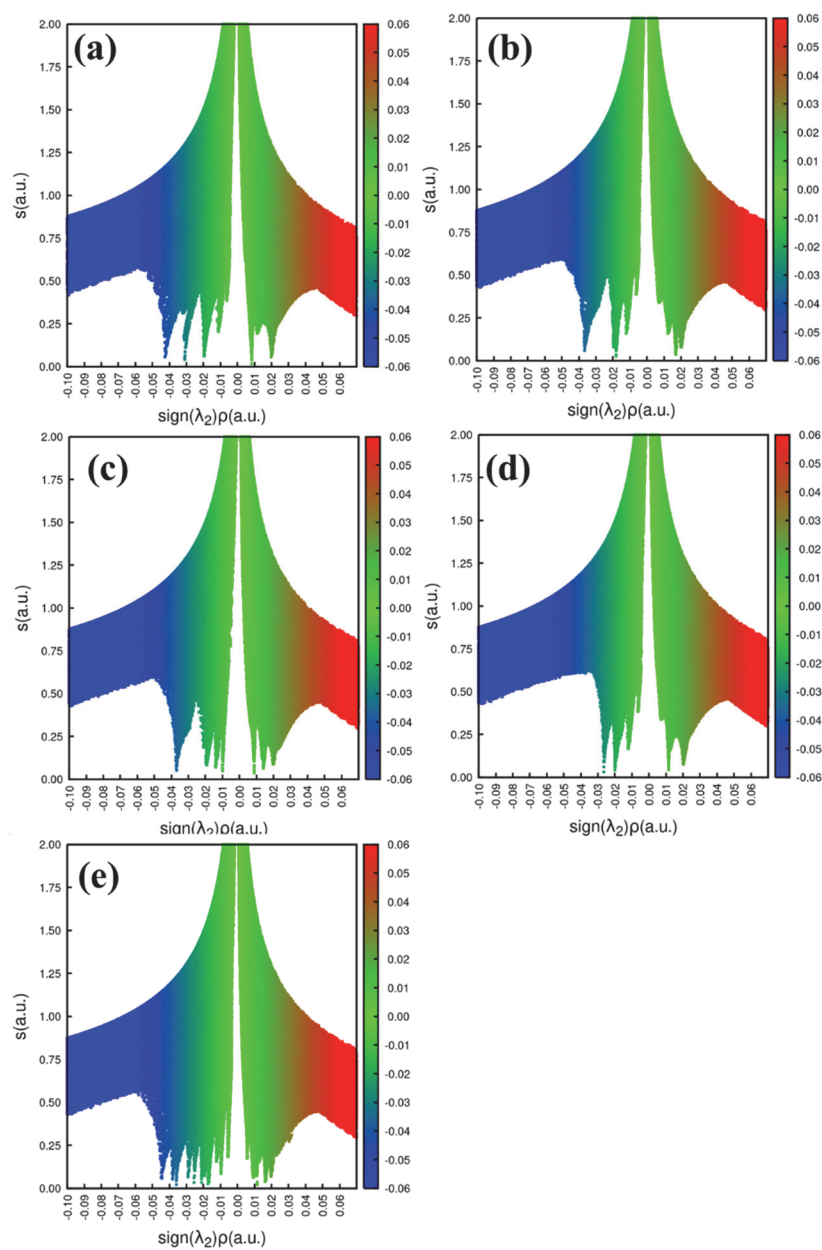


Fig. S-2. RDG plot of NCI analysis of (a) Met-wtr.P1, (b) Met-wtr.P2, (c) Met-wtr.P3, (d) Met-wtr.P4. and (e) Met-wtr.All using the structure optimized at B3LYP/def-TZVP level of theory.

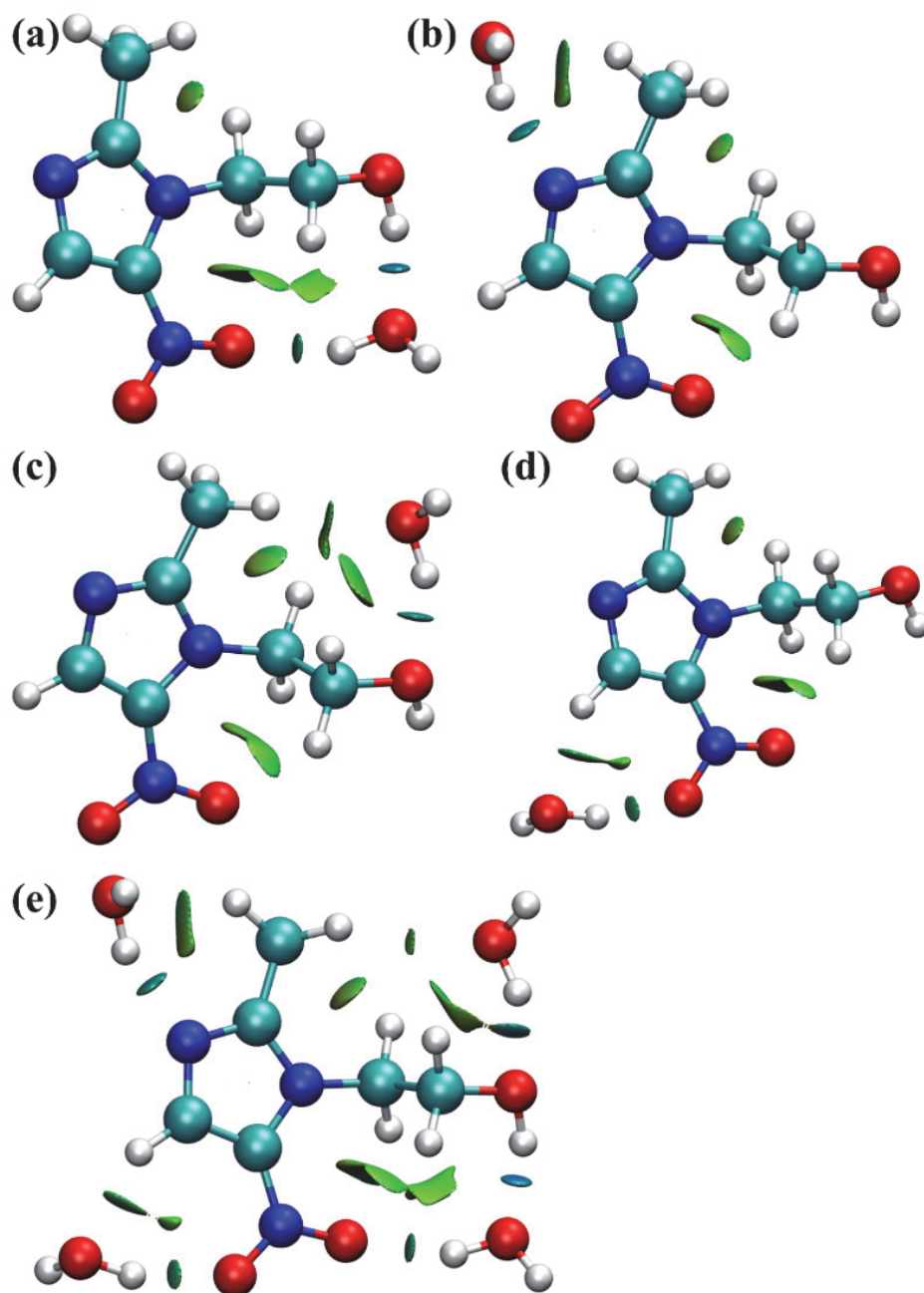


Fig. S-3. Isosurface extractions of RDG plots of NCI analysis of (a) Met-wtr.P1, (b) Met-wtr.P2, (c) Met-wtr.P3, (d) Met-wtr.P4. and (e) Met-wtr.All

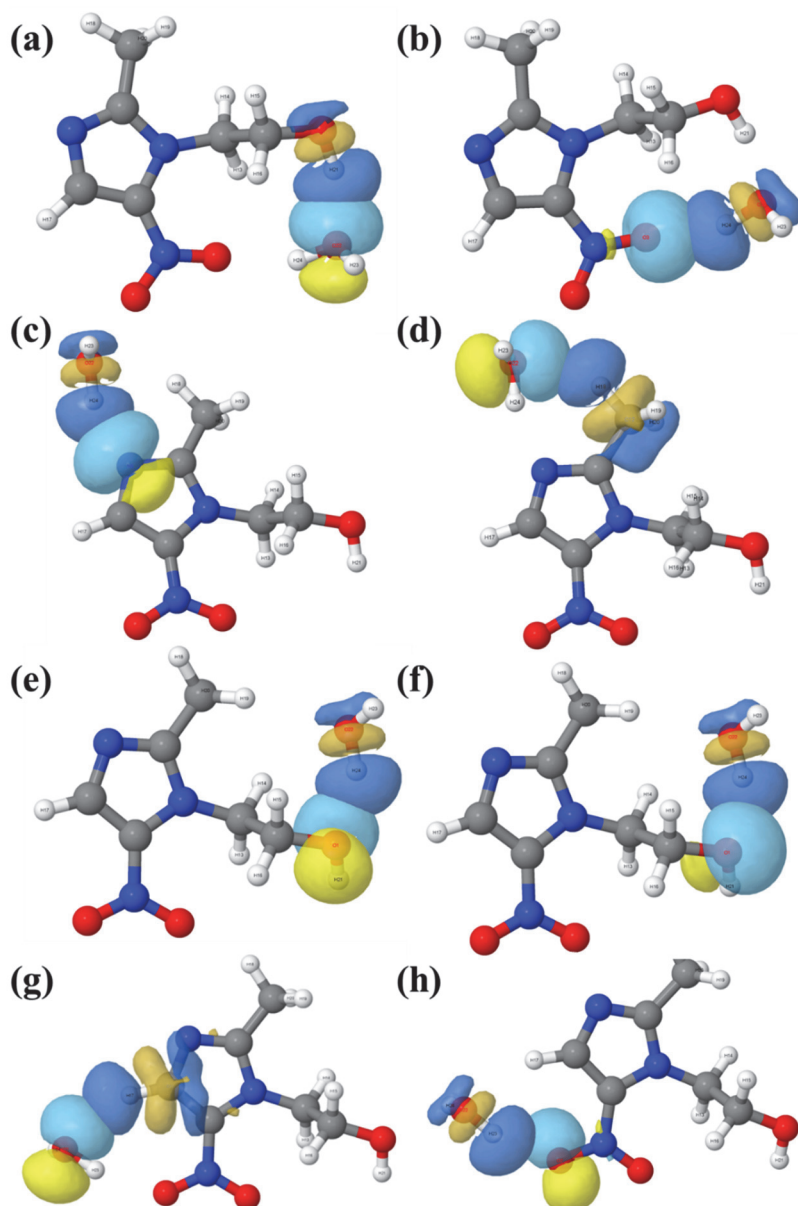


Fig. S-4. NBO interaction of (a) $\text{LP}(2)\text{O}22 \rightarrow \text{BD}^*(1)\text{O}1\text{-H}21$ and (b) $\text{LP}(1)\text{O}3 \rightarrow \text{BD}^*(1)\text{O}22\text{-H}24$ for Met-wtr.P1, (c) $\text{LP}(1)\text{N}5 \rightarrow \text{BD}^*(1)\text{O}22\text{-H}24$ and (d) $\text{LP}(2)\text{O}22 \rightarrow \text{BD}^*(1)\text{C}12\text{-H}18$ Met-wtr.P2, (e) $\text{LP}(2)\text{O}1 \rightarrow \text{BD}^*(1)\text{O}22\text{-H}24$ and (f) $\text{LP}(1)\text{O}1 \rightarrow \text{BD}^*(1)\text{O}22\text{-H}24$ Met-wtr.P3, and (g) $\text{LP}(2)\text{O}22 \rightarrow \text{BD}^*(1)\text{C}11\text{-H}17$ and (h) $\text{LP}(2)\text{O}2 \rightarrow \text{BD}^*(1)\text{O}22\text{-H}23$ for Met-wtr.P4.

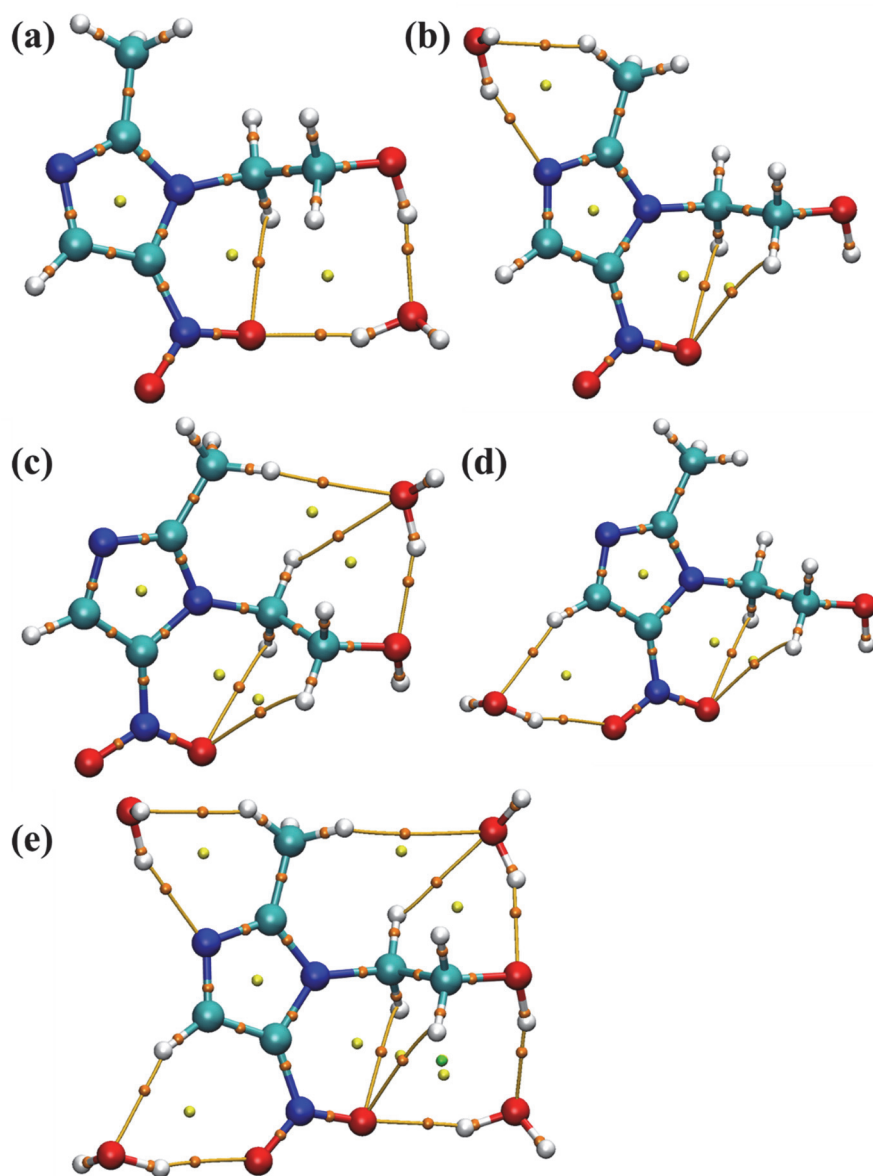


Fig. S-5. AIM study for (a) Met-wtr.P1, (b) Met-wtr.P2, (c) Met-wtr.P3, (d) Met-wtr.P4 and (e) Met-wtr.All.

TABLE S-I. Molecular characteristics of metronidazole, Met-wtr.P1, Met-wtr.P2, Met-wtr.P3, and Met-wtr.P4.

Molecule	Met	Met-wtr.P1	Met-wtr.P2	Met-wtr.P3	Met-wtr.P4	Met-wtr.All
LUMO (eV)	-2.441	-2.716	-2.664	-2.351	-2.699	-3.045
HOMO (eV)	-7.075	-7.023	-7.347	-6.939	-7.173	-7.293
Band gap (eV)	4.635	4.306	4.683	4.588	4.474	4.249
Ionisation energy (eV)	7.075	7.023	7.347	6.939	7.173	7.293
Electron affinity (eV)	2.441	2.716	2.664	2.351	2.699	3.045
Chemical hardness (eV)	2.317	2.154	2.342	2.294	2.237	2.124
Electronic chemical potential (eV)	-4.758	-4.870	-5.006	-4.645	-4.936	-5.169
Electrophilicity index (eV)	9.771	11.011	10.700	9.405	10.891	12.579
Softness (1/eV)	0.432	0.464	0.427	0.436	0.447	0.471

TABLE S-II. Decomposition of total interaction energy of the solvated metronidazole with water.

	ΔE_{ele}	ΔE_{ex}	ΔE_{rep}	ΔE_{pol}	ΔE_{disp}	ΔE_{tot}	$\Delta E_{\text{disp}}/\Delta E_{\text{ele}}$
	kJ mol ⁻¹						
Met-wtr.P1	-70.29	-32.55	122.63	-47.15	-40.29	-67.66	0.57
Met-wtr.P2	-47.95	-28.37	96.48	-38.74	-27.87	-46.40	0.58
Met-wtr.P3	-46.69	-21.13	87.32	-38.91	-35.86	-55.27	0.76
Met-wtr.P4	-30.96	-14.69	61.63	-31.46	-23.14	-38.62	0.74
Met-wtr.All	-199.07	-98.70	372.33	-154.26	-128.45	-208.20	0.64

TABLE S-III. Solvation model of metronidazole employed at PBE0-D3/def2-TZVP level of theory.

Solvent	Surface-charge	Charge-correction	Free-energy (cav+disp)	Dielectric constant
Water	-28.207	0.030	4.506	80.4
DMSO	-14.465	0.022	-1.829	47.2
DMF	-14.471	0.022	-2.932	38.3
Acetonitrile	-15.478	0.025	-2.282	36.6
Pyridine	-14.501	0.021	-3.906	12.5
tetrahydrofuran	-14.531	0.020	-3.774	7.3
Chloroform	-16.932	0.025	-2.807	4.9

TABLE S-IV. Nature bonding orbital (NBO) assay of metronidazole.

NAOs	Donor NBO (i)	NAOs	Acceptor NBO (j)	$E_{i \rightarrow j}^{(2)}$ (kJ mol ⁻¹)
Met-wtr.P1				
24	LP(2)O22	52	BD*(1)O1-H21	64.48
18	LP(1)O3	76	BD*(1)O22-H24	16.32
50	BD(1)O22-H24	55	BD*(1)O3-N6	2.97
20	LP(3)O3	76	BD*(1)O22-H24	2.18
29	BD(1)O3-N6	466	RY(2)H24	1.26
23	LP(1)O22	52	BD*(1)O1-H21	1.13
24	LP(2)O22	429	RY(1)H21	0.88
24	LP(2)O22	51	BD*(1)O1-C10	0.46
				0.00
Met-wtr.P2				
21	LP(1)N5	76	BD*(1)O22-H24	46.65
24	LP(2)O22	72	BD*(1)C12-H18	11.00
50	BD(1)O22-H24	61	BD*(1)N5-C11	1.88
35	BD(1)N5-C11	465	RY(1)H24	1.09
50	BD(1)O22-H24	181	RY(1)N5	0.63
21	LP(1)N5	467	RY(3)H24	0.59
50	BD(1)O22-H24	72	BD*(1)C12-H18	0.54
50	BD(1)O22-H24	414	RY(1)H18	0.54
41	BD(1)C9-C11	76	BD*(1)O22-H24	0.50
				0.00
Met-wtr.P3				
15	LP(2)O1	76	BD*(1)O22-H24	24.89
14	LP(1)O1	76	BD*(1)O22-H24	13.81
24	LP(2)O22	73	BD*(1)C12-H19	4.73
24	LP(2)O22	65	BD*(1)C7-H14	1.34
49	BD(1)O22-H23	394	RY(1)H14	0.79
26	BD(1)O1-H21	466	RY(2)H24	0.71
24	LP(2)O22	70	BD*(1)C10-H16	0.67
50	BD(1)O22-H24	52	BD*(1)O1-H21	0.67
				0.00
Met-wtr.P4				
24	LP(2)O22	71	BD*(1)C11-H17	15.61
17	LP(2)O2	75	BD*(1)O22-H23	13.51
16	LP(1)O2	75	BD*(1)O22-H23	7.53
49	BD(1)O22-H23	71	BD*(1)C11-H17	1.13
49	BD(1)O22-H23	53	BD*(1)O2-N6	1.09
18	LP(3)O2	75	BD*(1)O22-H23	1.00
24	LP(2)O22	61	BD*(1)N5-C11	0.46
49	BD(1)O22-H23	409	RY(1)H17	0.46
				0.00
Met-wtr.All				
27	LP(2)O22	67	BD*(1)O1-H21	69.91
25	LP(1)N5	93	BD*(1)O25-H27	44.18
18	LP(2)O1	97	BD*(1)O31-H33	25.19
31	LP(2)O28	86	BD*(1)C11-H17	18.03
17	LP(1)O1	97	BD*(1)O31-H33	15.94
22	LP(1)O3	91	BD*(1)O22-H24	14.06
20	LP(2)O2	94	BD*(1)O28-H29	12.47
29	LP(2)O25	87	BD*(1)C12-H18	9.92

NAOs	Donor NBO (i)	NAOs	Acceptor NBO (j)	$E_{i \rightarrow j}^{(2)}$ (kJ mol ⁻¹)
19	LP(1)O2	94	BD*(1)O28-H29	6.65
33	LP(2)O31	81	BD*(1)C7-H14	3.14
59	BD(1)O22-H24	69	BD*(1)O3-N6	2.72
61	BD(1)O25-H27	75	BD*(1)N5-C11	1.80
62	BD(1)O28-H29	86	BD*(1)C11-H17	1.26
37	BD(1)O3-N6	487	RY(2)H24	1.21
33	LP(2)O31	88	BD*(1)C12-H19	1.21
64	BD(1)O31-H32	415	RY(1)H14	1.13
43	BD(1)N5-C11	522	RY(1)H27	1.09
26	LP(1)O22	67	BD*(1)O1-H21	1.05
62	BD(1)O28-H29	68	BD*(1)O2-N6	1.00
32	LP(1)O31	81	BD*(1)C7-H14	1.00
65	BD(1)O31-H33	67	BD*(1)O1-H21	0.96
33	LP(2)O31	85	BD*(1)C10-H16	0.88

TABLE S-V. MPA charges of Met-wtr.P1, Met-wtr.P2, Met-wtr.P3, and Met-wtr.P4.

Atom No	Metronidazole	Met-wtr.P1	Met-wtr.P2	Met-wtr.P3	Met-wtr.P4
O1	-0.364881	-0.3896	-0.3629	-0.3965	-0.3641
O2	-0.280926	-0.2737	-0.2781	-0.2837	-0.3390
O3	-0.324958	-0.3833	-0.3201	-0.3290	-0.3163
N4	-0.013881	-0.0100	-0.0190	-0.0097	-0.0144
N5	-0.256843	-0.2565	-0.3140	-0.2576	-0.2620
N6	0.365079	0.3928	0.3654	0.3638	0.3946
C7	-0.253083	-0.2552	-0.2548	-0.2748	-0.2538
C8	0.151093	0.1558	0.1953	0.1480	0.1499
C9	0.027772	0.0301	0.0474	0.0269	0.0041
C10	0.018371	-0.0194	0.0178	-0.0279	0.0182
C11	-0.031026	-0.0270	-0.0378	-0.0301	-0.0034
C12	-0.322707	-0.3220	-0.3272	-0.3155	-0.3221
H13	0.162977	0.1720	0.1641	0.1684	0.1623
H14	0.140126	0.1413	0.1443	0.1626	0.1402
H15	0.106472	0.1155	0.1080	0.1322	0.1075
H16	0.106777	0.1031	0.1060	0.1350	0.1069
H17	0.148904	0.1483	0.1480	0.1475	0.1463
H18	0.130322	0.1293	0.1281	0.1248	0.1318
H19	0.121097	0.1243	0.1214	0.1189	0.1216
H20	0.1261	0.1272	0.1281	0.1295	0.1259
H21	0.243215	0.2616	0.2434	0.2538	0.2428
O22		-0.5027	-0.5073	-0.4858	-0.4877
H23		0.2542	0.2370	0.2377	0.2679
H24		0.2839	0.2673	0.2616	0.2426

TABLE S-VI. AIM analysis data for Met-wtr.P1

BCP	$\rho(r)$	$\nabla^2\rho_{\text{bcn}}(r)$	ELF	LOL
C10...H16	-0.2733	-0.95299	0.989361	0.906067
C10...O1	-0.38933	-0.71868	0.730268	0.621999
C11...C9	-0.35117	-0.94826	0.93623	0.793045
C12...C8	-0.24279	-0.70858	0.957553	0.826097
C12...H19	-0.26594	-0.90844	0.98593	0.893312
C7...C10	-0.20931	-0.61938	0.96297	0.836078
C7...H13	-0.28684	-1.00942	0.990628	0.91138
C8...N4	-0.48341	-0.94763	0.753954	0.63644
C9...N6	-0.38036	-0.84916	0.82946	0.688038
H13...O3	0.002227	0.062306	0.045079	0.1786
H14...C7	-0.28096	-0.981	0.989496	0.906618
H15...C10	-0.27718	-0.97181	0.990077	0.909023
H17...C11	-0.29277	-1.05068	0.993018	0.922661
H18...C12	-0.27281	-0.94355	0.988002	0.900766
H20...C12	-0.26418	-0.90101	0.985651	0.892359
H21...O22	-0.00364	0.111881	0.130611	0.279395
H24...O22	-0.64045	-2.30744	0.982795	0.883167
N4...C7	-0.29823	-0.61289	0.793272	0.662052
N4...C9	-0.40694	-0.88528	0.820371	0.681242
N5...C11	-0.49003	-1.12479	0.845012	0.700158
N5...C8	-0.52191	-1.23904	0.862654	0.714798
N6...O2	-0.70453	-1.24069	0.853356	0.706948
N6...O3	-0.6439	-1.12269	0.853679	0.707217
O1...H21	-0.6165	-2.19996	0.979329	0.873163
O22...H23	-0.64558	-2.30921	0.981684	0.879839
O3...H24	0.003184	0.093755	0.04836	0.184032

TABLE S-VII. AIM analysis data for Met-wtr.P2

BCP	$\rho(r)$	$\nabla^2\rho_{\text{bcn}}(r)$	ELF	LOL
C10...H16	-0.27786	-0.97674	0.99053	0.910957
C10...O1	-0.36786	-0.64682	0.707527	0.608676
C11...C9	-0.35802	-0.96373	0.935327	0.791809
C12...C8	-0.24285	-0.70063	0.954394	0.820636
C12...H19	-0.26524	-0.9031	0.985344	0.891322
C7...C10	-0.21386	-0.63651	0.96418	0.838428
C7...H13	-0.28508	-1.00299	0.99059	0.911215
C8...N4	-0.48379	-0.94725	0.752869	0.635764
C9...N6	-0.35902	-0.83579	0.855705	0.708911
H13...O3	0.002281	0.061292	0.043683	0.176201
H14...C7	-0.28156	-0.98496	0.989785	0.907805
H15...C10	-0.28073	-0.98825	0.990657	0.911508
H16...O3	0.001712	0.035909	0.022256	0.131251
H17...C11	-0.29334	-1.05309	0.993049	0.922821
H18...C12	-0.27352	-0.95579	0.989566	0.906901
H20...C12	-0.26371	-0.89756	0.985281	0.891112
H24...N5	-0.00042	0.080896	0.128494	0.277542
N4...C7	-0.29961	-0.62639	0.802227	0.668234
N4...C9	-0.41037	-0.88324	0.813596	0.676301
N5...C11	-0.48078	-1.09588	0.840936	0.696916
N5...C8	-0.52499	-1.23279	0.855268	0.708542
N6...O2	-0.70047	-1.22994	0.852957	0.706619
N6...O3	-0.66796	-1.1651	0.853132	0.706763
O1...H21	-0.65062	-2.32411	0.981498	0.879292
O22...H18	0.002167	0.048868	0.047364	0.182471
O22...H23	-0.63703	-2.25547	0.979283	0.873037
O22...H24	-0.62185	-2.21723	0.979352	0.873226

TABLE S-VIII. AIM analysis data for Met-wtr.P3

BCP	$\rho(r)$	$\nabla^2\rho_{\text{bcn}}(r)$	ELF	LOL
C10...H16	-0.28079	-0.99146	0.991146	0.913669
C10...O1	-0.34585	-0.57626	0.684808	0.595808
C11...C9	-0.35469	-0.95499	0.935358	0.791851
C12...C8	-0.2424	-0.70367	0.9561	0.823556
C12...H19	-0.26977	-0.93163	0.987758	0.89985
C7...C10	-0.21589	-0.6444	0.964839	0.839726
C7...H13	-0.28339	-0.99537	0.990355	0.910204
C8...N4	-0.4728	-0.92966	0.755858	0.63763
C9...N6	-0.36876	-0.84514	0.845898	0.700872
H13...O3	0.002248	0.059883	0.042364	0.173891
H14...C7	-0.28444	-1.00046	0.990579	0.911165
H15...C10	-0.28436	-1.00494	0.991207	0.913947
H17...C11	-0.29268	-1.04967	0.992936	0.92224
H18...C12	-0.2714	-0.93537	0.987429	0.898633
H19...O22	0.001266	0.027317	0.030058	0.149916
H20...C12	-0.26214	-0.89111	0.98508	0.890443
H24...O1	-0.00012	0.10487	0.094057	0.243763
N4...C7	-0.30153	-0.64076	0.811153	0.674548
N4...C9	-0.40913	-0.87978	0.813255	0.676056
N5...C11	-0.48154	-1.11004	0.848166	0.702701
N5...C8	-0.52914	-1.25035	0.859496	0.712099
N6...O2	-0.69585	-1.22127	0.853108	0.706744
N6...O3	-0.65964	-1.14821	0.853178	0.706801
O1...H21	-0.64995	-2.32855	0.982151	0.88122
O22...H14	0.001707	0.034301	0.019652	0.124183
O22...H23	-0.64374	-2.28934	0.980303	0.875865
O22...H24	-0.62824	-2.24433	0.98011	0.875324
O3...H16	0.001762	0.038455	0.026051	0.140714

TABLE S-IX. AIM analysis data for Met-wtr.P4

BCP	$\rho(r)$	$\nabla^2\rho_{\text{bcp}}(r)$	ELF	LOL
C10...H16	-0.27781	-0.97652	0.990522	0.910921
C10...O1	-0.36698	-0.64423	0.706789	0.608251
C11...C9	-0.35017	-0.94333	0.935335	0.79182
C12...C8	-0.24243	-0.7078	0.957692	0.826342
C12...H19	-0.26597	-0.90779	0.985781	0.892803
C7...C10	-0.21388	-0.63664	0.964224	0.838512
C7...H13	-0.28477	-1.00123	0.990498	0.910818
C8...N4	-0.47914	-0.9407	0.754895	0.637028
C9...N6	-0.38101	-0.84889	0.828301	0.68716
H13...O3	0.002306	0.062551	0.044259	0.177196
H14...C7	-0.28122	-0.98248	0.989594	0.907016
H15...C10	-0.28069	-0.98803	0.990642	0.911441
H16...O3	0.001731	0.036398	0.021956	0.13046
H17...C11	-0.29327	-1.06107	0.993937	0.927578
H17...O22	0.002441	0.05554	0.051483	0.189087
H18...C12	-0.2729	-0.9444	0.988096	0.90112
H20...C12	-0.26397	-0.89964	0.985527	0.891939
H23...O2	0.002546	0.079234	0.058272	0.199293
H24...O22	-0.63912	-2.27013	0.980044	0.875138
N4...C7	-0.30014	-0.63286	0.806799	0.671448
N4...C9	-0.40448	-0.87467	0.816918	0.678711
N5...C11	-0.484	-1.11093	0.844992	0.700142
N5...C8	-0.52767	-1.25338	0.862861	0.714976
N6...O2	-0.66864	-1.17122	0.85386	0.707367
N6...O3	-0.67251	-1.178	0.853764	0.707287
O1...H21	-0.65048	-2.32319	0.981457	0.879173
O22...H23	-0.64086	-2.29443	0.981297	0.878706

TABLE S-X. AIM analysis data for Met-wtr.all

BCP	$\rho(r)$	$\nabla^2\rho_{\text{BCP}}(r)$	ELF	LOL
C10...H16	-0.27648	-0.9678	0.989922	0.908371
C10...O1	-0.36817	-0.6578	0.716032	0.613602
C11...C9	-0.34761	-0.9409	0.936815	0.793851
C11...H17	-0.29341	-1.06326	0.9941	0.928494
C12...C8	-0.24505	-0.70261	0.952407	0.81732
C12...H19	-0.26795	-0.92055	0.986889	0.896676
C7...C10	-0.21225	-0.63066	0.96387	0.837821
C7...H13	-0.28519	-1.00098	0.990254	0.909771
C8...N4	-0.49678	-0.96882	0.749721	0.633811
C9...N6	-0.39543	-0.85196	0.808	0.672296
H13...O3	0.00217	0.060194	0.042969	0.174956
H14...C7	-0.28588	-1.00903	0.991056	0.913268
H14...O31	0.002102	0.045101	0.027781	0.144733
H15...C10	-0.2821	-0.99407	0.990809	0.912173
H16...O3	0.001665	0.036072	0.022248	0.131228
H17...O28	0.002482	0.05981	0.055658	0.195473
H18...C12	-0.27351	-0.95436	0.989354	0.906043
H19...O31	0.000693	0.014277	0.013668	0.105648
H20...C12	-0.26237	-0.89264	0.985207	0.890865
H21...O22	-0.00453	0.115585	0.137564	0.28546
H24...O22	-0.64182	-2.3144	0.983059	0.883974
H27...N5	-0.0002	0.080072	0.124589	0.274016
H29...O2	0.002687	0.07602	0.054745	0.194071
H30...O28	-0.63978	-2.27488	0.980285	0.875814
H33...O1	-0.00057	0.106228	0.099273	0.24931
N4...C7	-0.30117	-0.6116	0.786339	0.657363
N4...C9	-0.40026	-0.87967	0.826886	0.686093
N5...C11	-0.49507	-1.12297	0.83671	0.693603
N5...C8	-0.50693	-1.21287	0.866299	0.717959
N6...O2	-0.67572	-1.18677	0.854061	0.707533
N6...O3	-0.65297	-1.1444	0.854186	0.707637
O1...H21	-0.60803	-2.17217	0.979375	0.873289
O22...H23	-0.64747	-2.32062	0.982137	0.881179
O25...H18	0.002085	0.046243	0.044681	0.177965
O25...H26	-0.63726	-2.25667	0.979321	0.873138
O25...H27	-0.62357	-2.22421	0.979518	0.87368
O28...H29	-0.64136	-2.29624	0.981354	0.878873
O3...H24	0.003448	0.086886	0.041967	0.173152
O31...H32	-0.64402	-2.28862	0.980135	0.875392
O31...H33	-0.62267	-2.22425	0.979913	0.874774

TABLE S-XI. Cartesian coordinates of Metronidazole

O	3.245939	1.738927	-0.315804
O	-2.61276	1.588767	-0.029273
O	-0.607587	2.255099	0.376866
N	0.293796	-0.387844	0.315368
N	-0.95495	-2.160301	-0.165808
N	-1.434344	1.387593	0.1515
C	1.491219	0.385633	0.619786
C	0.256306	-1.729331	0.142129
C	-0.996791	0.042053	0.088333
C	2.087146	0.997859	-0.639813
C	-1.744973	-1.065966	-0.199515
C	1.442773	-2.609422	0.295035
H	1.238333	1.162294	1.329944
H	2.209315	-0.287841	1.07476
H	2.39526	0.208996	-1.321592
H	1.339262	1.612314	-1.136742
H	-2.793606	-1.097739	-0.424129
H	1.143373	-3.612492	0.01613
H	2.264387	-2.291443	-0.342684
H	1.795189	-2.621548	1.324744
H	2.980314	2.588193	0.041364

TABLE S-XII. Cartesian coordinates of Met-wtr.P1

O	-2.441894	-2.07175	0.496255
O	0.366797	3.091201	0.236281
O	-1.153231	1.661836	-0.276841
N	0.752805	-0.387674	-0.256454
N	2.915698	-0.112214	0.179035
N	0.002038	1.967313	-0.011737
C	-0.497775	-1.096945	-0.515604
C	1.961008	-0.978215	-0.128314
C	0.976586	0.948291	0.005775
C	-1.256579	-1.374367	0.777438
C	2.315158	1.089919	0.264091
C	2.187416	-2.433142	-0.31965
H	-1.108583	-0.513661	-1.191803
H	-0.24512	-2.03551	-0.996091
H	-0.647021	-1.996556	1.430159
H	-1.452785	-0.432106	1.29053
H	2.835458	1.998697	0.498432
H	3.22496	-2.636887	-0.084327
H	1.548922	-3.026401	0.330718
H	1.993989	-2.729712	-1.34869
H	-3.075933	-1.420948	0.163892
O	-3.689197	0.304461	-0.392859
H	-4.521448	0.707356	-0.14477
H	-3.020909	0.99452	-0.329069

TABLE S-XIII. Cartesian coordinate of Met-wtr.P2

O	2.746476	-2.684867	-0.372409
O	1.40934	3.025247	-0.146694
O	2.492091	1.206292	0.239135
N	0.087282	-0.217636	0.317412
N	-1.915924	0.635819	-0.087276
N	1.465169	1.834504	0.052472
C	1.103024	-1.225211	0.601887
C	-1.237074	-0.463038	0.21023
C	0.235127	1.129329	0.060447
C	1.767664	-1.713567	-0.677732
C	-1.010843	1.634146	-0.179952
C	-1.848559	-1.800228	0.418151
H	1.841257	-0.801435	1.270134
H	0.610866	-2.053763	1.098641
H	1.029364	-2.195632	-1.313744
H	2.18992	-0.868304	-1.217301
H	-1.266466	2.650218	-0.409884
H	-2.906379	-1.724299	0.190547
H	-1.390523	-2.548692	-0.224433
H	-1.733233	-2.122147	1.451567
H	3.54038	-2.241528	-0.068584
O	-4.708564	-0.223366	-0.225499
H	-5.059698	-0.099542	-1.10714
H	-3.881948	0.281288	-0.202513

TABLE S-XIV. Cartesian coordinate of Met-wtr.P3

O	-2.395849	-2.097383	-0.328003
O	3.407385	-1.068386	-0.084149
O	1.537375	-2.023501	0.386013
N	0.229829	0.439628	0.244502
N	1.1788	2.369137	-0.308827
N	2.214184	-1.045365	0.113045
C	-0.832504	-0.49754	0.582406
C	0.05166	1.764255	0.030356
C	1.570411	0.209679	0.012788
C	-1.306976	-1.240889	-0.657033
C	2.130481	1.412727	-0.320521
C	-1.256995	2.447959	0.186007
H	-0.467607	-1.193442	1.327614
H	-1.656955	0.075041	0.992592
H	-1.68768	-0.530098	-1.384177
H	-0.488607	-1.80395	-1.097584
H	3.15896	1.601559	-0.56118
H	-1.152445	3.450084	-0.212215
H	-2.058674	1.923208	-0.327089
H	-1.532447	2.516362	1.23748
H	-2.068793	-2.850039	0.168151
O	-3.919593	0.223371	0.102648
H	-4.845255	0.336977	-0.111605
H	-3.731187	-0.719461	0.010884

TABLE S-XV. Cartesian coordinate of Met-wtr.P4

O	3.620053	-1.550636	0.509436
O	-2.197102	-1.604185	-0.203006
O	-0.146443	-2.16575	-0.494758
N	0.65115	0.492604	-0.29627
N	-0.688412	2.212952	0.127592
N	-1.014562	-1.335797	-0.298309
C	1.894876	-0.232145	-0.525356
C	0.556187	1.826078	-0.099966
C	-0.640613	0.017565	-0.171226
C	2.412428	-0.861284	0.760544
C	-1.446708	1.098054	0.084798
C	1.719707	2.747806	-0.146277
H	1.729191	-0.993898	-1.276066
H	2.622571	0.478409	-0.901589
H	2.636195	-0.082484	1.485371
H	1.652179	-1.516214	1.181189
H	-2.511288	1.098239	0.238845
H	1.365093	3.734799	0.124784
H	2.499812	2.444817	0.548335
H	2.149965	2.790312	-1.145091
H	3.416372	-2.399215	0.112584
O	-4.528501	0.06212	0.58501
H	-3.932056	-0.665006	0.382301
H	-5.224588	0.013104	-0.07031

TABLE S-XVI. Cartesian coordinate of Met-wtr.All

O	3.523685	-0.064457	0.749351
O	-1.626322	-2.804421	-0.025312
O	0.464539	-2.442315	-0.328819
N	0.021725	0.312857	-0.353744
N	-1.952639	1.287666	-0.101028
N	-0.684014	-2.053224	-0.176168
C	1.472677	0.20582	-0.480025
C	-0.64943	1.479245	-0.29229
C	-0.93301	-0.672052	-0.177135
C	2.117011	-0.00923	0.884009
C	-2.14475	-0.041742	-0.02921
C	-0.009175	2.808789	-0.443476
H	1.717469	-0.606009	-1.152073
H	1.831096	1.13859	-0.899926
H	1.885187	0.834113	1.528997
H	1.734225	-0.919959	1.342189
H	-3.107196	-0.497477	0.123833
H	-0.744585	3.565439	-0.192886
H	0.872791	2.906806	0.184497
H	0.309365	2.95718	-1.474535
H	3.736968	-0.923749	0.355344
O	3.351862	-2.624826	-0.363894
H	3.844804	-3.436954	-0.245121
H	2.419043	-2.86222	-0.341898
O	-3.146525	3.960401	0.072811
H	-3.489659	4.082801	0.957866
H	-2.966594	3.01151	-0.001122
O	-4.486422	-2.224553	0.593564
H	-3.650716	-2.677351	0.447567
H	-5.10939	-2.627176	-0.011678
O	3.422257	2.642236	0.098499
H	4.080201	3.2886	0.352602
H	3.796956	1.773258	0.302102



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Molecular docking's pioneering role in materials science. Metal ion site-preference in fluorapatite

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Abstract: Originally created for biological systems, molecular docking has proven to be useful to predict metal ion binding locations and affinities in materials. By using this novel approach, it was possible to estimate the binding energies of Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} and Pr^{3+} for two different calcium sites in fluorapatite. The data obtained from the crystal structures of doped fluorapatites showed a good agreement with the docking research. The interpretation of docking results was made easier by combining results from DFT calculations with geometrical analysis of crystal structures of small molecules. Notably, this strategy is more favourable than previously used theoretical approaches due to the computational efficiency and the demonstrated reliability.

Keywords: electrostatic model; binding energy.

INTRODUCTION

Fluorapatite (FAP, $\text{Ca}_5(\text{PO}_4)_3\text{F}$) represents the prevailing form of calcium phosphate following hydroxyapatite (HAP) in biological matrices. Due to its presence in natural systems, synthetic fluorapatite is frequently employed as a bio-compatible material for applications such as bone replacement and the coating of bone prostheses.¹ The integration of materials incorporating both HAP and FAP holds significant promise in advancing dental surgery and conservative dentistry.

Fluorapatite comprises three ions (F^- , Ca^{2+} and PO_4^{3-}), classified under the space group $\text{P6}_3/\text{m}$. The compositions of fluorapatite minerals found in nature differ from these ideal compositions because the crystal lattice of FAP is tolerant

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of various isomorphic substitutions. Fluorapatite's structure and numerous ionic bonds make it an excellent host for various substitutes. Four distinct types of substitution can be defined based on earlier research.² The primary and universally acknowledged substitution within the context of fluorapatite is the F⁻ substitution. The singular substitution causing changes to the crystal structure involves Ca²⁺ substitution, which can manifest at two distinct sites, Ca(1) and Ca(2). In a general sense, the substitution process is contingent upon the relative size and charge of metal ion substituent. Specifically, for the Ca²⁺ sites, the substituent radius may vary within the range of 0.95 to 1.35 Å.⁴ Small cations in the M²⁺ category occupy the Ca(1) site, as larger substituents exhibit a preference for the Ca(2) site.^{3,4} Monovalent ions (K⁺, Na⁺) frequently engage in substitution at the Ca(1) site, resulting in the creation of vacancies within the F⁻ sites and occasionally within the Ca(1) site.³ The greater affinity of Ag⁺ for the Ca(1) site has been confirmed.⁵ In cases where the substituent is divalent, such as Sr²⁺, Pb²⁺ or Mg²⁺, the Ca(2) site is preferentially targeted for substitution.^{6,7} Furthermore, the substitution by a trivalent substituent is strategically employed in the fabrication of fluorescent materials tailored for diverse applications.^{6,8–10} Rare earth ions exhibit the capability to undergo substitutions in both cation positions.^{9,11}

Various theoretical methodologies have been developed for the precise evaluation of material properties and the prediction of thermodynamic data. The present research is oriented towards exploring the potential application of molecular docking to predict the site-preferences of particular metal ions to fluorapatite, as well as towards the changes in stability of the crystal structure induced by the doping of these metal ions were quantified. As a validation of applicability, the theoretically predicted parameters were compared with the experimentally determined values.

METHODOLOGY

CSD analysis

From the Cambridge Structural Database (CSD),¹² all structures containing the M–O fragment were extracted (M = Ca²⁺, Sr²⁺, Mn²⁺, Cu²⁺ or Pr³⁺). For the purposes of understanding the binding geometry of the mentioned ions to fluorapatite, two geometrical parameters were analysed: the lengths of the M–O bonds and the number of bonded X atoms to the metal ion (or the number of M–X bonds). To achieve the most reliable analysis of geometric data, only structures from the CSD that fulfil the following criteria were analysed: error-free coordinates according to the criteria used in the CSD; not disordered structures; no powder structures; no polymer structures; determined 3D coordinates; the crystallographic R factor is less than 10 %.

Density functional theory (DFT) calculations

Given that the interactions between metal ions and phosphate groups exert the most significant influence on metal ion binding, the chosen model system for the assessment of interaction strength comprises solely these two molecular species. Calculations at the wb97xd/x2c-TZVPall level of theory, which proved to be the most relevant (described in more detail in

Supplementary material to this paper, SM1), were conducted to assess the interaction strength of the phosphate group with other metal ions.

Molecular docking study

The rigid structures of the receptors corresponding to calcium binding sites 1 and 2 (Ca(1) and Ca(2)) were extracted from the crystal structure of hexagonal (P6₃/m) fluorapatite with the formula Ca₅(PO₄)₃F and refined to $R = 0.025$.¹³ The preparation of targets and ligands (metal ions) for the docking study was conducted using the AutoDockTools program, and the docking calculations were executed with the AutoDock program.¹⁴ A grid box of dimensions 12 Å×12 Å×12 Å, encompassing the entire target structure was employed to accommodate the investigated metal ions. Throughout the docking calculations, the structures of the targets were kept rigid, while the ligand (metal ion) had freedom of movement within the box. The Lamarckian genetic algorithm served as the search method, using 100 runs for each virtual screening. Analysis of the docking study results and visualization of outcomes, graphically representing the conclusions, were performed using the Discovery Studio software (BIOVIA Software product).¹⁵ There are two commonly used sets of charges (Gasteiger and Kollman) in Autodock. In contrast to the classical docking calculation in the AutoDock program (Gasteiger or Kollman charges are used), this research employed an electrostatic model. Specifically, in the pdbqt files of targets, the calcium ions' charge was set to +2, and the fluoride ion's charge was set to −1. The total charge of the phosphate group is −3, and the partial charges of the oxygen atoms and phosphorus were determined through quantum-mechanical calculations. The phosphate group structure was optimized at the wb97xd/x2c-TZVPall level of theory, and the natural population analysis (NPA) charges were calculated. The calculated partial charges (+2.488 for P and −1.372 for O) were then entered into the pdbqt files of the targets. The charges of metal ions as ligands were set to +2 (for calcium, strontium, manganese and copper) or +3 (for praseodymium).

RESULTS AND DISCUSSION

CSD analysis

Molecular docking is a fast and useful computational technique widely used in drug discovery for identifying the binding affinity and the binding mechanism of small molecules (ligands) to biomolecules (receptors). While originally devised for applications in biological systems, this investigation departs from the customary usage of molecular docking methodologies by employing them to assess the binding affinities of specific metal ions (Ca²⁺, Sr²⁺, Mn²⁺, Cu²⁺ and Pr³⁺) for two distinct calcium binding sites within fluorapatite, which is a material of interest. In addition, the further goal of this research is to determine whether the molecular docking is a suitable tool for materials and an adequate technique to explain the experimental results, based on X-ray diffraction, for doped fluorapatite. In the context of this investigation, the structural attributes of sites 1 and 2 are delineated as comprising Ca(1) and Ca(2) coordination polyhedra (Fig. 1).

Calcium binding site 1 manifests as a nine-coordinated Ca(1) polyhedron, characterized by six abbreviated Ca–O bonds (shorter than 2.5 Å), defining an approximate trigonal prism, and three elongated Ca–O bonds (bond lengths of 2.764 Å) extending through the prism faces. From the supramolecular point of view, six phosphate groups are coordinated to the Ca(1) ion in site 1 (Fig. 1).

Among these, three phosphate groups exhibit bidentate coordination to Ca (Ca–O bond lengths of approximately 2.434 and 2.763 Å), while the remaining three groups demonstrate monodentate coordination (Ca–O bond lengths of 2.376 Å). The average length of Ca–O bonds at Ca(1) is around 2.524 Å. The Ca(2) polyhedron can be conceptualized as a CaO_5F octahedron, with Ca–O bond lengths of 2.348, 2.349, 2.375, 2.501 and 2.502 Å, and Ca–F bond of 2.310 Å. Nonetheless, the octahedral geometry experiences distortion owing to the presence of an additional (seventh) Ca–O bond (Fig. 1b), which is comparatively weaker than the aforementioned bonds, evidenced by a bond length of 2.700 Å. At Ca(2), five phosphate groups coordinate with the calcium ion, with only one group engaging in bidentate coordination (Ca–O bond lengths of 2.501 and 2.502 Å), while the remaining groups exhibit monodentate coordination. The average length of Ca–O bonds at site 2 is notably shorter at 2.441 Å compared to Ca(1) (2.524 Å), signifying heightened sensitivity to doped ion size in the binding of metal ions at Ca(2).

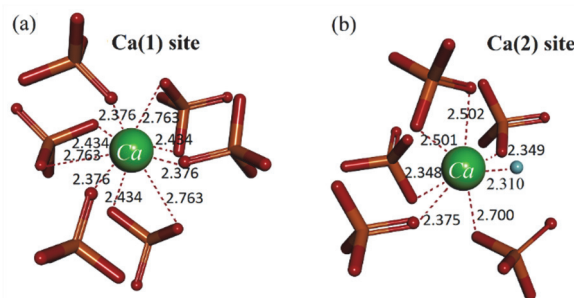


Fig. 1. Supramolecular profile of binding site 1 Ca(1) (a) and site 2 Ca(s) (b) in the crystal structure of fluorapatite.¹³

In order to determine whether the doping of fluorapatite with Sr^{2+} , Mn^{2+} , Cu^{2+} or Pr^{3+} lead to their coordination with phosphate groups, or the binding of metal ions has an electrostatically attractive character, the analysis of the crystal structures from the CSD was performed. The structures featuring M–O bonds (where M is Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} or Pr^{3+}) were extracted from the CSD, and the lengths of these M–O bonds, along with the metal ion environment, were systematically examined (Figs. 2 and S-1 of the Supplementary material). The environment was assessed by considering the number of M–X bonds (where X is any atom) achieved by the metal ion possessing at least one M–O bond. As previously explained, the binding of calcium ions to Ca(1) or Ca(2) site results in the formation of Ca–O and Ca–F bonds within the range of 2.3 to 2.8 Å (Fig. 1).

Taking this interval into consideration, it is deduced that all selected metal ions, with the exception of Mn^{2+} , can effectively substitute calcium ions in both sites. This substitution involves the establishment of covalent-coordination bonds with phosphate groups and fluoride ions in the respective sites (Fig. 2). Notably,

there is a lack of crystal structures in the CSD featuring Mn–O bonds longer than 2.6 Å, setting Mn^{2+} apart. This finding aligns with the analysis of the number of M–X bonds for individual metal ions (Fig. 2).

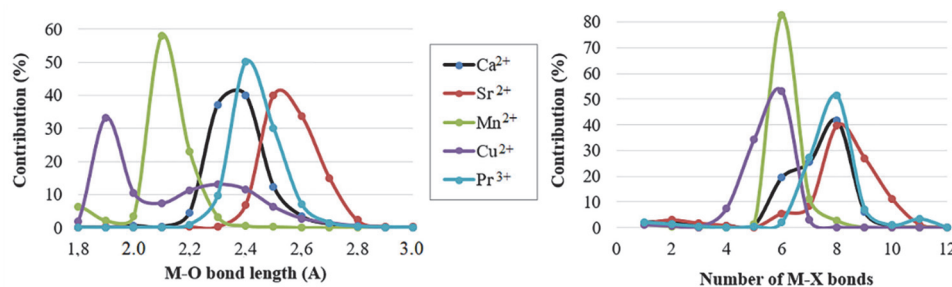


Fig. 2. Geometrical parameters for structures with M–O bonds (M: Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} or Pr^{3+}) extracted from crystal structures of small molecules, archived in the CSD.

Considering that Ca(1) site corresponds to a nine-coordinated polyhedron, while Ca(2) site corresponds to a seven-coordinated polyhedron, it is evident that all metal ions, excluding Mn^{2+} , can maintain the coordination geometry akin to the calcium ion following substitution. Specifically, there is an absence of crystal structures in the CSD depicting Mn^{2+} compounds concurrently featuring Mn–O bonds and Mn^{2+} with nine M–X bonds, a characteristic geometry associated with Ca(1) site.

DFT calculations

In a benchmark study, the model system comprised solely of Mn^{2+} and a phosphate group (Fig. S-2 of the Supplementary material) underwent examination using the wb97xd method with four distinct basis sets (def2-TZVP, jorge-TZP, x2c-TZVPall and dgauss-DZVP basis set). The investigation revealed that the x2c-TZVPall basis set exclusively produced a curve corresponding to the Lennard-Jones potential. This potential represents a mathematical framework elucidating interactions between two chemical entities at specific distances (Fig. S-2). Consequently, the x2c-TZVPall basis set was selected for assessing the strength of interactions between other metal ions (M^{n+}) and the phosphate group (Fig. 3).

Results derived from calculations at the wb97xd/x2c-TZVPall level of theory disclosed that the Ca^{2+} makes interactions with a strength ranging from -2550 to -2650 kJ mol^{-1} , within the distance range characteristic of Ca(1) and Ca(2) sites (2.3 to 2.8 Å). On the other hand, within the given distance range, the Sr^{2+} display slightly weaker interactions (from -2420 to -2550 kJ mol^{-1}). Interestingly, the Mn^{2+} exhibits interaction energies ranging from -3180 to -3390 kJ mol^{-1} , forming significantly stronger bonds with the phosphate ion. Cu^{2+} exhibits the strongest interactions of all the divalent ions, with interaction energies ranging from -3550 to -3770 kJ mol^{-1} . Pr^{3+} establishes the strongest interactions with phosphate group

(ranging from -4680 to -4940 kJ mol^{-1}), attributable to its elevated positive charge relative to other metal ions.

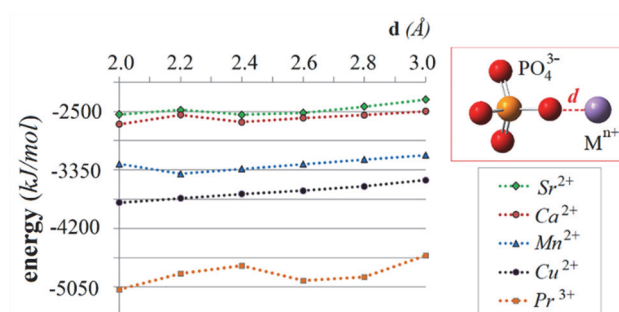


Fig. 3. Results of DFT calculations for evaluation of the interaction energies between the phosphate group and the metal M^{n+} (M^{n+} : Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} or Pr^{3+}). Parameter d represents the $M\cdots\text{O}$ distance, with the linear geometry of the interacting atoms and phosphorus atom ($M\cdots\text{O}-\text{P}$ angle is equal to 180°).

Molecular docking study

The molecular entities constituting Ca(1) and Ca(2) targets (Fig. 4), represent the phosphate groups and fluorides, with a cavity at the site of the central calcium ion. The incorporation of eight additional calcium ions on the surface of mentioned target structures (Fig. 4), resulted in the binding of metal ions only in the cavity. Calculated binding energies served as direct indicators of the investigated metal ions' affinities for specific sites, offering a refined focus on the interaction within the designated cavity. The results of docking studies on the mentioned targets are shown in Table I and Fig. S-3 (Supplementary material).

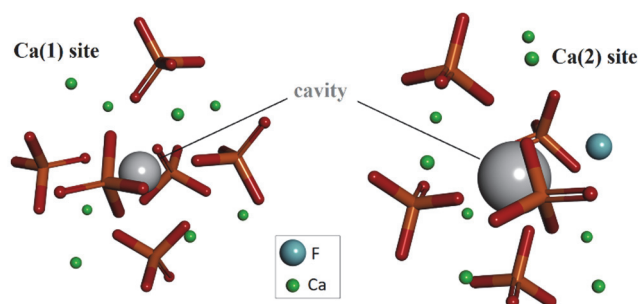


Fig. 4. The structures of targets, Ca(1) site and Ca(2) site, used for the docking study.

Results of docking studies with Ca^{2+}

The docking study showed that the Ca^{2+} has a slightly higher binding energy for Ca(2) site ($\Delta G_2 = -70.7$ kJ mol^{-1}) than for Ca(1) site ($\Delta G_1 = -70.0$ kJ mol^{-1}). The disparity in affinity towards specific sites is quantified by the $\Delta\Delta G_{1-2}$ para-

meter ($\Delta\Delta G_{1-2} = \Delta G_1 - \Delta G_2$), where a positive value denotes a heightened affinity for Ca(2) site. For the Ca^{2+} , the $\Delta\Delta G_{1-2}$ value is merely 0.7 kJ mol^{-1} , indicating a subtle predilection for binding to Ca(2) site. This outcome aligns with expectations derived from the analysis of parameters acquired through the CSD search. The distribution of Ca–O bond lengths (Fig. 2) reveals a peak in the range of 2.3 to 2.5 Å, supporting the notion of calcium ions favouring binding to Ca(2) site. The average Ca–O bond length in Ca(2) site (2.441 Å) is shorter than that in Ca(1) site (2.524 Å). The analysis of the number of atoms bound to calcium in crystal structures (Fig. 2) further reinforces the preference for binding to Ca(2) site. The structures with seven atoms bound to calcium (a geometric feature of Ca(2) site) are five times more prevalent than those with nine atoms bound to calcium (a geometric feature of Ca(1) site). DFT calculations indicate that the electrostatic interactions between the Ca^{2+} and the phosphate group at distances of 2.4 Å are stronger than interactions at approximately 2.6 Å (Fig. 3). This suggests that binding with shorter Ca–O distances, characteristic of Ca(2) site, is energetically favoured. Overall, the combination of docking studies, structural analysis, and DFT calculations provides comprehensive insights into the preferential binding behaviour of Ca^{2+} at distinct sites. The results of conventional molecular docking simulations are not consistent with the electrostatic model (Table I).

TABLE I. Gibbs energies changes (kJ mol^{-1}) for binding of investigated metal ions (ligands) to Ca(1) and Ca(2) sites, obtained by molecular docking with electrostatic model and conventional model

Ligand	ΔG_1	ΔG_2	$\Delta\Delta G_{1-2}$	$\Delta\Delta G_{11}$	$\Delta\Delta G_{22}$
Molecular docking with electrostatic model					
Ca^{2+}	–70.0	–70.7	+0.7	0	0
Sr^{2+}	–51.0	–54.5	+3.5	–19.0	–16.2
Mn^{2+}	–80.5	–78.9	–1.6	+9.5	+8.2
Cu^{2+}	–61.9	–68.8	+6.9	–8.1	–1.9
Pr^{3+}	–93.3	–100.4	+7.1	+23.3	+29.7
Conventional molecular docking model					
Ca^{2+}	–61.3	–53.1	–8.2	0	0
Sr^{2+}	–44.2	–14.1	–30.1	–17.1	–39.0
Mn^{2+}	–66.5	–59.0	–7.5	+5.2	+5.9
Cu^{2+}	–52.1	–44.9	–7.2	–9.2	–8.2
Pr^{3+}	–78.5	–66.5	–12.0	+17.2	+13.5

Results of docking studies with Sr^{2+}

To investigate the impact of an increase in ionic radius on the binding affinity, without altering the charge or nature of the ion, docking study was conducted with Sr^{2+} . Strontium is a metal of s-block elements, like calcium. Sr^{2+} is significantly larger (1.31 Å for nine-coordinated and 1.21 Å for seven-coordinated strontium) than Ca^{2+} (1.18 Å for nine-coordinated and 1.06 Å for seven-coordinated cal-

cium).¹⁶ In this case, there is no agreement in the prediction of binding affinity between the analysis of crystal structures from CSD and the results of DFT calculations. The distribution of Sr–O bond lengths (Fig. 2) peaks in the range of 2.5 to 2.7 Å, suggesting higher affinity of Sr²⁺ for binding to Ca(1) site. A threefold higher occurrence of crystal structures with nine bound atoms to Sr (Fig. 2) supports this prediction. Conversely, DFT calculations indicate the most stable orientation of the phosphate group and Sr²⁺ at 2.4 Å, indicating a higher binding affinity for Sr²⁺ to Ca(2) site, characterized by shorter M–O bonds than Ca(1) site. Docking study results further confirm a slightly higher binding energy for Sr²⁺ at Ca(2) site ($\Delta G_2 = -54.5 \text{ kJ mol}^{-1}$) compared to Ca(1) site ($\Delta G_1 = -51.0 \text{ kJ mol}^{-1}$), with a $\Delta\Delta G_{1-2}$ of 3.5 kJ mol^{-1} , signifying a slight affinity for Ca(2) site. The disparity in Sr–O bond lengths in crystal structures (ranging from 1.9 to 3.0 Å) is attributed to structural diversity and varying charges of oxygen species bound to strontium. The prevalence of neutral oxygen species leads to longer Sr–O bonds, affecting the reliability of Sr–O bond length distribution in crystal structures for predicting binding affinity in the molecular docking system. The $\Delta\Delta G_{1-2}$ parameter alone is insufficient for prediction of structural changes of materials due to doping. The additional parameters, $\Delta\Delta G_{11}$ and $\Delta\Delta G_{22}$ (Table I), represent the differences between ΔG values for calcium and doped ions in each site. The positive values indicate fluorapatite structure stabilization due to doping. Sr²⁺ doping induces slight destabilization, more pronounced for Ca(1) site. Lower binding free energies for Sr²⁺ in both sites ($\Delta\Delta G_{11}$ and $\Delta\Delta G_{22}$) align with weaker interactions observed in DFT calculations, where Sr²⁺ form fewer coordination bonds than Ca²⁺. Considering $\Delta\Delta G_{11}$, $\Delta\Delta G_{22}$ and $\Delta\Delta G_{1-2}$ parameters, Sr²⁺ exhibits a higher affinity for binding to Ca(2) site, but binding in both sites is possible. The results of the docking study have shown excellent agreement with the data obtained from the crystal structures of natural Sr-substituted fluorapatites, archived in the American Mineralogist Crystal Structure Database (AMCSD).¹⁷ Namely, 38 fluorapatite structures were found in the database, of which two structures have doped strontium ions.⁶ In these structures, the Sr²⁺ prefers binding in Ca(2) site, although a small number of ions are doped in Ca(1) site as well. In the first structure (database code amcsd 0001421), the site-occupancy of Sr²⁺ is 0.016 for Ca(1) site and 0.094 for Ca(2) site, while in the second structure (database code amcsd 0001422), the site occupancy for Ca(1) site is 0.002 and for Ca(2) site is 0.048. The results of conventional molecular docking simulations are not consistent with the experimental results, indicating a higher affinity of Sr²⁺ to the Ca(1) site (Table I).

Results of docking studies with Mn²⁺

In order to investigate the impact of reducing the ionic radius, while keeping the charge constant, on binding the affinity, a docking study was conducted using Mn²⁺. Manganese, belonging to the d-block elements, is situated in period 4 of the

Periodic Table, analogous to calcium. Analysis of data retrieved from the Crystal Structure Database (CSD) reveals that Mn^{2+} forms notably shorter M–O bonds, with a peak in the range of 2.1 to 2.3 Å (Fig. 2), compared to calcium ion in both binding sites. Manganese exhibits a propensity to form compounds with six bonded atoms, adopting an octahedral geometry (Fig. 2). Nevertheless, there exists the possibility of forming complexes with seven bonded atoms, corresponding to the geometry observed in Ca(2) site. In addition, there are no structures with nine bonded atoms for manganese (corresponding to Ca(1) site-geometry). Results of DFT calculations (Fig. 3) have shown that Mn^{2+} forms significantly stronger interactions with the phosphate group (interaction energy of $-3350 \text{ kJ mol}^{-1}$) than Ca^{2+} (interaction energy of $-2500 \text{ kJ mol}^{-1}$). This analysis suggests that Mn^{2+} is likely to exhibit a greater binding free energy than calcium, although specific predictions regarding its affinity to a particular binding site remain indeterminate. The results from the docking study (Table I) reveal a slightly elevated binding free energy for the Mn^{2+} at Ca(1) site ($\Delta G_1 = -80.5 \text{ kJ mol}^{-1}$) compared to Ca(2) site ($\Delta G_2 = -78.9 \text{ kJ mol}^{-1}$). The $\Delta\Delta G_{1-2}$ value of -1.6 kJ mol^{-1} indicates a marginal preference for binding to Ca(1) site, with the possibility of binding to Ca(2) site. However, this site-specific affinity is less pronounced when compared to Sr^{2+} ($\Delta\Delta G_{1-2}$ value of $+3.5 \text{ kJ mol}^{-1}$). Positive values of $\Delta\Delta G_{11}$ and $\Delta\Delta G_{22}$ parameters (9.5 and 8.2 kJ mol^{-1} , respectively) indicate stabilization of the fluorapatite structure due to doping of Mn^{2+} , which is slightly pronounced for site 1. Stabilization of fluorapatite structure upon doping with Mn^{2+} can be explained by stronger interactions with phosphate groups, compared to Ca^{2+} (Fig. 3). In Ca(1) site, the Mn^{2+} forms a tetrahedral complex (four bound atoms), with Mn–O bond lengths of 1.566, 1.908, 2.015 and 2.758 Å (mean value of 2.062 Å). In Ca(2) site, the Mn^{2+} forms a square-pyramidal complex (five bound atoms), with Mn–O bond lengths of 2.311, 2.507, 2.593 and 2.647 Å (mean value of 2.514 Å), as well as the Mn–F bond with length of 1.549 Å. Mn^{2+} is significantly smaller (0.66 Å for four-coordinated and 0.75 Å for five-coordinated manganese) than Ca^{2+} (1.18 Å for nine-coordinated and 1.06 Å for seven-coordinated calcium).¹⁶ Even though Ca(2) site has more bound atoms, the significantly longer bond lengths likely account for the diminished binding strength of Mn^{2+} to this site. The outcomes of the docking study exhibit the remarkable congruence with data derived from crystal structures of naturally occurring Mn-substituted fluorapatites within the American Mineralogical Crystal Structure Database (AMCSD). Two distinct structures featuring doped manganese ions are identified.⁶ In both structures, Mn^{2+} displays a preference for binding at Ca(1) site, with a minor presence at Ca(2) site. In the first structure (database code: 0001423), the site occupancy of Mn^{2+} is 0.205 for Ca(1) site and 0.065 for Ca(2) site. Similarly, in the second structure (database code: 0001424), the site-occupancy for Ca(1) site is 0.083, while that for Ca(2) site is 0.015. Although the results of conventional molecular docking simulations showed

that Mn^{2+} is bound stronger to both sites than Ca^{2+} , it is not consistent with the experimental results, because it refers to the tendency of Mn^{2+} to bound only to Ca(1) sites (Table I).

Results of docking studies with Cu^{2+}

Despite their comparable sizes, Cu^{2+} exhibits a distinct binding affinity compared to Mn^{2+} . Specifically, the Rietveld analysis of the hexagonal crystal structure of synthesized Cu^{2+} -doped fluorapatite indicates a preference for Cu^{2+} to selectively substitute for Ca^{2+} at Ca(2) site.¹⁸ An examination of data obtained from the CSD reveals that Cu^{2+} establishes considerably shorter M–O bonds than calcium ions in both binding sites. The distribution of M–O bond lengths exhibits a conspicuous peak within the range of 1.9 to 2.0 Å, accompanied by a secondary, less pronounced peak spanning from 2.3 to 2.4 Å (Fig. 2). Analogous to manganese, copper compounds predominantly feature six atoms bound to copper, while those with five bonded atoms are notably prevalent (Fig. 3). There exists a potential for forming complexes with seven and nine bound atoms, corresponding to geometries at Ca(2) site and Ca(1) site, respectively (Fig. 2). Geometrical analysis of data from the CSD base suggests a resemblance in the binding patterns of Cu^{2+} with Mn^{2+} to fluorapatite. The results derived from the docking study (Table I) reveal that Cu^{2+} manifests a significantly lower binding free energy at Ca(1) site ($\Delta G_1 = -61.9 \text{ kJ mol}^{-1}$) in comparison to Ca(2) site ($\Delta G_2 = -68.8 \text{ kJ mol}^{-1}$). The positive value of $\Delta\Delta G_{1-2}$ parameter (6.9 kJ mol^{-1}) indicates affinity for binding to Ca(2) site; however, as this value is greater than 5 kJ mol^{-1} , it should be expected that Cu^{2+} is bound only to Ca(2) site. The findings from the docking study align with the outcomes of Rietveld analysis,¹⁸ confirming the exclusive binding of Cu^{2+} to Ca(2) site. The negative values for $\Delta\Delta G_{11}$ and $\Delta\Delta G_{22}$ parameters (-8.1 and -1.9 kJ mol^{-1} , respectively) signify a destabilization effect on the fluorapatite structure resulting from the incorporation of Cu^{2+} . This destabilization phenomenon was observed in a crystal structure where the substitution of Ca^{2+} by Cu^{2+} occurred.¹⁹ Although the interaction energy of Mn^{2+} with the phosphate group (of $-3350 \text{ kJ mol}^{-1}$, Fig. 3) is lower than the energy of the interaction involving the Cu^{2+} (of $-3770 \text{ kJ mol}^{-1}$, Fig. 3), the binding free energy change of the Mn^{2+} is still higher. This disparity can be attributed to the geometric characteristics of the resulting complexes at both binding sites. In Ca(1) site, Cu^{2+} forms a tetrahedral complex characterized by four bound atoms, with Cu–O bond lengths measuring 2.225, 2.389, 2.561 and 2.606 Å (mean value of 2.445 Å). The average length of the Cu–O bond is notably longer than that observed for Mn^{2+} (mean value of 2.062 Å), which likely contributes to the higher binding free energy of Mn^{2+} at Ca(1) site. At Ca(2) site, Cu^{2+} adopts a square-pyramidal configuration involving five bound atoms, with Cu–O bond lengths of 1.940, 2.336, 2.489 and 2.643 Å (mean value of 2.352 Å). However, a significantly elongated Cu–F bond (length of 2.716

Å) is observed in comparison to Mn^{2+} (Mn–F bond with a length of 1.549 Å). Based on the findings from the docking study, it can be inferred that the binding of Mn^{2+} and Cu^{2+} induces a reduction in the M–O bond length compared to undoped fluorapatite. Notably, in nanocrystals of Cu^{2+} -doped fluorapatite, M–O distances were observed to be shorter with an increasing concentration of doped Cu^{2+} .¹⁹ The ionic radius of the Cu^{2+} in both binding sites (0.57 Å for four-coordinated and 0.65 Å for five-coordinated manganese) is marginally smaller than that of Mn^{2+} and significantly diminutive compared to the radius of the Ca^{2+} .¹⁶ Consequently, the substitution of Ca^{2+} by Cu^{2+} in Ca(2) site induces a reduction in the stability of the fluorapatite structure, leading to structural distortions. These alterations are discernible through a decrease in the height of the apatite peaks¹⁹ and an enhanced thermal stability observed in the fluorapatite sample in contrast to Cu^{2+} -doped fluorapatite.¹⁸ The destabilization of the fluorapatite structure, as evidenced by DTA–TGA studies, has also been observed in structures doped with Ag, Cd and Zn ions.^{20–22} The results of conventional molecular docking are not consistent with the experimental results, because it refers to the tendency of Cu^{2+} to bound only to Ca(1) sites (Table I).

Results of docking studies with Pr^{3+}

Pr^{3+} was chosen to determine the effect of increasing charge on binding affinity to a specific site in fluorapatite. In light of the distribution of Pr–O bond lengths, characterized by a peak within the range of 2.4 to 2.6 Å (Fig. 2), a preference for the binding of Pr^{3+} to any specific site cannot be established. This is due to the fact that the mean values of Ca–O bond lengths in Ca(1) site and Ca(2) site falls within this range, measuring 2.524 and 2.441 Å, respectively. Pr^{3+} forms slightly longer M–O bonds, compared to Ca^{2+} , whose distribution of M–O bonds has a maximum in the range from 2.3 to 2.5 Å (Fig. 2). Within crystal structures, praseodymium compounds featuring eight bonded atoms are predominant, as illustrated in Fig. 2. Notably, the incidence of structures with seven atoms bound to praseodymium, corresponding to the coordination geometry observed at Ca(2) site, significantly surpasses the occurrence of structures with nine atoms bound to praseodymium, corresponding to the coordination geometry observed at Ca(1) site. Based on this parameter, it can be assumed that Pr^{3+} has a higher binding affinity to Ca(2) site. The results of molecular docking have confirmed this assumption. Namely, the Pr^{3+} has a significantly smaller binding free energy to Ca(1) site ($\Delta G_1 = -93.3 \text{ kJ mol}^{-1}$) than to Ca(2) site ($\Delta G_2 = -100.4 \text{ kJ mol}^{-1}$). Positive value of $\Delta\Delta G_{1-2}$ parameter (7.1 kJ mol^{-1}) indicates the affinity for binding to Ca(2) site; however, as its value is greater than 5 kJ mol^{-1} , it should be expected that Pr^{3+} is bound only to Ca(2) site. The positive values recorded for the $\Delta\Delta G_{11}$ and $\Delta\Delta G_{22}$ parameters (23.3 and 29.7 kJ mol^{-1} , respectively) signify the stabilization of the fluorapatite structure resulting from the incorporation of Pr^{3+} . The prior assessments of site-

-occupancy ratios for rare earth elements in natural fluorapatites have been conducted, and the determined ratio for Pr^{3+} underscores a predominant preference for the Ca(2) site.^{9,23} Observationally, the binding free energy changes of Pr^{3+} , as documented in Table I, exhibit a notable increase compared to those of Ca^{2+} . This marked difference can likely be attributed to the more robust interactions of the Pr^{3+} with the phosphate group, denoted by an interaction energy of $-5000 \text{ kJ mol}^{-1}$ (Fig. 3), in contrast to the interactions of this group with Ca^{2+} , characterized by an interaction energy of $-2500 \text{ kJ mol}^{-1}$. The possible reason can be also found in the geometry of the resulting complexes in both sites. In Ca(1) site, Pr^{3+} forms a seven-coordinated polyhedron, with Pr–O bond lengths of 2.225, 2.251, 2.477, 2.511, 2.561, 2.606 and 2.772 Å (mean value of 2.486 Å). The mean Pr–O bond length is comparatively shorter than the mean Ca–O bond length observed at Ca(1) site, with a recorded mean value of 2.524 Å. Pr^{3+} adopts a six-coordinated polyhedron at Ca(2) site, forming Pr–O bond lengths of 1.940, 2.163, 2.336 and 2.843 Å, resulting in a mean bond length of 2.354 Å. Additionally, a Pr–F bond with a length of 2.716 Å is present. Notably, at Ca(2) site, the mean value of the Pr–O bond length is smaller than the corresponding value observed for Ca^{2+} , which is recorded at a mean value of 2.441 Å. The outcomes of DFT calculations reveal that the energetic strength of interactions between Pr^{3+} and the phosphate group is greater at a distance of 2.3 Å, compared to interactions at a distance of 2.4 Å. This observation potentially elucidates the elevated binding free energy change of Pr^{3+} at Ca(2) site. The reduced mean M–O bond lengths for Pr^{3+} , as compared to corresponding values for Ca^{2+} , arise from the higher positive charge of the praseodymium ion, leading to intensified electrostatic interactions between metal ions and negatively charged species (phosphate groups and fluoride ions) within both sites. This phenomenon may provide a plausible explanation for alterations in lattice parameters and reductions in crystallite size observed in Pr^{3+} -doped fluorapatite samples relative to pure fluorapatite.²⁴ Although the results of conventional molecular docking simulations showed that Pr^{3+} is bound stronger to both sites than Ca^{2+} , it is not consistent with the experimental results, because it refers to the tendency of Pr^{3+} to bound only to Ca(1) sites (Table I).

CONCLUSION

The presented findings unequivocally demonstrate the successful attainment of the primary research objective. Notably, this study establishes, for the first time, the efficacy of molecular docking with electrostatic model in making material-related predictions, extending its utility beyond the conventional realm of assessing small molecule (ligand) binding to biomolecules (targets). Although the conventional molecular docking simulations are commonly used for predicting the binding of small molecules to materials, such as metal–organic frameworks (MOFs),^{25–27} in our case, they proved to be ineffective. However, the application

of the molecular docking with an electrostatic model yielded results that exhibited excellent agreement with experimental data. This not only lends an innovative character to our approach but also demonstrates its scientific potential in predicting binding to materials representing ionic compounds, such as fluorapatite. The investigation focused on evaluating the binding strength of fluorapatite with five metal ions (Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} and Pr^{3+}) for the purpose of predicting site-preference and discerning alterations in the stability of crystal structures resulting from fluorapatite doping with these ions. The selected metal ions exhibit varying ionic radii – smaller in the case of Mn^{2+} and Cu^{2+} , larger for Sr^{2+} , and comparable but with a higher charge for Pr^{3+} , when contrasted with Ca^{2+} . These differences in ionic radii and charges significantly influence the site-preference of metal ion binding, as determined through site-occupancy, and contribute to changes in the stability of doped fluorapatite crystal structures, as indicated by alterations in the height of the apatite X-ray diffraction (XRD) peaks. The molecular docking was here employed to predict site-preferences and quantitatively characterize the stability of structures, demonstrating a surprising concordance with experimentally measured values in doped fluorapatites. This remarkable agreement strongly supports the applicability of molecular docking in materials research. Utilizing molecular docking in the biomaterials domain can facilitate the discovery of novel structures with multifunctional attributes for biomedical uses. Moving forward, the team plans to expand the repertoire of metal ions for theoretical binding predictions to fluorapatite and explore the applicability of molecular docking in other material systems.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13235>, or from the corresponding author on request.

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ИЗВОД

ПИОНИРСКА УЛОГА МОЛЕКУЛСКОГ ДОКИНГА У НАУЦИ О МАТЕРИЈАЛИМА. ПРЕФЕРЕНТНА ВЕЗИВНА МЕСТА ЈОНА МЕТАЛА У ФЛУОРАПАТИТУ

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Првобитно намењен за биолошке системе, молекулски докинг се се показао као вишенаменски, јер се може користити и за предвиђање места везивања јона метала и

њихових афинитета у материјалима. Коришћењем овог новог приступа, могуће је проценити енергије везивања Ca^{2+} , Sr^{2+} , Mn^{2+} , Cu^{2+} и Pr^{3+} за два различита места везивања јона метала у флуорапатиту. Подаци добијени из кристалних структура допираних флуорапатита показали су добру сагласност са докинг студијом. Интерпретација резултата молекулског докинга је употпуњена комбиновањем са резултатима DFT прорачуна, и са геометријском анализом кристалних структура малих молекула. Очигледно је да је ова нова стратегија погоднија од других теоријских приступа, због своје рачунарске сврсисходности, брзине и поузданости.

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SUPPLEMENTARY MATERIAL TO
**Molecular docking's pioneering role in materials science. Metal ion
site-preference in fluorapatite**

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METHODOLOGY

SM1. DFT calculations

The wb97xd method, a long-range corrected hybrid density functional, was employed for the calculations, providing the satisfactory accuracy for thermochemistry, kinetics, covalent systems, and both short-range and long-range non-covalent interactions. All density functional theory (DFT) calculations were executed using the Gaussian09 program. To ensure the generation of reliable interaction strength data, four distinct basis sets (def2-TZVP, jorge-TZP, x2c-TZVPall, and dgauss-DZVP basis set) were applied to the model system, consisting solely of a Mn²⁺ ion and a phosphate group. The distance between the metal ion and the nearest oxygen atom of the phosphate group was systematically varied during the calculations, from 2.0 to 3.0 Å. Importantly, this variation was performed in a manner that maintained the linear orientation of the interacting atoms, with the phosphorus atom forming a fixed angle (M···O-P angle fixed at 180°).

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RESULTS AND DISCUSSION

SM2. Analysis of crystal structures

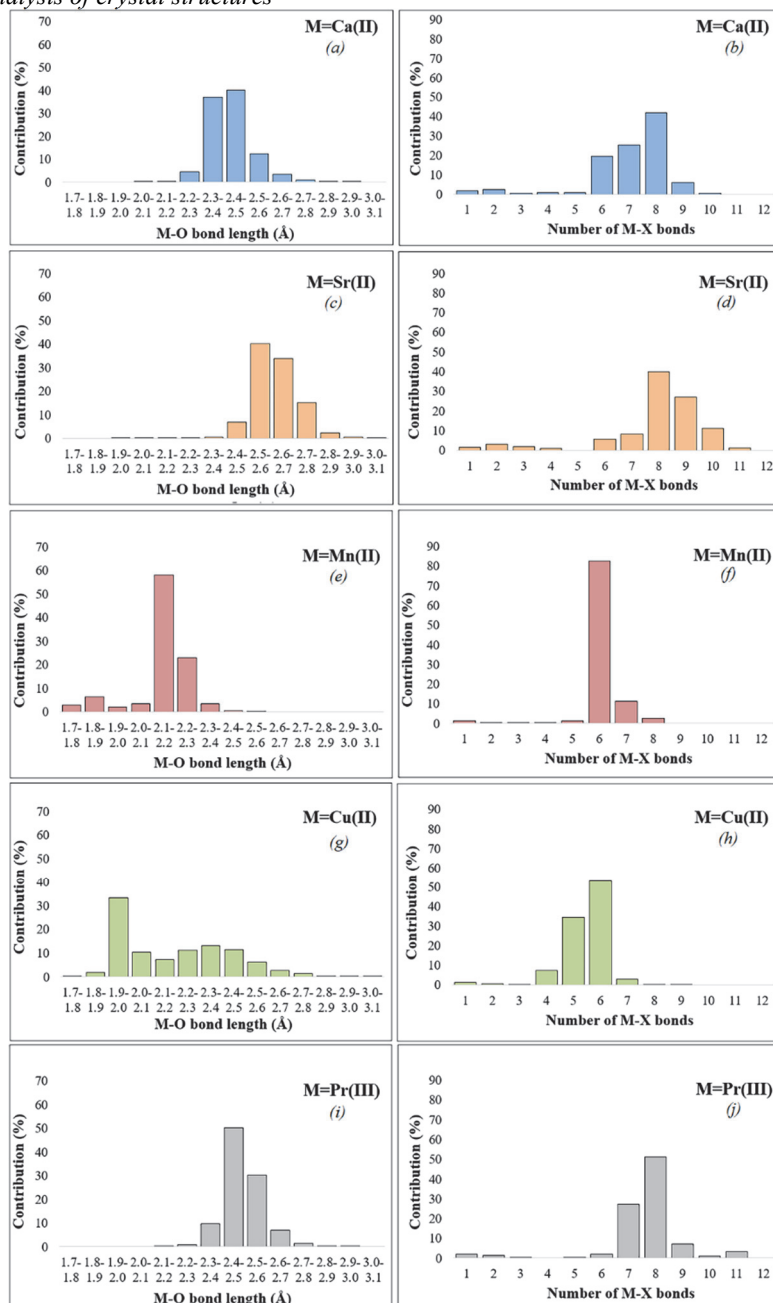


Fig. S-1. Geometrical parameters for M-O bonds ($M = \text{Ca}^{2+}$, Sr^{2+} , Mn^{2+} , Cu^{2+} , and Pr^{3+}) extracted from crystal structures of small molecules, archived in the CSD.

SM3. DFT calculations

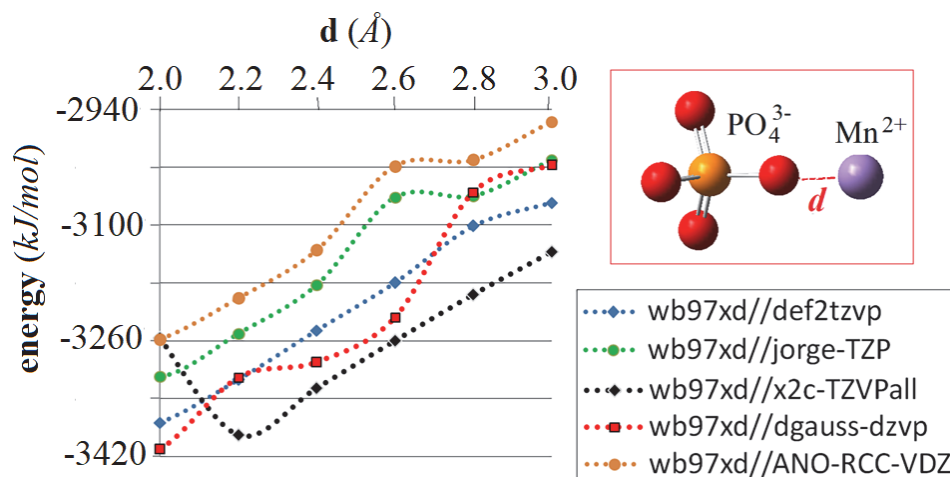


Fig. S-2. Results of DFT calculations for evaluation of the interaction energies between the phosphate group and the metal M ion ($M = \text{Ca}^{2+}$, Sr^{2+} , Mn^{2+} , Cu^{2+} or Pr^{3+} ion). Parameter d represents the distance between the metal ion and the nearest oxygen O atom, with the linear geometry of the interacting atoms and phosphorus atom ($M\cdots\text{O}-\text{P}$ angle is equal to 180°).

SM4. Molecular docking study

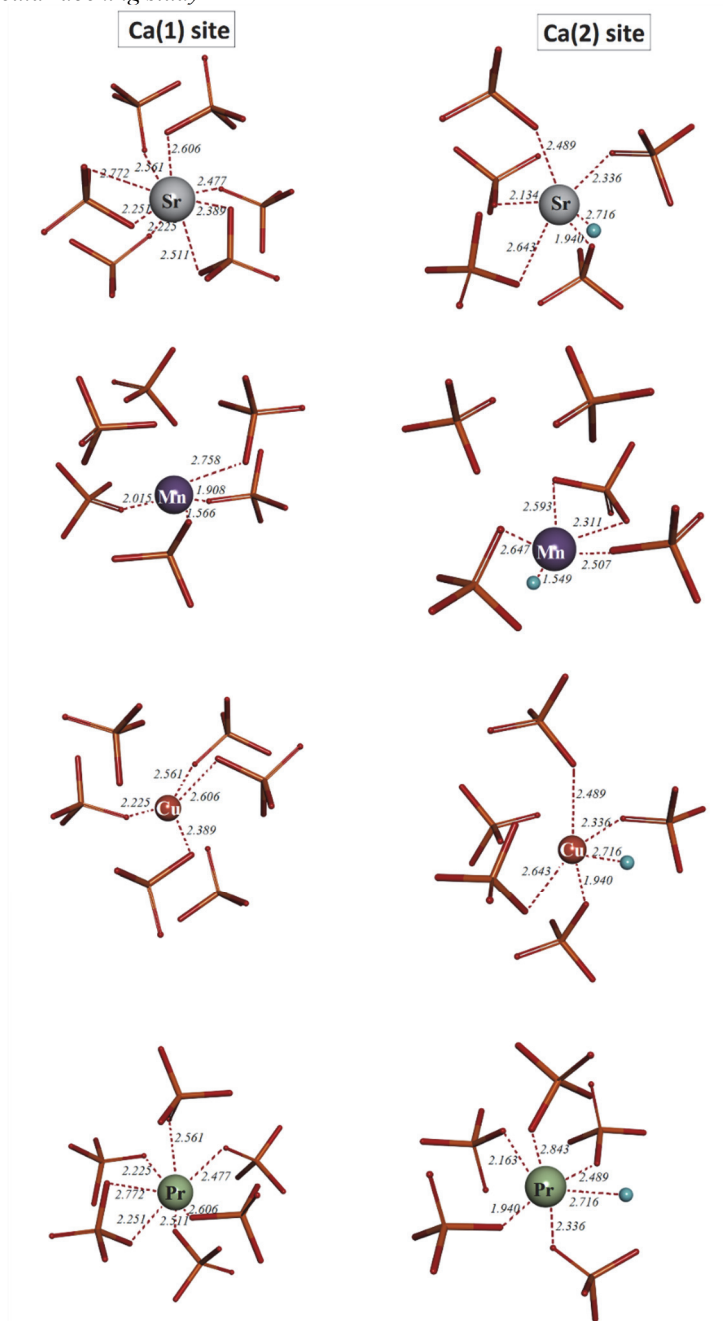


Fig. S-3. Interactions of metal ions (Sr^{2+} , Mn^{2+} , Cu^{2+} , and Pr^{3+}) in site 1 (left) and site 2 (right), obtained by the docking study.



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Influence of pressure and mixing with air on the equilibrium composition of laser-induced plasma on ODS steel

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Abstract: Laser induced plasma (LIP) is transient, the part of the time evolution of its emission used for the Laser induced breakdown spectroscopy (LIBS) technique corresponds to plasma pressures that are comparable to atmospheric, where a certain degree of mixing of the ablated material and air must be taken into account. The aim of this work was to discuss the influence of pressure change and air mixing of laser ablated material on the equilibrium composition of plasma obtained on an ODS steel target, which is a material of great interest for nuclear technology. Special care is devoted to the influence of pressure and mixing on the equilibrium relationship between the temperature T and the electron number density N_e , which is important for plasma diagnostics. The obtained results show that the concept of local thermodynamic equilibrium (LTE) can be used for the LIBS plasma diagnostics and the optimization of spectrochemical analysis parameters. The conclusion refers to the expected interval of plasma pressure during its evolution (10–0.1 bar) and the expected degree of mixing of the ablated material and air, in the temperature range 5,000–15,000 K, relevant for the LIBS technique.

Keywords: equilibrium plasma composition; LIBS; ODS steel; pressure, air mixing.

INTRODUCTION

In order to describe the gas plasma state in detail, it is necessary to know the population distribution of electronic levels of various atoms and ions, as well as the distribution of kinetic energy of various particles, including electrons. In the general case, to fulfil this task, it is necessary to carefully take into account all elementary processes in the plasma, together with their reaction rates and cross sections for collisions. If the plasma fulfils the conditions of local thermodynamic equilibrium (LTE), it can be completely described much easier, by applying the

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appropriate equilibrium distributions, without the need to treat the kinetics of elementary processes. Of particular interest is the knowledge of plasma composition at different temperatures. Under the conditions of validity of LTE, the equilibrium composition of the plasma as a function of temperature can be calculated by solving a system of equations consisting of the Saha equations, the mass conservation law and the electroneutrality equation. This approach is successfully applied to describe the equilibrium composition of the laser-induced plasma (LIP), for the Laser-Induced Breakdown Spectroscopy (LIBS) technique.^{1,2} In somewhat colder plasmas, where the existence of molecules cannot be ignored, a more optimal approach to calculating the equilibrium composition is by minimizing the total free energy of the particles, taking into account the appropriate mass and charge conservation relations.^{3,4} LIBS is a modern technique of elemental chemical analysis, which uses a plasma induced on the analysed sample as a source of excitation. Plasma is induced by a high-power density laser pulse. Usually, the nanosecond laser pulses are used for LIBS, but lasers with shorter pulses can also be used. The interaction of a laser pulse and a solid sample leads to its ablation and the formation of a hot and dense plasma that spreads usually in air, at atmospheric pressure.⁵ For analytical applications, a part of the time evolution of the plasma between 1 and 10 μs is usually used, when the plasma expansion slows down almost completely, and the pressure becomes close to the ambient pressure. Careful experimental measurements and theoretical modelling of laser-induced plasma expansion have shown that during the time evolution of the plasma, near the end of its expansion, the pressure can even be lower than ambient.^{6,7} Cristoforetti *et al.* showed that the LTE concept can be applied to LIP, in the stage of evolution used for LIBS technique.⁸

The oxide dispersion strengthened steels (ODS) are promising materials in nuclear technology, due to their favourable properties when they are exposed to high temperatures and intense radioactive radiation.⁹ Different types of ODS steel are considered for potential application both in fission technology, for fuel cladding, and in nuclear fusion reactors, where they should play the role of first wall material (known also as plasma face material, PFM). Due to the possibility of remote analysis, the LIBS technique is of particular interest for the application in nuclear technology.

The aim of this work was to clarify in more detail the influence of pressure on the equilibrium composition and the relationship between temperature and electron number density during the time evolution of laser-induced plasma. Since it is difficult to monitor the temporal evolution of the plasma pressure during its expansion with plasma diagnostic methods, it is usually assumed that the plasma pressure in the time interval used for LIBS, is close to atmospheric pressure. Given that the acquisition of spectra for LIBS is performed within time interval of 1–10 μs after the laser pulse with an ICCD detector (or from 1 μs to 10 ms or more with a CCD

camera), it is of interest to take into account the pressures higher than atmospheric, as well as slightly lower values.^{6,7} Also, the influence of the mixing of the ablated material and the surrounding air should be taken into account. The equilibrium composition was calculated using the program described in our recent publication,¹⁰ where the influence of the ionization potential lowering (IPL, known also as continuum lowering) was taken into account. ODS steel was chosen to illustrate the effect of pressure and mixing with air, because it contains mostly metals but also non-metal carbon, which will significantly affect the effect of mixing with air.

METHOD

The equilibrium composition of the plasma is calculated using the previously developed program, which is described in details recently and will only be briefly outlined here.¹⁰ First, the local thermal equilibrium is assumed to be established on a given temperature T for the initial atomic number densities of all the elements that constitute plasma. Each plasma component is assumed to enter subsequent ionization processes and thus multiple ionization stages are reached. For every atomic species i , the ionization–recombination equilibrium between atoms in ionization stage j and $j+1$ is described by Saha equation:

$$S_{ij} = \frac{N_{i,j+1}N_e}{N_{ij}} = 2\left(\frac{2\pi mk_B T}{h^2}\right)^{3/2} \frac{Z_{i,j+1}(T)}{Z_{ij}(T)} \exp\left(-\frac{E_{ij} - \Delta E}{k_B T}\right) \quad (1)$$

where S_{ij} is the Saha coefficient, N_{ij} and $N_{i,j+1}$ are the number densities of species i in j and $j+1$ ionization stage, respectively, N_e is the electron number density, m is the mass of an electron, h is the Plank constant, k_B is the Boltzmann constant and E_{ij} is the corresponding ionization energy, while ΔE is ionization potential lowering. $Z_{ij}(T)$ and $Z_{i,j+1}(T)$ are the electronic partition functions of species i in j and $j+1$ ionization stages, respectively. These partition functions are calculated from the database of statistical weights and energies adopted from NIST.¹¹ The partition function of an atom or ion is defined as:

$$Z_{ij}(T) = \sum_{k=1}^n g_k \exp\left(-\frac{E_k}{k_B T}\right) \quad (2)$$

where g_k and E_k are, respectively, the statistical weight and energy of electronic level k (relative to the ground level energy), while n is the highest occurring level.

Based on the plasma quasi-neutrality conditions, the partial pressure of free electrons p_e will be:

$$p_e = \sum_{i=1}^N \sum_{k=1}^M (j-1) p_{ij} \quad (3)$$

where N is the number of elements that constitute plasma, M is the maximal number of ionization stages while p_{ij} is the partial pressure of atomic species i , in ionization stage j ($j = 1$ for neutral atom, $j = 2$ for single charged ion, *etc.*). Also, the Dalton law of partial pressures applies, so the total pressure P is:

$$P = p_e + \sum_{i=1}^N \sum_{k=1}^M (j-1) p_{ij} \quad (4)$$

All Saha equations are mutually dependent through the p_e (or N_e) value, and after N_e is obtained the program calculates all N_{ij} values.¹² Based on this set of N_e and N_{ij} values, the ionization potential lowering is determined by Stewart–Pyatt model,^{13,14} via the following equation:

$$\Delta E = \frac{3}{2} \frac{j e^2}{4 \pi \epsilon_0 \lambda_D} \frac{((n_{IS} / \lambda_D)^3 + 1)^{2/3} - 1}{(n_{IS} / \lambda_D)^3} \quad (5)$$

r_{IS} being the ion-sphere radius:

$$r_{IS} = \left(\frac{3j}{4 \pi N_e} \right)^{1/3} \quad (6)$$

while λ_D is the Debye length, defined as:

$$\lambda_D = \sqrt{\frac{\epsilon_0 k_B T}{e^2 (N_e + \sum_i \sum_j (j-1)^2 N_{ij})}} \quad (7)$$

ϵ_0 being vacuum permittivity and e is the elementary charge.

After that, the iterative procedure follows and all Saha equilibria now need to be recalculated (this time with IPL included) in order to obtain a new, more accurate set of N_e and N_{ij} values. This again leads us to a new and more accurate value of ΔE . Pressure reduction due to the ionization potential lowering, ΔP is also applied:¹⁵

$$\Delta P = \frac{e^2 (N_e + \sum_i \sum_j j(j-1) N_{ij})}{24 \pi \lambda_D} \quad (8)$$

The iterations are further performed until the difference between two adjacent iterations of N_e value is lower than 10^5 m^{-3} . Reduction of the partition functions can occur due to the lowering of the ionization potential, if certain high-lying electronic states remain above the new ionization potential. In present calculations, the reduction of partition functions due to IPL is taken into account in order to obtain more reliable results. Validation of the used program was performed by comparison with the literature data, and can be found in our recent publication.¹⁰

RESULTS AND DISCUSSION

Effect of pressure on plasma composition

The equilibrium composition of the plasma in the range of temperatures relevant for the LIBS technique, from 5,000 to 15,000 K, is given in Figs. 1 and 2, for pressures of 0.1 and 5 bar*, respectively. The pressure of the LIP ranges between those two values in the final phase of its time evolution, when the spectral emission is registered for elemental analysis and characterization of the plasma. The equilibrium composition of the plasma corresponds to the starting composition

* 1 bar = 10^5 Pa

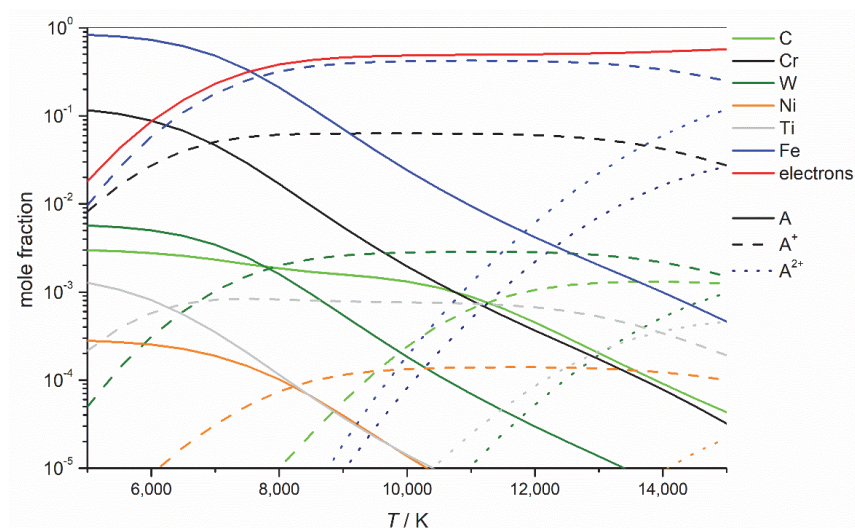


Fig. 1. Equilibrium plasma composition as a function of temperature, which corresponds to the ablation of ODS steel at pressure of 0.1 bar.

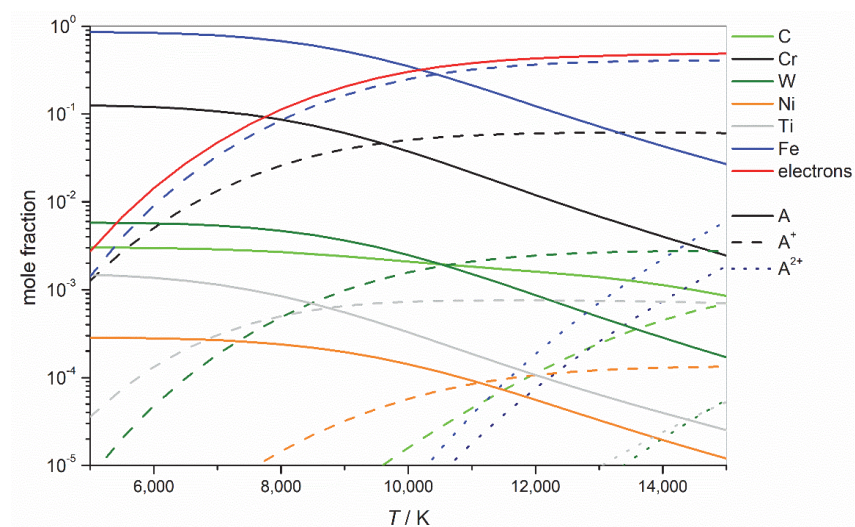


Fig. 2. Equilibrium plasma composition as a function of temperature, which corresponds to the ablation of ODS steel at pressure of 5 bar.

typical for ODS steel, Table I. Since ODS steel contains large amount of Fe, we have assumed that the selective evaporation does not occur during the ablation, as shown earlier by Pershin *et al.*¹⁶ For that starting composition and temperature range, the presence of molecules in plasma is negligible. The concentration of single charged ions becomes higher than the concentration of the corresponding

atom in the temperature interval from 8,000 to 9,700 K for metals depending on their first ionization energy (from 6.77 eV for Cr to 7.90 eV for Fe), for plasma at atmospheric pressure. As a non-metal, carbon has a significantly higher first ionization energy (11.26 eV), so its ion and atomic mole fractions become equal at higher temperature of 13,500 K. With the increase of plasma pressure, the proportion of ions decreases, and thus it equals the fraction of atoms at higher temperatures. On the contrary, at pressures lower than atmospheric, the degree of ionization increases. For example, the temperature at which the atom and single charged ion mole fractions are the same for Ti, shifts from approximately 6,290 K at a pressure of 0.1 bar, over 7,548 K at 1 bar, to 8,750 K at 5 bar. The significant shift of the ionization–recombination equilibrium with pressure indicates that this fact must also be taken into account during the spectroscopy of LIP, especially if the emission is integrated over a long period of time. Even in time-resolved measurements, when an ICCD camera is used, where the integration time can go well below 1 μ s, the decision of whether it is better to use an atomic or an ion line for spectrochemical analysis also depends on the pressure evolution during the camera exposure. The concentrations of doubly charged ions are significant at higher temperatures, at low pressure (Fig. 1), while they become negligible as the pressure increases.

TABLE I. Composition of the ODS sample with first ionization energies of its constituents

Element	Mole fraction	$E_{\text{ion}} / \text{eV}$
C	0.00303	11.26
Cr	0.12700	6.77
W	0.00584	7.87
Ni	0.00028	7.64
Ti	0.00152	6.83
Fe	0.86233	7.90

The effect of pressure on the ionization–recombination equilibrium of the analyte is actually related to the dependence of the electron number density on pressure, which is why this dependence should be discussed in more detail. In Fig. 3, the mole fraction of electrons as a function of temperature is presented, for the ODS steel plasma, at different pressures. As can be seen, for a given temperature, the mole fraction of electrons strongly depends on pressure: with a decrease in pressure (followed by the decrease in the number densities of all particles), the mole fraction of electrons increases. If the mole fractions of electrons for the corresponding pressures are translated into the electron number densities N_e , the dependence as shown in Fig. 4 is obtained. The increase in electron number density with pressure is not linear, because it increases more slowly at higher pressure. The reason for this behaviour lies in the fact that the increase in the electron number density favours recombination.

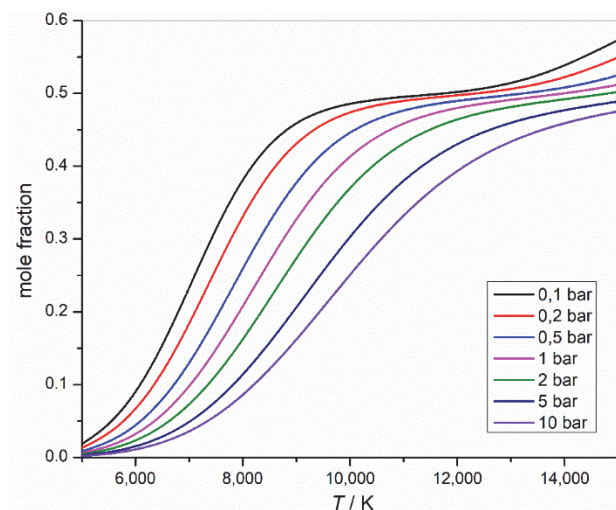


Fig. 3. Mole fraction of electrons in ODS plasma at various pressures.

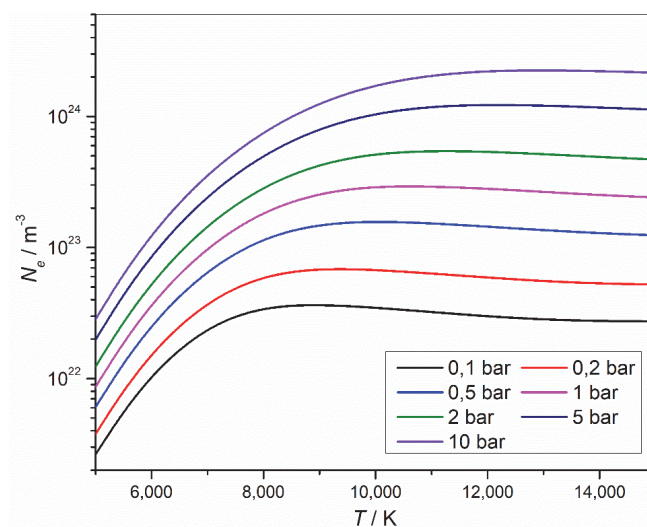


Fig. 4. Number density of electrons in ODS plasma at various pressures.

The Fig. 4 is very interesting from the plasma diagnostics point of view. If the plasma is in LTE (for a given plasma composition and pressure) each temperature corresponds to exactly one value of N_e , considering the lower temperature region in which (at a given pressure) N_e increases. For higher temperatures, where N_e reaches maximum, it starts to fall and changes very slowly, the approach cannot be applied. It follows that the pressure could be determined using Fig. 4, based on the previously measured values for N_e and temperature. Or *vice versa*: if the pressure value is known, based on the previously determined N_e , the temperature

of plasma can be determined. For pressures (up to 10 bar, including the development phase of plasma plume with a pressure lower than the ambient) and temperatures relevant for the LIBS technique (5,000–15,000 K), the dependence curves of $N_e(T)$ are sufficiently different that from them one can, at least roughly, estimate the pressure in plasma that emitted spectral lines, based on which T and N_e were determined.

In plasmas where the pressure changes with time, such as LIPs, there is no simple diagnostic method to measure the pressure, especially not by non-invasive diagnostic methods of optical emission spectroscopy. Therefore, any possibility of even a rough estimation of the pressure value is of interest. The aforementioned possibility of applying Fig. 4. to determine T or N_e , if one of those two values is previously determined by some diagnostic method, is also interesting for the plasma diagnostics, especially in cases when N_e is determined first (from the Stark broadening of spectral lines, for example) and then the temperature is estimated using the curves from Fig. 4. This is because the determination of temperature by emission methods (Boltzmann plot, emission line-intensity-ratio method) is somewhat unreliable, due to large gradients of plasma parameters, especially if the spectral measurements are not time-resolved.

The effect of mixing with air on plasma composition

The transient nature of LIP results in several additional problems for the applications in optical spectroscopy. In addition to the unknown plasma pressure during the time interval of the spectrum acquisition, the plasma composition is also unknown. It is undeniable that, while the plasma pressures during its expansion are significantly higher than the ambient pressure, there is practically no mixing of the ablated material with particles from the surrounding atmosphere. In other words, the composition of the plasma corresponds to the composition of the ablated material, taking into account, of course, the ionization processes. However, knowing the typical time interval of the time evolution of LIP and the expected pressures, the mixing of the ablated material with the surrounding air must also be taken into account. Therefore, the influence of mixing air and the ablated material must also be considered in the context of the results and discussion in the previous chapter.

When it comes to LIP plasma that is formed in air at atmospheric pressure, considering that the drop-in pressure is accompanied by a drop-in temperature, *i.e.*, the extinction of optical emission, it is reasonable to assume that in the stage of plasma evolution when the LIBS signal is registered, complete mixing of the air and ablated material (50 mol. % sample + 50 mol. % air) is not achieved. Therefore, we took into consideration the following degrees of mixing: 5, 10 and 20 mol. % of air, in order to evaluate the influence of mixing on the degree of dissociation of analytes and the connection of $N_e(T)$ in the LIP plasma. Figs. 5 and 6 show the

distribution of the equilibrium plasma composition as a function of temperature, at a pressure of 1 bar, without mixing with air and for a mixture with 20 vol. % of air and 80 vol. % of ablated material (with the composition given in Table I).

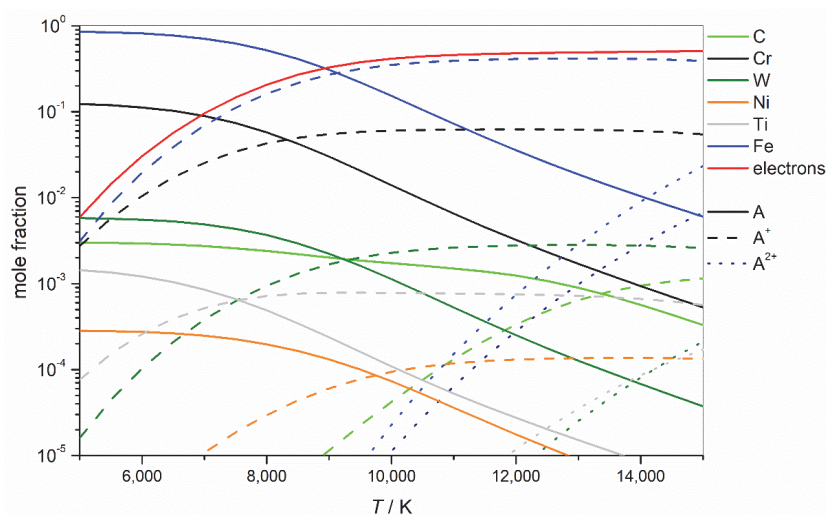


Fig. 5. Equilibrium plasma composition as a function of temperature at 1 bar, which corresponds to the ablation of pure ODS steel.

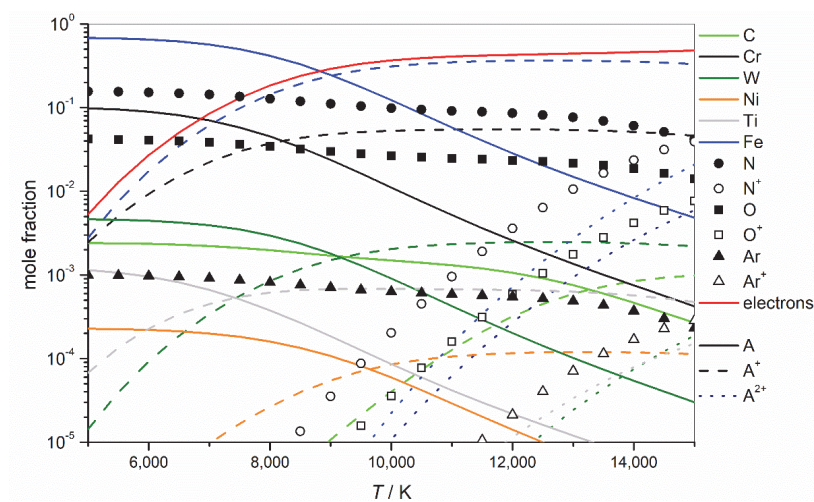


Fig. 6. Equilibrium plasma composition as a function of temperature at 1 bar, which corresponds to the ablation of 80 vol. % ODS steel mixed with 20 vol. % air.

The introduction of 20 mol. % of heavier ionizing elements (the first ionization energy for N is 14.53 eV, for O 13.62 eV and for Ar 15.76 eV) in relation to the metals present (6.67–7.90 eV), favours a slightly higher metal ionization, so

that the temperatures on which the mole fractions of atoms and ions are equalized, move to lower values: 100 K for C, up to 122 K for Ti (see Table II), with the shift being smaller for higher ionization energies. At pressures lower than the atmospheric this effect is somewhat smaller, while at higher pressures, it is greater. However, one should not lose sight of the fact that an even more significant proportion of air in the plasma can only occur when the plasma pressure approaches the surrounding pressure. It can therefore be concluded that possible mixing of ablated material and air does not significantly affect the choice of optimal lines (atomic or ionic) for the LIBS technique.

TABLE II. Temperatures at which mole fractions of atoms and ions of all ODS constituents are equalized, for pure ablated ODS and for mixing with 20 vol. % of air, at pressure of 1 bar. The differences between these two temperatures are given in ΔT column

Element	T / K		$\Delta T / \text{K}$
	ODS	ODS + 20 vol. % air	
C	13,375	13,275	100
Cr	8,327	8,209	118
W	9,281	9,177	104
Ni	9,747	9,644	103
Ti	7,548	7,426	122
Fe	9,130	9,025	105

The effect of mixing the ablated material with air on the ionization-recombination balance is illustrated in Fig. 7. Due to the overall decrease in the portion of metals, their degree of ionization is lower, and so is the mole fraction of electrons. However, the decrease in the mole fraction of electrons is less than the decrease in the mole fraction of the ablated material due to mixing with air: the introduction of heavier ionizing elements from the atmosphere favours the metal ionization. For the plasma diagnostics, the influence of mixing with air on the number density of electrons is very important. With the increase of temperature and the number density of electrons, the influence of mixing with air increases, until at around 9,000 K it reaches approximately 5 %, compared to a mixture with 20 % air, *i.e.*, 8 % at 10,000 K. This means that curves on Fig. 7. can be used to determine T if N_e is known, and *vice versa* for the atomic lines of metals, because those in the part of the time evolution of the LIP plasma used for analysis usually have emission maxima at temperatures below 8,000 K. On the other hand, the lines of metal ions have emission maxima at temperatures higher than 10,000 K. If the temperature is measured experimentally, the electron number density can be determined from the curves from Fig. 7. in the entire temperature interval, therefore also for the ionic lines of metals, with an accuracy better than 10 %.

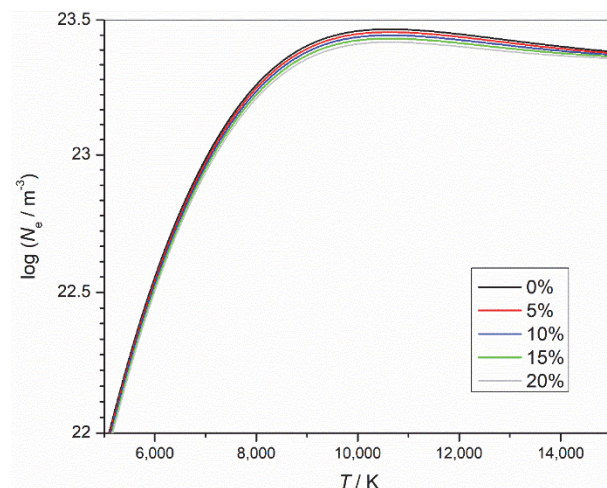


Fig. 7. Logarithm of the electron number density as a function of temperature in ODS plasma with various portions of air indicated (in vol. %).

CONCLUSION

With the increasing pressure, the equilibrium composition of the plasma shifts towards recombination, *i.e.*, the degree of ionization decreases. Depending on the first ionization energy of the observed element, the temperature at which the mole fractions of atoms and ions are equalized shifts to higher values: this shift is significant, for metals it can be up to 1,500 K, when the pressure changes by an order of magnitude. With an increase in the pressure, the electron number density for a given temperature also increases, with the increase in N_e being slower than the increase in pressure, because the increase in pressure favours recombination. The dependence of N_e on T at different pressures is sensitive enough that, based on previously measured T and N_e , one can at least roughly estimate the pressure. In other words, by monitoring the time evolution of N_e and T , the time evolution of the plasma pressure can be roughly estimated. This fact is particularly important considering the lack of suitable emission spectroscopic methods for the plasma pressure determination.

Considering the fact that the emission of LIP plasma is recorded at pressures close to atmospheric, the influence of the mixing of the ablated material with the surrounding air on the ionization-recombination balance of the analyte elements, as well as on the relationship between N_e and T , cannot be completely ignored. Air into the plasma increases the degree of ionization, so that the temperatures at which the mole fractions of atoms and ions are equalized move to higher values to a certain extent. The effect of mixing with air on the dependence of $N_e(T)$ can be neglected up to 9,000 K, even for a mixture with 20 % air, which is the upper limit of the mixing relevant for LIBS. In the interval of 9,000–15,000 K, the LTE

concept can still be used to determine N_e from the previously measured temperature.

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ИЗВОД

УТИЦАЈ ПРИТИСКА И МЕШАЊА СА ВАЗДУХОМ НА РАВНОТЕЖНИ САСТАВ ЛАСЕРСКИ ИНДУКОВАНЕ ПЛАЗМЕ НА ODS ЧЕЛИКУ

МИРОСЛАВ РИСТИЋ, НИКОЛА КРСТЕВСКИ, МИЛИЦА МАРКОВИЋ, АЛЕКСАНДРА ШАЈИЋ
и МИРОСЛАВ КУЗМАНОВИЋ

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Ласерски индукована плазма је променљива, пошто део временске еволуције њене емисије који се користи за технику спектроскопије ласерски индуковане плазме (LIBS) одговара притисцима плазме који су упоредиви са атмосферским, при чему се мора узети у обзир и изванредан степен мешања аблираног материјала и ваздуха. Циљ овог рада је да испита утицај промене притиска и мешања са ваздухом ласерски аблираног материјала на равнотежни састав плазме добијене на мети од ODS челика, материјала који је од великог интереса за нуклеарну технологију. Посебна пажња је посвећена утицају притиска и мешања на зависност између температуре T и концентрације електрона N_e , која је од великог значаја за дијагностику плазме. Добијени резултати показују да се концепт локалне термодинамичке равнотеже може користити за дијагностику LIBS плазме и оптимизацију параметара спектрохемијске анализе. Закључак је да је интервал притиска плазме током њене еволуције 10–0,1 bar и да је очекивани степен мешања аблираног материјала и ваздуха, у опсегу температура 5.000–15.000 K, релевантан за LIBS технику.

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Innovation in printer ink based on carbon quantum dots (CQDs) derived from styrofoam waste for anti-counterfeiting document technology

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Abstract: In response to the dual challenges of the environmental plastic pollution and the rise of document forgery, this study presents a novel fluorescent printer ink based on nitrogen and boron co-doped carbon quantum dots (N,B-codoped CQDs) derived from styrofoam waste. The CQDs were synthesized using a microwave-assisted green synthesis approach, and their photoluminescent properties were tailored *via* co-doping with urea and boric acid. The resulting ink formulation not only exhibited strong dual-colour fluorescence under 365 and 532 nm excitation but also demonstrated effective printability and stability on standard paper substrates. Characterization techniques, including UV–Vis spectrophotometry, fluorescence emission, Fourier-transform infrared spectroscopy and high-resolution transmission electron microscopy confirmed the successful incorporation of heteroatoms and the formation of nanoscale amorphous CQDs with rich surface functionality. Compared to the commercial printer ink, the N,B-codoped CQDs printer ink offered superior anti-counterfeiting features through fluorescence activation, albeit with a slightly lower ink absorption rate (0.47 ± 0.14 vs. 0.74 ± 0.06 cm/min). This study introduces a low-cost, eco-friendly and scalable strategy for producing smart ink with potential applications in secure printing and sustainable nanomaterial development.

Keywords: N,B-codoped CQDs printer ink; styrofoam; fluorescence.

INTRODUCTION

The document forgery is a criminal act involving the use of falsified documents.¹ Such forgery may include counterfeit signatures, falsified document content, or forged origins of the documents.² This issue represents a global problem,

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resulting in significant financial losses and posing security threats to individuals, companies, governments and society at large.³ The Supreme Court of Indonesia recorded 37,757 cases of document forgery between 2021 and 2023.⁴ Consequently, the need for anti-counterfeiting technology has become increasingly crucial to protect documents against increasingly sophisticated forgery attempts.

In parallel with this security concern, the issue of environmental sustainability – particularly plastic waste pollution – has also become a pressing global challenge. One major contributor to this problem is the increasing use of styrofoam as food packaging, driven by the rising demand for online food purchases, especially among street vendors and small-scale traders. This popularity stems from styrofoam's affordability, light weight, water resistance, practicality and accessibility. However, despite its practical advantages, styrofoam poses significant environmental threats because it is a non-degradable plastic that often accumulates in landfills or pollutes oceans.⁵ Styrofoam is a significant contributor to environmental pollution and is categorized as the fifth largest waste material globally.^{6,7}

Interestingly, the recent advances in nanotechnology have revealed that this problematic waste material can be transformed into high-value nanomaterials. Styrofoam, made from polystyrene polymer, contains styrene monomers that can be used to synthesize carbon quantum dots (CQDs). Several studies have successfully synthesized carbon-based quantum dots from styrofoam waste.⁸ This approach does not only reduces environmental pollution but also creates value by enabling the development of eco-friendly fluorescent inks for anti-counterfeiting applications. CQDs are particularly well-suited for such applications due to their low toxicity, ease of synthesis, and cost-effectiveness.⁹ Furthermore, styrofoam's high carbon content (approximately 62.6–92.2 %) and its low cost make it an ideal precursor for sustainable CQDs synthesis.¹⁰

To understand their application potential, it is essential to examine the fundamental characteristics of CQDs. These nanomaterials, typically smaller than 10 nm, are composed of fluorescent graphite-like cores.^{11,12} Their fluorescence originates from π -conjugated bandgap transitions and surface defect states.^{13,14} However, undoped CQDs generally exhibit low fluorescence quantum yields (*FQY*), around 10 %.¹⁵ Doping with heteroatoms such as nitrogen can significantly enhance their *FQY*, reaching up to 49 %.¹⁶ Co-doping strategies – such as combining nitrogen from ethylenediamine and boron from boric acid – have achieved *FQY* values around 39 %. In addition, sulphur and oxygen co-doping has been shown to enable dual-color emission (blue and red).¹⁵ Eda *et al.*¹⁷ reported that nitrogen and boron co-doped carbon quantum dots (N,B-codoped CQDs) emit distinct blue and yellow fluorescence, respectively. These findings underscore the tunable optical properties of heteroatom-doped CQDs, which are particularly valuable in multi-color anti-counterfeiting technologies.

The recent studies have increasingly explored the use of CQDs for anti-counterfeiting applications, focusing on their tunable fluorescence and low toxicity. For example, Deng *et al.*⁹ synthesized CQDs *via* a solvothermal method using ascorbic acid as a carbon source, but his work did not explore the use of eco-friendly waste materials or their scalability in industrial printing. Similarly, Eskalen *et al.*¹⁸ successfully derived blue-fluorescent CQDs from *Laurus nobilis* leaves, yet his study lacked an evaluation of ink absorption behaviour. Eda *et al.*¹⁷ research demonstrated promising dual-color emission from N,B-codoped CQDs but did not assess printing performance, particularly the crucial parameter of ink absorption rate.

This study seeks to bridge these gaps by synthesizing N,B-codoped CQDs from styrofoam waste and formulating them into printer ink. It focuses not only on the sustainable and low-cost production of fluorescent N,B-codoped CQDs but also on evaluating their performance in anti-counterfeiting document printing, specifically regarding ink absorption efficiency.

EXPERIMENTAL

Materials

Locally collected styrofoam waste (Bengkulu, Indonesia) was used as the primary carbon source. Monohydrate citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, $210.14 \text{ g mol}^{-1}$), boric acid (H_3BO_3 , 61.83 g mol^{-1}), and urea ($(\text{NH}_2)_2\text{CO}$, 60.06 g mol^{-1}), all analytical grade, served as dopant precursors. These chemicals were procured from Emsure® Merck and used without further purification. Activated charcoal and technical grade ethanol (96 %) were used, with ethanol serving as the solvent.

Preparation of styrofoam waste

Styrofoam waste was washed thoroughly under running water to remove surface impurities and air-dried. The dried styrofoam was chopped into small pieces and heated in an oven at 240°C for varying durations of 30, 60, 90, 120 and 150 min. After softening, the material was ground into fine powder using a laboratory grinder and sieved through a 100-mesh sieve to ensure uniform particle size. The resulting powder was stored in an airtight container for subsequent use.

Synthesis of N,B-codoped CQDs

N,B-codoped CQDs were synthesised *via* microwave-assisted synthesis based on the method reported by Alkian *et al.*¹⁹ with slight modifications in precursor ratios and microwave exposure time. 1 g of styrofoam powder was mixed with 1 g each of citric acid, boric acid and urea in a 250 mL glass beaker. To facilitate homogenisation, 2 mL of distilled water was added to the mixture, which was then heated in a microwave oven for 5 min. After cooling, 25 mL of 96 % ethanol was added, and the mixture was stirred thoroughly. The solution was filtered using standard filter paper to separate the filtrate containing the N,B-codoped CQDs from the solid residue. UV irradiation (365 nm) of the filtrate produced a cyan-blue emission, while irradiation with a green laser (532 nm) yielded yellow fluorescence, confirming successful N,B-codoped CQDs formation.

Characterization of N,B-codoped CQDs

The optical properties of the synthesised CQDs were analysed using a Thermo Scientific Genesys 50 UV-Vis spectrophotometer in the wavelength range of 250–800 nm. Fluorescence

characteristics were assessed using a Horiba FluoroMax hybrid fluorescence spectroscopy steady state and life time system. All samples for UV–Vis and fluorescence measurements were prepared using identical dilution volumes, without quantitative concentration calibration. Functional group analysis was conducted using a Bruker Alpha II FTIR spectrometer. The particle morphology, interplanar spacing, and selected area electron diffraction (SAED) patterns were investigated using a high-resolution transmission electron microscope (HRTEM) Talos F200X.

Preparation of N,B-codoped CQDs-based printer ink

The printer ink formulation was adapted from the method reported by Sari *et al.*²⁰ with adjustments made to the concentrations of activated charcoal and the gum arabic-to-water ratio to improve stability and fluorescence. A 10 mL portion of the N,B-codoped CQDs solution was mixed with 2 g of activated charcoal under constant stirring. Separately, 3.5 g of gum arabic was dissolved in 20 mL of distilled water. The two solutions were combined and homogenised. UV irradiation at 365 nm confirmed the successful formulation through the observation of blue fluorescence.

Testing of N,B-codoped CQDs-based printer ink

The ink absorption rate was evaluated using a capillary rise method adapted from previous studies.²⁰ A strip of standard A4 printing paper was cut to dimensions of 2 cm×15 cm and vertically immersed to a depth of 1 cm into each ink formulation for precisely 2 min. Immediately upon removal, the total length of ink rise (*i.e.*, the distance from the base of the strip to the furthest visible color front) was measured in cm using a ruler. This experiment was repeated five times for each ink type to ensure reproducibility.

The absorption rate (cm/min) was calculated using the formula:²⁰

$$\text{Absorption rate} = \text{Absorption length} / \text{Immersion time} \quad (1)$$

For the printing test, the printer ink was loaded into an empty Canon iP2700 ink cartridge. The printed outputs were then exposed to UV light at 365 nm to observe fluorescence, confirming the presence and activity of the N,B-codoped CQDs within the printed material.

RESULTS AND DISCUSSION

Qualitative evaluation of N,B-codoped CQDs using laser illumination

The successful synthesis of N,B-codoped CQDs is visually confirmed under various lighting conditions, as illustrated in Fig. 1.

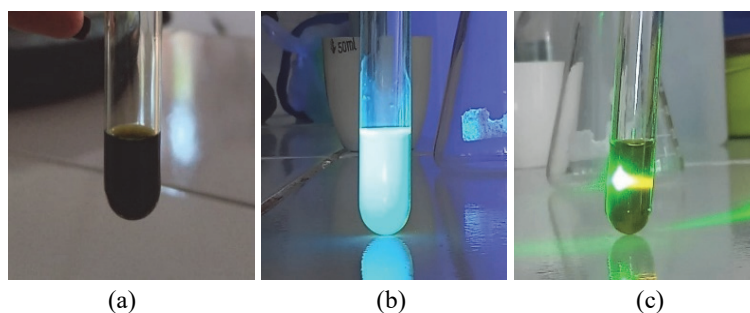


Fig. 1. Visual appearance of N,B-codoped CQDs solution under: a) normal lighting, b) UV laser (365 nm) and c) green laser (532 nm).

Under ambient lighting (Fig. 1a), the N,B-codoped CQDs solution exhibits a dark green color. Upon excitation with a 365 nm UV laser (Fig. 1b), it emits a strong cyan-blue fluorescence, which is attributed to the surface defect states and functional groups that facilitate radiative recombination of photoexcited electron–hole pairs. This phenomenon is consistent with prior studies highlighting the pivotal role of surface chemistry in determining carbon dot emission behaviour, particularly the involvement of sp^2 domains and defect-related energy states in generating blue-shifted luminescence.²¹ Upon the exposure to a 532 nm green laser (Fig. 1c), the solution displays a yellow emission, suggesting longer-wavelength transitions associated with deeper surface states or boron-induced mid-gap trap levels. This excitation-dependent, multicolor emission behaviour underscores the tunable photoluminescence of the N,B-codoped system, in line with observations by Feng *et al.*²² who emphasized the role of heteroatom doping in modulating the emissive characteristics of CQDs.

Morphological and structural analysis via HRTEM

Fig. 2. presents the morphology, crystallinity and size distribution of the synthesized N,B-codoped CQDs.

The HRTEM images in Fig. 2a and b reveal the quasi-spherical morphology and uniform dispersion of the N,B-codoped CQDs. A magnified HRTEM image (Fig. 2c) displays a clear dark-light fringe pattern with an interplanar spacing of 0.28 nm. This dark-light pattern signifies the presence of sp^2 hybridization in the core carbon atoms, further supporting the structural integrity of the N,B-codoped CQDs.²³ This spacing is comparable to those reported by Eda *et al.*¹⁷ (0.25 nm) and Qu *et al.*²³ (~0.21 nm), suggesting partial graphitization of the carbon core.²³

The SAED pattern (Fig. 2d) exhibits the diffuse diffraction rings characteristic of amorphous carbon, consistent with synthesis conducted at 240 °C. This observation agrees with the findings of Yadav *et al.*¹⁴ who reported that synthesis temperatures below 300 °C favour amorphous carbon formation, whereas higher temperatures promote the development of graphitic crystallites. The particle size distribution (Fig. 2e) reveals an average diameter of 5.7 nm, which falls within the typical size range for CQDs (<10 nm).¹⁴ Collectively, the HRTEM analysis confirm the successful synthesis of amorphous N,B-codoped CQDs with the desired structural features, interplanar spacing, and nanoscale size distribution.

Optical properties via UV–Vis spectrophotometry

The UV–Vis absorbance spectra of the N,B-codoped CQDs at varying synthesis times are shown in Fig. 3.

The UV–Vis spectrum of the N,B-codoped CQDs displays a prominent absorption peak at 348 nm, attributed to the $n-\pi^*$ transition of the C=O bond. This electronic transition typically occurs within the 300–500 nm range and aligns with

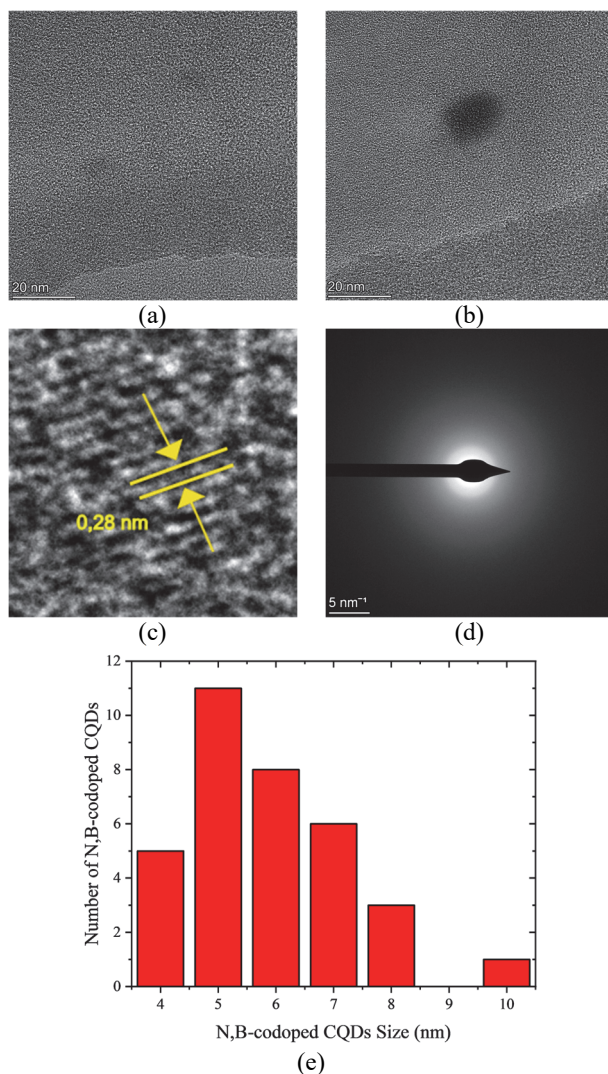


Fig. 2. A and b) HRTEM images of N,B-codoped CQDs; c) magnified HRTEM image showing interplanar spacing; d) SAED pattern of N,B-codoped CQDs; e) particle size distribution.

previous findings by Yuniarti *et al.*²⁴ and Kasmiarno *et al.*²⁵ In addition, a π - π^* transition associated with C=C bonds is observed between 250–300 nm range.^{25,26} These features reflect the intrinsic optical characteristics of N,B-codoped CQDs, which are influenced by both surface functional groups and the core structure.²⁷

The synthesis duration plays a critical role in tuning the surface chemistry and, consequently, the optical absorption behaviour of the CQDs.^{14,25} As illustrated in Fig. 3, the optimal synthesis time is identified at 90 min. At shorter durations, the

N,B-codoped CQDs exhibit insufficient surface development, resulting in reduced absorbance intensity. In contrast, N,B-codoped CQDs synthesised for 90 min demonstrate enhanced structural homogeneity and a higher density of functional groups, particularly those contributing to $n-\pi^*$ transitions, thereby yielding a more pronounced absorption peak.^{25,26} Beyond this optimal synthesis time, extended thermal treatment induces surface degradation, possibly due to the over-oxidation or the particle aggregation, leading to a decline in absorbance. These observations are consistent with the report by Kasmiarno *et al.*,²⁵ which highlights the adverse effects of prolonged synthesis on the structural and electronic integrity of CQDs. Hence, precise control of synthesis time is essential for optimizing the optical performance of N,B-codoped CQDs.

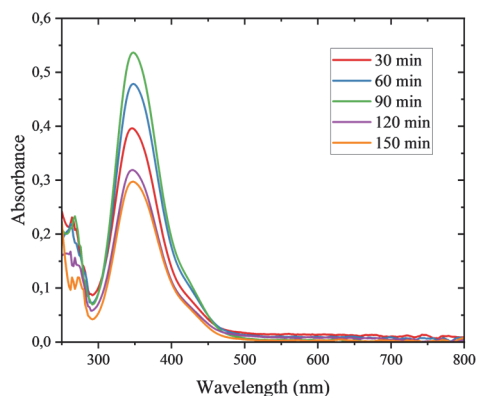


Fig. 3. UV-Vis absorbance spectra of N,B-codoped CQDs synthesized at different durations.

Photoluminescence analysis via fluorescence spectroscopy

The fluorescence spectra of the N,B-codoped CQDs are presented in Fig. 4.

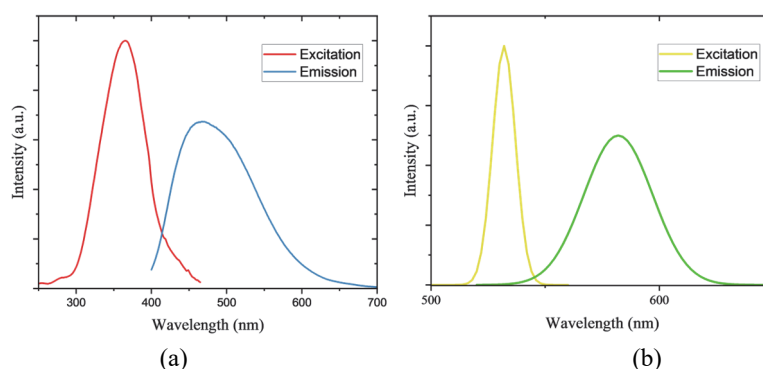


Fig. 4. Fluorescence emission spectra of N,B-codoped CQDs: a) at 469 nm ($\lambda_{\text{ex}} = 365$ nm), b) at 582 nm ($\lambda_{\text{ex}} = 532$ nm).

Under 365 nm excitation, a prominent emission peak is observed at 469 nm, corresponding to the cyan-blue fluorescence, which is consistent with the visual observation under UV laser irradiation (Fig. 1b). In contrast, excitation at 532 nm results in an emission peak at 582 nm, yielding a yellow fluorescence, as visually demonstrated under green laser illumination (Fig. 1c). These emissions originate from the radiative recombination of photoexcited electron–hole pairs, facilitated by surface states and functional groups introduced through nitrogen and boron co-doping.^{27,28} These dopant-related functional moieties play a significant role in enhancing the FQY and tuning the emission characteristics of the CQDs.^{29–31} This finding is consistent with the study by Eda *et al.*¹⁷ who reported a similar cyan-blue emission peak at 477 nm under 365 nm excitation, along with a secondary emission peak at 575 nm under 532 nm excitation, corresponding to yellow emission. Such dual-colour emission behaviour underscores the wavelength-dependent photoluminescent properties of N,B-codoped CQDs.

Functional group identification via FTIR

FTIR spectra of the N,B-codoped CQDs solution confirm, Fig. 5, the incorporation of nitrogen and boron functional groups.

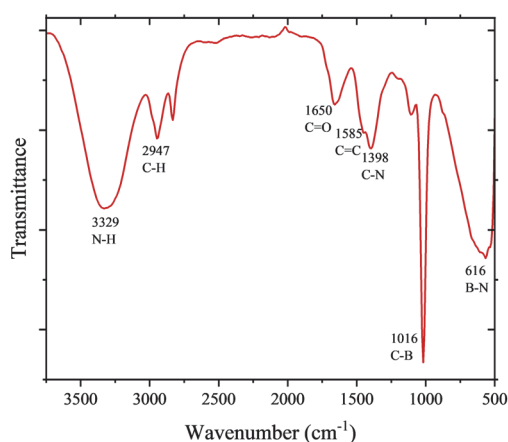


Fig. 5. FTIR spectrum of N,B-codoped CQDs.

The FTIR spectrum of the N,B-codoped CQDs solution exhibits the characteristic absorption peaks corresponding to various functional groups. The N–H stretching vibration appears at 3329 cm^{−1}, while the C–H stretching vibration is observed at 2947 cm^{−1}. Peaks at 1650 and 1585 cm^{−1} indicate the presence of C=C bonds and C=O stretching vibrations, respectively. Additional absorption bands at 1398, 1016 and 616 cm^{−1} correspond to C–N, C–B and B–N bonds, confirming the successful incorporation of nitrogen and boron during doping. The previous studies by Eda *et al.*¹⁷ and Liu *et al.*³² reported that the formation of N–H, C–N,

C–B and B–N bonds is characteristic of N,B-codoped CQDs, resulting from interactions between precursor materials and dopant atoms during synthesis. The presence of N–H and C–N groups suggests the effective nitrogen integration, which enhances the hydrophilicity and the surface reactivity of the CQDs. Similarly, the C–B and B–N functional groups confirm boron incorporation, playing a crucial role in modulating the electronic properties and surface chemistry.

These FTIR features further verify successful N and B doping, as evidenced by distinct absorption bands associated with these functional groups. The introduction of nitrogen and boron atoms is expected to generate some additional active sites, improving the optical performance of CQDs. Moreover, these functional groups contribute to the stabilization of surface states and influence the FQY of the nanomaterials.^{15–17,29–31}

Qualitative comparison of N,B-codoped CQDs and commercial printer ink

The distinct fluorescent properties of N,B-codoped CQDs-based printer ink are compared with commercial printer ink in Fig. 6.



Fig. 6. Comparison under: a) normal light and b) 365 nm UV laser between N,B-codoped CQDs and commercial printer ink.

Under normal lighting conditions, both the N,B-codoped CQDs ink and commercial printer ink appear black, exhibiting no discernible differences in visual appearance (Fig. 6a). However, upon exposure to a 365 nm UV laser, a marked contrast is observed: the N,B-codoped CQDs ink emits a bright cyan-blue fluorescence, whereas the commercial ink remains non-fluorescent (Fig. 6b). This cyan-blue emission directly stems from the inherent photoluminescent properties of the N,B-codoped CQDs,^{17,33} confirming their successful incorporation into the ink formulation. The fluorescence observed in the N,B-codoped CQDs printer ink corresponds to the emission peak at 469 nm detected in the fluorescence spectrum of the CQDs solution (Fig. 4). In contrast, the absence of fluorescence in the commercial ink reflects the lack of N,B-codoped CQDs or analogous fluorescent components, further emphasizing the distinctive properties of the N,B-codoped CQDs printer ink.

Ink absorption rate comparison

Table I summarizes the ink absorption test comparing commercial and N,B-codoped CQDs printer ink formulations.

TABLE I. Comparison of absorption rate (cm/min) between commercial and N,B-codoped CQDs printer ink

Experiment no.	Material	
	Commercial printer ink	N,B-codoped CQDs printer ink
1	0.75	0.60
2	0.80	0.35
3	0.70	0.40
4	0.65	0.35
5	0.80	0.65
Mean $\pm$ SD, cm/min	0.74 $\pm$ 0.065	0.47 $\pm$ 0.144

The data presented in Table I reveal a notable difference in the absorption rates between commercial and N,B-codoped CQDs-based printer inks. The commercial ink exhibits a higher average absorption rate (0.74 $\pm$ 0.065 cm/min) compared to the CQDs-based ink (0.47 $\pm$ 0.144 cm/min). This discrepancy is primarily attributed to the compositional differences between the two formulations. Specifically, the absorption behaviour is influenced by the nature of the added compounds, particularly the surface-functionalised CQDs present in the latter.

Commercial printer inks typically contain organic dyes dispersed in solvent systems tailored to maximise penetration and drying efficiency on cellulose-based substrates.³⁴ These formulations often include the surfactants and humectants that enhance interaction with paper fibres, thereby increasing the absorption rate.³⁵ In contrast, the N,B-codoped CQDs-based ink incorporates these nanomaterials as its main functional component, significantly altering the ink's physicochemical profile. The structured and functionalised surfaces of the doped CQDs can reduce the mobility of the ink, thereby slowing its capillary-driven absorption into the paper matrix.

Furthermore, the presence of hydrophilic functional groups on the N,B-codoped CQDs surfaces modulates the interaction with the substrate, contributing to the observed lower absorption rate,³⁶ which because of slower absorption may initially be perceived as a drawback, it can actually enhance print quality by reducing ink feathering and improving colour uniformity. These characteristics make N,B-codoped CQDs-based inks promising candidates for the high-resolution or the anti-counterfeiting printing applications, where precision and stability are essential.

Printing test of ink

Printed samples were tested under normal light and UV 365 nm in Fig. 7.



Fig. 7. Printed paper samples using N,B-codoped CQDs-based printer ink (top) and commercial printer ink (bottom) under: (a) normal lighting, and (b) UV 365 nm illumination.

Fig. 7 compares the visual and fluorescent properties of printed paper using N,B-codoped CQDs and commercial printer ink. Under the normal lighting (Fig. 7a), the prints exhibit no discernible difference in appearance. However, upon exposure to the UV illumination at 365 nm (Fig. 7b), the N,B-codoped CQDs ink emits a distinct cyan-blue fluorescence, whereas the commercial printer ink shows no fluorescence. The observed cyan-blue emission arises from the photoluminescent characteristics of the N,B-codoped CQDs incorporated into the ink, which are attributed to their surface functional groups and the electronic transitions, consistent with the previous reports by Deng *et al.*⁹ and Eskalen *et al.*¹⁸ This fluorescence behavior corroborates the findings presented in Fig. 6, confirming the unique luminescent properties of the N,B-codoped CQDs printer ink under similar excitation.

The absence of fluorescence in the commercial ink highlights the enhanced functional capabilities of the N,B-codoped CQDs printer ink, which, unlike conventional inks relying solely on dyes or pigments,^{35,36} incorporates nanomaterials that impart fluorescence and improved photostability. These properties present the N,B-codoped CQDs printer ink as particularly advantageous for applications in security printing, anti-counterfeiting and high-resolution imaging. Overall, the results validate the successful formulation of a fluorescent N,B-codoped CQDs-based printer ink.

CONCLUSION

This study successfully synthesized the N,B-codoped CQDs from styrofoam waste *via* a microwave-assisted method. The resulting N,B-codoped CQDs exhibited quasi-spherical morphology with an average size of 5.7 nm and an interplanar spacing of 0.28 nm, indicating partial graphitization. The fluorescence analysis revealed the emission peaks at 469 nm ($\lambda_{\text{ex}} = 365$ nm) and at 582 nm ($\lambda_{\text{ex}} = 532$ nm). FTIR confirmed the incorporation of N–H, C–N, C–B and B–N functional groups. The formulated N,B-codoped CQDs-based ink demonstrated strong cyan-blue fluorescence under UV light and effective printability on standard paper. Despite a lower average ink absorption rate (0.47 ± 0.14 cm/min) compared to the commercial ink (0.74 ± 0.06 cm/min), it provided superior anti-counterfeiting feat-

ures. In conclusion, the modified ink offers enhanced functionality and sustainability, making it a competitive and eco-friendly alternative to conventional commercial inks.

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ИЗВОД

НОВИНА У ТОНЕРУ ЗА ШТАМПАЧ БАЗИРАНА НА УГЉЕНИЧНИМ КВАНТНИМ ТАЧКАМА (CQDs) ДОБИЈЕНИМ ИЗ ОТПАДА ОД СТИРОПОРА ЗА ТЕХНОЛОГИЈУ ПРОТИВ ФАЛИСИФИКОВАЊА ДОКУМЕНАТА

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Као одговор на два проблема, загађење животне средина пластиком и пораст фалсификовања докумената, ова студија представља нови флуоресцентни тонер за штампач базиран на угљеничним квантним тачкама ко-допираним азотом и бором (N,B-кодипиране CQDs) синтетисаним из отпада од стиропора. CQDs су добијене приступом зелене синтезе подпомогнуте микроталасима, а њихове фотолуминесцентне особине су мењане ко-допирањем са уреом и борном киселином. Резултујућа формулација за тонер не само да је показала интензивну двобојну флуоресценцију под дејством побуђивачких таласних дужина од 365 и 532 nm, већ је показала и ефективну могућност штампања и стабилност на стандардним папирним супстратима. Техникама за карактеризацију које укључују UV-Vis спектrophотометрију, флуоресцентну емисију, FTIR спектроскопију и трансмисиону електронску микроскопију високе резолуције (HRTEM), потврђена је успешна инкорпорација хетероатома и формирање аморфних на нанометарској скали CQDs са функционалном површином. У поређењу са комерцијалним тонером за штампач, тонер на бази N,B-кодипираних CQDs показује супериорне карактеристике за спречавање фалсификовања докумената кроз активацију флуоресценције, додуше уз нешто нижу брзину апсорпције тонера ($0,47 \pm 0,14$ наспрам $0,74 \pm 0,06$ cm/min). Ова студија представља економичну, еколошки прихватљиву и прилагодљиву стратегију за производњу тонера са могућношћу примене у сигурном штампању и одрживом развоју наноматеријала.

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Acetic acid liquid–liquid extraction and UHPLC-DAD detection of polycyclic aromatic hydrocarbons in toasted and fried foods

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic compounds containing two or more fused aromatic rings. They have very low water solubility and are highly lipophilic. Acetic acid is a polar protic solvent that is often used in reactions involving carbocation intermediates. In this study, acetic acid, coupled with other organic solvents, was optimized and used for the extraction of eleven PAHs in two types of foods: toasted and fried. The UHPLC-DAD method, coupled with a C18 column, was validated and applied to analyze eleven PAHs in the food samples. The *LOD* and *LOQ* values obtained ranged from 0.0049 to 0.373 $\mu\text{g L}^{-1}$. The recoveries of the PAHs ranged from 47.3 to 119.7 %. The analysis results show that light PAHs were commonly found in both types of food. Some fried foods are highly carcinogenic due to the presence of BaP and Group 2B PAHs. Generally, toasted foods are safe to consume.

Keywords: Malaysian food; organic solvent; matrix effect; validation; HPLC.

INTRODUCTION

Prolonged thermal food processing, such as grilling, roasting, and frying, may induce the production of polycyclic aromatic hydrocarbons (PAHs). Many studies have shown that some PAHs are highly carcinogenic, and long-term exposure to them may increase the risk of human cancers.¹ Animal studies have indicated that certain PAHs may affect the hematopoietic and immune systems and can cause reproductive, neurological and developmental effects.² Due to the carcinogenic effects of PAHs, the International Agency for Research on Cancer (IARC) classifies benzo[*a*]pyrene (BaP) as carcinogenic to humans (group 1), while com-

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pounds such as benzo[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF) and chrysene (Chr) are classified as possibly carcinogenic to humans (group 2B).³ Accordingly, the maximum limits for BaP and the sum of group 2B PAHs in foodstuffs were set at 2.0 and 12.0 $\mu\text{g kg}^{-1}$, respectively.⁴ Due to BaP's carcinogenicity, it is often used as a marker for the presence and carcinogenic effects of PAHs in food. However, the committee also emphasized that the analysis of multiple PAHs is still necessary to gather more information on contamination levels and possible changes in the PAHs formation profile in foods.⁵

Various extraction methods have been used to recover PAHs from foods, including conventional techniques like liquid–liquid extraction (LLE), solid–phase extraction (SPE) and supercritical fluid extraction (SFE), as well as more advanced methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), liquid–liquid microextraction (LLME) and the QuEChERS method (quick, easy, cheap, effective, rugged and safe).¹ The main disadvantages of conventional techniques are the large amounts of organic solvents consumed, lengthy extraction times and high energy use.⁶ The introduction of LLME has addressed many of these drawbacks, as it uses microliter volumes of solvent. LLME is a miniaturized version of LLE, where an extraction solvent is mixed with a dispersive solvent and rapidly injected into the aqueous sample. The resulting cloudy solution is centrifuged to separate it into a two-phase system, allowing for easy recovery of the extraction solvent for analysis. MAE uses electromagnetic radiation to break the cellular matrix, releasing intracellular compounds into the extraction solvents.⁷ UAE enhances mass transfer rates and solvent penetration, leading to higher extraction yields and shorter extraction times.⁶ PLE employs liquid solvents at temperatures above their atmospheric boiling points but below their critical points, improving solubility and mass transfer properties.⁷ MAE, UAE and PLE are considered the most practical methods for industrial-scale use due to the availability of equipment, short extraction times and low solvent consumption rates.⁸ The QuEChERS technique is a type of dispersive solid-phase extraction (dSPE) used for sample preparation. It effectively uses adsorbent fillers to interact with impurities in the matrix, achieving high impurity removal.⁹

The PAHs profiles of various foods, especially meat variants such as grilled and smoked meats, have been widely studied.^{1,10} However, limited research has been conducted on the PAHs profiles of satay (grilling), roasted chicken (roasting) and Youtiao or Chinese doughnut sticks (frying). Acetic acid, one of the most widely used carboxylic acids, is often employed in reactions like the synthesis of acetic esters.¹¹ It is also used as a solvent, for example, in the production of cellulose acetate.¹² In this study, acetic acid, coupled with selected organic solvents, was applied for the first time in the extraction of PAHs from selected foods. The effects of acetic acid composition and the types of organic solvents, used for the

extraction, were optimized. The PAHs content and profile in the foods were determined using the UHPLC-DAD method. Separation was performed using a traditional C18 column rather than a more selective and expensive PAH column. The UHPLC-DAD method was developed and validated before being applied to determine eleven PAHs in the selected foods. The focus was on indigenous toasted and fried foods, commonly prepared from or mixed with flour, which are popular as appetizers or snacks in Southern and Southeast Asia. These foods are typically served for breakfast or afternoon tea. They can be categorized into toasted (Roti Canai, Chapati, Thosai, Kuih Tayap and Apam Balik) and fried (Youtiao, Keropok Lekor, Pisang Goreng, Cekodok and fried chicken) foods. The appearance of these foods is shown in Fig. S-1 of the Supplementary material to this paper. The determination of PAHs in these items has not been previously reported. The PAHs analyzed (ANT, FLR, FLT, PHE, PYR, CHR, BaA, BbF, BkF, BaP, Ghi) are among the most prevalent PAHs found in smoked, grilled and fried foods.

EXPERIMENTAL

Preparation of standard solutions

0.05 g of each PAH (ANT, FLR, FLT, PHE, PYR, CHR, BaA, BbF, BkF, BaP, Ghi) (Table S-I of the Supplementary material) was measured to prepare a stock solution (100 mg L^{-1}) containing the eleven PAHs in a 500 mL volumetric flask. Acetonitrile was used to dissolve the PAHs, and the solution was made up to 500 mL. The flask was left overnight at room temperature to ensure complete dissolution of the PAHs stock solution. This stock solution was further diluted 100-fold to prepare a working solution of 1 mg L^{-1} . From this, a series of standard solutions (25 mL each) with concentrations of 10, 50, 200, 800 and $1000 \text{ } \mu\text{g L}^{-1}$ were prepared, which were used to obtain calibration curves of peak areas as a function of PAH standard concentrations. These standard solutions were covered with aluminum foil and stored at 4°C until analysis.

Optimization of acetic acid extraction

Different acetic acid compositions (0, 30, 50 and 70 %) combined with an organic solvent (*i.e.*, acetonitrile) were prepared for the optimization study. Two selected food samples (3 g each), Chapati and Keropok Lekor (representing the toasted and fried categories, respectively), were placed into separate beakers and spiked with eleven PAHs at a concentration of $5000 \text{ } \mu\text{g L}^{-1}$ (50 mL). The samples were stirred overnight at 300 rpm using a mechanical stirrer. The spiked food samples were filtered and subsequently extracted with 10 mL of a mixed solvent containing different acetic acid compositions, stirred at 300 rpm for 30 min. After stirring, the food solids were removed by vacuum filtration. One mL of the filtrate was diluted to 5 mL with acetonitrile and then injected into the HPLC-DAD system for PAH analysis. Once the optimum acetic acid composition was determined, the most suitable solvent for extraction was evaluated by replacing acetonitrile with four other solvents: toluene, dichloromethane, ethyl acetate and ethanol. A 10 mL extraction solvent consisting of acetic acid and one of the selected solvents was added separately to each type of food sample. The samples were stirred at 300 rpm for 30 min, filtered and then diluted fivefold before analysis.

Liquid–liquid extraction (LLE)

Two selected food samples, Chapati and Keropok Lekor, were spiked with the eleven PAHs at concentrations of $5000 \mu\text{g L}^{-1}$. The spiked food samples were extracted with a 10 mL mixed solvent of acetonitrile and acetic acid (70:30 volume ratio) using a magnetic stirrer for 30 min. After stirring, the food solids were removed by vacuum filtration. One mL of the filtrate was diluted to 5 mL with acetonitrile and then injected into the HPLC-DAD system for PAH analysis. This procedure is referred to as acetic acid extraction without SPE.

Solid phase extraction (SPE)

In a separate experiment, after LLE, the two selected food samples (Chapati and Keropok) underwent SPE clean-up. The SPE method followed Hamidi *et al.*¹⁰ with modifications and it was conducted using a Supelco C18 SPE cartridge (ENVI-18, 6 mL/0.5 g). The PAH clean-up was evaluated based on the percentage recovery when passed through the SPE cartridge (VacElut, 16 mm×150 mm, Agilent, Palo Alto, CA, USA). The cartridge was conditioned with 10 mL of the respective mixed solvents (acetic acid and acetonitrile in varying ratios) used for extraction prior to the clean-up procedure. Elution was performed using 3 mL of toluene, and the process was repeated three times. Toluene was chosen due to its potential for π – π interactions with better aromatic selectivity. Toluene was evaporated using a rotary evaporator, and the residue was diluted with 4 mL of HPLC-grade acetonitrile. The solution was filtered through a membrane before injection into the HPLC system. This procedure is referred to as acid extraction with SPE.

Sample extraction

A total of 10 different foodstuffs were purchased from various food stalls in the Klang Valley region, Malaysia. These included 5 types of toasted foods (Roti Canai, Chapati, Thosai, Kuih Tayap and Apam Balik) and 5 types of fried foods (Youtiao, Keropok Lekor, Pisang Goreng, Cekodok and fried chicken). All samples were carefully packed in polyethylene bags and stored at 4 °C until analysis. For the extraction, 3 g of each food sample was cut into equal sizes of 1 cm×1 cm. A 10 mL solvent mixture of acetic acid and acetonitrile (30:70) was added to the food sample, which was then sonicated for 5 min, followed by mechanical stirring for 30 min at 300 rpm at room temperature. The food sample was separated from the filtrate by vacuum filtration using a Buchner funnel. The filtrate was diluted fivefold prior to analysis. Details of the food descriptions are summarized in Table I.

Instrumentation and chromatographic conditions

The separation and quantification of PAHs were performed using an HPLC system (Thermo Technologies, Anderson, CA, USA) consisting of a Thermo UltiMate 3000 quaternary pump, an Agilent 1260 Infinity diode-array detector, a Thermo UltiMate 3000 standard autosampler injector with a 10 μL loop, and a reversed-phase Ascentic 5 μm C18 column (250 mm×4.6 mm). A gradient elution using ultrapure water and HPLC-grade acetonitrile was varied from 0 to 40 % acetonitrile over 30 min. The flow rate, wavelength and column temperature were set at 1.0 mL/min, 259 nm, and 30°C, respectively.

TABLE I. Description of food samples analyzed

Food type	Name of food	Description
Toasted food	Roti Canai	Indian flatbread dish served by the Indian-Muslim community popularly as breakfast or for tea time. The dough is made out of fat, flour and water and is flattened and oiled before toasting.

TABLE I. Continued

Food type	Name of food	Description
Toasted food	Chapati	A type of bread served by the Indian community for breakfast. It is made out of wheat flour, also known as “atta” flour mixed into a dough.
	Thosai	It is a type of “pancake” that is originated from India which is made from mainly rice and black gram. This food resembles a crepe in appearance and is normally served as breakfast.
	Kuih Tayap	Also known as pandan pancake rolls. It is a Nyonya sweet desert made from crepe batter
	Apam Balik	A type of pancake served as a sweet desert, originating in the Chinese cuisine. The batter is made from flour, eggs, sugar, baking soda, coconut milk and water.
Fried food	Youtiao	It is a “fried bread stick” which is a popular breakfast food among the Chinese society. It is normally eaten as a side dish and always consume together with porridge.
	Keropok Lekor	Also known as fish crackers. It is a traditional Malay snack and is made by grinding fish, mixing with sago and deep frying it.
	Pisang Goreng	Also known as goreng pisang. It is a traditional Malay snack made by coating sliced bananas in flour and deep frying them. Sometimes served with soy sauce.
	Cekodok	Also known as fried banana balls served by the Malay community as snacks. The batter is made by mashing bananas together with plain flour and rice flour and subsequently deep fried.
	Fried chicken	The chicken pieces are floured and deep-fried. Commonly consumed as a side dish together with other types of foods.

RESULTS AND DISCUSSION

Development and optimization of liquid–liquid extraction

Effect of acid composition. The primary goal of this section was to develop an effective extraction method for PAH analysis. Various acid compositions and solvent types were tested for extracting PAHs from two selected food samples, Chapati and Keropok Lekor. The effect of solid phase extraction (SPE) was examined. The optimized conditions were applied to determine the PAH profiles in selected toasted and fried foods.

The tested acid compositions were 0, 30, 50 and 70 vol. % of acetic acid. The solvents tried included toluene, dichloromethane, ethyl acetate and ethanol, all of which are miscible with acetic acid. Two sets of extraction experiments were conducted for each acetic acid composition: one without SPE (direct acetic acid extraction), and one with SPE following acetic acid extraction. In the primary trials, acetonitrile with different acetic acid compositions was used as the extraction solvent, as acetonitrile is the most polar solvent (polarity index, 5.8) compared to ethyl acetate (4.4), toluene (2.6), dichloromethane (3.1) and ethanol (5.2).

The extraction method's effectiveness was evaluated by the percentage recovery after spiking a mixture of eleven PAHs into two food samples (Chapati and Keropok Lekor). Results showed that acetonitrile with 30 % acetic acid, without SPE, yielded higher percentage recoveries (mean 70.9 and 109.5 % for Chapati and Keropok Lekor, respectively) for the eleven PAHs (Fig. 1a and b). Other acetic acid compositions (0, 50 and 70 %) resulted in lower recoveries (mean 55.8, 44.0 and 38.2 % for Chapati; 101.0, 56.2 and 94.1 % for Keropok Lekor, respectively). Similar trends were observed for Chapati with SPE (Fig. 1a). However, for Keropok Lekor, the recovery for 30 % acetic acid with SPE (50.5 %) was slightly lower than for other compositions (58.3, 55.3 and 69.2 % for 0, 50 and 70 % acetic acid, respectively, Fig. 1b). Details on the effects of acetic acid composition on recoveries are provided (Table S-II of the Supplementary material).

Overall, recoveries without SPE were higher than those with SPE, likely due to the semi-adsorption of PAHs onto the SPE adsorbent. The higher recoveries without SPE may be attributed to acetic acid enhancing PAH solubility in organic solvents, with maximum solubility occurring at 30 % acetic acid. In contrast, higher acetic acid concentrations (50 and 70 %) resulted in lower recoveries, possibly due to increased solvent polarity reducing PAH solubility. In the presence of water, acetic acid dissociates into acetate and H^+ , but without water, it acts as a polar covalent compound, binding to other compounds via covalent bonding.¹³ The protic nature of acetic acid (dielectric constant 6.2) may also facilitate the dissolution of both polar and non-polar compounds like PAHs.¹⁴ This explains why 30% acetic acid yielded optimal solubility for PAHs, and this condition will be used to select the best organic solvent for PAH extraction in the next section.

Effect of organic solvent. In this study, different organic solvents were used to extract spiked PAHs from the selected food samples (Chapati and Keropok Lekor), using the optimum 30 % acetic acid composition without SPE. The percentage recoveries of the solvents in descending order were: acetonitrile (70.9 %), ethyl acetate (52.9 %), toluene (45.0 %), dichloromethane (36.8 %) and ethanol (25.5 %) (Fig. 1c). Acetonitrile gave the highest percentage recovery. Detailed recovery data for the solvents are presented in Table S-III of the Supplementary material.

When correlating solvent extraction ability to their polarity indices, the expected order (from highest to lowest) would be acetonitrile ($P = 5.8$), ethanol ($P = 5.2$), ethyl acetate ($P = 4.4$), dichloromethane ($P = 3.1$) and toluene ($P = 2.6$) (Zarrinmehr *et al.*, 2022).¹⁵ However, toluene showed higher recovery than expected and ethanol showed lower recovery. The higher extraction power of toluene compared to ethanol may result from π - π interactions between toluene and PAHs, while ethanol's poor performance could be due to the unfavorable interaction between its highly polar hydroxyl group ($-OH$) and the non-polar aromatic rings of PAHs.¹⁵

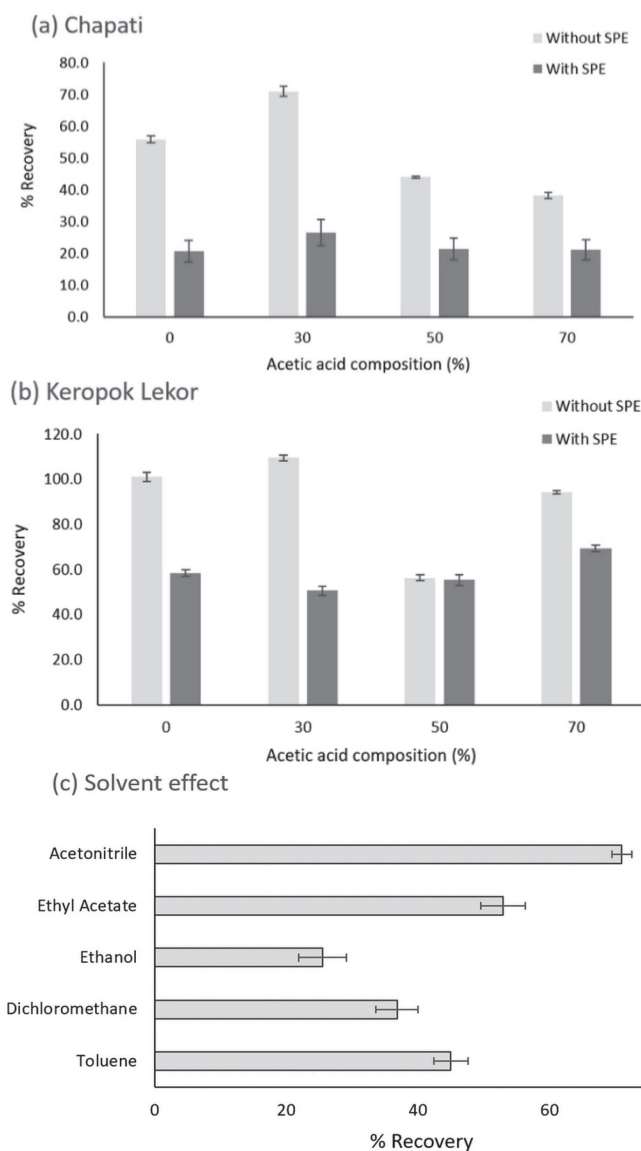


Fig. 1. Effects of different acetic acid compositions on the percentage recoveries of eleven PAHs with and without SPE for Chapati (a) and Keropok Lekor (b); c) the effects of different organic solvents on the percentage recoveries of eleven PAHs after spiking to a selected food sample (acetic acid composition is 30 %).

Acetonitrile's high extraction power can be explained by the presence of the relatively less polar cyano group ($-\text{CN}$) compared to ethanol's hydroxyl group ($-\text{OH}$). The smaller difference in electronegativity between carbon and nitrogen (2.5 and 3.0) in $-\text{CN}$, compared to oxygen and hydrogen (3.5 and 2.1) in $-\text{OH}$,

combined with acetonitrile's higher polarity index and shorter carbon chain, may account for its superior performance.¹⁶ Thus, acetonitrile with 30 % acetic acid provided the highest extraction power for PAHs.

HPLC method development and optimization

Before analyzing PAHs using a gradient reversed-phase HPLC-DAD method, an isocratic elution with the following solvent compositions was tested: solvent A (40–80 % acetonitrile, ACN) and solvent B (60–20 % H₂O) on an Ascentic ODS column (250 mm×4.6 mm, 5 µm, USA). However, this method was unsatisfactory due to the co-elution of CHR and BaA, as well as BbF and BkF. To address this issue, gradient elution was employed, achieving complete separation with good resolution and sensitivity using the following solvent program: 0–5 min 40 % ACN, 5–10 min 30 % ACN, 10–15 min 20 % ACN, 15–16.5 min 30 % ACN, 16.5–18 min 20 % ACN, 18–25 min 30 % ACN, 25–30 min 0 % ACN, 30–35 min 40 % ACN. To our knowledge, the complete separation of CHR and BaA (both with four aromatic rings) and BbF and BkF (with similar 4.5-ring structures) on a C18 column with DAD detection has not been reported in the literature, except when using selective columns for PAHs or C18 columns with fluorescence detection.^{1,10} The optimized conditions for the best compromise between resolution and sensitivity were: a flow rate of 1.0 mL/min, a column temperature of 30 °C, and a detection wavelength of 259 nm. Fig. 2a shows the chromatogram for the eleven PAHs standards using the optimized gradient method, while representative chromatograms for food samples are shown in Fig. 2b and c.

Validation and comparison with previously reported method

The presence of PAHs in the food samples was identified by comparing the peak retention times with those of the corresponding standards. The chromatographic method's reliability was confirmed by validating parameters such as linear range, repeatability, reproducibility, limits of detection (*LOD*) and quantitation (*LOQ*) and recovery. These validation results are summarized in Tables S-IV and S-V of the Supplementary material. Excellent linearity was observed, with a range of 10–1000 µg L⁻¹ for all eleven PAHs, and correlation coefficients exceeding 0.99 (*R*²: 0.9984–0.9997), showing a strong relationship between peak area and analyte concentration.

The *LOD* and *LOQ* were calculated using the formulae $3.3 s/S$ and $10 s/S$, where *s* is the standard deviation of the *y*-intercept, and *S* is the slope of the regression analysis.⁸ The *LOD* values for the PAHs ranged from 0.0049 to 0.1232 µg L⁻¹ and the *LOQ* values ranged from 0.0149 to 0.373 µg L⁻¹, aligning with those reported by others, such as Mahmoudpour *et al.*,¹⁷ who analyzed smoked rice using HPLC-UV with C18 columns, and Hamidi *et al.*,¹⁰ who studied grilled meat using HPLC-FL with PAH-specific columns. Mahmoudpour *et al.*¹⁷ found *LOD*

and *LOQ* values of 0.05–0.12 $\mu\text{g L}^{-1}$ and 0.14–0.38 $\mu\text{g L}^{-1}$, respectively, and Hamidi *et al.*¹² reported values of 0.03–3.00 $\mu\text{g L}^{-1}$ and 0.08–9.00 $\mu\text{g L}^{-1}$, respectively.

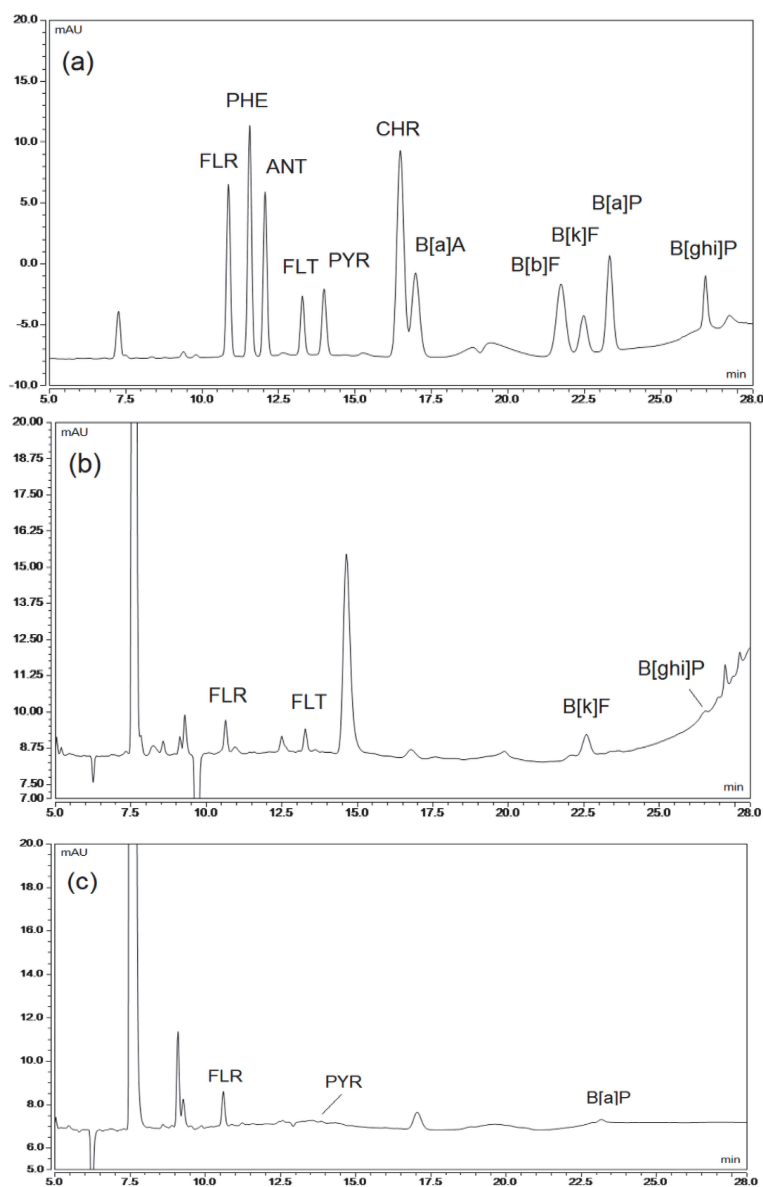


Fig. 2. Chromatogram of PAHs obtained using the HPLC-DAD method under the optimum condition: a) standard PAHs (5000 $\mu\text{g/L}$), b) Thosai, sample 3 and c) Youtiao, sample 3.

Repeatability and reproducibility were assessed using relative standard deviation (*RSD*) values from three consecutive injections of seven standard PAH solutions (10–1000 $\mu\text{g L}^{-1}$). For intraday repeatability (same-day analysis), *RSD* ranged from 0.03 to 0.76 % for retention time and 1.10 to 3.78 % for peak area. Interday repeatability (over five days) showed similarly satisfactory results. Recovery tests were done by spiking the food samples with PAH standards at three concentrations (50, 200 and 400 $\mu\text{g/L}$). Recovery values ranged from 47.3 to 119.7 % in Chapati and from 77.3 to 119.6 % in Keropok Lekor (Table S-V). Comparable results with low recoveries for some PAHs were also observed by Zachara *et al.*² in tea samples and by Hamidi *et al.*¹² in charcoal-grilled beef and chicken. These low recoveries may be attributed to differences in food type and composition, as suggested by Navarro *et al.*,¹⁸ who noted that whole-wheat flour may adsorb PAHs, leading to incomplete desorption in Chapati samples. All eleven PAHs were successfully resolved and eluted in under 28 min using a C18 column, a satisfactory result in line with other studies.^{1,10} The elution time was faster compared to those reported in similar studies.^{1,2}

In general, PAHs extraction from food samples involves liquid–liquid extraction (LLE) followed by solid-phase extraction (SPE).¹⁹ Commonly used solvents like *n*-hexane and toluene benefit from π – π interactions with PAHs, and SPE cartridges are often used to purify the extracted solvent, reducing solvent use and improving recoveries. However, SPE can be time-consuming and require multiple steps.⁶ Newer microextraction techniques like solid phase microextraction (SPME), stir bar sorptive extraction (SBSE) and liquid phase microextraction (LPME) address some limitations of LLE and SPE, such as high solvent consumption and extended extraction times. SPME, for instance, uses minimal solvent but has issues with limited fiber life and sample carryover. SBSE is selective toward non-polar solutes and requires a long equilibrium time. LPME, particularly dispersive liquid–liquid microextraction (DLLME), is a simpler and more cost-effective approach but uses halogenated solvents that can pose environmental and health risks.⁶

The method developed in this study improves upon LLE without requiring SPE or SPME, achieving higher PAH recoveries (mean recoveries of 70.9 % for Chapati and 109.5 % for Keropok Lekor) using acetonitrile with 30 % acetic acid. This is notably better than when SPE was employed (recoveries of 26.4 and 50.5 %, respectively), likely due to the partial adsorption of PAHs onto the SPE adsorbent. This method is simpler and faster as it avoids the additional time and steps associated with SPE or SPME. Its analytical performance in terms of linearity, *LOD*, *LOQ*, repeatability, reproducibility and recovery are comparable to other reported methods.^{17,19} Moreover, the exclusion of macromolecules such as proteins and fats were made easier by their unfavorable interaction with the protic acidic solvent.²⁰

Matrix effect

The matrix effect (*ME*) was evaluated by comparing the slopes of the linear equations for the eleven PAHs in both the solvent and the matrix.⁸ This effect, calculated using the following formula:

$$ME = 100 \frac{\text{Slope with matrix} - \text{Slope without matrix}}{\text{Slope without matrix}} \quad (1)$$

was investigated for two food categories: Chapati and Keropok Lekor. The results, provided in Table S-VI of the Supplementary material, indicated a more significant matrix effect in Chapati than in Keropok Lekor. For Chapati, several PAHs, including FLR (−58.39 %), PHE (−54.92 %), ANT (−49.85 %), CHR (42.62 %), B[b]F (29.58 %) and B[a]P (29.93 %), showed substantial *ME*, exceeding the cut-off value of ±20 %.⁸ In contrast, only PHE (33.40 %) and CHR (24.70 %) exhibited significant *ME* in Keropok Lekor.

Statistical analysis using the *t*-test revealed significant differences ($p < 0.05$) in the slopes of PAHs spiked to Chapati but not to Keropok Lekor (Table S-VI), likely due to flour adsorption in Chapati.¹⁸ Food products like Roti Canai, Thosai and Youtiao, which contain high amounts of dehydrated flour, are expected to experience similar flour adsorption.²¹ Conversely, food items such as Kuih Tayap, Apam Balik and Pisang Goreng, which have lower flour content and higher levels of other ingredients (*e.g.*, coconut, banana), are less prone to this effect.²² Based on these findings, food samples suspected of flour adsorption were quantitated using the linear plot for Chapati, while samples with less flour adsorption were quantitated using the Keropok Lekor model. The *LOD* and *LOQ* values obtained in the food matrices (Chapati: mean *LOD* 0.3544 mg L^{−1}, mean *LOQ* 1.0742 mg L^{−1}; Keropok Lekor: mean *LOD* 0.3447 mg L^{−1}, mean *LOQ* 1.0445 mg L^{−1}) were higher than those in the solvent (mean *LOD* 0.2847 mg L^{−1}, mean *LOQ* 0.8630 mg L^{−1}). Thus, the matrix-derived *LOD* and *LOQ* values were used for all analyses.

Analysis of food samples

The developed method was applied to analyze PAHs in 10 food samples, categorized as toasted (Roti Canai, Chapati, Thosai, Kuih Tayap, Apam Balik) and fried (Youtiao, Keropok Lekor, Pisang Goreng, Cekodok, fried chicken). Each sample was collected from different food stalls and analyzed in triplicate. Quantification was performed using the external standard method, with calibration plots fitted by least squares linear regression. The analysis results are summarized in Table S-VII of the Supplementary material.

As shown in Table S-VII, light PAHs (compounds with two to four fused benzene rings, including FLR, PHE, ANT, FLT, PYR, CHR and BaA) were detected in both toasted and fried foods. Heavy PAHs (compounds with four or more aromatic rings, such as BbF, BkF, BaP and Ghi) were found in fried foods.

Light PAHs accounted for 82.3 % of the total PAHs (mean total: 3525.2 $\mu\text{g kg}^{-1}$), while heavy PAHs contributed 17.7 % (mean total: 757.5 $\mu\text{g kg}^{-1}$) to the total PAHs (mean total: 4282.7 $\mu\text{g kg}^{-1}$).

Fried foods generally contained more PAHs than toasted foods. About 64.4 % of light PAHs were detected in fried foods, compared to 35.6 % in toasted foods. Similarly, 86.5 % of heavy PAHs were found in fried foods, whereas only 13.5 % were detected in toasted foods. This suggests that fried foods contain higher amounts of both light and heavy PAHs.

Light PAHs are generally less toxic, as the International Agency for Research on Cancer (IARC) classifies them as group 3 (not carcinogenic).³ These include FLR, PHE, ANT, FLT, PYR and B[ghi]P. In contrast, B[a]P, a group 1 carcinogen, and other heavy PAHs like BaA, BbF, BkF and CHR, classified as group 2B (possible carcinogens), pose higher toxicity concerns. Fried foods were found to have higher levels of Group 2B PAHs (mean total: 487.7 $\mu\text{g kg}^{-1}$) compared to toasted foods (mean total: 193.2 $\mu\text{g kg}^{-1}$). Additionally, the total PAH content in fried foods (mean total: 2925.3 $\mu\text{g kg}^{-1}$) was significantly higher than in toasted foods (mean total: 1357.4 $\mu\text{g kg}^{-1}$). This could be attributed to the higher temperatures involved in frying, which promotes PAH formation.

CONCLUSION

This study introduced a novel PAH extraction method using acetic acid coupled with organic solvents for analyzing toasted and fried foods. The method optimized acid compositions and solvent types, with 30 % acetic acid coupled with acetonitrile showing improved extraction efficiency and eliminating the need for SPE. The analysis of 10 food samples revealed that light PAHs are commonly found in both toasted and fried foods, while heavy PAHs are more prevalent in fried foods. Fried foods were found to contain higher levels of both light and heavy PAHs, with some fried items posing higher carcinogenic risks due to the presence of BaP and group 2B PAHs. Overall, this extraction method provides a simple and effective alternative for PAH analysis in food samples.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13125>, or from the corresponding author on request.

ИЗВОД

ТЕЧНО–ТЕЧНА ЕКСТРАКЦИЈА СИРЋЕТНОМ КИСЕЛИНОМ И UHPLC-DAD
ОДРЕЂИВАЊЕ ПОЛИЦИКЛИЧНИХ АРОМАТИЧНИХ УГЉОВОДОНИКА У
ТОСТИРАНОЈ И ПРЖЕНОЈ ХРАНИCHEN SON YUE¹, CHUI FUNG LOKE¹, SIEW HONG TEO¹, HAN HONG TEO¹, SARAH HANGOVAN¹
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Полициклические ароматические углеводороды (ПАУ) относятся к классу органических соединений, которые содержат два или более соединенных ароматических прстенов. Они имеют очень низкую растворимость в воде и являются липофильными. Сирчатая кислота является полярным протонным растворителем, который часто используется в реакциях, включающих карбокатионные интермедиаты. В этой работе смесь сирчатой кислоты и других органических растворителей была оптимизирована и использовалась для экстракции ПАУ из полициклических ароматических углеводородов в тостированной и жареной пище. Метод UHPLC-DAD с колонкой C18, является валидированным и применяется для анализа ПАУ в образцах пищи. Полученные значения LOD и LOQ составили от 0,0049 до 0,373 мкг Л⁻¹, а recovery от 47,3 до 119,7 %. Результаты анализа показывают, что легкие полициклические ароматические углеводороды обычно встречаются в обеих видах пищи. Нежареная пища является очень канцерогенной из-за присутствия ВПР и полициклических ароматических углеводородов группы 2Б. Тостированная пища является безопасной для употребления.

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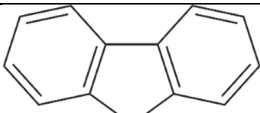
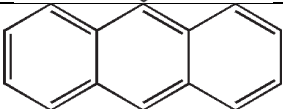
SUPPLEMENTARY MATERIAL TO
**Acetic acid liquid–liquid extraction and UHPLC-DAD detection
of polycyclic aromatic hydrocarbons in toasted and fried foods**

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Table S-I: List of selected PAHs with structures, molecular weights and IARC group classification.

Molecular arrangement	Type of PAH	Structure	MW	IARC Group
Linear	Flourene (FLR)		166	3
	Anthracene (ANT)		178	3
	Phenanthrene (PHE)		178	3

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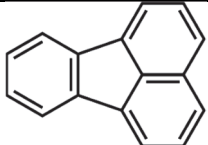
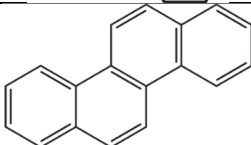
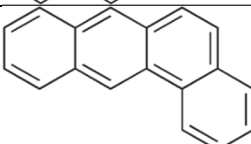
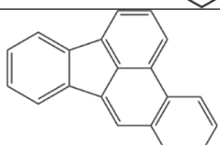
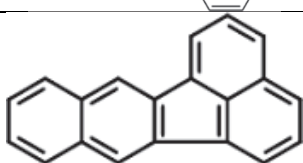
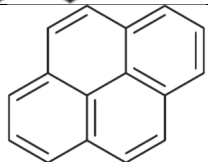
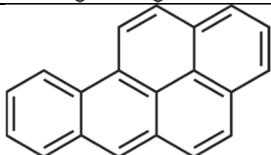
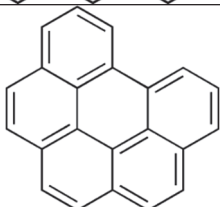
Molecular arrangement	Type of PAH	Structure	MW	IARC Group
Angular	Fluoranthene (FLT)		202	3
	Chrysene (CHR)		228	2B
	Benzo[a]anthracene (BaA)		228	2B
	Benzo[b]fluoranthene (BbF)		252	2B
	Benzo[k]fluoranthene (BkF)		252	2B
Cluster	Pyrene (PYR)		202	3
	Benzo[a]pyrene (BaP)		252	1
	Benzo[ghi]perylene (BghiP)		276	3

Table S-II: Acid composition effects on the percentage recoveries of Chapati and Keropok Lekor

E	F	G	H	I	J	Q	R	S	T	U	V
b4 spe 0% acid (Chapati)						b4 spe 30% acid					
Retention times	Types of PAH	Area	Area	Area	Mean	Retention times	Types of PAH	Area	Area	Area	Mean
11.082 - 11.085	FLR	0.5179	0.5267	0.5340	0.5262	10.697 - 10.696	FLR	0.5829	0.6003	0.5934	0.5922
11.735 - 11.754	PHE	0.5925	0.5708	0.5869	0.5834	11.384 - 11.383	PHE	0.6006	0.6155	0.6076	0.6079
12.208 - 12.212	ANT	0.4709	0.4703	0.4722	0.4711	11.877 - 11.883	ANT	0.5257	0.5355	0.5450	0.5354
13.375 - 13.376	FLT	0.4882	0.4878	0.4893	0.4884	13.077 - 13.076	FLT	0.8150	0.8930	0.8870	0.8650
14.068 - 14.069	PYR	0.4841	0.4829	0.4835	0.4835	13.744 - 13.749	PYR	0.9461	0.9091	0.9568	0.9373
16.448 - 16.450	CHR	0.7286	0.7115	0.7323	0.7241	16.031 - 16.036	CHR	0.7350	0.7458	0.7246	0.7351
16.915 - 16.917	B[a]A	0.4792	0.4874	0.4832	0.4833	16.471 - 16.476	B[a]A	0.6844	0.6941	0.6843	0.6876
18.802 - 18.805	B[b]F	0.6631	0.6936	0.6798	0.6788	20.958 - 20.963	B[b]F	1.0389	1.0632	1.0498	1.0506
21.462 - 21.467	B[k]F	0.9648	0.9798	0.9565	0.9670	22.838 - 22.843	B[k]F	0.8031	0.8058	0.8029	0.8039
23.128 - 23.129	B[a]P	0.6947	0.6980	0.6864	0.6930	26.171 - 26.176	B[a]P	0.7736	0.7771	0.7754	0.7754
26.288 - 26.290	B[ghi]P	0.6025	0.6098	0.6035	0.6053	27.118 - 27.123	B[ghi]P	0.6762	0.6718	0.6772	0.6751
b4 spe 50% acid						b4 spe 70% acid					
Retention times	Types of PAH	Area	Area	Area	Mean	Retention times	Types of PAH	Area	Area	Area	Mean
11.038 - 11.039	FLR	0.5691	0.5723	0.5685	0.5700	10.687 - 10.689	FLR	0.4461	0.4573	0.4501	0.4512
11.698 - 11.701	PHE	0.6116	0.6278	0.6220	0.6205	11.374 - 11.375	PHE	0.4821	0.4859	0.4903	0.4861
12.178 - 12.182	ANT	0.4341	0.4356	0.4358	0.4352	11.874 - 11.876	ANT	0.4155	0.4344	0.4256	0.4252
13.118 - 13.119	FLT	0.3679	0.3682	0.3675	0.3679	13.067 - 13.069	FLT	0.3553	0.3558	0.3565	0.3559
14.038 - 14.040	PYR	0.3648	0.3645	0.3653	0.3649	13.734 - 13.735	PYR	0.3562	0.3578	0.3567	0.3569
16.405 - 16.407	CHR	0.6202	0.6214	0.6223	0.6213	16.007 - 16.011	CHR	0.4992	0.4865	0.4799	0.4885
16.865 - 16.867	B[a]A	0.4547	0.4564	0.4553	0.4555	16.447 - 16.479	B[a]A	0.3516	0.3524	0.3535	0.3525
18.945 - 18.947	B[b]F	0.4706	0.4712	0.4709	0.4709	20.901 - 20.902	B[b]F	0.5964	0.5898	0.5877	0.5913
21.418 - 21.420	B[k]F	0.6728	0.6565	0.6674	0.6656	22.814 - 22.817	B[k]F	0.4730	0.4645	0.4685	0.4687
23.105 - 23.106	B[a]P	0.4519	0.4579	0.4543	0.4547	25.794 - 25.803	B[a]P	0.4741	0.4799	0.4695	0.4745
26.285 - 26.286	B[ghi]P	0.3977	0.3986	0.3965	0.3976	26.714 - 26.716	B[ghi]P	0.3584	0.3568	0.3575	0.3576
E	F	G	H	I	J	P	Q	R	S	T	U
after spe 0% acid (chapati)						30%					
Retention times	Types of PAH	Area	Area	Area	Mean	Retention times	Types of PAH	Area	Area	Area	Mean
11.082	FLR	1.0554	1.1069	1.0721	1.0781	10.697	FLR	1.0560	1.0965	1.1132	1.0881
11.735	PHE	1.5543	1.6754	1.5596	1.5631	11.384	PHE	1.5687	1.6589	1.7231	1.6501
12.208	ANT	1.0219	1.1254	1.0965	1.0811	11.877	ANT	1.0409	1.1004	1.2583	1.1331
13.375	FLT	0.6428	0.6457	0.7032	0.6639	13.077	FLT	0.6490	0.6543	0.6994	0.6671
14.068	PYR	0.6524	0.6555	0.6987	0.6689	13.744	PYR	1.0611	1.1665	1.2312	1.1521
16.448	CHR	3.0426	3.1067	3.0945	3.0811	16.031	CHR	3.0597	3.0954	3.1287	3.0941
16.915	B[a]A	1.0284	1.1002	1.0765	1.0684	16.471	B[a]A	1.0683	1.1798	1.0954	1.1141
18.802	B[b]F	5.0795	5.1324	5.1209	5.1111	20.958	B[b]F	5.3740	5.4744	5.7562	5.5341
21.462	B[k]F	2.5481	2.6745	2.7863	2.6611	22.838	B[k]F	3.0156	3.1743	3.2133	3.1331
23.128	B[a]P	1.0557	1.1243	1.1112	1.0971	23.541	B[a]P	5.0673	5.1639	5.2004	5.1431
26.288	B[ghi]P	0.3567	0.3499	0.3754	0.3605	26.171	B[ghi]P	0.6091	0.6111	0.6324	0.6171
50%						70%					
Retention times	Types of PAH	Area	Area	Area	Mean	Retention times	Types of PAH	Area	Area	Area	Mean
11.038	FLR	1.1293	1.2435	1.1897	1.1875	10.966	FLR	1.2609	1.3002	1.2856	1.2821
11.698	PHE	1.0747	1.1360	1.1422	1.1175	11.619	PHE	1.0992	1.1003	1.1760	1.1251
12.178	ANT	1.0147	1.0648	1.0993	1.0596	12.099	ANT	1.0189	1.0675	1.0483	1.0441
13.338	FLT	0.5941	0.5842	0.5681	0.5808	13.252	FLT	0.5942	0.6012	0.5895	0.5951
14.038	PYR	1.0059	1.0102	1.0566	1.0239	13.932	PYR	1.0057	1.0105	1.0099	1.0081
16.405	CHR	2.9540	2.7932	3.0020	2.9161	16.219	CHR	2.2442	2.3222	2.3451	2.3031
16.865	B[a]A	0.4644	0.4986	0.4761	0.4777	16.659	B[a]A	0.6945	0.6351	0.6744	0.6681
21.418	B[b]F	4.6202	4.7843	4.9037	4.7694	21.092	B[b]F	4.6834	4.6633	5.0734	4.8061
22.996	B[k]F	2.4443	2.5637	2.6424	2.5501	22.906	B[k]F	2.3394	2.4531	2.5211	2.4371
23.105	B[a]P	2.4402	2.6597	2.5553	2.5518	24.013	B[a]P	2.3453	2.3456	2.6759	2.4551
26.285	B[ghi]P	1.0626	1.1165	1.0954	1.0915	26.166	B[ghi]P	1.0542	1.1029	1.0931	1.0831

E	F	G	H	I	J
b4 spe 0% acid (keropok)					
Retention times	Types of PAH	Area	Area	Area	Mean
10.787	FLR	2.0210	2.1431	2.0653	2.07
11.480	PHE	2.6664	2.6576	2.6897	2.67
11.987	ANT	1.7003	1.7898	1.7789	1.75
13.187	FLT	0.6936	0.6795	0.6884	0.68
13.914	PYR	0.8855	0.8663	0.8923	0.88
16.267	CHR	2.4523	2.4442	2.4002	2.43
16.727	B[a]A	0.2017	0.2135	0.2069	0.20
21.374	B[b]F	1.8814	1.9856	1.8790	1.91
22.180	B[k]F	0.7925	0.7734	0.7888	0.78
23.140	B[a]P	1.8112	1.8537	1.8945	1.85
26.360	B[ghi]P	0.7488	0.7675	0.7590	0.75
b4 spe 50%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.852	FLR	0.8336	0.8365	0.8456	0.83
11.546	PHE	1.0769	1.0993	1.0841	1.08
12.052	ANT	0.7972	0.7870	0.7534	0.77
13.259	FLT	0.3491	0.3297	0.3354	0.33
13.986	PYR	0.4174	0.4099	0.4200	0.41
16.439	CHR	1.1752	1.1657	1.1865	1.17
16.926	B[a]A	0.3683	0.3798	0.3743	0.37
21.639	B[b]F	0.7790	0.7854	0.7812	0.78
22.399	B[k]F	0.3893	0.3907	0.3976	0.39
23.286	B[a]P	0.8466	0.8543	0.8632	0.85
26.446	B[ghi]P	0.8044	0.8102	0.8089	0.80
P	Q	R	S	T	U
b4 spe 30%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.856	FLR	2.1226	2.1354	2.1456	2.1345
11.549	PHE	2.7972	2.7786	2.7893	2.7884
12.056	ANT	1.7751	1.7884	1.7902	1.7846
13.276	FLT	0.7238	0.7343	0.7297	0.7293
13.982	PYR	0.9254	0.9854	0.9533	0.9547
16.476	CHR	2.6558	2.6699	2.6705	2.6654
16.976	B[a]A	0.2659	0.2765	0.2831	0.2752
21.729	B[b]F	2.0089	2.1001	2.0065	2.0385
22.469	B[k]F	0.8337	0.8376	0.8431	0.8381
23.322	B[a]P	1.8609	1.8953	1.8532	1.8698
26.456	B[ghi]P	0.9653	0.9703	0.9876	0.9744
b4 spe 70%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.874	FLR	1.7364	1.7675	1.7743	1.7594
11.568	PHE	2.2987	2.2876	2.2697	2.2853
12.074	ANT	1.6265	1.6345	1.6299	1.6303
13.288	FLT	0.5847	0.5987	0.5905	0.5913
14.014	PYR	0.7454	0.7555	0.7398	0.7469
16.501	CHR	2.5537	2.5435	2.5421	2.5464
16.994	B[a]A	0.3143	0.3131	0.3203	0.3159
21.714	B[b]F	1.5903	1.5876	1.5799	1.5859
22.481	B[k]F	0.6642	0.6543	0.6621	0.6602
23.348	B[a]P	1.4723	1.4689	1.4645	1.4686
26.474	B[ghi]P	0.9172	0.9108	0.9143	0.9141
E	F	G	H	I	J
after spe 0% acid (keropok)					
Retention times	Types of PAH	Area	Area	Area	Mean
10.901	FLR	4.1033	4.2121	4.2198	4.1784
11.594	PHE	4.6047	4.6298	4.6423	4.6256
12.101	ANT	4.2324	4.3354	4.3043	4.2907
13.321	FLT	3.5295	3.5505	3.6465	3.5755
14.047	PYR	3.7780	3.8912	3.7856	3.8183
16.581	CHR	6.2677	6.3232	6.3797	6.3235
17.087	B[a]A	3.4819	3.5921	3.4869	3.5203
21.854	B[b]F	2.7390	2.7456	2.8632	2.7826
22.574	B[k]F	4.1808	4.2997	4.2044	4.2283
23.414	B[a]P	5.9994	5.9812	6.0736	6.0181
26.514	B[ghi]P	2.7229	2.7354	2.8429	2.7671
after spe 50%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.941	FLR	3.7355	3.8876	3.9821	3.8684
11.635	PHE	4.1588	4.1698	4.3001	4.2096
12.141	ANT	3.8868	3.9945	3.7612	3.8808
13.368	FLT	3.3927	3.3021	3.2980	3.3309
14.095	PYR	3.5552	3.4673	3.5025	3.5083
16.668	CHR	5.5764	5.5551	5.7322	5.6212
17.195	B[a]A	2.5830	2.8431	2.6983	2.7081
21.968	B[b]F	4.9925	4.9824	4.7527	4.9092
22.661	B[k]F	3.8552	3.7982	3.6548	3.7694
23.475	B[a]P	5.1392	5.0012	5.2234	5.1213
26.541	B[ghi]P	3.4720	3.4985	3.5109	3.4938
P	Q	R	S	T	U
after spe 30%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.937	FLR	3.1960	3.2823	3.1905	3.2229
11.630	PHE	3.3080	3.3156	3.4249	3.3495
12.137	ANT	3.2385	3.3467	3.4378	3.3410
13.364	FLT	3.1018	3.2099	3.2156	3.1758
14.090	PYR	3.1486	3.1973	3.1739	3.1733
16.664	CHR	3.8360	3.5765	3.6522	3.6882
17.190	B[a]A	3.3819	3.2991	3.3795	3.3535
21.964	B[b]F	3.4950	3.5864	3.5790	3.5535
22.664	B[k]F	3.2179	3.3223	3.2014	3.2472
23.477	B[a]P	3.5340	3.6542	3.5829	3.5904
26.544	B[ghi]P	3.7683	3.6954	3.7721	3.7453
after spe 70%					
Retention times	Types of PAH	Area	Area	Area	Mean
10.922	FLR	5.3326	5.3698	5.4002	5.3675
11.651	PHE	6.6239	6.7453	6.6984	6.6892
12.122	ANT	5.7056	5.7239	5.8015	5.7437
13.348	FLT	4.2066	4.2951	4.3011	4.2676
14.075	PYR	4.7304	4.9346	4.8762	4.8471
16.635	CHR	7.8997	7.6497	7.7767	7.7754
17.155	B[a]A	3.6984	3.7003	3.8654	3.7547
21.942	B[b]F	8.1168	8.1254	8.2288	8.1570
22.642	B[k]F	2.5707	2.6503	2.7050	2.6420
23.462	B[a]P	6.5675	6.6732	6.7899	6.6769
26.542	B[ghi]P	3.0936	3.1231	3.1457	3.1208

Table S-III: The extraction percentage recovery of different solvents

(c) Solvent effect

Solvent	% Rec
Acetonitrile	100
Ethyl Acetate	100
Ethanol	25
Bromomethane	35
Toluene	100

Table S-IV: Analytical characteristics of the HPLC method

PAH	Linear range (µg/L)	Regression equation	R ²	Intraday (%RSD) (n=3)		Interday (%RSD) (n=3)		LOD (µg/L)	LOQ (µg/L)
				t _R	Area	t _R	Area		
FLR	10 - 1000	y = 0.0011x + 0.0018	0.99	0.75	2.64	0.84	1.70	0.045	0.138
			97	60	99	00	71	7	4
PHE	10 - 1000	y = 0.0011x + 0.0131	0.99	0.10	1.55	0.12	1.02	0.047	0.143
			94	98	23	20	80	4	8

ANT	10 -	$y = 0.0010x +$	0.99	0.07	1.81	0.10	1.82	0.033	0.101
	1000	0.0039	97	06	86	85	17	5	4
FLT	10 -	$y = 0.0009x +$	0.99	0.08	2.44	0.20	2.41	0.004	0.014
	1000	0.0058	87	60	71	96	80	9	9
PYR	10 -	$y = 0.0017x +$	0.99	0.07	1.98	0.17	3.27	0.083	0.252
	1000	0.0415	90	16	28	96	23	5	9
CHR	10 -	$y = 0.0010x +$	0.99	0.09	3.78	0.10	1.13	0.104	0.429
	1000	0.4640	85	27	24	30	36	3	9
BaA	10 -	$y = 0.0014x +$	0.99	0.03	1.26	0.09	1.31	0.058	0.176
	1000	0.0581	91	01	31	35	22	4	9
BbF	10 -	$y = 0.0016x +$	0.99	0.03	1.98	0.05	3.48	0.105	0.319
	1000	0.0533	97	41	95	79	07	6	9
BkF	10 -	$y = 0.0011x +$	0.99	0.10	3.03	0.11	1.34	0.111	0.339
	1000	0.0331	91	79	79	99	49	9	1
BaP	10 -	$y = 0.0011x +$	0.99	0.06	1.10	0.07	3.11	0.097	0.296
	1000	0.0515	84	86	13	62	74	7	0
Ghi	10 -	$y = 0.0012x +$	0.99	0.35	1.76	0.59	2.54	0.123	0.373
	1000	0.0052	85	48	88	43	33	2	2

x, concentration of amine (mg/L); y, peak area of SPAs; R^2 , square of regression coefficient; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

Table S-V: Recoveries and RSD of PAHs (5000 $\mu\text{g/L}$) when spiked to selected food samples using 30:70 acetic acid and acetonitrile extraction solvent

PAHs	% Recovery					
	50 $\mu\text{g L}^{-1}$		200 $\mu\text{g L}^{-1}$		400 $\mu\text{g L}^{-1}$	
	Chapati	Keropok Lekor	Chapati	Keropok Lekor	Chapati	Keropok Lekor
FLU	49.5 $\pm$ 0.5	80.9 $\pm$ 1.2	57.9 $\pm$ 0.4	109.3 $\pm$ 0.8	65.7 $\pm$ 0.8	90.4 $\pm$ 0.9
PHE	47.3 $\pm$ 0.4	84.6 $\pm$ 1.0	56.9 $\pm$ 0.6	95.7 $\pm$ 1.1	70.7 $\pm$ 0.7	110.5 $\pm$ 0.6
ANT	59.2 $\pm$ 0.25	100.2 $\pm$ 1.3	66.3 $\pm$ 0.3	119.6 $\pm$ 1.0	87.5 $\pm$ 0.3	111.8 $\pm$ 0.7
FLT	93.4 $\pm$ 0.58	89.3 $\pm$ 0.7	83.8 $\pm$ 0.5	113.3 $\pm$ 1.0	103.0 $\pm$ 0.6	102.9 $\pm$ 0.4
PYR	87.0 $\pm$ 0.96	99.3 $\pm$ 0.9	95.3 $\pm$ 0.5	116.1 $\pm$ 0.5	111.6 $\pm$ 0.9	85.8 $\pm$ 0.7
CHR	61.9 $\pm$ 0.7	87.2 $\pm$ 0.3	79.2 $\pm$ 0.1	119.4 $\pm$ 0.7	89.7 $\pm$ 0.7	107.9 $\pm$ 0.2
BaA	104.7 $\pm$ 0.6	115.9 $\pm$ 0.7	114.2 $\pm$ 0.3	116.0 $\pm$ 0.3	119.7 $\pm$ 0.8	104.8 $\pm$ 1.0
BbF	73.3 $\pm$ 0.5	101.3 $\pm$ 1.2	114.2 $\pm$ 0.8	101.6 $\pm$ 0.2	116.2 $\pm$ 0.5	96.7 $\pm$ 0.4
BkF	110.5 $\pm$ 0.8	99.6 $\pm$ 0.8	114.9 $\pm$ 0.4	106.2 $\pm$ 0.3	115.3 $\pm$ 0.6	86.7 $\pm$ 0.5
BaP	69.6 $\pm$ 1.0	116.6 $\pm$ 0.3	84.6 $\pm$ 0.1	98.7 $\pm$ 0.5	86.0 $\pm$ 0.9	78.3 $\pm$ 0.7
Ghi	114.8 $\pm$ 0.4	108.5 $\pm$ 0.7	119.7 $\pm$ 0.5	77.3 $\pm$ 0.8	107.7 $\pm$ 1.0	85.2 $\pm$ 0.8

All results reported in $\bar{x} \pm \text{SD}$

Table S-VI. Comparison between slopes of the developed microwave HPLC method with and without matrix effects by linear regression lines

Method	Sample	Concentration range (mg L ⁻¹)	Regression equation of each PAH	R ²	LOD (mg L ⁻¹)	LOQ (mg L ⁻¹)	Matrix effect (%)
Without matrix	-	5 – 80	y = 86478x + 186310 (FLR)	0.9764	0.5126	1.5533	-
			y = 48935x + 61678 (PHE)	0.9786	0.4880	1.4790	-
			y = 0.20 y = 362022x - 132927 (ANT)	0.9924	0.2893	0.8768	-
			y = 8512.2x - 7013.1 (FLT)	0.9963	0.2833	0.8585	-
			y = 57761x + 45872 (PYR)	0.9958	0.3640	1.1030	-
			y = 27152x - 64306 (CHR)	0.9980	0.1493	0.4527	-
			y = 374719x + 415886 (B[a]A)	0.9925	0.2859	0.8665	-
			y = 13775x - 16546 (B[b]F)	0.9961	0.2064	0.6257	-
			y = 130905x - 91267 (B[k]F)	0.9961	0.2067	0.6264	-
			y = 106025x - 28987 (B[a]P)	0.9975	0.1662	0.5038	-
			y = 32510x - 12084 (B[ghi]P)	0.9970	0.1808	0.5480	-
With matrix	Chapati	5 – 80	y = 35985x + 45014 (FLR)	0.9841	0.4191	1.2700	-58.39
			y = 22062x + 4409.4 (PHE)	0.9761	0.5164	1.5649	-54.92

			$y = 0.20 y$ $= 181558x$ $+ 162794$ (ANT)	0.9679	0.6010	1.8212	-49.85
			$y = 9838.7x$ $- 14320$ (FLT)	0.9975	0.1658	0.5024	15.58
			$y = 56788x$ $+ 103031$ (PYR)	0.9943	0.2500	0.7577	1.68
			$y = 15581x$ $- 57197$ (CHR)	0.9987	0.1202	0.3643	-42.62
			$y = 424549x$ $- 768308$ (B[a]A)	0.9911	0.3119	0.9452	13.29
			$y = 9699.7x$ $- 6290.3$ (B[b]F)	0.9856	0.3987	1.2082	-29.58
			$y = 133466x$ $+ 138210$ (B[k]F)	0.9677	0.6033	1.8283	1.95
			$y = 74297x$ $- 164049$ (B[a]P)	0.9980	0.1465	0.4440	-29.93
			$y = 35606x$ $- 70824$ (B[ghi]P)	0.9986	0.3664	1.1104	9.52
With matrix	Keropok lekor	5 – 80	$y = 89997x$ $+ 216395$ (FLR)	0.9975	0.5010	1.5181	4.06
			$y = 65280x$ $- 31335$ (PHE)	0.9823	0.4434	1.3438	33.40
			$y = 0.20 y$ $= 431559x -$ 365265 (ANT)	0.9996	0.0676	0.2049	19.20
			$y = 9299x -$ 10469 (FLT)	0.9944	0.2475	0.7501	9.24
			$y = 58663x$ $+ 96555$ (PYR)	0.9887	0.3527	1.0688	1.56

$y = 33858x$ - 71182 (CHR)	0.9914	0.3067	0.9295	24.70
$y = 330608x$ - 634396 (B[a]A)	0.9957	0.2164	0.6558	11.77
$y = 14375x$ - 21463 (B[b]F)	0.9972	0.1735	0.5257	4.35
$y = 132511x$ + 201298 (B[k]F)	0.9754	0.5235	1.5865	1.22
$y = 113716x$ - 107985 (B[a]P)	0.9849	0.4086	1.2381	7.25
$y = 34440x$ + 51027 (B[ghi]P)	0.9729	0.5508	1.6692	5.93

TRP, tryptamine; PEA, phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine.

Table S-VII: Concentrations of PAHs found in toasted and fried food samples (n=3)

Concentration, $\mu\text{g kg}^{-1}$														
Toasted Food	Light PAHs							Heavy PAHs						
<i>Roti Canai</i>	FLR	PHE	ANT	FLT	PYR	CHR	BaA	Sub-total	BbF	BkF	BaP	Ghi	Sub-total	Total
1	N.D.	N.D.	2.62 ± 0.8	5.9 ± 0.2	N.D.	N.D.	N.D.	38.5 ± 0.3	N.D.	N.D.	N.D.	N.D.	0	38.5 ± 0.3
2	N.D.	N.D.	10.2 ± 0.3	8.1 ± 0.4	N.D.	N.D.	N.D.	68.3 ± 0.6	N.D.	N.D.	N.D.	N.D.	0	68.3 ± 0.6
3	N.D.	N.D.	N.D.	34.5 ± 0.1	N.D.	N.D.	N.D.	34.5 ± 0.1	N.D.	156.8 ± 2.4	N.D.	N.D.	156.8 ± 2.4	191.3 ± 2.4
$\bar{x}$	0	0	4.3 ± 0.1	76.2 ± 0.1	0	0	0	180.5 ± 0.1	0	52.3 ± 0.8	0	0	12.3 ± 0.1	32.7 ± 1.1
<i>Chapati</i>														
1	143.3 ± 0.9	N.D.	250.0 ± 0.6	N.D.	N.D.	N.D.	35.5 ± 0.4	128.8 ± 1.5	N.D.	N.D.	N.D.	N.D.	0	128.8 ± 1.5
2	N.D.	N.D.	5.1 ± 0.7	6.2 ± 0.4	N.D.	N.D.	N.D.	101.3 ± 1.1	N.D.	N.D.	N.D.	N.D.	0	101.3 ± 1.1
3	48.4 ± 0.3	89.3 ± 0.8	N.D.	41.3 ± 1.1	N.D.	N.D.	N.D.	179.0 ± 2.1	N.D.	N.D.	N.D.	N.D.	0	179.1 ± 2.1
$\bar{x}$	159.4 ± 0.4	29.8 ± 0.3	35.0 ± 0.4	9.2 ± 0.5	0	0	11.8 ± 0.1	165.2 ± 1.1	0	0	0	0	0	165.2 ± 1.1
<i>Thosai</i>														
1	1.4 ± 0.6	6.1 ± 0.8	N.D.	N.D.	N.D.	N.D.	N.D.	7.5 ± 1.4	N.D.	N.D.	N.D.	28.2 ± 0.4	8.2 ± 0.3	35.7 ± 1.8
2	N.D.	3.7 ± 1.9	N.D.	100.3 ± 1.1	N.D.	N.D.	367.2 ± 1.4	571.2 ± 4.6	N.D.	N.D.	N.D.	84.0 ± 2.4	4.0 ± 2.1	55.2 ± 7.6
3	94.1 ± 0.1	N.D.	N.D.	51.0 ± 0.2	N.D.	N.D.	N.D.	155.1 ± 0.1	N.D.	20.2 ± 0.1	N.D.	12.0 ± 2.2	0.1 ± 0.1	87.3 ± 0.1

	$\bar{x}$	0.1													0.4
		31.8 ± 0.2	3.3 ± 0.9	0	37.1 ± 0.5	0	0	122.4 ± 0.5	144.6 ± 2.1	0	6.7 ± 0.0	0	41.5 ± 1.1	8.2 ± 1.1	92.8 ± 3.1
Kuih Tayap															
1		13.4 ± 0.3	N.D.	N.D.	75.8 ± 3.3	N.D.	N.D.	N.D.	89.2 ± 3.6	N.D.	N.D.	5.8 ± 0.1	N.D.	5.8 ± 0.1	95.2 ± 3.6
2		106.1 ± 1.2	N.D.	308.3 ± 3.2	37.0 ± 0.6	N.D.	N.D.	N.D.	151.4 ± 5.0	N.D.	N.D.	N.D.	N.D.	0	151.4 ± 5.0
3		27.3 ± 0.4	N.D.	43.0 ± 1.3	44.4 ± 0.2	N.D.	N.D.	N.D.	114.7 ± 1.5	N.D.	N.D.	N.D.	N.D.	0	14.7 ± 1.5
	$\bar{x}$	48.9 ± 0.6	N.D.	117.1 ± 1.5	52.4 ± 1.4	0	0	0	118.4 ± 3.4	0	0	1.9 ± 0.0	0	1.9 ± 0.0	120.4 ± 3.4
Apam Balik															
1		27.1 ± 1.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	27.1 ± 1.5	N.D.	N.D.	N.D.	N.D.	0	27.1 ± 1.5
2		107.6 ± 2.1	N.D.	245.1 ± 0.4	N.D.	N.D.	N.D.	N.D.	152.7 ± 2.4	N.D.	N.D.	N.D.	N.D.	0	152.7 ± 2.4
3		143.9 ± 0.8	N.D.	215.0 ± 0.6	N.D.	N.D.	N.D.	N.D.	158.9 ± 1.4	N.D.	N.D.	N.D.	N.D.	0	158.9 ± 1.4
	$\bar{x}$	92.9 ± 1.5	N.D.	153.4 ± 0.3	N.D.	N.D.	N.D.	N.D.	146.3 ± 1.8	N.D.	N.D.	N.D.	N.D.	0	146.3 ± 1.8
Mean total		133 ± 2.1	33.1 ± 1.2	359.8 ± 2.3	94.9 ± 2.1	0	0	134.2 ± 0.6	1255.0 ± 9.1	0	59.0 ± 0.8	1.9 ± 0.0	41.5 ± 1.7	102.4 ± 2.5	1357.4 ± 11.6
Mean		66.6 ± 0.5	6.6 ± 0.2	71.9 ± 0.5	78.9 ± 0.5	0	0	26.8 ± 0.1	151.0 ± 1.8	0	11.8 ± 0.2	0.4 ± 0.0	1.3 ± 0.0	10.5 ± 0.4	142.9 ± 2.1
Fried food															
		Light PAHs										Heavy PAHs			
Youtiao															
1		N.D.	2.9 ± 0.1	N.D.	23.6 ± 0.1	N.D.	N.D.	N.D.	126.5 ± 0.6	N.D.	N.D.	N.D.	N.D.	0	26.5 ± 0.6
2		691.4 ± 2.2	1.6 ± 0.3	N.D.	111.6 ± 0.1	273.7 ± 0.2	N.D.	35.5 ± 0.2	1178.3 ± 3.2	N.D.	1305.1 ± 1.2	N.D.	506.1 ± 0.3	811.2 ± 1.5	2989.5 ± 4.7
3		245.6 ± 1.0	N.D.	N.D.	N.D.	8.4 ± 0.1	N.D.	N.D.	154.0 ± 1.1	N.D.	N.D.	5.8 ± 0.5	N.D.	N.D.	154.0 ± 1.1
	$\bar{x}$	315.2 ± 1.1	1.5 ± 0.1	0	11.7 ± 0.1	91.2 ± 0.1	0	11.8 ± 0.1	131.4 ± 1.1	0	435.0 ± 0.1	1.9 ± 0.2	168.7 ± 0.1	605.6 ± 0.4	1137.0 ± 2.1
Keropok															
Lekor															
1		404.8 ± 2.1	N.D.	N.D.	1032.2 ± 1.6	N.D.	N.D.	N.D.	1437.0 ± 3.7	N.D.	N.D.	25.8 ± 1.1	N.D.	15.8 ± 1.1	1462.9 ± 4.8
2		N.D.	N.D.	19.2 ± 1.1	198.1 ± 1.1	N.D.	N.D.	N.D.	117.3 ± 2.1	N.D.	N.D.	N.D.	N.D.	0	117.3 ± 2.1
3		N.D.	N.D.	213.3 ± 3.4	103.7 ± 0.1	N.D.	N.D.	N.D.	117.0 ± 4.1	N.D.	N.D.	N.D.	N.D.	0	117.0 ± 4.1
	$\bar{x}$	134.9 ± 0.7	0	77.5 ± 1.5	144.7 ± 1.1	0	0	0	157.1 ± 3.4	0	0	8.6 ± 0.4	0	8.6 ± 0.4	145.7 ± 3.1
Pisang Goreng															
1		N.D.	N.D.	N.D.	46.3 ± 3.1	681.5 ± 0.5	N.D.	N.D.	127.8 ± 3.1	N.D.	93.8 ± 0.5	N.D.	N.D.	13.8 ± 0.1	121.7 ± 4.1
2		N.D.	N.D.	116.7 ± 1.1	14.8 ± 1.1	N.D.	N.D.	N.D.	131.5 ± 1.8	N.D.	N.D.	N.D.	N.D.	0	131.5 ± 1.8

t-Test results (Chapati)

[illegible]

data	with Matrix	without M	calculation ANT1	ANT2
conc	ANT1	ANT2	slope =	181557.9964 362022.4960
5	705750.7	1485790.9	n =	5 5
10	1761697.8	3420772.4	SE(reg) =	1164362.2655 1117795.2605
20	3320245.4	6324276.9	SE(slope) =	19090.4714 18326.9753
40	9198921.8	15998124.8	difference =	180464.4996
80	13968843.3	28219885.5	SE(difference) =	26463.6377
			t-stat =	6.8193
			df =	6
			p =	0.0005



Figure S-1: The toasted and fried foods analyzed in this study.

Ruthenium(III)-monosubstituted Keggin-type polyoxotungstate: Synthesis, characterization and application in the catalytic methanation of carbon dioxide

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Abstract: Cs₅[α -SiW₁₁O₃₉Ru^{III}(H₂O)]·8H₂O heteropolysalt with a Keggin structure was successfully synthesized, and its physicochemical characteristics were determined *via* X-ray diffraction, UV–Vis spectroscopy, Fourier-transform infrared and Brunauer, Emmett and Teller surface area measurements. The acid–base properties were evaluated *via* isopropanol decomposition. The catalytic performance for the CO₂ methanation reaction was evaluated in a fixed-bed reactor at atmospheric pressure by varying the process parameters, which included the reaction temperature (200–450 °C), reactant mole ratio H₂/CO₂ (1, 2 and 4) and flow rate (1, 1.5 and 2 L/h). The experimental results showed that Cs₅[α -SiW₁₁O₃₉Ru^{III}(H₂O)]·8H₂O exhibited the best compromise between conversion and selectivity at 350 °C, with a H₂/CO₂ mole ratio of 4 and a flow rate of 1 L/h.

Keywords: Keggin-type polyoxometalate; hydrogenation; CO₂; methane.

INTRODUCTION

Polyoxometalates (POMs) are a large class of anionic metal oxygen clusters consisting of a transition metal called an “addenda atom” (*e.g.*, Mo, W and V in higher oxidation states), a heteroatom of a p-block element (usually Si or P) and a counteraction from group 1 (H⁺, Li⁺, Na⁺, K⁺ and Cs⁺). POMs are known for their wide range of physicochemical properties, including strong acidity, redox behaviour, thermal stability and unique catalytic properties. These properties can be fine-tuned by the choice of heteroatoms, incorporated transition metals and counteractions, allowing the synthesis of a wide variety of structures.^{1,2} These

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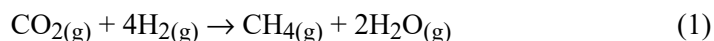
unique properties of POMs make them a class of materials with potential for catalytic applications.^{3,4}

Owing to its excellent performance, Keggin, a well-known structure of POMs, has been tested as a catalyst for various reactions, such as oxidative dihydrogenation (ODH) of alkanes to alkenes^{5,6} and arylalkanes to arylalkenes,⁷ partial oxidation of methane to methanol,⁸ oxidation of isobutane to methacrylic acid⁹ and partial oxidation of propane to acrylic acid.¹⁰

Owing to the unique redox and catalytic properties of the ruthenium (Ru) atom, monoruthenium (Ru)-substituted Keggin-type heteropolytungstates have recently received considerable attention,¹¹ and they have been evaluated as catalysts for oxidation^{12,13} and reduction reactions.¹⁴

Carbon dioxide is usually produced by combustion and is considered to be an important factor in the greenhouse effect and climate change. For this reason, carbon dioxide fixation has attracted much interest in recent years.^{15,16} Although CO₂ has high thermodynamic stability, making it a particularly inert molecule, the hydrogenation reactions currently produce a number of useful chemical products, such as formic acid,¹⁷ methanol¹⁸ and methane,¹⁹ depending on the different reaction conditions.

The Sabatier reaction (Eq. (1)), which converts carbon dioxide to methane, is the most thermodynamically favourable among carbon dioxide conversion reactions, with a negative change in Gibbs energy ($\Delta G_{298} = -130.8$ kJ/mol). However, owing to its high exothermicity ($\Delta H_{298} = -165$ kJ/mol), the complete reduction of carbon dioxide to methane needs low temperatures. Additionally, CO₂ reduction is an eight-electron process with notable kinetic constraints, requiring a suitable catalyst to achieve high reaction rates and methane selectivity:²⁰



Earlier studies have shown that transition metals such as Ru, Rh, Ni and Pd, particularly in groups VIII to X, are effective catalysts for producing methane through CO₂ hydrogenation, especially at lower temperatures.^{20–22} Ni-based catalysts are commonly used because of their high activity, methane selectivity and low cost.^{23,24} Ruthenium, while pricier, is more active than Ni and has high methane selectivity and low coke formation.²⁵

In the present study, a Ru-POM catalyst was synthesized, characterized by various physicochemical techniques and tested for the CO₂ methanation reaction. The optimum conditions for CO₂ methanation were obtained by studying the effects of the reaction temperature, H₂/CO₂ molar ratio and gas mixture flow rate on the prepared catalyst.

EXPERIMENTAL

Materials

All chemicals and reagents used were of analytical grade and were acquired from Sigma Aldrich.

Preparation of $Cs_5[\alpha-SiW_{11}O_{39}Ru^{III}(H_2O)] \cdot 8H_2O$

The title compound was prepared *via* the use of Keggin-type monolacunary silicotungstate $\alpha-K_8SiW_{11}O_{39} \cdot xH_2O$.

The monolacunary salt $\alpha-K_8SiW_{11}O_{39} \cdot xH_2O$ (abbreviated SiW_{11}) was prepared *via* the following method.²⁶

A 4 M solution of hydrochloric acid (165 mL) was added dropwise over 30 min, with vigorous stirring, to a solution of $Na_2WO_4 \cdot 2H_2O$ (182 g) in 300 mL of boiling distilled water. To the resulting mixture, $Na_2SiO_3 \cdot 5H_2O$ (11 g) in distilled water (100 mL) was added dropwise with stirring. The final solution was boiled for 1 h. After cooling to room temperature, KCl (150 g) was added to precipitate $K_8[SiW_{11}O_{39}]$. The white solid product was filtered, washed and finally dried in air. A polyhedral representation of a Keggin monolacunary anion, $\alpha-SiW_{11}O_{39}^{8-}$ is shown in Fig. 1.

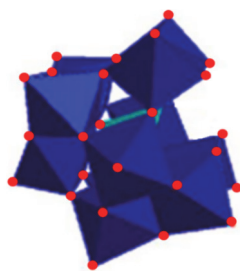


Fig. 1. Polyhedral representation of a Keggin monolacunary anion, $\alpha-SiW_{11}O_{39}^{8-}$. Color code: WO_6 = dark blue octahedra, SiO_4 = teal tetrahedra and O = red.

The Keggin-type cesium salt of mono-ruthenium (III)-substituted silicotungstate, $Cs_5[SiW_{11}O_{39}Ru^{III}(H_2O)] \cdot 8H_2O$ (abbreviated as $Cs-SiW_{11}Ru$), was prepared *via* the literature method²⁷ with slight modifications. $RuCl_3$ (0.12 g) was dissolved in a minimal amount of water and added dropwise, with vigorous stirring, to a solution of 1.5 g of $[SiW_{11}O_{39}]^{8-}$ in 30 mL of water heated at 70 °C. The mixture was stirred for 60 min and then cooled to room temperature. Then, solid caesium chloride was added while stirring, and the black–brown precipitate was isolated, washed with methanol and dried under vacuum. A polyhedral representation of a Keggin anion $[\alpha-SiW_{11}O_{39}Ru^{III}(H_2O)]^{5-}$ is shown in Fig. 2.

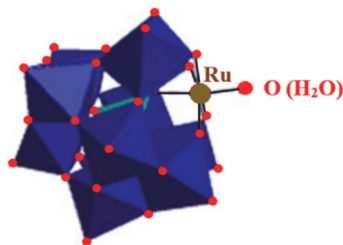


Fig. 2. Polyhedral representation of a Keggin anion $[SiW_{11}O_{39}Ru^{III}(H_2O)]^{5-}$; colour code: WO_6 = dark blue octahedra; SiO_4 = teal tetrahedron; Ru = brown and O = red.

Catalyst characterization techniques

The Brunauer, Emmett and Teller (BET) surface area (S_{BET}) was determined through N_2 adsorption–desorption analysis at a temperature of liquid N_2 (77 K) via a Quantachrome NOVA 4000e instrument. Approximately 10 mg of the catalyst sample was loaded into the U-shaped sample holder and degassed for 1 hour at 150 °C and 10^{-5} Pa in a N_2 gas flow to eliminate any adsorbed vapours. The surface area and pore size were determined by the BET theory and the Barrett–Joyner–Halenda (BJH) method, respectively.

The IR spectra of the samples were recorded on a Bio-Rad FTS 165 instrument via the KBr pellet technique in the region from 4000 to 400 cm^{-1} .

The fresh catalyst was analysed via X-ray diffraction (XRD) with a Siemens “D5000” diffractometer with a filtered $\text{CuK}\alpha$ X-ray source ($\lambda = 1.5406 \text{ \AA}$). The diffractogram was collected within a 2θ range of 0 to 60°.

The UV–Vis spectra of a 2×10^{-5} mol/L solution of the catalyst sample in neat acetonitrile were recorded with a Shimadzu UV-2100 PC spectrophotometer in the 190–400 nm range via quartz cuvettes with an optical path length of 10 mm.

The acid–base properties of the catalyst were determined through an indirect method, specifically the isopropanol (IPA) decomposition test. The test was conducted in a continuous-flow fixed-bed reactor at temperatures ranging from 100 to 200 °C under atmospheric pressure. Nitrogen (as the carrier gas) was passed through a saturator that contained IPA and maintained at 0 °C during the test. The catalyst weighed 200 mg, and the flow rate was 40 mL/min. The reaction products, which included propene, di-isopropylether and acetone, were analysed via a Shimadzu 14B gas chromatograph with a flame ionization detector (FID). The products were separated via 10 % Carbowax 20 M on Chromosorb W packed columns.

Catalytic tests

Catalytic experiments were conducted using 0.2 g of catalyst in a stainless-steel tubular fixed bed reactor measuring 6 mm in diameter and 400 mm in length for the gas-phase hydrogenation of carbon dioxide to methane. The reaction was carried out at atmospheric pressure by means of reaction mixtures of H_2/CO_2 with different molar ratios (1, 2 and 4) at temperatures ranging from 200 to 450 °C, and the flow rate of the reaction mixture ranged from 1 to 2 L/h. Prior to the activity tests, the catalyst was pre-treated in an oxygen stream at a flow rate of 2 L/h for 1 h at the reaction temperature. The reactants and products were analysed online by a Shimadzu GC-14 B gas chromatograph equipped with a TCD detector using a molecular sieve 5 Å packed column for the separation of H_2 , CO_2 , CO and CH_4 . Data were collected after an 8-h reaction period. The experimental setup for the methanation of CO_2 is illustrated in Fig. S-1 of the Supplementary material to this paper. The performance of the catalyst was evaluated in terms of CO_2 conversion (X_{CO_2}) and CH_4/CO selectivity ($S_{\text{CH}_4/\text{CO}}$), which were determined via Eqs. (2) and (3), respectively:

$$X_{\text{CO}_2} (\%) = 100(\text{moles of CO}_{2\text{in}} - \text{moles of CO}_{2\text{out}})/\text{moles of CO}_{2\text{in}} \quad (2)$$

$$S_{\text{CH}_4/\text{CO}} (\%) = 100(\text{moles of CH}_4/\text{CO}/\text{moles of all products}_{\text{out}}) \quad (3)$$

RESULTS AND DISCUSSION

Characterization of the catalyst

FT-IR Study. IR spectroscopy is a crucial and commonly utilized technique for characterizing POMs and confirming their structural integrity. Fig. 3 shows the

FT-IR spectra of SiW_{11} and $\text{Cs-SiW}_{11}\text{Ru}$. The IR spectrum of the mono-lacunary silicotungstate SiW_{11} exhibited five characteristic bands at 1000 cm^{-1} $\nu_{\text{as}}(\text{W}=\text{O}_{\text{t}})$, 954 cm^{-1} $\nu_{\text{as}}(\text{Si}-\text{O})$, 890 cm^{-1} $\nu_{\text{as}}(\text{W}-\text{O}_{\text{b}}-\text{W})$ and 796 and 742 cm^{-1} $\nu_{\text{as}}(\text{W}-\text{O}_{\text{c}}-\text{W})$, which agreed with those mentioned in the literature.²⁶

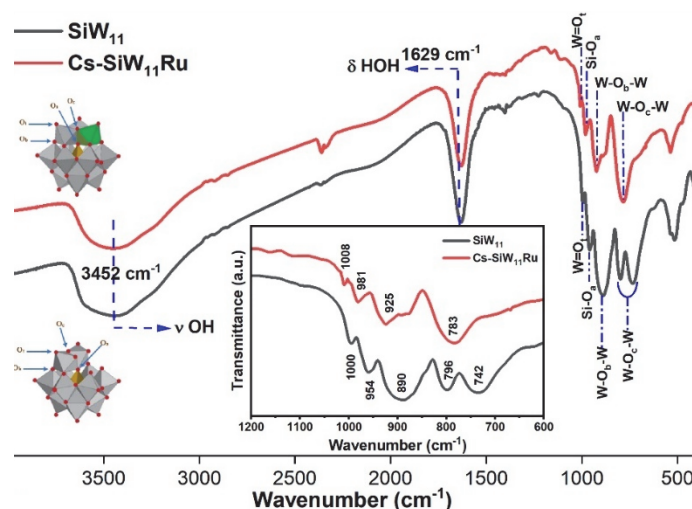


Fig. 3. FTIR spectra of $\text{K}_8(\alpha\text{-SiW}_{11}\text{O}_{39})$ (shown in black) and $\text{Cs}_5[\text{SiW}_{11}\text{O}_{39}\text{Ru}^{\text{III}}(\text{H}_2\text{O})]$ (shown in red); insets: polyhedral representations of SiW_{11} and $\text{Cs-SiW}_{11}\text{Ru}$ with the corresponding metal–oxygen bonds were O_{a} : oxygen atoms attached to the silicon atom, O_{t} : terminal oxygen atoms bonded to the tungsten atom, O_{b} : oxygen atom belonging to the WO_6 octahedral units sharing corners and O_{c} oxygen atoms at the edges.

The IR spectrum of the monoruthenium-substituted Keggin silicotungstate $\text{Cs-SiW}_{11}\text{Ru}$ reveals that the POM anions are in the basic Keggin structure.^{27,28} The four typical peaks at 1008 , 980 , 925 and 784 cm^{-1} are associated with $\nu_{\text{as}}(\text{W}=\text{O}_{\text{t}})$, $\nu_{\text{as}}(\text{Si}-\text{O}_{\text{a}})$, $\nu_{\text{as}}(\text{W}-\text{O}_{\text{b}}-\text{W})$ and $\nu_{\text{as}}(\text{W}-\text{O}_{\text{c}}-\text{W})$, respectively. The corresponding metal-to-oxygen bonding is displayed in the presented images. Furthermore, two prominent bands are observed at approximately 1630 and 3451 cm^{-1} , which can be attributed to the bending vibrations of water molecules ($\text{H}-\text{O}-\text{H}$) and the stretching vibrations of water hydroxyl groups (OH), respectively.

In the FT-IR spectrum of SiW_{11} , the stretching vibration of the $\text{W}-\text{O}_{\text{c}}-\text{W}$ band was showed two distinct bands at 796 and 742 cm^{-1} . This splitting was caused by the decrease in structure symmetry (Cs symmetry) after the loss of one $[\text{W}=\text{O}]^{4+}$ group from the parent Keggin ion ($\text{SiW}_{12}\text{O}_{40})^{4-}$, characterized by Td symmetry.²⁹ In the FT-IR spectrum of $\text{Cs-SiW}_{11}\text{Ru}$, the main absorption bands were shifted toward higher wavenumbers and a only single band was observed for

the stretching vibration of W–Oc–W (*ca.* 783 cm^{−1}), which was due to the restoration of the classical α -Keggin type structure with Td symmetry,³⁰ indicating that the vacancy of the SiW₁₁ was occupied by Ru(III).

UV–Vis spectra. Fig. 4 shows the UV spectra of SiW₁₁ and Cs-SiW₁₁Ru, which display two absorption bands between 200 and 400 nm. The absorption peak at 227 nm is linked to charge transfer from terminal oxygen to metal atoms (Ot → W), whereas the absorption peak at 255 nm is associated with charge transfer from bridging oxygen to metal atoms (Ob/Oc → W). These distinct bands are characteristic of Keggin-type heteropolytungstates.^{29,31} However, the absorption peak at 250 nm increased in intensity and became more distinct following the addition of the metal cation in the lacunary position, providing the evidence of the combination of α -SiW₁₁ and Ru^{III}.

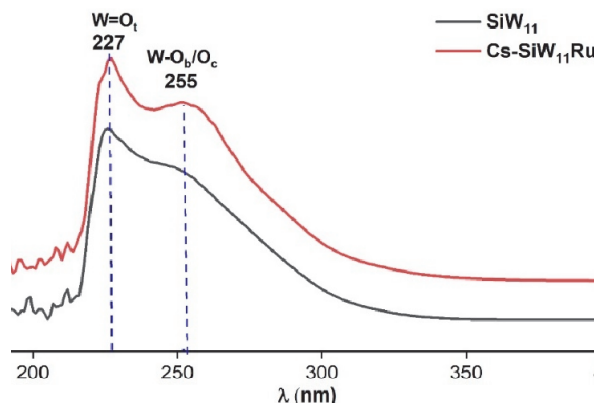
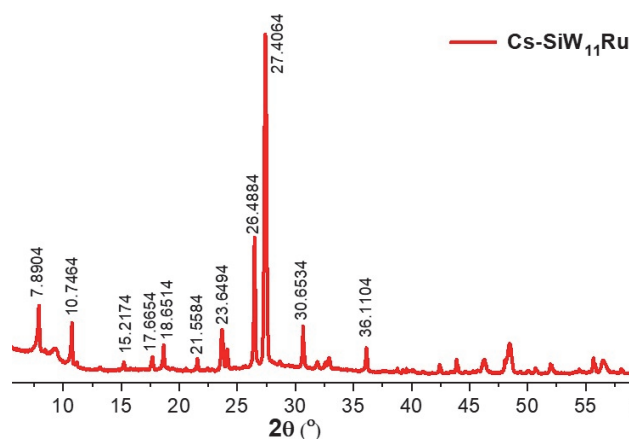


Fig. 4. UV–Vis spectra of K₈(α -SiW₁₁O₃₉) and Cs₅[SiW₁₁O₃₉Ru^{III}(H₂O)].

Powder XRD study. X-ray diffraction analysis is used extensively to study the structure of POMs. Fig. 5 shows the XRD patterns of solid CsSiW₁₁Ru. The sharp peaks demonstrate that Cs-SiW₁₁Ru is crystalline. The diffractogram of Cs-SiW₁₁Ru shows four groups of peaks at 2 θ ranges of 7–10°, 16–22°, 25–30° and 33–38°, which are consistent with the characteristic diffraction peaks of the Keggin structure.^{30,32} According to the literature,²⁸ the XRD pattern of Cs-SiW₁₁Ru agrees with the existence of a single crystallographic phase with a pattern typical of a tetragonal system (space group *P42/ncm*) with *a* = 20.9299 Å, *c* = 10.3603 Å.

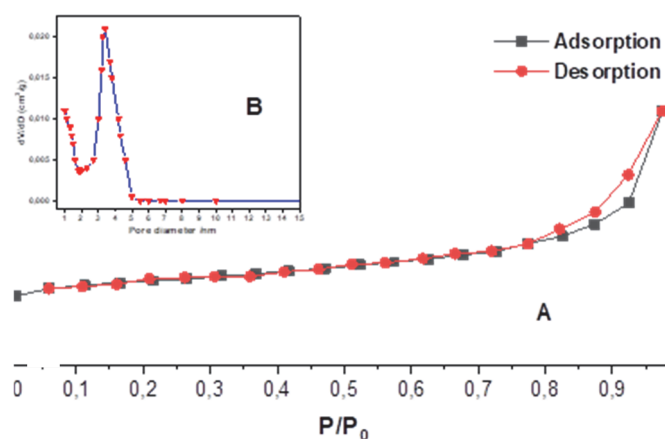
Textural properties of Cs-SiW₁₁Ru

N₂ adsorption–desorption isotherms and pore diameter distribution diagrams are depicted in Fig. 6, and the main results are shown in Table I. According to the IUPAC classification, the isotherm shape (Fig. 6A) of the synthesized salt was categorized as type IV, typical for mesoporous materials, with a hysteresis loop due

Fig. 5. X-ray diffraction pattern of the Cs-SiW₁₁Ru catalyst.TABLE I. Textural properties of the Cs-SiW₁₁Ru catalyst

Solid	Specific surface $\text{m}^2 \text{g}^{-1}$	Pore volume, V_{IP} $10^{-2} \text{cm}^3 \text{g}^{-1}$	Pore diameter, d_{m} nm
Cs-SiW ₁₁ Ru	150.2	4.7	3.4

to the vaporization of nitrogen condensed in the mesopores.^{31,33} Another interesting feature is the steep increase in adsorption at low pressures ($P/P_0 < 0.1$), which also suggests the presence of micropores in this sample.^{32,34} From Fig. 6B, the pore size distribution curve shows a maximum at 3.4 nm, confirming that the salt was a mesoporous solid (*i.e.*, mesoporous solids between 2 and 50 nm). The textural parameters ($S_{\text{BET}} \approx 150 \text{ m}^2 \text{g}^{-1}$, $V_{\text{p}} \approx 0.47 \text{ cm}^3 \text{g}^{-1}$) of the sample are in agreement with reported values for Keggin-type silicotungstic cesium salt.^{33,35}

Fig. 6. N₂ adsorption–desorption isotherms (A) and BJH pore size distribution (B) diagrams of the Cs-SiW₁₁Ru sample.

Isopropanol decomposition

The decomposition of isopropanol (IPA) without oxygen is frequently used as a chemical probe reaction to assess the acid–base characteristics of catalysts.^{36,37} The decomposition of IPA leads to the formation of propene, diisopropyl ether (DIPE) or acetone. If the solid exhibits acidic properties, only the dehydration products (DIPE and propene) are obtained, whereas if the catalyst displays basic or redox properties, the dehydrogenation of the IPA occurs, resulting in the formation of acetone.^{36,38}

Table II shows the results obtained from the IPA decomposition reaction using Cs-SiW₁₁Ru as a catalyst as a function of reaction temperature. As shown in Table II, the conversion of isopropanol increases with the increase of temperature. At all reaction temperatures, the catalyst exhibited high selectivity toward propene (only traces of acetone and diisopropylether were detected). This selectivity reached 99 % at a reaction temperature of 200 °C (Table II), indicating the presence of strong Lewis and/or Brønsted acid sites.^{37,39} According to the literature,⁴⁰ the Lewis acidity of heteropoly salts result from metal ions, whereas the Brønsted acidity result from the presence of the residual unchanged protons and/or from water molecules coordinating metal cation that were introduced into heteropoly salts.

TABLE II. Results of the catalytic activity of Cs-SiW₁₁Ru at different temperatures in IPA decomposition

<i>t</i> / °C	Conversion, %	Selectivity of products, %		
		Propene	Diisopropyl ether	Acetone
100	50	97	2	1
150	80	98	2	–
200	100	99	1	–

In other words, the solid Cs-SiW₁₁Ru favoured dehydration over dihydrogenation; therefore, the solid Cs-SiW₁₁Ru can essentially be assumed to be acidic.

Catalytic behavior

Effect of reaction temperature. The effect of the reaction temperature on CO₂ methanation was investigated in the range of 200–450 °C (in increments of 50 °C) at atmospheric pressure. The H₂/CO₂ ratio and the flow rate of the gas mixture were fixed at 4 and 1 L/h, respectively. The catalytic performance as a function of the reaction temperature is shown in Fig. 7, and the detailed catalytic results are summarized in Table S-I of the Supplementary material.

As shown in Fig. 7 and Table S-I, CO₂ conversion increases as the reaction temperature increases from 200 to 450 °C, reaching a maximum of 59 % at 450 °C. Fig. 7 also shows that at lower temperatures, *i.e.*, < 350 °C, the selectivities of methane and CO were 100 and 0 %, respectively. These results indicate that only the direct methanation mechanism occurs at low temperatures (from 200 to 300

°C). However, at the highest reaction temperatures (≥ 350 °C), there is a decrease in methane selectivity and an increase in CO selectivity, which can be attributed to the reverse water gas shift (RWGS) reaction ($\text{CO}_2 + \text{H}_2 \rightleftharpoons \text{CO} + \text{H}_2\text{O}$, $\Delta H_{298 \text{ K}} = 41 \text{ kJ/mol}$), which becomes more favourable as the reaction temperature increases due to its endothermic nature. This result is in agreement with the results obtained in the literature.⁴¹ Increasing the temperature is unfavourable since CO_2 methanation is highly exothermic, and it is important to operate at temperatures below 350 °C to achieve high methane selectivity at atmospheric pressure.

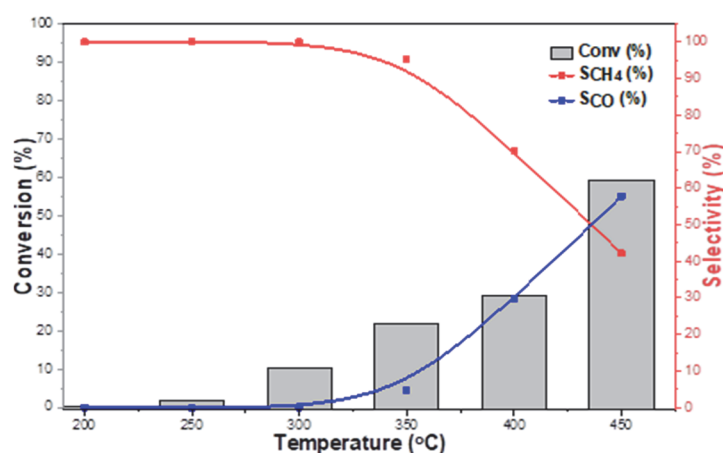


Fig. 7. Effect of temperature on CO_2 conversion and CH_4 selectivity. The reaction conditions were as follows: catalyst weight, 0.2 g; reaction temperature, 200–450 °C; H_2/CO_2 , 4; flow rate, 1 L/h; and atmospheric pressure.

Effect of the flow rate of the reaction mixture. The effects of the reactant flow rate on CO_2 conversion and methane selectivity were studied in a reactor containing Cs-SiW₁₁Ru at 300 °C with a stoichiometric reactant ratio ($\text{H}_2/\text{CO}_2 = 4$) by varying the flow rate of the reactant gases between 1 and 2 L/h. The results are shown in Fig. 8 and Table S-II of the Supplementary material.

As shown in Fig. 8, CO_2 the conversion and the CH_4 selectivity generally decreased as the reactant flow rate increased. Table S-II shows that when the flow rate was 1 L/h, the CO_2 conversion and methane selectivity obtained were 10 and 100 %, respectively. However, with a further increase in the flow rate to 2 L/h, the CH_4 selectivity and CO_2 conversion significantly decreased to 85.3 and 3 %, respectively. These observations could be explained by the shorter contact time between the reagents and the catalytically active sites, thus reducing the amount of reactants adsorbed onto the surface of the catalyst when the flow rate was increased. These results are in agreement with those of previous studies.^{42,43}

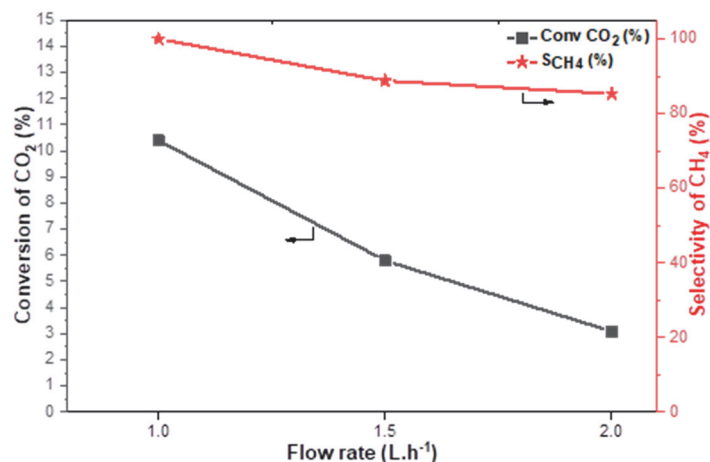


Fig. 8. Effect of the total flow rate on the performance of the Cs-SiW₁₁Ru catalyst. Reaction conditions: catalyst weight, 0.2 g; H₂/CO₂, 4; reaction temperature, 300 °C; atmospheric pressure.

Effect of the CO₂/H₂ ratio. To study the effect of the composition of the reaction mixture on the Cs-SiW₁₁Ru catalyst, several tests were carried out at 300 °C with a flow rate of 1 L/h, varying only the ratio of the reactive gases H₂/CO₂ from 1 to 4.

The results of the carbon dioxide conversion and methane selectivity as a function of the H₂/CO₂ ratio are shown in Fig. 9, and the detailed catalytic results are summarized in Table S-III of the Supplementary material. Fig. 9 shows that the conversion of CO₂ increases almost linearly with increasing H₂/CO₂ mole ratio from 1 to 4. For example, the conversion of CO₂ increases from 8 to 11 % when the H₂/CO₂ mole ratio is changed from 1 to 2 and reaches a maximum of 22.5 % when the H₂/CO₂ ratio is further increased from 2 to 4 (Table S-III). This result suggests that the increment of the H₂ content in the reactant mixture significantly enhances CO₂ conversion. Fig. 9 also shows the effect of the H₂/CO₂ mole ratio on CH₄ selectivity. The CH₄ selectivity slightly changed with increasing mole ratio. Table S-III shows that when the H₂/CO₂ mole ratio varies from 1 to 4, the CH₄ selectivity slightly increases from 98.1 % at a H₂/CO₂ ratio of 1 and reaches 100 % at a H₂/CO₂ ratio of 4. A further increase in the H₂ content slightly affects the CH₄ selectivity. These results show that the probability of CO₂ reacting with H₂ to form CH₄ is greater in the presence of a large amount of hydrogen. On the other hand, the formation of CO is favoured with a limited amount of hydrogen, as the methanation reaction remains incomplete. This observation could reinforce the results of earlier studies proposing a two-step reaction mechanism for the dissociation of CO₂ into CO_{ads} and O_{ads} before further reduction to CH₄.^{44,45}

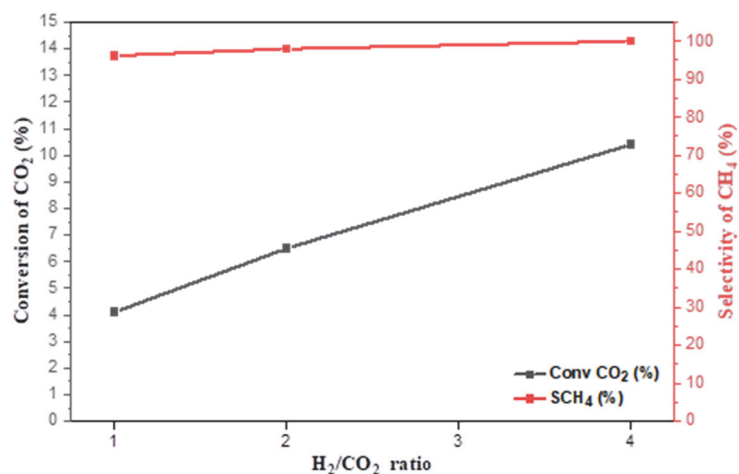


Fig. 9. Effect of the H₂/CO₂ ratio on the performance of the Cs-SiW₁₁Ru catalyst. The reaction conditions were as follows: catalyst weight, 0.2 g; flow rate, 1 L/h; reaction temperature, 300 °C; atmospheric pressure.

Effect of conversion on selectivity. Fig. 10 shows the evolution of methane and CO selectivities as a function of CO₂ conversion on the Cs-SiW₁₁Ru catalyst.

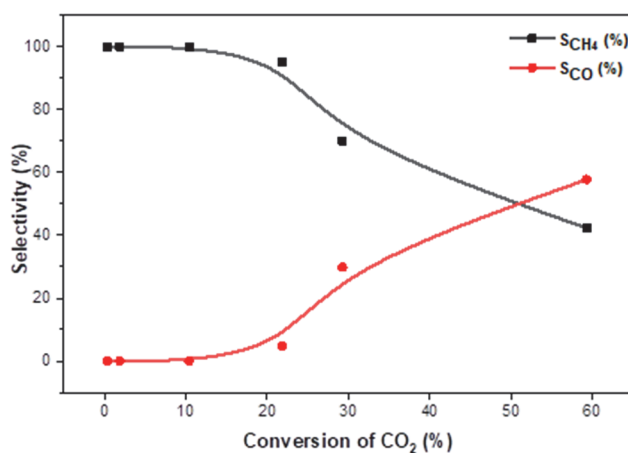


Fig. 10. Evolution of CH₄ and CO selectivities as a function of CO₂ conversion. Reaction conditions: catalyst weight, 0.2 g; temperature, 200–450°C; H₂/CO₂, 4; *P*, 1 atm; flow rate, 1 L/h.

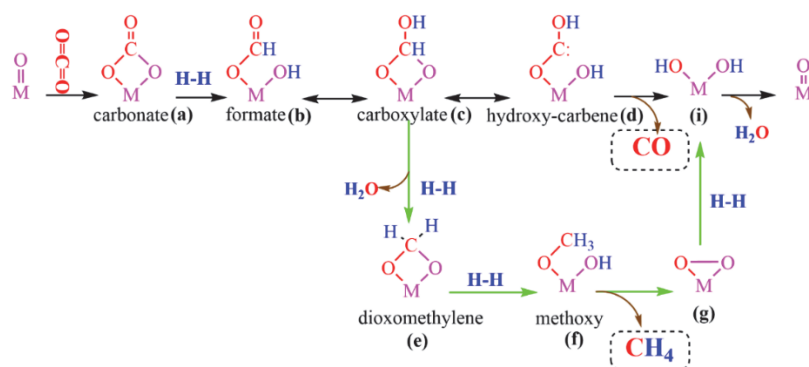
Fig. 10 shows that when the conversion of CO₂ is less than 10 %, the selectivity for methane is 100 %, and that for CO is 0 %. However, the CO₂ conversion increased from 10 to 57.73 %, the selectivity of CH₄ drastically decreased to 42.27 %, and the CO selectivity increased from 0 to 57.73 %. This shows

that for the methanation of CO_2 , CH_4 is the preliminary reaction product and is obtained by a series of consecutive reactions, whereas CO is the secondary reaction product.

Mechanism of CO_2 methanation

Although CO_2 methanation is a relatively simple reaction, its mechanism has not yet been clearly established. In general, two different mechanisms have been proposed for CO_2 methanation. The first mechanism involves the formation of CO as an intermediate product through the RWGS reaction, which is later converted into methane. The second mechanism involves the direct reaction of CO_2 with H_2 to form methane.^{46,47} Several species, such as carbonates, hydrogen carbonates, formates and methoxys, have been identified on the surface of the catalyst. Various techniques, such as the temperature programmed desorption (TPD) and the Fourier transform infrared spectroscopy (FT-IR), have been used to identify all of these species.⁴⁸

On the basis of effect of conversion on selectivity results (CH_4 was the preliminary reaction product) and the literature,⁴⁹ a mechanism involving the formation of surface is highly likely. Once the two reactants (CO_2 and H_2) are adsorbed on the surface of the catalyst, methane is obtained by the successive hydrogenation of several intermediates, including formate, dioxomethylene, and methoxy,⁴⁹ as shown in Scheme 1.



Scheme 1. Plausible mechanism of CO_2 methanation on $\text{Cs-SiW}_{11}\text{Ru}$.

After the surface is highly likely adsorption of CO_2 in the form of surface carbonate (a), the first step, which corresponds to the formation of formate species (b), is carried out relatively easily because it is energetically favourable. However, the successive hydrogenation of the formate to dioxomethylene (e) and then methoxy (f) groups is the rate-determining step (RDS) in methane synthesis. This step requires the most energy and is endothermic. The methoxy species (f) that leads to methane being strongly adsorbed on the surface of the catalyst, and the

temperature causes the methane to be desorbed by a radical process that leads to the formation of a peroxide species (g). Hydrogen addition enables the catalyst to be regenerated after a molecule of water has been eliminated.

Stability test

A catalytic test of 8 h at a reaction temperature of 300 °C was performed to assess the stability of the Cs-SiW₁₁Ru catalyst as a function of time in the CO₂ methanation reaction. The results of the stability experiment are presented in Fig. S-3 of the Supplementary material. The Cs-SiW₁₁Ru catalyst exhibited good stability, maintaining CO₂ conversion at approximately 10 % under operating conditions over the entire investigated time range. The CH₄ selectivity remained constant at 100 % throughout the stream. These results indicate that the catalyst was not subjected to deactivation during the tested period.

CONCLUSION

In the present study, a Cs-SiW₁₁Ru catalyst was prepared by the reaction of the monolacunary silicotungstate (SiW₁₁) with RuCl₃ and CsCl. The product was characterized by FT-IR, UV and X-ray diffraction, indicating that it possesses Keggin structure. Finally, the catalyst was tested in a CO₂ methanation reaction at atmospheric pressure. The effects of several CO₂ methanation parameters, including the reaction temperature, H₂/CO₂ mole ratio and flow rate, on Cs-SiW₁₁Ru were also investigated. With respect to the reaction temperature, an optimum range was found between 200 and 300 °C, because methane is the only molecule formed. In terms of the H₂/CO₂ molar ratio studied, our study revealed that CO₂ conversion increases continuously with the mole ratio, and the highest conversion is obtained for a stoichiometric ratio of 4. With respect to the flow rate, our study revealed that CH₄ selectivities remained unaffected while conversion decreased continuously with increasing flow rate, and the highest conversion was obtained for a flow rate of 1 L/h. In addition, this catalyst exhibited stable performance for 8 h of reaction. From this study, it can be concluded that the heteropolyanion system Cs-SiW₁₁Ru is a promising catalyst for the conversion of CO₂ to methane, depending on the reaction conditions. The formate pathway proposed, where the CO₂ is converted to methane, involves: *i*) the adsorption of CO₂ in the form of surface carbonates which are rapidly hydrogenated to formate intermediates, followed by *ii*) the successive hydrogenation of the formate to dioxomethylene then to methoxy species, and finally to methane.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13256>, or from the corresponding author on request.

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ИЗВОД

РУТЕНИЈУМ(III) МОНОСУПСТИТУИСАНИ ПОЛИОКСОВОЛФРАМАТ КЕГИНОВОГ ТИПА: СИНТЕЗА, КАРАКТЕРИЗАЦИЈА И ПРИМЕНА ЗА КАТАЛИТИЧКУ МЕТАНАЦИЈУ УГЉЕН-ДИОКСИДА

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Cs₅[α-SiW₁₁O₃₉Ru^{III}(H₂O)]·8H₂O со хетерополикиселине Кегинове структуре је успешно синтетисана, и њене физикохемијске карактеристике су одређене дифракцијом X-зрака, UV-Vis спектроскопијом, инфрацрвеном спектроскопијом са Фуријеовом трансформацијом и Brunauer, Emmett и Teller анализом површине. Кисело-базне особине су одређене праћењем реакције разлагања изопропанола. Каталитичке перформансе за реакцију метанације CO₂ су испитане у реактору са фиксним слојем на атмосферском притиску уз варирање процесних параметара и то реакционе температуре (200–450 °C), молског односа реактанта H₂/CO₂ (1, 2 и 4) и брзине протока (1, 1,5 и 2 L/h). Експериментални резултати су показали да Cs₅[α-SiW₁₁O₃₉Ru^{III}(H₂O)]·8H₂O показује најбољи компромис између конверзије и селективности на 350 °C, при молском односу H₂/CO₂ од 4 и брзином протока 1 L/h.

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SUPPLEMENTARY MATERIAL TO
**Ruthenium(III)-monosubstituted Keggin-type polyoxotungstate:
 Synthesis, characterization and application in the catalytic
 methanation of carbon dioxide**

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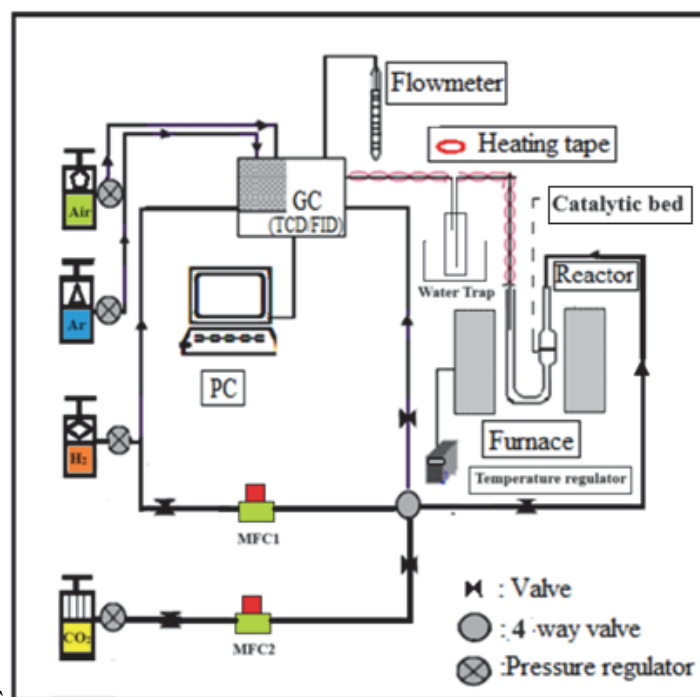


Fig. S-1. Schematic diagram of the experimental setup for methanation of CO₂

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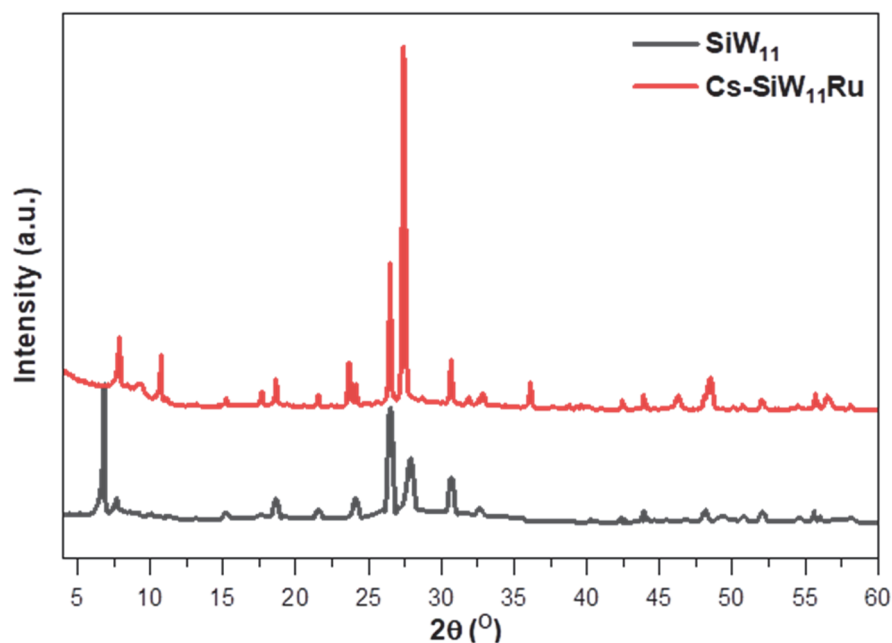


Fig. S-2. X-ray diffraction (XRD) patterns of $K_8(\alpha\text{-SiW}_{11}\text{O}_{39})$ (black) and $\text{Cs}_5[\text{SiW}_{11}\text{O}_{39}\text{Ru}^{\text{III}}(\text{H}_2\text{O})]$ (red).

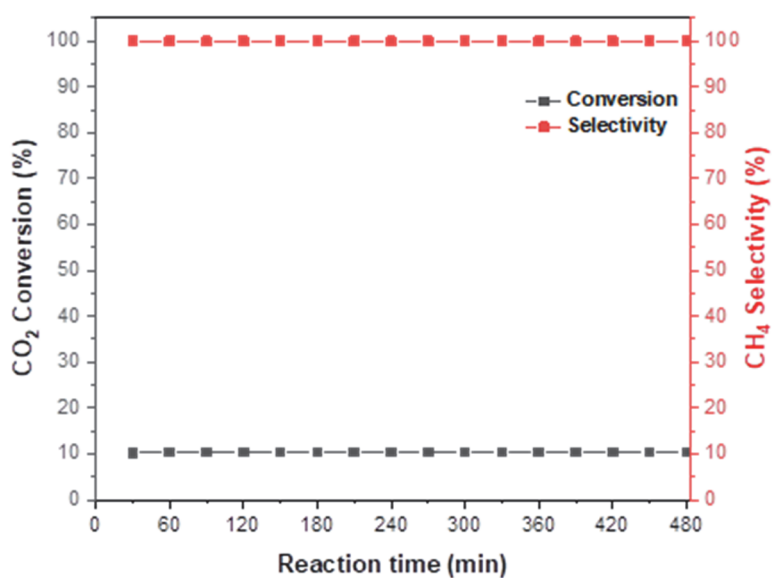


Fig. S-3. Stability of the prepared $\text{Cs-SiW}_{11}\text{Ru}$ catalyst for CO_2 methanation. Reaction conditions: flow rate, 1 L/h; H_2/CO_2 , 4; reaction temperature, 300 °C; and atmospheric pressure.

TABLE S-I. Effect of the reaction temperature on CO₂ methanation over the Cs-SiW₁₁Ru catalyst

Catalyst	T (°C)	Conversion (%)	Selectivity of products (%)	
			CH ₄	CO
Cs-SiW ₁₁ Ru	200	0.4	100	0
	250	2	100	0
	300	10	100	0
	350	22	95.31	4.69
	400	29	70.10	29.90
	450	59	42.27	57.73

TABLE S-II. Effect of the total flow rate on the reactivity of the Cs-SiW₁₁Ru catalyst

Flow rate (l/h)	T (°C)	Conversion (%)	Selectivity of products (%)	
			CH ₄	CO
1	300	10	100	0
1.5		06	88.8	11.2
2		03	85.3	14.7

TABLE S-III. Effect of the H₂/CO₂ ratio on the reactivity of the Cs-SiW₁₁Ru catalyst

T (°C)	Flow rate (L/h)	H ₂ /CO ₂ ratio	Conversion (%)	Selectivity of products (%)	
				CH ₄	CO
300	1	1	04	98.5	1.5
		2	06.5	99	01
		4	10	100	0

Influence of dissolved organic carbon nature on adsorption of ibuprofen, caffeine and diclofenac by powdered activated carbon from water

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Abstract: The impacts of structurally different dissolved organic carbon surrogates (DOC) on the adsorption of ibuprofen, caffeine and diclofenac (C_0 2–3 $\mu\text{g L}^{-1}$) onto powdered activated carbon (PAC) were studied. The efficiency of PAC in a synthetic matrix (SM) was tested when low or high molecular weight DOC surrogates were added. Additionally, the impact of a potential natural coagulant was investigated. Different DOC surrogates differently affected the adsorption of tested organic micropollutants (OMPs). In the first 30 min, a positive impact of all types of surrogates on the adsorption of neutral and hydrophilic caffeine was observed, while for negatively charged ibuprofen and diclofenac, the impacts varied. The low molecular weight DOC (containing amino acids and resorcinol) positively impacted the adsorption of ibuprofen, while effects were not clear for diclofenac. The high molecular weight DOC decreased the degree of adsorption for both compounds. The mass transfer calculations showed that low molecular weight DOC accelerated the transport of OMPs. High molecular weight DOC slowed reaching equilibrium (within 48 h). Thermogravimetry confirmed that the DOC coating qualitatively changes during the adsorption, which also affects the OMPs removal. The addition of a natural coagulant affected ibuprofen and diclofenac adsorption. For neutral caffeine the effects were not clear.

Keywords: dissolved organic carbon surrogates; pharmaceuticals; coagulation.

INTRODUCTION

It is well known¹ that some pharmaceuticals and their metabolites are detected in the effluents of treated wastewater at concentrations ranging from several ng L^{-1} to $\mu\text{g L}^{-1}$.¹ The efficiency of wastewater treatment is influenced by the effluent organic matter (EfOM), which has a very complex structure.² It is measured as the

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content of dissolved organic carbon (DOC). DOC affects the adsorption of organic micropollutants (OMPs) by blocking adsorbent pores with sufficiently large DOC molecules and/or direct binding of low molecular weight DOC to adsorption sites within the pores.³ Various studies in pure, surface, and groundwater have demonstrated that DOC negatively impacts the adsorption efficiency of OMPs.^{4–7} In the study of de Ridder *et al.*⁶ the impact of DOC on pharmaceuticals adsorption ($C_0 = 2 \mu\text{g L}^{-1}$) on granular activated carbon (GAC) treated with wastewater and surface water was investigated. It was found that the hydrophobicity of DOC originating from wastewater and the presence of smaller molecular fractions in its structure compete with micropollutants more than DOC originating from surface water. Zietzschmann *et al.*⁸ also demonstrated that the size of DOC molecules in wastewater treatment plant effluents affects OMPs adsorption, with small DOC molecules causing the most significant competition effects. Guillosoy *et al.*^{9,10} showed that the contact time of carbon with water plays an important role and that the nature of micropollutants affects DOC influence on adsorption. They proved that a combination of ozonation and activated carbon adsorption is more effective for removing 28 different OMP from wastewater treatment plant effluents compared to adsorption alone, due to the oxidation of DOC and thus reduced absorbability and competition potential. It was observed that the presence of DOC negatively affected the removal of 12 tested OMP (atenolol, carbamazepine, erythromycin, ofloxacin, roxithromycin, trimethoprim, acetaminophen, diclofenac, ibuprofen, lorazepam, oxazepam and sulfadiazine) after 30 min of adsorption (4–60 %) in wastewater treatment plant effluent compared to ultrapure water, in which high removal ($\geq 77 \%$) was achieved within 30 min and 72 h.¹⁰ The positively charged and neutral substances were better removed than negatively charged ones. After 72 h, similar removal efficiency was achieved for 6 of above mentioned 12 OMPs (atenolol, carbamazepine, erythromycin, ofloxacin, roxithromycin and trimethoprim), as in ultrapure water. The difference in removal for positively charged and neutral substances between 30 min and 72 h is explained by insufficient contact time and the possibility that during the 72 h, the competition between OMP and DOC only slows down diffusion transport but does not prevent adsorption. A mechanism of DOC–OMP complex formation was proposed. They gradually adsorb onto the surface of activated carbon over time. These findings highlight the complexity of interactions between DOC and OMP during the adsorption process on activated carbon, which is important for understanding and improving the process of removing micropollutants from wastewater.¹⁰ Anielak *et al.*¹¹ confirmed that for pollutants with a molecular weight higher than 230 g L^{-1} , interactions with humic substances in the solution become significant. Some studies have used different specific surrogates for DOC molecules, such as methylene blue,¹² congo red¹³ and methyl orange¹⁴ to investigate these effects. For example, in the study by Dittmar *et al.*¹⁴ the addition of methyl orange to deionized water

reduced the removal efficiency of carbamazepine ($C_0 = 10 \mu\text{g L}^{-1}$; from 60 to 30 % for a contact time of 30 min and from 90 to 60 % for a contact time of 48 h) using powdered activated carbon (PAC, 10 mg L^{-1}). π - π interactions between OMPs and DOC, as well as complex formation, have also been mentioned by other authors.^{15–17} The recent research¹⁸ has shown that higher aromaticity and lower polarity both enhanced dissolved organic matter (DOM) adsorption, with aromaticity being the dominant factor for adsorption of low molecular weight DOM ($<350 \text{ g L}^{-1}$).¹⁸ Wang *et al.*¹⁷ studied the impact of specific low molecular weight DOC molecules (aliphatic and aromatic compounds with hydroxyl, phenolic and carboxyl groups) on OMPs adsorption onto PAC. The study showed that unsaturated structures such as benzene rings, double and triple bonds, as well as DOC hydrophobicity, increase its competition ability, even for structures that are less adsorbable (unsaturated aliphatic compounds) or hydrophilic (polyphenols, alcohols). Besides these naturally occurring structures, the presence of amino acids in real systems is inevitable but insufficiently studied according to our findings in the literature. The amino acids contain both amino and carboxyl functional groups that engage in specific interactions with pollutants and adsorbents (hydrogen bonds and electrostatic interactions). These small molecules (L-serine, L-leucine and resorcinol) are recognized in the literature as surrogates for DOC remaining in water treatment after the frequently applied coagulation process.¹¹ Thus, a particular goal of this work was to examine how their mixture affects the adsorption of frequently detected pharmaceuticals (ibuprofen, caffeine and diclofenac) in the presence and absence of more complex humic acid (HA). In addition to these surrogates, DOC influence was assessed also in the systems where an innovative natural coagulant, a crude extract from bean seeds, was added. It was investigated as an alternative to commercial iron and aluminum-based coagulants in water treatment^{1,19,20} and it was confirmed that the addition of an optimal dose for wastewater treatment plant effluent ($37.5 \mu\text{L L}^{-1}$) does not contribute to an increase in organic carbon content measured by chemical oxygen demand, but may contribute to the removal of total DOC when applied together with the activated carbon adsorption. One closing parenthesis is missing at the end of the sentence/incorrect: In the previous studies it was found that the application of the extract, which in the solution has partially negatively and positively charged molecules, can negatively affect the adsorption of different pollutants from wastewater treatment plant effluent, when a low dose of carbon (5 mg L^{-1}) is applied (e.g. caffeine, the more hydrophobic neutral benzophenone (log D at pH 7.4: 2.96) and benzophenone-3 which also has a negative charge (log D at pH 7.4: 3.64))./correct: In the previous studies it was found that the application of the extract, which in the solution has partially negatively and positively charged molecules, can negatively affect the adsorption of different pollutants from wastewater treatment plant effluent, when a low dose of carbon (5 mg L^{-1}) is applied (e.g. caffeine, the more hydrophobic

neutral benzophenone (log *D* at pH 7.4: 2.96) and benzophenone-3 which also has a negative charge (log *D* at pH 7.4: 3.64). At a dose of 20 mg L⁻¹, such effects were absent for the mentioned three substances, as well as for neutral molecules of carbamazepine (log *D* at pH 7.4: 2.28) and triclosan (log *D* at pH 7.4: 5.13). In this study it was examined whether the addition of such a material affects the adsorption of ibuprofen, caffeine, and diclofenac in cleaner water matrices, enriched only with DOC surrogates, without other components considered as EfOM.

EXPERIMENTAL

The influence of DOC nature on activated carbon efficiency was examined by testing the capability of PAC to remove pharmaceuticals in 30 min, in several different water matrices: without the addition of DOC surrogates – synthetic matrix (SM), and with the addition of different low and high molecular weight DOC surrogates, into SM, both separately and in combination, to achieve DOC concentrations resembling natural levels (2.5–3.5 mg L⁻¹, Table I). All details related to materials used (activated carbon, natural coagulant, water matrices, DOC surrogates, OMPs aqueous solution preparation) are provided in the Supplementary material of this paper under the section heading materials. All details related to materials used (activated carbon, natural coagulant, water matrices, DOC surrogates, OMPs aqueous solution preparation) are provided in the Supplementary material of this paper under the section heading Materials.

TABLE I. The aqueous matrices used in the experiments

Type of matrix	Abbreviation in text	DOC mg L ⁻¹
Synthetic matrix (SM)	SM	<0.5
SM with the addition of low molecular weight DOC surrogates – mixture of L-serine, L-leucine and resorcinol	SRL	2.47
SM with the addition of high molecular weight DOC surrogates – humic acid	HA	3.46
SM with the addition of both high and low molecular weight DOC surrogates (humic acid and the mixture of L-serine, L-leucine and resorcinol)	HA-SRL	2.53

Testing was performed with and without the simultaneous dosing of natural coagulant isolated from the bean seeds (dosage 37.5 µL L⁻¹ selected based on its efficiency in removing organic matter from wastewater treatment plant effluent, details presented in Marjanović *et al.*¹). The choice of the PAC contact time was based on real applications rather than reaching equilibrium. However, further tests that were 48 h long were performed to assess the kinetics of the adsorption in the different water matrices. In addition, the thermogravimetric analysis was used for the characterization of sorbents that were soaked for different times in solutions containing DOC surrogates in order to investigate the coating of DOC on the surface of PAC.

All the details related to experimental methodologies applied are provided in the Supplementary material under the section heading methods.

RESULTS AND DISCUSSION

The efficiency of the 30-min adsorption onto PAC

In Fig. 1 the efficiency of powdered activated carbon (dose 5 mg L^{-1} , contact time 30 min) is presented separately and in combination with a natural coagulant (dose $37.5 \text{ }\mu\text{L L}^{-1}$) for the removal of ibuprofen, caffeine and diclofenac (C_0 2–3 $\mu\text{g L}^{-1}$) from a synthetic matrix, a synthetic matrix with added low molecular weight DOC surrogates, high molecular weight DOC surrogates and their combination. All experiments were performed in duplicate, and the results are presented accordingly, indicating satisfactory repeatability in the vast majority of cases. Only in the case of ibuprofen in the SM, and in the case of diclofenac in the HA-SRL matrix, was poorer reproducibility of the experiments observed.

It has been shown that without the addition of surrogates, 30 min adsorption on PAC in SM in one case did not achieved significant removal for ibuprofen while in duplicate experiment it was 28 %. The values for caffeine and diclofenac were more consistent – 48 and 50 % for caffeine and 83 and 87 % of diclofenac. A relevant positive effect of DOC on the adsorption was observed for ibuprofen a hydrophilic and negatively charged molecule, in matrices containing DOC exclusively of low molecular weight (removal increased up to 90 %, analytical method bias 4.6–14 %, Supplementary material, Table S-III) and in combination with HA (up to 40–60 % removal, analytical method bias 7.2–10 %, Table S-III). Considering the analytical method bias (Table S-III), HA alone reduced the ibuprofen adsorption, likely due to the lack of specific interactions with small molecules (*e.g.*, hydrogen bonds and complex formation with amino acids) and competition of negatively charged molecules during adsorption on positively charged carbon (the isoelectric point of the PAC is 9.81).²¹ For hydrophilic, but neutral caffeine, all types of surrogates positively influenced the removal efficiency. The reason for the absence of commonly observed DOC competition effects, confirmed in the literature, may be the unimpeded sorption of caffeine on DOC coatings. For negatively charged diclofenac, expected negative effects on adsorption were observed in matrices containing HA, probably due to competition with negative HA molecules, while the positive effects of the SRL matrix could not be observed. The reason for this is unknown. Based on $\log D$ at relevant pH 7.4, diclofenac is less hydrophilic than ibuprofen (Supplementary material, Table S-I).²² However, some literature data show that there are no large differences between $\log D$ values when other software is used for the calculations.^{23,10} In any case, diclofenac is a larger molecule with two aromatic rings, containing nitrogen and chlorine which are both capable of electron withdrawal, while ibuprofen has only one aromatic ring and has no other heteroatoms than oxygen. Relevant positive effects in terms of improved adsorption with the addition of a natural coagulant were also observed only for ibuprofen, both in the absence of DOC surrogates and in the presence of HA

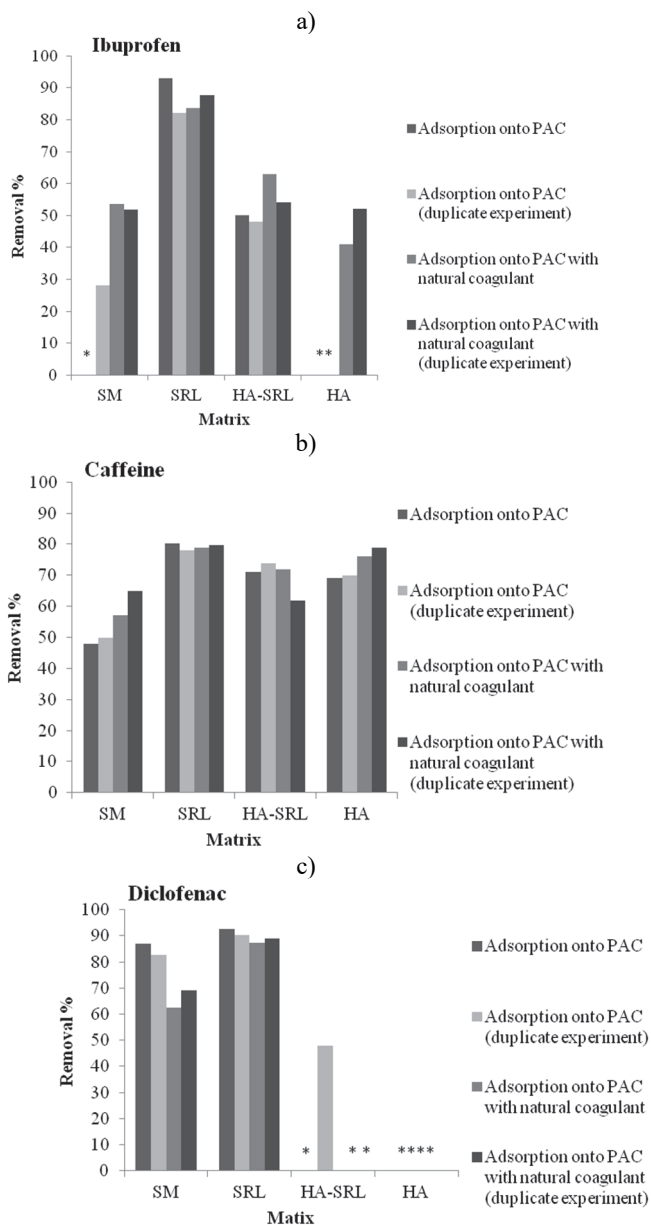


Fig. 1. Results of the study on the efficiency of PAC (dose 5 mg L^{-1}) in combination with a natural coagulant (dose $37.5 \text{ }\mu\text{L L}^{-1}$) for the removal of: a) ibuprofen, b) caffeine and c) diclofenac, C_0 $2\text{--}3 \text{ }\mu\text{g L}^{-1}$, contact time 30 min; * – no removal achieved; SM – synthetic matrix, SRL – synthetic matrix with added low molecular weight DOC surrogates (a mixture of L-serine, L-leucine and resorcinol), HA-SRL – synthetic matrix with the addition of the aforementioned low molecular weight DOC mixture and humic acid, HA – synthetic matrix with added high molecular weight DOC surrogates (humic acid).

alone (Fig. 1a). In the case of diclofenac (Fig. 1c), the addition of the coagulant to the matrix without surrogates had the opposite effect, reducing the adsorption, likely due to the possibility of preventing the large diclofenac molecule from reaching the adsorption sites due to competition with small molecules of the SRL matrix.

In cases of matrices containing HA, observed differences were of the order of analytical method bias (7–40%) and cannot be considered relevant. It can be concluded that the structure of the negatively charged molecules and the size of the DOC surrogates affect the degree of pollutant removal, and that the natural coagulant induces the same type of effect as HA in the given system. Some of the obtained results are opposite to most frequently reported result in literature that DOC decrease the removal of OMPs due to competition effects. It should be noted that in comparison to literature, very low concentrations of OMPs are used in this study as well as the low PAC dose. As mentioned before, the possible specific interactions between the selected pollutants and the chosen DOC surrogates, might be the reason for this. Table II summarizes the results of the impact of the natural coagulant on the adsorption of ibuprofen, caffeine, and diclofenac in matrices with different types of DOC surrogates.

TABLE II. The impact of a natural coagulant on the adsorption of ibuprofen, caffeine and diclofenac in matrices with different types of dissolved organic carbon (DOC) surrogates (only differences higher than analytical method bias taken into account); +: positive impact; -: negative impact; *: no impact; SM – synthetic matrix, SRL – synthetic matrix with added low molecular weight DOC surrogates (a mixture of L-serine, L-leucine and resorcinol), HA-LSRL – synthetic matrix with the addition of the aforementioned low molecular weight DOC mixture and humic acid, HA – synthetic matrix with added high molecular weight DOC surrogates (humic acid)

Type of matrix	Ibuprofen	Caffeine	Diclofenac
SM	+	*	–
SRL	*	*	*
HA-SRL	*	*	–
HA	+	*	*

The previous work with wastewater treatment plants effluent¹ and same carbon dose, showed mainly negative effects of the natural coagulant addition on the removal of caffeine and the more hydrophobic neutral benzophenone and negatively charged benzophenone-3. In the cleaner water matrices used in this work, no negative effects for caffeine were observed (Fig. 1c). However, similar to the previous case, negative effects were observed for diclofenac in the SM and HA-SRL matrices. Conversely, for ibuprofen, a positive effect was observed, but it was significant only when there were no small molecule DOC surrogates in the matrix. Prodanović²⁴ demonstrated that the isoelectric point (pI) of the raw bean extract is 3.61. This means that most macromolecules in the raw bean extract are

negatively charged at the pH at which the experiments were conducted (pH around 7.5). However, the electrostatic interactions with negative OMPs are possible due to the partially positive charge of peptide bonds and the probable formation of hydrogen bonds, which can also be influenced by the size of the molecules (ibuprofen is smaller than diclofenac, Table S-I). A similar mechanism could be proposed for the observed effects of DOC surrogates on adsorption due to the presence of positively charged amino groups in the DOC surrogates. Such interactions most probably can form complexes that may be more easily adsorbed onto activated carbon, provided that the interactions between the carbon surface and other parts of the molecule in the formed complex are favourable (*e.g.*, electrostatic, van der Waals interactions, hydrogen bonds and π - π interactions). This confirms the earlier hypotheses about the complexity of the pollutant and the DOC interactions in the adsorption process, which require deeper investigation to potentially utilize their existence for the improvement of water treatment process performance.^{10,15–17}

Adsorption kinetics of ibuprofen, caffeine and diclofenac under different water matrices

The removal efficiencies of ibuprofen, caffeine and diclofenac in kinetic experiments at a PAC dose of 5 mg L⁻¹ are presented in Table S-III. It was found that different DOC surrogates affect the rate of establishing adsorption equilibrium differently. In some cases, desorption and adsorption occurred over 48 h. For all three OMPs equilibrium was established after 2 h in the synthetic matrix (SM). The addition of small DOC molecules (SRL) did not affect the time needed to reach it. However, the addition of HA extended the time required to establish equilibrium for all three substances to 24–48 h. In the synthetic matrix, with added high molecular weight DOC, adsorption equilibrium is established only after 24 h or, in the case of ibuprofen, even later. In the synthetic matrix with added DOC surrogates of both small and large molecules, the concentration of ibuprofen (one of the two experiment duplicates) and caffeine (both experiment duplicates) is already below the practical quantitation limit of the method after 2 h of contact, while for diclofenac, the adsorption equilibrium is established later, after 24 h.

Table III shows products of film mass transfer coefficients (k_F / m min⁻¹) of ibuprofen, caffeine and diclofenac and total surface area of the adsorbent mass available in the reactor (a_m / m² g⁻¹). Based on the experiments within the first 15–30 min (depending on whether linearity is obtained in the initial part of the curve) and based on Eq. (S-1) in the Supplementary Material, describing the $\ln(C/C_0)$ vs. time, it was possible to compare the product of the multiplication of the a_m and k_F .

The carbon mass and the reactor volume were the same in all experiments. Since the a_m can be considered the same in all experiments due to the same PAC dose, the products of two numbers can be used for the relative comparison in

different water matrices. It is important to note that the contact times (0, 15 and 30 min) used for this purpose were relatively long, but this approach was the only possible having in mind the time needed for the manipulation of a larger sample volume (500 mL), which was necessary at the chosen concentration level.

TABLE III. Product of the total surface area of the adsorbent mass available in the reactor ($a_m / \text{m}^2 \text{g}^{-1}$) and mass transfer coefficient ($k_F / \text{m min}^{-1}$) through the film for ibuprofen, caffeine and diclofenac

Type of matrix	Ibuprofen		Caffeine		Diclofenac	
	t / min	r^2	t / min	r^2	t / min	r^2
SM	0; 15	0.9530	0; 15; 30	0.9969	0; 15	0.9986
		0.014		0.004		0.025
SRL	0; 15;	0.9988	0; 15;	0.9998	0; 15;	0.9999
		0.027		0.022		0.031
HA	0; 15;	1.0000	0; 30;	1.0000	0; 15;	1.0000
		0.006		0.008		0.005
HA-SRL	0; 15; 30;	0.9845	0; 15; 30;	0.9839	0; 15; 30;	0.8051
		0.005		0.008		0.003

The differences in mass transfer coefficients between the tested pharmaceuticals are most pronounced in the synthetic matrix where there are no interacting DOC surrogates. The largest molecule, diclofenac, which is negatively charged exhibits the fastest transport through the film. Conversely, caffeine transports most slowly toward positively charged carbon particles. The calculations showed an acceleration in the SRL matrix, whereas slowdown was observed in matrices containing HA for negatively charged compounds in comparison to SM. In the presence of small DOC molecules, all have 1.2–5 times higher mass transfer coefficients through the film. The given fact is particularly pronounced for ibuprofen and caffeine, contrary to the results reported by Zeitzschmann *et al.*⁸ where small DOC molecules caused the greatest competitive effects. According to Wang *et al.*²⁵ low molecular weight molecules do not universally compete with organic micropollutant adsorption onto activated carbon. The competitive impact varied significantly depending on the absorbability of the specific OMPs (caffeine, carbamazepine, sulfamethoxazole). It was reported that poorly adsorbable DOC even can contribute to adsorption of OMPs due to their complexation. Further investigation of Wang *et al.*¹⁸ showed that aromatic DOM did not significantly compete with OMPs (caffeine, sulfamethoxazole and carbamazepine) since the highly aromatic and polar fraction of DOM exhibited lower adsorption competitiveness than the less aromatic and less polar fraction.¹⁸ This confirms that in addition to the DOC size, the type of DOC molecule affects competition. It is hypothesized that DOC molecules such as small amino acids containing amino groups can facilitate additional interactions between OMPs and DOC in solution, thereby accelerating transport through the film to the particle surface. Ibuprofen and diclofenac

(negatively charged ions, Table S-I) can engage in electrostatic interactions with the carbon itself (applied PAC isoelectric point 9.8, indicating positive charge at pH 7.5),²¹ whereas transport of the neutral molecule caffeine accelerates up to five times likely due to different interactions such as SRL–carbon and SRL–caffeine. Conversely, the addition of large DOC molecules (HA) slows down ibuprofen and diclofenac transport 2–5 times compared to the synthetic matrix, while caffeine transport still can speed up 2 times. Similar effects were observed when small DOC molecules were present in the matrix along with large molecules (HA–SRL). Overall, these interactions affect the time to establish adsorption equilibrium, which is achieved after 48 h, consistent with the findings of Guillosoy *et al.*¹⁰

Thermogravimetric (TG) characterization of native sorbent and sorbent coated with various types of DOC surrogates

The results of TG measurements are presented in Supplementary material, Fig. S-1a, b and c. TG and derivative thermogravimetric (DTG) curves are shown for carbons that were exposed to different types of DOC surrogates for short-term (30 min) and long-term (24 h). The freshly filtered model matrices at first lose water up to ~100 °C. Since the water content of the samples highly depends on the temperature, relative humidity and time between filtering and thermal measurement, only the properties after water evaporation above 100 °C are analysed here. The sample of native PAC without coating showed no change in weight and therefore was omitted from further discussion. The PAC exposed to model matrix with low molecular weight DOC (PAC–SRL), analysed after 30-min contact, loses 1.2 and 1.3 % of the weight in two low-intensity steps through a wide temperature range with DTG maxima at 191 and 359 °C, respectively (Fig. S-1a). Differently, the same mixture after 24 h contact shows only one weight loss step of 3.3 % with DTG maximum at 356 °C (Fig. S-1a). This weight decrease is also detected in a wide range. The difference in between the two materials indicates that the sorbent surface changes during 24 h. The higher weight loss in only one step suggests that a coat of higher amount of low molecular weight DOCs is formed on the surface of PAC after longer contact.

Fig. S-1b shows the results for the PAC exposed to the model matrix with high molecular weight DOC (PAC–HA), after 30 min and 24 h. After 30 min contact weight loss happens in two steps, like in the previous case. In the first one 0.8 % with DTG maximum at 207 °C, and in the second step 8.6 % with DTG maximum at 432 °C. After 24 h contact in the first step lost a higher amount of weight (1.3 %) and in the second one, a somewhat lower weight (5.8 %) than after 30 min contact. The overall weight loss tendency of this sample suggests that up to ~400 °C the contact time between the PAC and HA does not affect the thermal properties of the sample. Above this temperature, the lower weight loss in the sample after

24 h may be the consequence of the change in interactions between the PAC and the HA during 24 h in comparison to 30 min contact time.

In the model matrix with both types of DOC (PAC-HA-SRL) the weight loss steps were distinguished only in the sample after 30 min contact their DTG maxima are at 148, 208 and 399 °C (Fig. S-1c). In the first two steps, the overall weight loss is 1.1 %, and in the third 7.8 %. After 24 h contact, only one step can be distinguished with DTG maximum at 403 °C and weight loss of 7.1 % (Fig. S-1c). In this matrix, the missing DTG peak at 207 °C, which is detected on the DTG curve of PAC-HA for 24 h, and the different shape of the DTG peak at 403 °C compared to PAC-HA and PAC-SRL suggest some intermolecular interactions between HA and SRL. This confirms the very complex dynamic nature of DOC–PAC interactions which may also affect the adsorption of pharmaceuticals during different contact times in addition to PAC interaction with pharmaceuticals and DOC interactions with pharmaceuticals in solution.

CONCLUSION

This study highlights the role of dissolved organic carbon in the removal of organic micropollutants and its complex interactions with the micropollutants and the adsorbents. The results show that the presence of DOC enhanced the initial adsorption of caffeine independently of molecular weight of DOC surrogates. In contrast, the ibuprofen adsorption increased only in low molecular weight DOC matrices while the adsorption of diclofenac was not affected by this kind of matrix. The high molecular weight DOC (*e.g.*, humic acid) reduced the adsorption of negatively charged compounds (ibuprofen and diclofenac), suggesting competitive interactions. It has been demonstrated that the lower molecular weight DOC surrogates enhance the adsorption rates, whereas higher molecular weight ones slow the process. The natural coagulant affects the adsorption of only negatively charged compounds, but in different ways for different compounds. The thermogravimetric analysis revealed that DOC coatings on powdered activated carbon (PAC) evolved over time, providing the opportunity for altering interactions with pollutants and affecting adsorption efficiency.

Having in mind the observed effects of DOC quality on adsorption of OMPs, the next interesting topic for the research can be the evaluation of PAC's reuse across multiple cycles in water matrices containing DOC of different characteristics. This should include the aspects of harvesting the PAC after the adsorption stage in water treatment train and its preparation (conditioning) for the reuse not only in water treatment but also in other environmental applications (*e.g.*, soil amendments).

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/13067>, or from the corresponding author on request.

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ИЗВОД

УТИЦАЈ ПРИРОДЕ РАСТВОРЕНОГ ОРГАНСКОГ УГЉЕНИКА НА АДОРПЦИЈУ
ИБУПРОФЕНА, КОФЕИНА И ДИКЛОФЕНАКА ПРИМЕНОМ АКТИВНОГ УГЉА У
ПРАХУ ИЗ ВОДЕ

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У овом раду је испитан утицај структурно различитих сурогата растворене органске материје (DOC) на адсорпцију ибупрофена, кофеина и диклофенака (C_0 2–3 $\mu\text{g L}^{-1}$) применом активног угља у праху (РАС). Испитана је ефикасност РАС у синтетичком матриксу (SM) са додатком сурогата DOC мале или велике молекулске масе. Такође, истраживан је и утицај додатка природног коагуланта. Сурогати DOC су различито утицали на адсорпцију различитих органских микрополутаната (OMP). У првих 30 min примећен је позитиван утицај свих врста сурогата на адсорпцију неутралног и хидрофилног кофеина, док је за негативно наелектрисани ибупрофен и диклофенак утицај варирао. Матрикс са сурогатима DOC мале молекулске масе (који садржи аминокиселине и резорцинол) позитивно је утицао на адсорпцију ибупрофена, док за диклофенак ефекти нису били јасни. Сурогати DOC велике молекулске масе умањили адсорпцију за оба једињења. Апроксимација брзине транспорта OMP кроз филм указује да DOC мале молекулске масе убрзава транспорт ибупрофена, кофеина и диклофенака у односу на синтетички матрикс. Сурогати DOC велике молекулске масе узроковали су спорије достизање равнотеже (у року од 48 h). Термогравиметрија је потврдила да се DOC превлака на РАС квалитативно мења током адсорпције што такође утиче на уклањање OMP. Додавање природног коагуланта утицало је на адсорпцију ибупрофена и диклофенака. За неутрални кофеин, ефекти нису били јасни.

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SUPPLEMENTARY MATERIAL TO
**Influence of dissolved organic carbon nature on adsorption of
ibuprofen, caffeine and diclofenac by powdered
activated carbon from water**

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MATERIALS

Activated carbon

The activated carbon used in the experiments was NORIT SAE (according to the manufacturer's specifications, with a BET surface area of 1150 m² g⁻¹ and a particle size of D50 15 µm). The isoelectric point of the powdered activated carbon (PAC) is 9.81,¹ indicating a positive surface charge.

Natural coagulant

The natural coagulant was isolated from the seeds of the Gradištanac variety of beans (*Phaseolus vulgaris*) in the laboratories of the Faculty of Technology in Novi Sad.²

Synthetic matrix (SM)

The synthetic matrix without or with dissolved organic carbon (DOC) surrogates was prepared in laboratory-grade pure water (conductivity 16 µS) by adding individual stock solutions of NaHCO₃ (Centrohem, p.a. >99 %) at a concentration of 0.02 mol L⁻¹, CaCl₂·2H₂O (Centrohem, p.a. >99 %) at a concentration of 0.03 mol L⁻¹, and MgSO₄·7H₂O (Centrohem, p.a. >99 %) at a concentration of 0.02 mol L⁻¹ in accordance with DIN EN 12 902:2004.³ The measured pH value of the prepared synthetic water was 7.5.

SM was further enriched first DOC surrogates and then with aqueous solution of selected organic micropollutants (OMPs) to achieve initial concentrations of ibuprofen (IB), caffeine (CF), and diclofenac sodium salt (DCF) (Sigma Aldrich, purity ≥99 %) of 2–3 µg L⁻¹.

SM with the addition of low molecular weight DOC surrogates - mixture of L-serine, L-leucine, and resorcinol

The solutions of L-serine (Reagent Plus®, ≥99 %, Sigma Aldrich), L-leucine (Reagent grade, ≥98%, Sigma Aldrich) and resorcinol (Reagent Plus®, ≥99 %, Sigma Aldrich) were prepared by weighing 20 mg of each substance into 50 mL volumetric flasks and dissolving

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them in laboratory-grade pure water. The equal volumes of these solutions were mixed to enrich the matrix with small molecular weight DOC surrogates to achieve total DOC concentrations of approximately 3 mg C L⁻¹.

SM with the addition of high molecular weight DOC surrogates - humic acid

The humic acid solution (HA, technical grade, Sigma Aldrich) was prepared according to the procedure⁴ by weighing 0.3 g of HA and dissolving it in 250 mL of laboratory-grade pure water. The pH was adjusted to 10 by adding NaOH solution (0.1 mol L⁻¹). The solution was then mixed on a magnetic stirrer at 300 rpm for 24 hours. After mixing, it was filtered first through glass fibre filter paper (0.6 µm, MACHEREY-NAGEL, Germany), and then through cellulose nitrate filter paper (0.45 µm, Sartorius, US) to remove undissolved HA.

SM with the addition of both high and low molecular weight DOC surrogates (humic acid and the mixture of L-serine, L-leucine, and resorcinol)

In the case of SM with the addition of both high and low molecular weight DOC surrogates (humic acid and a mixture of L-serine, L-leucine, and resorcinol), low and high molecular weight DOC molecules were added at equal concentrations. Specifically, 1.5 mg L⁻¹ of low molecular weight DOC molecules (a mixture of equal volumes of mixture of L-serine, L-leucine, and resorcinol solutions) and 1.5 mg L⁻¹ of humic acid were combined to achieve a total DOC concentration of 3 mg C L⁻¹.

This solution was further used to enrich the matrix with large molecular weight DOC surrogates to achieve total DOC concentrations of approximately 3 mg C L⁻¹. The concentration of dissolved organic carbon in synthetic matrices with and without the addition of DOC surrogates was determined using a TOC analyzer (liquiTOCII, Elementar, Germany) and ranged from <0.5 to 3.5 mg C L⁻¹.

Organic micropollutants (OMPs) aqueous solution preparation

The stock aqueous solution of IB, CF, and DCF (prepared from diclofenac sodium salt) in laboratory pure water was used to enrich samples with organic micropollutants in experiments. The solution was prepared by weighing the appropriate amounts of substances (around 10 mg of IB, CF, DCF) on an analytical balance, dissolving them, and transferring the solution to a 2 L volumetric flask, which was then filled with laboratory water up to the mark.

The dissolution process was enhanced by ultrasound treatment for 3 hours, after which the solution was left overnight in a refrigerator. Afterwards the solution was filtered through a cellulose nitrate membrane filter paper (0.45 µm, Sartorius, USA). In subsequent experiments, the stock aqueous solution was diluted as needed to achieve the desired initial concentration (around 2.00 µg L⁻¹). The variations in concentration (2.02–2.66 µg L⁻¹) arose due to the precision of the substance weighing. In the **TABLE S-I**, the nominal concentrations of OMPs are shown depending on the type of matrix.

TABLE S-I. Nominal concentrations of OMPs ($\mu\text{g L}^{-1}$) in experiments

Type of matrix	Nominal concentrations of OMPs ($\mu\text{g/L}$)		
	IB	CF	DCF
SM	2.16	2.42	2.10
SRL	2.66	2.56	2.12
HA-SRL	2.16	2.02	2.02
HA	2.06	2.10	2.42

SM-synthetic matrix, SRL-synthetic matrix with added low molecular weight DOC surrogates (a mixture of L-serine, L-leucine, and resorcinol), HA-SRL-synthetic matrix with the addition of the aforementioned low molecular weight DOC mixture and humic acid, HA - synthetic matrix with added high molecular weight DOC surrogates (humic acid);

EXPERIMENTAL METHODOLOGIES

The efficiency of the 30 min adsorption onto PAC

The efficiency of the adsorption process onto PAC (5 mg L^{-1}) with and without dosing of natural coagulant was conducted on the JAR apparatus FC6S VELP scientific. Simultaneous dosing of PAC and natural coagulant was tested at a contact time of 30 minutes. Experiments were performed in four types of matrices with different DOC surrogates (SM, SRL, HA-SRL, and HA). After the mixing time elapsed, the sample was filtered through a cellulose nitrate filter paper ($0.45 \mu\text{m}$, Sartorius, US) previously rinsed with 300 mL of laboratory-grade water. The first aliquot of 250 mL was discarded, and the remaining portion (250 mL) was analysed for the concentration of OMPs using gas chromatography with mass spectrometry (GC / MS). All experiments were conducted in duplicate.

Adsorption kinetics of ibuprofen, caffeine, and diclofenac in different water matrices

The adsorption kinetics study was conducted using a shaker (KS 501-IKA, shaking intensity 180 rpm). In 500 mL of each matrices enriched with pharmaceuticals, PAC (5 mg L^{-1}) was added. The contact time with carbon was 15, 30, 60, 120 minutes, 24 hours, and 48 hours. The sample was filtered through a cellulose nitrate filter paper ($0.45 \mu\text{m}$, Sartorius, US) previously rinsed with 300 mL of laboratory-grade water. The first aliquot of 250 mL was discarded, and the remaining portion (250 mL) was analysed for the concentration of OMPs using GC / MS. All experiments were conducted in duplicate. Based on the experiments within the first 15-30 min, corresponding to the time frame where film diffusion is the dominant process and the kinetic curve exhibits linearity and based on the Equation 1 describing the $\ln c / c_0$ vs. time, it was possible to compare product of multiplication of the $a_m / \text{m}^2 \text{g}^{-1}$, the total surface area of the adsorbent mass available in the reactor, and $k_f / \text{m min}^{-1}$ the mass transfer coefficient through the film. The carbon mass and the reactor volume were the same in all experiments. Since the $a_m / \text{m}^2 \text{g}^{-1}$ can be considered same in all the experiments due to the same PAC dose, the products of two numbers can be used for relative comparison.

$$\ln \frac{c}{c_0} = \frac{m_A}{V_L} k_f a_m t \quad (\text{S-1})$$

Where m_A (g) is the mass of adsorbent in the reactor, V_L (m^3) is the total volume of the reactor, $a_m / \text{m}^2 \text{g}^{-1}$ is the total surface area of the adsorbent mass available in the reactor, and $k_f / \text{m min}^{-1}$ is the mass transfer coefficient through the film.

Analytical method for OMPs analysis

Physicochemical characteristics of OMPs are shown in **TABLE S-II**.

TABLE S-II. Physicochemical characteristics of the selected organic micropollutants

Organic Micropollutants	Molecular weight (g mol ⁻¹) ^a	pK _a ^b	log D ^c (pH 7.4)	Charge ^d
Ibuprofen	206.3	4.91	0.45	Negative
Caffeine	194.2	14.0	0.28	Neutral
Diclofenac	296.1	4.15	1.37	Negative

Source: a⁵; b, c, d⁶

For the preparation of samples for OMPs analysis, solid-phase extraction (60 mg, Oasis® HLB, Waters) and derivatization using N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Synthesis grade, Sigma-Aldrich) were applied. In a sample aliquot (250 mL), the pH value is first adjusted (pH=2) using concentrated HCl (p.a >37 %, Centrohem). Then, a solution of the internal standard mecoprop (PESTANAL®, analytical 99.6 % (HPLC), Sigma-Aldrich) in methanol was added so that the final concentration in the water sample is 2 µg L⁻¹. After that, the solid-phase extraction (60 mg, Oasis® HLB, Waters) was performed by conditioning the cartridge using 3x0.5mL dichloromethane (for HPLC, ≥99.8 %, Sigma-Aldrich), 3x0.5mL methanol (for Pesticide Residue Analysis, Chromasolv™, Honeywell), and 3x0.5 mL acidified laboratory-grade water, sequentially. After passing the sample (3 mL / min), the cartridge was dried under vacuum for 1 hour and eluted using 3x1 mL dichloromethane. The obtained eluate was evaporated to dryness under a stream of nitrogen and reconstituted in 0.5 mL toluene (99.85 %, for pesticide residue analysis, Thermo Scientific™). Prior to transferring the reconstituted eluate to the vial, 5 µL of a methanol solution of the internal standard phenanthrene-d10 (c= 100 µg mL⁻¹; analytical standard, Supelco) was added and evaporated to dryness. Then, the derivatization follows by adding 100 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Synthesis grade, Sigma-Aldrich) and heated up to 60 °C for 1 hour.⁷ Gas chromatography-mass spectrometry (GC / MS) is used for separation, detection, and quantification. A capillary column DB-5 MS (30 m × 0.25 mm × 0.25 µm, Agilent, USA) was used, with helium as the carrier gas at a constant flow rate of 1 mL min⁻¹. A 2 µL sample was injected in splitless mode (Purge flow 15 mL min⁻¹ at 0.75 min) at an injector temperature of 250 °C. The analysis is performed in SIM mode (selected ion monitoring), tracking ions for quantification (TABLE SII). In all synthetic matrices, the initial concentrations of substances and concentrations after the applied processes were determined using a standard calibration method with internal standards. Separate calibrations were used for each type of matrix. In the synthetic matrix and synthetic matrix with the addition of high molecular weight DOC surrogates, internal validation of the analytical method for OMP analysis was performed (linearity, method and instrument repeatability, analytical method bias, extraction efficiency, method detection limit (MDL), and practical quantitation limit (PQL).

The method detection limit is calculated according to Equation S2:

$$MDL = SD \cdot t(n - 1) \quad (S-2)$$

Where:

SD - standard deviation of the measured concentrations of ibuprofen, caffeine, and diclofenac; t(n-1) - Student's coefficient for a 95 % confidence level, for (n-1) degrees of freedom, where n represents the number of repetitions for determining the MDL (Student's coefficient for a 95 % confidence level for four measurements, n-1= 3 is 3.182)

The practical quantification limit is calculated according to Equation S3:

$$PQL = 3 \cdot MDL \quad (S-3)$$

Where:

SD - standard deviation of the measured concentrations of ibuprofen, caffeine, and diclofenac.

Method repeatability is determined in a synthetic matrix without added DOC and a synthetic matrix with added HA as the relative standard deviation of three measurements of different extracts.

Instrument repeatability is determined as the relative standard deviation of three measurements in the same extract.

Analytical method bias is determined in a synthetic matrix without added surrogate DOC and a synthetic matrix with added HA. It is expressed as the ratio of the measured concentration to the expected concentration for measurement in triplicate.

Extraction efficiency is based on the ratio of the signal intensity for substances in the sample undergoing solid-phase extraction and in the sample representing the matrix extract spiked with substances after the solid-phase extraction. In both cases, the signal intensity for substances is normalized by the peak area of the internal standard (mecoprop, $c=2 \mu\text{g L}^{-1}$) added to the matrix extract. Both types of measurements were performed in triplicate and the RSD of the measurements was calculated.

The method validation is presented in Table SIII. It is important to note that correction of the results is not performed in the cases where matrix influence was noticed (for example, in some samples containing HA, higher bias was observed) since it is considered constant for the initial solution and solution after adsorption experiments. All the samples for one batch of experiments were analysed in one sequence at once in order to avoid the possible interference of changes in matrix influence during the both sample preparation and sample analysis by GC / MS.

Removal efficiency

The removal efficiency (%) of the selected OMPs in all experiments was calculated using Equation (S4):

$$\text{Removal efficiency, \%} = C_0 - C_e / C_0 \cdot 100 \quad (\text{S-4})$$

where C_0 is the initial concentration of OMPs in matrix before the treatment and C_e is the concentration of compounds after sample treatments.

“no removal achieved”- the calculated removals were lower than method bias and not taken into consideration

TABLE S-III. Method validation

Type of matrix	OMP ^a	Target ion, ion	MDL ^b ($\mu\text{g L}^{-1}$) n=4	PQL ^c ($\mu\text{g L}^{-1}$) n=4	Method repeatability		Instrument repeatability		Bias		Extraction efficiency (E)	
					n=3		n=3		n=2			
					Conc. level $\mu\text{g L}^{-1}$	RSD %	Conc. level $\mu\text{g L}^{-1}$	RSD %	Conc. level $\mu\text{g L}^{-1}$	RSD %	Conc. level $\mu\text{g L}^{-1}$	RSD %
Synthetic Matrix (SM)	IB	160 , 263, 117	0.019	0.056	0.20	14.3	0.20	1.44	0.20	14	0.20	106 (3.0)
					2.00	10.0	2.00	0.95	2.00	4.6	2.00	101 (4.0)
	CF	194 , 109, 82	0.037	0.111	0.20	7.75	0.20	5.67	0.20	20	0.20	14.6 (2.0)
					2.00	12.5	2.00	2.28	2.00	4.5	2.00	14.1 (7.0)
SM with the addition of large molecular weight DOC surrogates - humic acid (HA)	DCF	214 , 242, 367	0.028	0.084	0.20	7.11	0.20	6.14	0.20	21	0.20	13.1 (6.0)
					2.00	13.5	2.00	4.75	2.00	1.1	2.00	19.2 (13)
	IB	160, 263, 117	0.008	0.023	0.20	8.6	0.20	0.32	0.20	7.2	0.20	107 (5.0)
					2.00	12	2.00	1.5	1.5	10	2.00	16.2 (6.0)
	CF	194, 109, 82	0.003	0.008	0.20	11	0.20	0.54	0.20	4.2	0.20	81.3 (12)
					2.00	8.0	2.00	5.0	1.5	1.9	2.00	13.5 (9.0)
	DCF	214 , 242, 367	0.005	0.016	0.20	14	0.20	4.1	0.20	40	0.20	32.1 (12)
					2.00	15	2.00	0.99	1.5	7.0	2.00	15.9 (6.0)

TABLE S-IV. The removal efficiency (%) of ibuprofen, caffeine, and diclofenac in kinetic experiments at a PAC dose of 5 mg L^{-1}

	Type of matrix	SM	Ibuprofen			SM	Caffeine			SM	Diclofenac		
			SR L	HA	HA-SRL		SR L	HA	HA-SRL		SRL	HA	HA-SRL
Time	15min	59	86	77	26	30	81	98	41	84	90	45	26
	15min D*	69	87	57	32	72	82	85	49	85	90	20	20
	30 min	5.0	93	-11	50	48	80	69	71	87	93	-9.0	30
	30 min D*	28	82	-15	48	50	78	70	74	83	90	27	48
	1h	56	94	-3,0	**	75	81	78	71	34	94	31	33
	1h D*	37	96	8.0	**	75	83	90	74	51	>95	70	30
	2h	84	94	**	83	85	79	82	>97	93	>95	35	64
	2h D*	84	93	45	>92	87	79	95	>97	92	>95	73	61
	24h	84	>97	79	>92	88	85	>98	>97	92	>95	80	90
	24h D*	84	>97	74	-	89	84	>98	-	92	>95	83	-
	48h	86	>97	89	>92	91	84	>98	>97	92	>95	76	88
	48h D*	85	>97	88	-	89	85	>98	-	92	>95	85	-

*Experiment duplicate; ** Analysis failed; - No experiment duplicate; SM - synthetic matrix, SRL - synthetic matrix with added low molecular weight DOC surrogates (a mixture of L-serine, L-leucine, and resorcinol), HA-SRL - synthetic matrix with the addition of the aforementioned low molecular weight DOC mixture and humic acid, HA - synthetic matrix with added high molecular weight DOC surrogates (humic acid); Note: The bolded values in the table fall outside the analytical method bias and cannot be considered relevant.

TG characterization of native and sorbents coated with various types of DOC surrogates

In order to investigate the coating of DOC on the surface of PAC, thermogravimetric (TG) characterization of the native sorbent (soaked in synthetic matrix, SM) and the sorbents coated with various types of DOC surrogates (after 30 minutes and 24 hours soaking in solution) was performed.

2.5 mg of PAC was added to 500 mL of each matrix and agitated using a shaker (KS 501–IKA, at 180 rpm). After the agitation period, the sorbent was separated by filtration through pre-washed cellulose nitrate filter paper (0.45 μm , Sartorius, US) using 300 mL of laboratory-grade water. The residual PAC cake on the filter paper was further analyzed. Thermal data were collected using TA Instruments SDT Q600 thermal analyzer. The decomposition was followed from room temperature to 600 $^{\circ}\text{C}$ at a 10 $^{\circ}\text{C min}^{-1}$ heating rate in the argon carrier gas (flow rate 50 $\text{cm}^3 \text{min}^{-1}$). Sample holder/reference: alumina crucible/empty alumina crucible. Sample mass 1.5–3 mg. The sample of native PAC without coating after drying is stable in the temperature range of measurement, and no weight loss was observed.

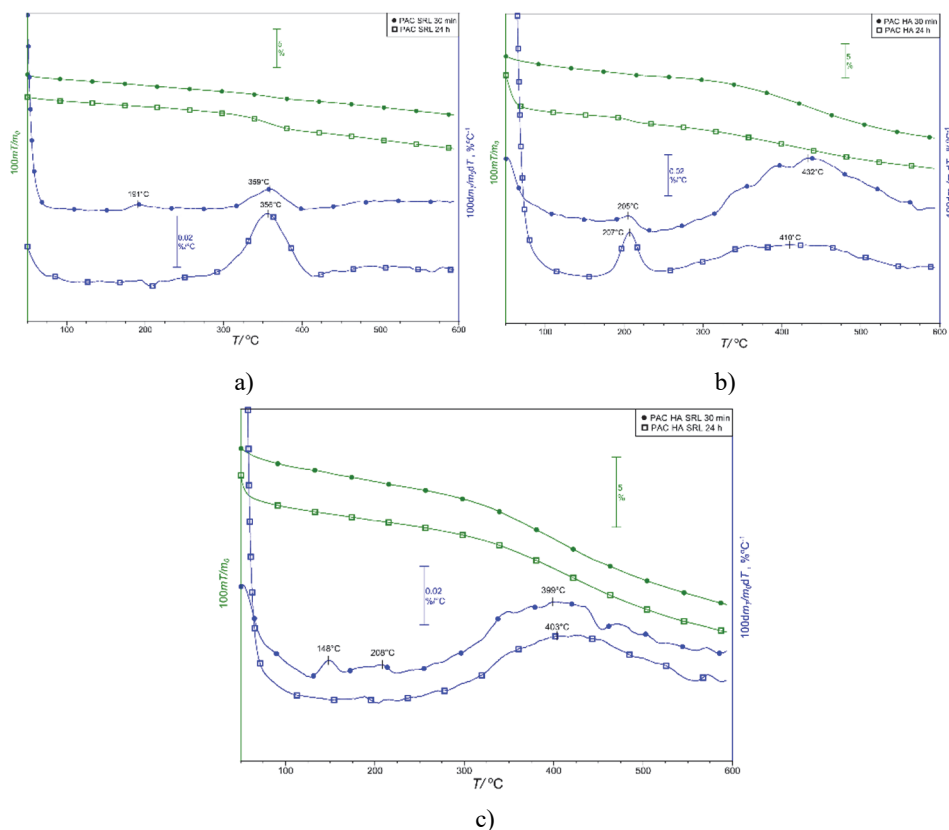


Fig. S-1. TG and DTG curves of PAC coated with a) SRL DOC surrogate, b) HA and c) HA-SRL.

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Arsenic in Bosnia and Herzegovina market seafood: Effects of matrix modifiers on measured concentration

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Abstract: Arsenic concentration in seafood could potentially reach very high levels and represent a significant health risk for humans. In this study, the concentration of arsenic in various seafood: crabs (shrimp, prawns), molluscs (mussels) and cephalopods (squid) available both fresh on the market and frozen in supermarkets in Sarajevo, Bosnia and Herzegovina, were determined by the electrothermal atomic absorption spectrometry (ETAAS). The results obtained using different matrix modifiers: $\text{Mg}(\text{NO}_3)_2$, $\text{Ni}(\text{NO}_3)_2$, $\text{Pd}(\text{NO}_3)_2$, and mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ were compared. The best recovery rate of 98.4 % arsenic for the reference material ERM-CE278k, was achieved after the addition of the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$. The mean arsenic concentrations were 1.551 ± 0.836 , 1.298 ± 0.410 and $2.794 \pm 0.958 \text{ mg kg}^{-1}$ for crustaceans, molluscs and cephalopods, respectively, using $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ as the matrix modifier. The arsenic concentrations in the same sample measured using different matrix modifiers varied widely, even above 70 %. With the current consumption rate of seafood products, both cancerogenic and non-cancerogenic risks associated with exposure to arsenic through seafood are very low for the residents of Bosnia and Herzegovina.

Keywords: ETAAS; matrix modifier; target hazard quotient; cancer risk.

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INTRODUCTION

Seafood is a well-known source of numerous nutrients. Many countries have issued national guidelines that support the regular intake of seafood. According to the U.S. Department of Agriculture and U.S. Department of Health and Human Services recommendations, an optimal seafood intake should be an app. 225 g per week.¹ The main reasons that support these claims are based on the content of high-quality proteins found in the seafood. Those proteins are easily digestible because the levels of connective tissue in seafood are significantly lower compared to red meat or chicken. In addition, seafood contains a particular type of polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids that positively impact human health.² Consumption of seafood 1–2 times a week is desirable for adults, while consumption of 3–4 times a week during pregnancy is associated with better functional outcomes of neurodevelopment in children.³ Seafood may or may not be contaminated with toxic elements. The accumulation of this elements in human body from marine organisms, including seafood, depends primarily on their habitat and species and internal factors such as size, weight, age, sex, sexual maturity and stress.⁴

The lethal doses of arsenic for adults are between 100 and 300 mg.⁵ Numerous epidemiological studies have reported a strong association between exposure to arsenic and systemic health effects.⁶ Long-term exposure to arsenic can lead to neurological and cognitive dysfunction in children and adults.⁷ Knowing that most of As ingested with food are very fast excreted from the body,⁸ adverse effects for human health can arise only after excessive exposure.⁹ The International Agency for Research on Cancer (IARC) classifies inorganic arsenic compounds as cancerogenic for humans. On the other hand, organic arsenic compounds are only potentially cancerogenic.¹⁰ However, the cancer risk in humans due to exposure to As through seafood consumption is considered to be low.¹¹

Arsenic toxicity in humans is mainly related to exposure to inorganic arsenic, which through binding to thiol or sulfhydryl groups on proteins can inactivate over 200 enzymes.¹² Most likely, this is the mechanism of action underlying the effects of arsenic on various organic systems. At the same time, inorganic arsenic has been shown to inhibit mitochondrial respiration, which may cause DNA mutation and the development of cancer.¹³ The percent of inorganic arsenic in seafood is, according to different literature sources, less than 2–3 %^{14–18} up to 10–13 %.^{16,19,20} It is generally accepted that inorganic arsenic is critical for human health risk assessment.²¹ However, some findings indicate that organic arsenic compounds undergo biotransformation, leading to trivalent toxic arsenic intermediates or some end products that are more toxic than the parent arsenic.¹³

The use of precise and reliable analytical techniques to determine arsenic at 0.01 mg kg⁻¹ levels has become a standard requirement. Inductively coupled plasma mass spectrometry (ICP-MS) and electrothermal atomic absorption spec-

trometry (ETAAS) are detection techniques that meet this requirement.²² Better control of chemical processes and reduction of interference of the present components of the matrix are the most important goals for analysts who deal with these techniques. They can be achieved by adding certain chemical compounds, known as matrix modifiers that reduce interference by separating the test compound from the matrix.²³

The matrix modifiers achieve their effect by binding to the analyte element, thus preventing its loss at high pyrolysis temperatures, which are sufficient to remove most of the matrix. In general, this can be achieved by two main mechanisms: decreasing the analyte's volatility or increasing the matrix's volatility. Arsenic compounds are highly volatile; therefore, samples can lose arsenic during the preparation phase.²⁴ Test method for ETAAS issued by U.S. EPA²⁴ recommend adding nickel nitrate or palladium nitrate prior to analysis to minimize volatilization losses of arsenic during drying and ashing. Adding nickel enables char temperatures up to 1500 °C can be achieved without an arsenic loss.²⁵ Achieving the highest pyrolysis temperature is not the only prerequisite for the optimal effect of the matrix modifier.²⁶ Therefore, some authors prefer palladium which allows pyrolysis temperatures up to 1300 °C with high sensitivity.²⁷ Palladium nitrate belongs to the platinum group matrix modifiers that catalyse analyte reduction under high pyrolysis temperatures. It forms low volatility intermetallic compounds with the analyte.²⁶ Palladium nitrate is often used to determine arsenic in seafood.²⁸ Given that the classes above of matrix modifiers have different modes of action, an attempt was made to combine the modifiers from both groups. For this purpose, the mixture of palladium nitrate and magnesium nitrate is most often used, which is also considered a universal matrix modifier.²⁷ Although some research has been conducted to examine exposure to heavy metals through fish and seafood in the adult population of Bosnia and Herzegovina,²⁹ there is still insufficient information on the arsenic concentrations in seafood and related health risks.

This study aimed to determine the concentration of arsenic in various seafood: crabs (shrimp, prawns), molluscs (mussels) and cephalopods (squid) available both fresh on the market and frozen in supermarkets in Sarajevo, Bosnia and Herzegovina. In addition, the effect of the use of different matrix modifiers on As concentration was investigated. Finally, the risk to human health from arsenic intake through seafood was assessed.

EXPERIMENTAL

Chemicals

Standard arsenic solution (1 g L⁻¹) in HNO₃ (0.5 mol L⁻¹), CertiPUR®, was obtained from Merck. Matrix modifiers stock solution: magnesium nitrate (10 g L⁻¹), palladium nitrate (2 g L⁻¹) and nickel nitrate (10 g L⁻¹), trace element analyses purity, were obtained from Carlo Erba Reagents, Italy. Nitric acid, HNO₃ (65 %), Suprapur™, was purchased from Thermo

Fisher Scientific. Ultrapure water was obtained from Mili-Q-Direct 8, Millipore, USA. Certified reference material ERM-CE278k (mussel tissue) was purchased from Sigma–Aldrich.

Instruments

Microwave digestion system producer Berghof MWS-3+, Germany; Electrothermal atomic absorption spectrometer – ETAAS – PinAAcle 900T AAS THGA, producer Perkin Elmer, supported by the software WinLab 32.

Samples

Analysis has been performed on a total of 61 samples of three different types of seafood: *crustaceans* ($n = 29$), the molluscs ($n = 17$) and the cephalopods ($n = 15$). More than half of the samples (59 %) were procured fresh on the fish market and stored in the fridge at 4 °C. The other samples (41 %) were acquired frozen from the supermarket and kept in the freezer. All samples were collected between June and the end of September 2019. This study did not take into account seasonal variations and different suppliers in the market. Before the analysis, all the samples were removed from the fridge and freezer to reach room temperature, after which they were prepared by separating the edible portion from the inedible (guts, scales, heads and bones). The animal's commonly edible part (tissue) was selected for analysis and were homogenised by grinding in stainless steel chopper. Arsenic concentration was determined in each sample using all investigated matrix modifiers.

Sample preparation

All samples were treated following the applicable standard methods EN 13804:2015 and EN 13805:2015. The frozen samples were thawed at room temperature. About 0.5 g of comminuted and homogenized sample was taken for microwave digestion in PTFE vessels. After that, 5 ml HNO₃ were added, and the samples were placed on microwave digestion. The digested samples were transferred into polypropylene volumetric flasks, diluted to 25 ml with ultrapure water and used for total As determination.

Electrothermal atomic absorption spectrometry

The determination of arsenic was performed by ETAAS, supported by the software WinLab 32 and following the standard EN 14332:2004. The operating parameters of the ETAAS used during the analysis are shown in Table I. program given by producer of ETAAS for determination arsenic. The injection temperature was 20 °C, and the samples were analysed in duplicate. The injected volume of the sample was 20 µl, while the injected volume of matrix modifiers was 5 µl. The concentrations of all matrix modifiers were 1 µg µL⁻¹.

TABLE I. ETAAS operating parameters

Step		Temperature °C	Ramp time min	Hold time min	Flow of argon mL/min
1a.	Drying	110	1	30	250
1b.		130	15	30	250
2.	Pyrolysis	1200	10	20	250
3.	Atomization	2000	0	3	0
4.	Cleaning	2450	1	3	250

Calibration

Standard arsenic solution (1 g L⁻¹), was diluted to get a calibration curve. The calibration was done with 5 different concentrations (0, 1, 5, 10, 15 and 20 µg L⁻¹) and blank, it was lin-

ear, with correlation coefficients above 0.991. The real samples were analysed without and with adding matrix modifiers. Stock solution matrix modifiers were diluted and used in amount of: 5 µg Mg(NO₃)₂, 5 µg Pd(NO₃)₂, 8 µg mix of 5µg Pd(NO₃)₂ + 3µg Mg(NO₃)₂ and 5µg Ni(NO₃)₂.

Quality control

The certified reference material ERM-CE278k was analysed in each sample series, to perform quality control. Certified As value for ERM-CE278k, given by producer is 6.7±0.4 mg kg⁻¹. Also, solvents and blank were included in each series of digestion and analysis. *LOQ* value of methods is 0,01 mg kg⁻¹. The certified and analysed As values and trueness (recoveries) with different matrix modifiers were presented in Table II. The recoveries were calculated as:

$$\text{Trueness (Recovery), \%} = 100(\text{Analysed As value/Certified As value}) \quad (1)$$

TABLE II. Analysed As values and trueness (recoveries) ERM-CE278k obtained without and with different matrix modifiers;

Matrix modifier (MM)	Analyzed value ^a , mg/kg	Recovery, %
Without MM	2.29±0.1	34.2
Mg(NO ₃) ₂	1.28±0.1	19.1
Pd(NO ₃) ₂	7.09±0.2	105.8
Ni(NO ₃) ₂	3.32±0.2	49.5
Pd(NO ₃) ₂ + Mg(NO ₃) ₂	6.50±0.1	98.4

^aThe data are presented as means ± standard deviation

Risk assessment

Target hazard quotient (*THQ*) and cancer risk (*CR*) have been determined for assessing non-carcinogenic and carcinogenic risk. *THQ* and *CR* were calculated as:³⁰

$$THQ = 10^{-3} E_{fr} \times ED \times FIR \times C / R_{fd} \times BW \times TA \quad (2)$$

$$CR = 10^{-3} E_{fr} \times ED \times FIR \times C \times CSF / BW \times TA \quad (3)$$

In formulas (2) and (3), *E_{fr}* refers to exposure frequency (365 days/year), and *ED* is exposure duration (years), where the average human lifetime of 70 years was used. *FIR* stands for fish (food) ingestion rate (g per day). Since there is no available official data regarding seafood consumption in Bosnia and Herzegovina, *FIR* was estimated by using FAOSTAT food supply quality data for 2018, the latest year with available records. According to this source, 0.04, 0.05, and 0.2 kg per capita per year was the consumption for crabs, molluscs and cephalopods, respectively.³¹ The *C* is the element concentration in the sample (mg kg⁻¹). The concentration of inorganic arsenic, which represents the most toxic arsenic form, was calculated as 3 % of the mean for total arsenic concentration, in line with the approach from other authors from Bosnia and Herzegovina.¹⁷ *BW* stands for average body weight (kg), where an average adult weight of 70 kg was used and *TA* refers to exposure time for non-carcinogens (365 days per year×*ED*). *R_{fd}* refers to oral reference dose mg kg⁻¹ per day), and *CSF* is a carcinogenic slope factor. According to United States Environmental Protection Agency the *R_{fd}* and *CSF* for arsenic are 0.0003 and 1.5 mg kg⁻¹ per day respectively.³²

This research gives a snapshot of the current arsenic levels in seafood available to the citizens of Bosnia and Herzegovina. The performed risk assessment based on the collected

data provides an initial understanding of the situation. To accurately assess the population's exposure to arsenic through this diet, several years of monitoring are needed.

RESULTS AND DISCUSSION

The results of quality control (Table II) showed that recoveries were between 19.1 % (the lowest) with $\text{Mg}(\text{NO}_3)_2$ and 105.8 % (the highest) with $\text{Pd}(\text{NO}_3)_2$ as the matrix modifier (Table II). The recovery obtained with a mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ as matrix modifier was 98.4 %, and this result was considered representative since arsenic concentration was closest to the certified value. Seafood samples were analysed first without matrix modifiers and then with various matrix modifiers as a CRM material. The results are presented in Table III. The arsenic concentrations are shown as mean values with standard deviation, and range for crustaceans ($n = 29$), molluscs ($n = 17$) and cephalopods ($n = 15$). Based on the results obtained by measuring recovery of the reference material, which we consider representative, the As results obtained with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ were compared with the results obtained by measuring with the other matrix modifiers. None of the samples, regardless of the matrix modifier used during the analysis, showed an arsenic concentration higher than 15 mg kg^{-1} , defined as the upper limit by state regulation on maximum levels of specific contaminants in food.³³

TABLE III. Total arsenic concentration (mg/kg) in seafood measured with different matrix modifiers and risk assessment results; *THQ* – target hazard quotient; *CR* – cancer risk; * – comparison with mixture $\text{Pd}(\text{NO}_3)_2$ and $\text{Mg}(\text{NO}_3)_2$

Sample	Parameter	No matrix modifier	$\text{Mg}(\text{NO}_3)_2$	$\text{Pd}(\text{NO}_3)_2$	$\text{Ni}(\text{NO}_3)_2$	$\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$
Crustaceans $n = 29$	Mean $\pm$ SD	1.281 $\pm$ 0.908	0.773 $\pm$ 0.458	1.601 $\pm$ 0.908	0.795 $\pm$ 0.340	1.551 $\pm$ 0.836
	Range	0.010–3.570	0.020–1.976	0.225–4.144	0.086–1.740	0.252–3.741
	<i>p</i> value*	1.000	< 0.003	0.173	< 0.001	N/A
	<i>THQ</i> /10 ⁻⁴	2.0	1.2	2.5	1.2	2.4
	<i>CR</i> /10 ⁻⁷	0.9	0.5	1.1	0.6	1.1
Cephalopods $n = 15$	Mean $\pm$ SD	1.225 $\pm$ 1.023	0.902 $\pm$ 0.311	1.346 $\pm$ 0.453	0.582 $\pm$ 0.242	1.298 $\pm$ 0.410
	Range	0.020–2.752	0.325–1.487	0.490–1.818	0.135–0.852	0.405–1.706
	<i>p</i> value*	0.108	< 0.001	0.663	< 0.001	N/A
	<i>THQ</i> /10 ⁻⁴	9.6	7.1	10.5	4.6	10.2
	<i>CR</i> /10 ⁻⁷	4.3	3.2	4.7	2.1	4.6
Molluscs $n = 17$	Mean $\pm$ SD	1.367 $\pm$ 0.740	0.774 $\pm$ 0.316	2.537 $\pm$ 0.793	1.042 $\pm$ 0.219	2.794 $\pm$ 0.958
	Range	0.218–2.710	0.163–1.288	1.070–3.851	0.686–1.363	1.106–4.301
	<i>p</i> value*	< 0.001	< 0.001	0.061	< 0.001	N/A
	<i>THQ</i> /10 ⁻⁴	2.7	1.5	5.0	2.0	5.5
	<i>CR</i> /10 ⁻⁷	1.2	0.7	2.2	0.9	2.5

A risk assessment analysis has been performed based on the gained arsenic concentrations to estimate the probability of adverse health effects potentially caused by exposure to arsenic-containing seafood. *THQ* and *CR* were calculated

using the methodology initially developed to determine concentrations of toxic substances ingested through the consumption of edible fish.³⁰ However, this methodology has been used by many authors for assessing the risk from the consumption of seafood as well.^{15,34,35} For the *THQ* values < 1, adverse health effects are not expected. If the *THQ* value is > 1, non-carcinogenic adverse health effects could be experienced. On the other hand, according to U.S. EPA,³⁶ carcinogenesis is a phenomenon for which risk evaluation based on the presumption of a threshold is inappropriate. Therefore, *CR* results are evaluated upon acceptable lifetime risk level (*ARL*) equal to 1×10^{-5} as defined by U.S. EPA,³⁷ representing a risk for developing cancer over a human lifetime of 1 in 100,000.

Crustaceans

It is not surprising that a high concentration of this heavy metal can be found in seafood shrimps.¹⁵ However, that was not the case in this research, where the gained arsenic concentration for crustaceans was in the range 0.010–4.144 mg kg⁻¹ (wet weight) with a mean concentration of 1.551 ± 0.836 mg kg⁻¹ observed for the samples analysed with the mixture Pd(NO₃)₂ + Mg(NO₃)₂ as a matrix modifier. The highest observed mean arsenic concentration was 1.601 ± 0.908 mg kg⁻¹ with Pd(NO₃)₂. In contrast, the lowest one was 0.773 ± 0.458 mg kg⁻¹ determined with the Mg(NO₃)₂. It can be noticed that the mean As concentrations obtained with Pd(NO₃)₂ and with a mixture Pd(NO₃)₂ + Mg(NO₃)₂ are very similar (1.601 ± 0.908 and 1.551 ± 0.836 mg kg⁻¹, respectively). This finding indicates that the effect of Pd(NO₃)₂ on reducing the interference of the present matrix components is crucial. Similar mean As values were also obtained with Mg(NO₃)₂ and Ni(NO₃)₂ (0.773 ± 0.458 and 0.795 ± 0.340 mg kg⁻¹, respectively), indicating that these two modifiers work similarly. The mean As concentration obtained without matrix modifier was 1.281 ± 0.908 mg kg⁻¹ and was between the values obtained with Pd (NO₃)₂/(Pd(NO₃)₂ + Mg(NO₃)₂) and Mg(NO₃)₂/Ni(NO₃)₂. Based on these findings, it can be concluded that Mg(NO₃)₂ and Ni(NO₃)₂ interfere with the determination of arsenic and should not be used alone for these purposes.

The most extensive range of concentrations was found for the samples analysed without matrix modifiers, while the smallest was in the group with Ni(NO₃)₂ (Fig. 1). For the individual samples, the highest arsenic concentration of 4.144 mg kg⁻¹ was recorded using Pd(NO₃)₂. The lowest concentration was 0.010 mg kg⁻¹ in the sample without a matrix modifier. Several literature sources reported the mean arsenic values for shrimp in a similar range of 0.27–2.18 mg kg⁻¹.^{38,39} In Olmedo *et al.* work,³⁸ fresh samples had the higher mean As concentrations of 0.739 mg kg⁻¹ than 0.509 mg kg⁻¹ recorded in frozen samples. Other studies have reported much higher arsenic values for shrimp in the range of 19.13–51.18 mg kg⁻¹.¹⁵

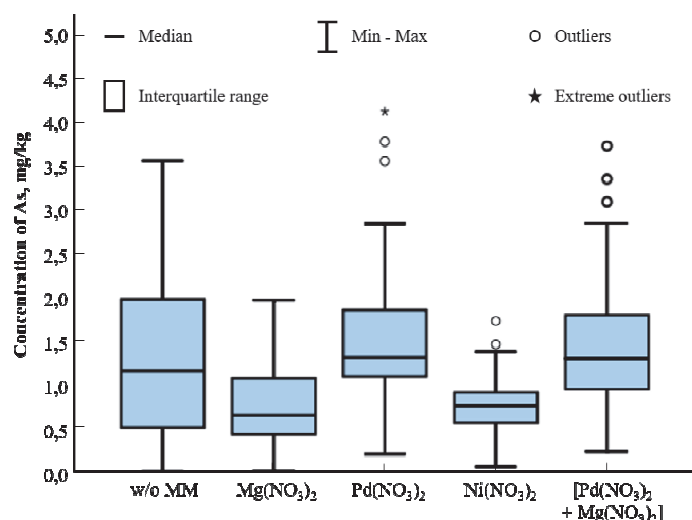


Fig. 1. Arsenic concentration (mg kg^{-1} , wet weight) for crustaceans, measured with different matrix modifiers.

In comparison with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$, the results with $\text{Pd}(\text{NO}_3)_2$ and without matrix modifier did not show a significant difference ($p = 0.173$ and $p = 1.000$, respectively). The mean arsenic concentrations were 3.22 % higher and 17.41 % lower, respectively, compared with the values obtained with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$. In contrast, the differences between As the concentrations obtained with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ and the concentrations obtained with $\text{Mg}(\text{NO}_3)_2$ and $\text{Ni}(\text{NO}_3)_2$ modifiers have shown statistical significance ($p < 0.05$). The mean arsenic concentration measured with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ was 50.17 % higher than the concentration observed with $\text{Mg}(\text{NO}_3)_2$ and 48.74 % higher than the value noticed with $\text{Ni}(\text{NO}_3)_2$.

Estimates for *THQ* and *CR* have shown that consumption of crustaceans does not pose significant non-carcinogenic or carcinogenic health risks. The calculated *THQ* results are far below the limit of 1 regardless of the matrix modifier used and the range between 1.2×10^{-4} and 2.5×10^{-4} . These values are significantly lower than those observed by the Bonsignore *et al.* study, ranging from 0.200 to 0.140.¹⁵ In addition to the lower levels of arsenic reported in this study, a significantly lower ingestion rate strongly contributed to the observed difference. These two factors notably influenced *CR*, calculated as 0.5×10^{-7} – 41.1×10^{-7} . Once again, these levels are much lower than those reported by Bonsignore *et al.*¹⁵

Cephalopods

Results of arsenic concentration in cephalopods are shown in Fig. 2. The mean arsenic concentration was $1.298 \pm 0.410 \text{ mg kg}^{-1}$ for the samples treated

with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$, which was the lowest value compared to crustaceans and molluscs (Table III). The observed mean As concentrations ranged from $0.582 \pm 0.242 \text{ mg kg}^{-1}$ for $\text{Ni}(\text{NO}_3)_2$ to $1.346 \pm 0.453 \text{ mg kg}^{-1}$ for $\text{Pd}(\text{NO}_3)_2$ modifier. In contrast, the lowest and the highest single sample arsenic concentrations were 0.020 and 2.752 mg kg^{-1} , respectively, observed in the samples not treated with matrix modifiers (Fig. 3). Pronounced diversity can also be found in the values of As concentration published in the literature. Some authors have reported relatively low arsenic levels below 5 mg kg^{-1} ,¹⁶ while the others have reported a high concentration of 36.63 mg kg^{-1} .⁴⁰ Several authors have reported arsenic levels in broad ranges, including the low and high boundaries mentioned above.^{14,18}

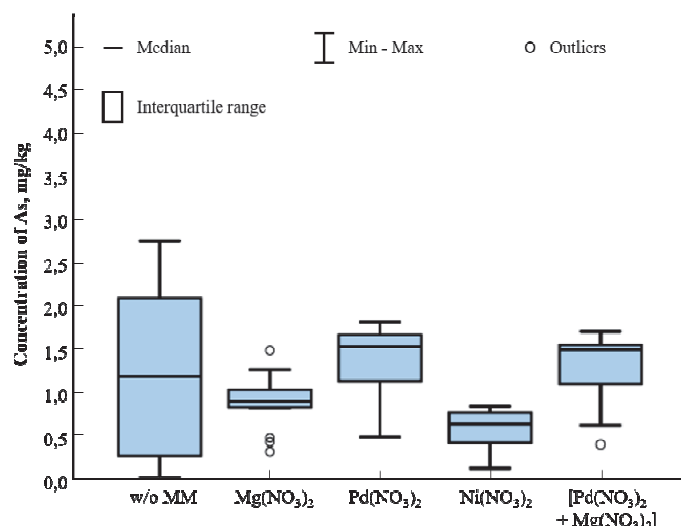


Fig. 2. Arsenic concentration (mg kg^{-1} , wet weight) for cephalopods, measured with different matrix modifiers.

In the same way as for crustaceans, a significant difference ($p < 0.05$) between the mean arsenic concentration measured with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ and both $\text{Mg}(\text{NO}_3)_2$ and $\text{Ni}(\text{NO}_3)_2$ modifiers was observed. The mean As value obtained with the mixture $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ was higher for 30.51 and 44.84 % compared with those measured separately with $\text{Mg}(\text{NO}_3)_2$ and $\text{Ni}(\text{NO}_3)_2$ modifiers. As expected, no significant difference has been determined when the mean As concentration obtained with $\text{Pd}(\text{NO}_3)_2$ was compared to the concentration obtained with $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ mixture ($p = 0.663$). The mean As value with $\text{Pd}(\text{NO}_3)_2$ was just 3.70 % higher than the value with the modifiers mixture. Similarly, the result without the matrix modifier does not show statistical significance ($p = 0.108$). Still, the mean As concentration

obtained with the modifiers mixture was higher by 5.62 % compared with concentration without matrix modifier.

Although the values of arsenic were the lowest in *cephalopods*, both carcinogenic and non-carcinogenic risks are higher comparing to crustaceans and molluscs due to the highest consumption. *THQ* scores were ranged from 1.5×10^{-4} to 5.5×10^{-4} , which is still well below the cut-off value of 1. The results are much lower than those reported by Bonsignore *et al.*, which have ranged from 0.15 to 2.00.¹⁵ A considerable difference was also observed in the results of *CR* scores. Calculated values for inorganic arsenic in this research were in the range 2.1×10^{-7} – 4.7×10^{-7} , whereas the *CR* score for cephalopods reported in the literature was considerably higher, amounted 2.6×10^{-4} – 8.1×10^{-4} .¹⁵

Molluscs

The results of arsenic concentration in molluscs are shown in Fig. 3. The obtained concentration ranged 0.163–4.301 mg kg⁻¹. The highest mean concentration of 2.794 ± 0.958 mg kg⁻¹ was observed in the group of results obtained with the mixture Pd(NO₃)₂ + Mg(NO₃)₂ as a matrix modifier. The lowest one of 0.774 ± 0.316 mg kg⁻¹ was obtained in the case of Mg(NO₃)₂ as a matrix modifier. The single sample's highest and lowest arsenic concentrations were also found in those two groups (4.301 and 0.163 mg kg⁻¹, respectively).

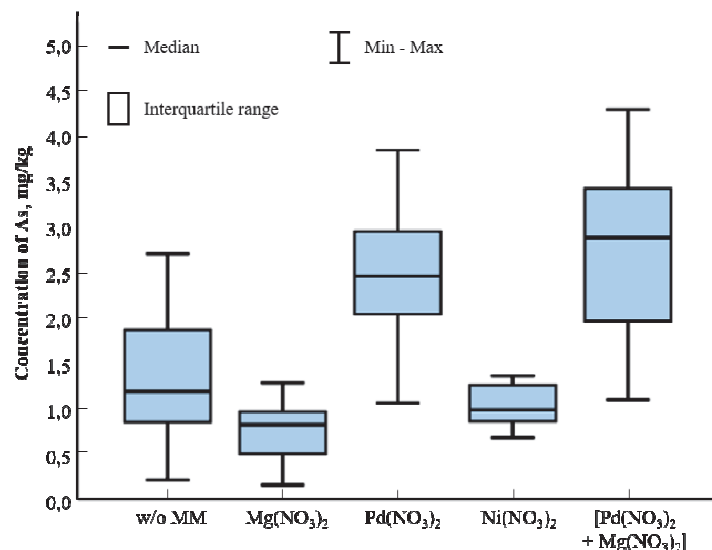


Fig. 3. Arsenic concentration (mg kg⁻¹, wet weight) for molluscs, measured with different matrix modifiers.

Similar to the results for crustaceans, it was again observed that the mean concentrations of As obtained with Pd(NO₃)₂ and a mixture of Pd(NO₃)₂ +

Mg(NO₃)₂, were quite close (2.537±0.793 and 2.794±0.958 mg kg⁻¹, respectively). Relatively close mean concentrations of As were also obtained when Mg(NO₃)₂ and Ni(NO₃)₂ were used (0.774±0.316 and 1.042±0.219 mg kg⁻¹, respectively). This finding again indicates that the mechanism of action of these modifiers, Mg(NO₃)₂ and Ni(NO₃)₂, which primarily eliminate interference from inorganic chlorides, in our research was not helpful. Indeed, according to Welz *et al.* sodium chloride caused very few problems in determining arsenic.²⁷

Most available literature sources have reported arsenic concentrations in molluscs with a similar range as found in our research. Several locations of the Adriatic Sea are the main source of fresh seafood products available at markets in Bosnia and Herzegovina. Mussels collected along the Croatian coast contained total arsenic in 2.33–2.56 mg kg⁻¹,^{28,40} while mussels at the coast of Montenegro contained 1.73–2.41 mg kg⁻¹.⁴² Recent research has shown that the main arsenic concentration in fresh and frozen mussels marketed in Serbia was 3.97 and 1.56 mg kg⁻¹, respectively.⁴³ The differences in arsenic concentration between fresh and frozen products are also described by Olmedo *et al.*, reporting lower As levels in both publications.³⁸

No statistically significant difference was observed between the results with the mixture Pd(NO₃)₂ + Mg(NO₃)₂ in comparison to the results with Pd(NO₃)₂ modifier ($p = 0.061$). The mean As concentration obtained with Pd(NO₃)₂ + Mg(NO₃)₂ mixture was slightly higher (9.20 %). Compared to other results, the mean concentration of As obtained with the mixture (Pd(NO₃)₂ + Mg(NO₃)₂) was significantly higher: 51.07 % than the mean concentration without matrix modifier, 72.30 % than with Mg(NO₃)₂ and 62.71 % than with Ni(NO₃)₂ modifier. All differences were statistically significant ($p < 0.05$). The low range with low mean values observed for the results with Mg(NO₃)₂ and Ni(NO₃)₂ clearly shows that these modifiers are not suitable for determining As.

Compared to crustaceans and cephalopods, molluscs had the highest observed As concentrations (Table III). Nevertheless, those concentrations are still well below the upper limit of 15 mg kg⁻¹ defined by the local Bosnia and Herzegovina regulation.³³ A low consumption rate of only 0.05 kg per capita per year³¹ resulted in very low *THQ* and *CR* scores. Ferrante *et al.* have determined a strong impact of the level of exposure to As through molluscs consumption on both non-carcinogenic and carcinogenic risks.³⁴ Calculated *THQ* scores from this work were in the range of 1.5×10^{-4} – 5.5×10^{-4} , which was much lower than 0.331–2.320 reported by Ferrante *et al.*³⁴ and also than 0.209–0.262 reported by Conte *et al.*¹⁹ Similarly, *THQ* scores calculated *CR* results in the range of 0.7×10^{-7} – 2.5×10^{-7} are still much lower than 1.49×10^{-4} , which was found to be the lowest level of exposure in Conte *et al.*¹⁹

CONCLUSIONS

This research has provided arsenic concentration in seafood available on markets in Sarajevo, the capital of Bosnia and Herzegovina. The obtained results have shown low levels of arsenic, toxic element associated accordingly with low health risks arising from seafood consumption. Since the portion of inorganic arsenic, which has a critical role in the impairment of human health, is minor in seafood, only the excessive exposure could increase health risks. With the current very low consumption rate of seafood products, both cancerogenic and non-cancerogenic risks associated with exposure to arsenic through seafood are very low for the residents of Bosnia and Herzegovina. Under the described conditions, different matrix modifiers did not show a considerably significant impact on the health risk assessment. However, it is essential to emphasize that some differences in As concentrations of the same samples measured using different matrix modifiers were more than 70 %. Additional research is needed to determine why some modifiers had better efficacy than others and to explain the intensive, beneficial response of seafood samples on $\text{Pd}(\text{NO}_3)_2$ matrix addition.

ИЗВОД

АРСЕН НА ТРЖИШТУ МОРСКИХ ПЛОВОДА У БОСНИ И ХЕРЦЕГОВИНИ: ЕФЕКТИ МОДИФИКАТОРА МАТРИЦЕ НА ИЗМЕРЕНУ КОНЦЕНТРАЦИЈУ

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Концентрација арсена у морским плодовима може потенцијално достићи веома високе вредности и представљати значајан здравствени ризик за људе. У овој студији, концентрација арсена у разним морским плодовима, као што су: ракови (шампи, козице), мекушци (дагње) и главоношци (лигње), доступним у свежем и смрзнутом стању на тржишту у Сарајеву, Босна и Херцеговина, одређена је електротермалном атомском апсорпционом спектрометријом (ETAAS). Упоредени су резултати добијени коришћењем различитих модификатора матрице: $\text{Mg}(\text{NO}_3)_2$, $\text{Ni}(\text{NO}_3)_2$, $\text{Pd}(\text{NO}_3)_2$ и смеше $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$. Најбоља стопа уклањања арсена концентрације 98,4 % за референтни материјал ERM-CE278k, је постигнута након додавања смеше $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$. Просечне концентрације арсена биле су $1,551 \pm 0,836$ и $1,298 \pm 0,410$, односно $2,794 \pm 0,958 \text{ mg kg}^{-1}$, за ракове, мекушце и главоношце, уз примену смеше $\text{Pd}(\text{NO}_3)_2 + \text{Mg}(\text{NO}_3)_2$ као модификатора матрице. Концентрације арсена у истом узорку мерене коришћењем различитих модификатора матрице су се веома разликовале, чак и преко изнад 70 %. Уз тренутну стопу потрошње производа од морских плодова, канцерогени и неканцерогени ризици повезани са изложеношћу арсену кроз морске плодове су веома ниски за становнике Босне и Херцеговине.

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Teaching chemical reactions to upper secondary school students: A laboratory approach

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Abstract: This study investigates the performance of upper secondary school students in the unit of chemical reactions following the implementation of a laboratory course in the context of the new Greek Chemistry Curriculum. The teaching intervention was run as a pilot program amongst 21 students and after the evaluation of the preliminary results, to another 76 students who were the final sample. Our findings show that most students who followed this laboratory course, were actively involved in the study of chemical reactions irrespectively of their performance in the school chemistry course. Students' overall school performance only played a role in the process of completing the chemical equations. Most of the students developed a solution strategy in the open inquiry stage. All students were enthusiastic with the teaching strategy employed in this teaching intervention and had a positive response when asked if they have a better understanding of chemical reactions.

Keywords: upper secondary school; chemical reactions; laboratory course.

INTRODUCTION

Chemical reactions are a central topic in chemistry education. Students' understanding of chemical reactions, including the transformation of substances and the distinction between chemical and physical changes, helps define the boundaries of chemistry in relation to other physical sciences.

A key factor is, for the students, to be able to link the observation of a chemical reaction at the macro level, with the chemical process occurring at the micro level, and also to comprehend the symbolism through a chemical equation. Johnstone

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first proposed the idea of three levels of chemistry knowledge (macro, micro and representation) as a useful framework for the understanding and the teaching of chemistry.¹ For the same reason Gilbert and Treagust used the terms sub micro, macro and symbolic to identify the three levels of representation that constitute the basis for the understanding of chemical concepts.² Although this framework has contributed significantly to the understanding of chemical phenomena, in the research conducted by Chandrasegaran *et al.* certain misconceptions between macroscopic and sub microscopic representations as well as a limited understanding of the symbolic representation system have been observed.³ In chemistry, there is no “perfect” chemical reaction in which the model approximates reality in the experimental process.⁴ A major impediment to conceptual understanding can be attributed to students’ inappropriate application of macroscopic reasoning to explain phenomena at the microscopic abstract level.⁵ Cheng and Gilbert have proposed two models of chemical reactions in the school chemistry curriculum: the “Particle model” of reactions in which chemical reactions involve a simple rearrangement of particles, which can be likened to a rearrangement of Lego blocks and the “Atomic model” in which the role of electrons in the chemical reaction is highlighted.⁶

Students’ understanding of the connection of chemical phenomena with chemical reactions has been the subject of limited research in Greece.^{7,8} These works have shown that students find it difficult to recognize when there is a new product formation, and also to transfer the concept of chemical reaction to the context of everyday life. The authors of this paper suggest that in order to address these difficulties, the use of the school laboratory is essential. Deficiencies that may exist in the laboratory equipment can be overcome by utilizing augmented reality, “Augmented chemical reactions”.⁹ In this direction, animations have also been utilized, but the problem that has been identified is that students tend to accept the animations as “correct” explanations without question or consideration for their limitations.¹⁰ We are going to show in this paper that the best possible solution is to involve the students in hands on experiments in the school laboratory in accordance with the new Chemistry Curriculum in Greece that is to be implemented, where the Inquiry-Based Method based on laboratory practice is proposed.¹¹

In Greece, chemistry is taught in grades 8 and 9 in the lower secondary school (1 hour per week), in grades 10 and 11 in upper secondary school (2 hours per week), and in grade 12 (6 hours per week) as an elective subject for those students who will choose the sciences orientation.¹² A serious problem is the number of teaching hours allocated with respect to the extent of the chemistry curriculum. Especially in upper secondary school, chemistry teaching is characterized as incomplete, and the laboratory character of the course is undermined.¹³ This is also the case in other countries, leading to a teacher-centred teaching model in which students have few opportunities to do experiments or solve problems.¹⁴

More specifically, chemical reactions as a unit are first taught in the eighth grade and are taught again in the tenth grade (first grade of upper secondary school). Unfortunately, the teaching of chemical reactions in upper secondary school is mainly carried out in the classroom through face-to-face teaching, although the percentage of school units, which have an adequately equipped science laboratory, is very high (90.4 %). Although the current syllabus include laboratory exercises, even in the form of a demonstration, teachers face a great difficulty in their implementation. This is because, in addition to the problem of time and insufficient teaching materials, most chemistry teachers do not have the appropriate laboratory teaching experience.¹⁵

The result of the above is that, during the teaching of chemical reactions, the concept of chemical reaction is identified in the student's mind with that of the chemical equation. The student practices ending is assessed on completing the products of a chemical equation and balancing it, given the reactants. It is an educational process that is more about solving mathematical equations than mastering chemical phenomena.¹⁶ Chemical concepts such as the production of a new substance are difficult to teach in the sense of direct consequence derived from an initial idea.¹⁷ The formation of a precipitate, the evolution of a gas or a change in colour is simply described by the teacher in class. In the best case, the student visits the school laboratory, but the chemical reactions are carried out in the form of a demonstration by the teacher. Students simply observe, and even get impressed in the view of some impressive chemical reaction, but perceive chemistry as something distant, as a science that can be dealt with by some experts, far from the direct experience of the students. The result is that the students approach chemistry through two worlds: that of the classroom in which they participate in the process, and that of the school laboratory and real life, where they watch an expert as spectators. Given these difficulties, this research investigates the performance of upper secondary school students in the unit of chemical reactions following the implementation of a laboratory course, making use of inquiry learning. More specifically, we investigate how students engage in inquiry and what are their responses and their attitudes when they are involved with this method in the chemistry laboratory.

Inquiry-based science education (IBSE)

Inquiry-based science education is recommended as a method for the teaching of Science in most western world science curricula. In the USA the National Research Council states that through the method of inquiry, students are led to master scientific concepts through research and develop their skills in designing and conducting research.¹⁸ In a European Commission survey on science education, IBSE emerges as the method that has increased student interest and achievement.¹⁹

For the application of IBSE it is necessary to use the school laboratory. During Lab sessions students understand science better, can ask more and better questions by observing chemical phenomena and develop high-level learning skills.²⁰ Initially, obstacles may arise because students and teachers are more familiar with the traditional way of performing laboratory exercises that is in the form of a cooking recipe. That is why teaching models that gradually lead students from lower to higher levels of inquiry need to be implemented.²¹

Unfortunately, while several IBSE projects have been developed in recent years, there is not much research on its application in school classrooms, so that there isn't a large amount of data to be used by teachers.²² The results from the survey of prospective chemistry teachers on the application of IBSE to chemical change were encouraging. As mentioned in the Introduction, it would be interesting to investigate the results of applying the method to students when these prospective teachers start working in schools.²³

Despite its clear advantages, the implementation of IBSE can create difficulties in terms of learning outcomes, especially when the student approaches new scientific concepts. It cannot be the solution for every problem in the teaching of sciences. While it is effective for developing skills and attitudes when learners must apply existing knowledge in new contexts, it is less effective for understanding new concepts. As a teaching strategy it does not necessarily lead to more successful learning outcomes.²⁴ Especially when we move from guided to open inquiry, students' degree of freedom increases but they can hardly process and draw conclusions.²⁵ Student's ability to design the solution to an unknown problem is not a given fact. Even in guided inquiry, a proper balance of student freedom and teacher support is needed.²⁶

The desirable result is that with the use of IBSE, in addition to attracting the students' interest which occurs due to the student-centred character of the method, students can be involved in inquiry and assisted in understanding new scientific concepts. The key to achieving this goal is careful planning and appropriate preparation of students. The topic and the activities must be carefully selected to adequately prepare students for their participation in inquiry-based learning (IBL) and design the connection between the research activities so that the desired learning outcome is achieved.²⁷ Adequate time is required for both the preparation and the implementation of the activities, which is often not the case.²⁸

Considering the difficulties arising from the implementation of IBSE based on the literature, the age of the students, and the need to understand the concepts in a fundamental category for the science of chemistry such as chemical reactions, we chose to include a preparatory session for students. The preparation of the students before the exploratory process in the laboratory was exclusively targeting the understanding of the general concepts of the chemical reaction, the changes (*e.g.*, energy) and the different categories of chemical reactions. There was nothing

taught about the specific reactions that the students exploratory observed and the chemical equations completed, as well as the process of determining the unknown solutions.

In new curriculum of Chemistry, in Greece, which will be implemented in 2025, for the chemical reactions' unit in the 10th grade, laboratory exercises are provided for redox, ion exchange and neutralization reactions in the form of a demonstration by the teacher. Students in groups simply record their observations for the relevant discussion that will follow. The paradox is that although the inquiry-based method through laboratory investigation is recommended, the students are once again limited to observation.

Research aim and research questions

The aim of this study is to explore students' ability to develop fundamental chemistry concepts, such as the concept of chemical reactions, through both guided and open-inquiry laboratory activities.

In this study we apply a laboratory approach with the direct involvement of students in the teaching chemical reactions. In the laboratory part in the first phase of our research, through guided research, students are asked to experimentally carry out chemical reactions, giving them clear instructions, to observe their result, complete the corresponding chemical equation, and identify the products.

In the second phase, students, like "young researchers", are asked to face an experimental problem in the school laboratory, think of a solution plan, follow it and considering their observations, evaluate the results and solve it. More specifically, the students were asked to design the procedure and carry out experiments to detect (qualitative analysis) the anions (or cations) present in three "unknown" solutions. Thus, students can approach the scientific method, get into the process of explaining experimental data and provide answers to the questions posed to them through an experimental process.

More specifically, this research will answer the following research questions:

1. Can students accurately complete chemical reactions in the school laboratory based on the instructions given to them?
2. Can students observe the chemical reactions taking place, record their observations and correctly complete the corresponding chemical equations?
3. Can students develop a strategy to solve an unknown problem in the school laboratory with appropriate preparation?
4. What is the students' attitude towards the applied teaching process?

EXPERIMENTAL

Sample

For the implementation of the present research, we chose students of the 1st Lyceum year (tenth grade), because in this class, chemical reactions are essentially introduced in the chemistry lesson. In addition, it is the first class of upper secondary education, so the level of the

students as well as their age allows their substantial participation in the specific teaching process.

For the present research, we initially piloted the specific teaching proposal in a section of the 1st Lyceum year located in the largest school complex in the country. Twenty-one (21) students participated.

After evaluating the results and making some small interventions in the teaching proposal, we proceeded to implement it in two schools: in a public school in the centre of Athens (whose students come from the lower sections of the working class, several from of them first or second-generation immigrants and some refugees) and in a private school in the south-eastern district of Athens. The research was conducted with a total of 76 first-year Lyceum students (aged 16), 52 from a public school and 24 from a private school. We secured the consent of the school and all students who participated in the research.

Experimental design

To carry out the didactic proposal, we prepared: a) lesson plans; b) a layout for classifying chemical reactions; c) a booklet with the rules of safety and behaviour in the school laboratory; d) two worksheets, one for each experimental procedure; e) a questionnaire consisting of five questions.

Means and materials we used for the workshop:

- Solutions: $\text{Pb}(\text{NO}_3)_2$, AgNO_3 , KI , Na_2CO_3 , NaCl , FeCl_3 , 1 M NaOH , HCl ($C = 1 \text{ mol/L}$)
- Cu wire, Fe fasteners and Zn filings
- Protective goggles, test tubes, dropper vials.

First teaching session (45 minutes). We brought back to the students' memory the difference between physical and chemical changes from the high school material, the principle of conservation of mass (whatever atoms participate in the reactants are also found in the products), the symbolism of a chemical reaction through a chemical equation, and the role played by the stoichiometric coefficients.

Second teaching session (45 minutes). We developed the four basic questions concerning a chemical reaction: when a chemical reaction takes place, how quickly it occurs and what factors affect its rate (concentration, temperature, contact surface, catalysts), and what energy changes accompany the chemical reaction.

Third teaching session (45 minutes). We categorized chemical reactions and taught redox reactions (synthesis, decomposition, single replacement) and substitution reactions (double replacement, neutralization).

Fourth teaching session (45 minutes). Acquaintance with the workshop, division into groups. A leaflet was distributed to the students and the rules of behaviour and safety in the laboratory were thoroughly discussed and the various instruments they would use were introduced.

Fifth teaching session (45 minutes). First laboratory exercise (guided inquiry). The students took their places at the laboratory benches, on which we had placed the necessary chemicals and laboratory equipment and worksheet I (Supplementary material to this paper, Table S-I). We ask students to mix the two reactants indicated in each case in the table below, in a test tube, and to write down the changes they observe, if any (change in color, formation of gas or precipitate, color of precipitate, *etc.*). We asked the students to complete the first worksheet.

Sixth teaching session (45 minutes). We discussed with the students the results of the first laboratory exercise and appropriate feedback was given.

Seventh teaching session (45 minutes). Second laboratory exercise (open inquiry). The students returned to their seats where we had placed the necessary chemicals, laboratory equipment and worksheet II (Supplementary material, Table S-II). We ask students to identify the anions found in the unknown solutions (X_1 , X_2 , X_3) by mixing them with the known solutions given to them. We asked the students to complete the second worksheet.

Eighth teaching session (45 minutes). We discussed with the students the results of the second laboratory exercise and appropriate feedback was given. Students completed a questionnaire based on methodology suggested by Bryman in 2004:²⁹

- 1) Have you visited the school laboratory again?
- 2) Did the school lab activities help you better understand chemical reactions?
- 3) Before performing the experiments, did you think you could do it?
- 4) Describe your experience in the school laboratory.

Due to the particularity of the student composition of the public school, the students filled in the questionnaire with their nationality and years of residence in Greece.

RESULTS AND DISCUSSION

In the following tables and figures we present the data from the worksheets and the questionnaire completed by the students. Table I shows the number of groups of public-school students (16 in total) who made correct or incorrect observations and who completed the chemical equations correctly or incorrectly for each of the 10 chemical reactions listed on the first worksheet. Mistakes made when completing the chemical equations are also noted.

TABLE I. Accuracy of observations and chemical equation completion by public school student groups for ten reactions in Worksheet 1, Laboratory Exercise 1, Question 1 ($N = 16$ groups)

Chemical reaction	Observation		Chemical equation		Mistakes		
	Correct	False	Right	Wrong	Blank	Oxidation number – chem. equation inscription	Coefficients
1	13	3	12	4	1	2	1
2	10	6	10	6	3	3	–
3	14	2	12	4	1	3	–
4	14	2	7	9	3	4	2
5	15	1	12	4	1	3	–
6	16	0	12	4	1	3	–
7	16	0	5	11	3	3	5
8	15	1	4	12	4	5	3
9	14	2	4	12	3	6	3
10	15	1	5	11	11	–	–

Table II shows the number of groups of public-school students (16 in total) who answered correctly, incorrectly, or did not answer the second and third questions listed on the first worksheet.

Table III presents the solution strategies developed by each group of public-school students during the open inquiry, their relevance to the results of the first

exercise and whether the chemical equations were completed correctly or incorrectly.

TABLE II. Public school student group responses to the second and third questions on the first worksheet ($N = 16$ groups)

Question	Right	Blank	Wrong
2	11	5	–
3	11	4	1 (colour of AgCl, PbI ₂)

TABLE III. Solution strategies of public school student groups: Laboratory exercise 2

Class	Group	x ₁	x ₂	x ₃	Solving strategy	Relevance to exercise 1	Chemical equations
A1	2	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively (partially)	Excellent	Wrong coefficients
	3	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Correct
	4	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Good	Correct
	1	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Correct
A2	1	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively (partially)	Moderate	Wrong oxidation number
	2	✓	✓	✓	Reaction of X1, X2, X3 with each other and with the known solutions	Very good	Correct
	3	–	–	–	They tried incompletely, making correct observations	Unsuccessfully	–
	4	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Correct
A3	4	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Wrong coefficients
	3	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Excellent	Just one wrong coefficient
	2	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Good	Wrong oxidation number
	1	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	–
A4	3	✓	✓	✓	Reaction of X1, X2 with all known solutions successively. X3 by exclusion	Moderate	Correct
	4	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Good	Wrong oxidation number
	2	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Unsuccessfully	–
	1	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	–

Fig. 1 shows the number of public-school students (52 in total) who answered yes, no or perhaps to the three closed-ended questions in the questionnaire.

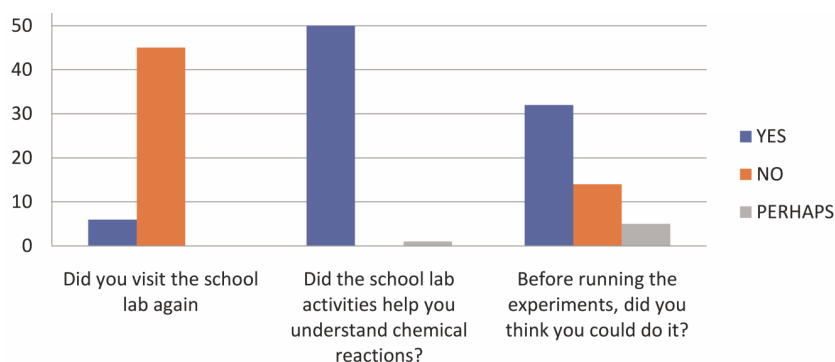


Fig. 1. Public school student responses to three closed-ended questionnaire questions ($N = 52$).

Table IV shows the number of private-school student groups (6 in total) who made correct or incorrect observations and who completed the chemical equations correctly or incorrectly for each of the 10 chemical reactions listed on the first worksheet. Mistakes made during the completion of the chemical equations are also noted.

TABLE IV. Accuracy of observations and chemical equation completion by private school student groups for ten reactions in Worksheet 1, Laboratory Exercise 1, Question 1 ($N = 6$ groups)

Chemical reaction	Observation		Chemical equation		Mistakes		
	Correct	False	Right	Wrong	Blank	Oxidation number – chem. equation inscription	Coefficients
1	2	4	6	–	–	–	–
2	2	4	6	–	–	–	–
3	2	4	3	3	–	3	–
4	6	–	5	1	–	–	1
5	6	–	6	–	–	–	–
6	6	–	6	–	–	–	–
7	6	–	5	1	–	1	–
8	6	–	5	1	–	–	1
9	6	–	4	2	–	–	2
10	6	–	2	4	4	–	–

Table V shows the number of private-school student groups (6 in total) who answered correctly, incorrectly, or did not answer the second and third questions listed on the first worksheet.

Table VI presents the solution strategies developed by each group of private-school students during the open inquiry, their relevance to the results of the first

exercise, and whether the chemical equations were completed correctly or incorrectly.

TABLE V. Private school student group responses to the second and third questions on the first worksheet ($N = 6$ groups)

Question	Right	Blank	Wrong
2	4	1	1 (colour AgCl)
3	4	1	1 (order of activity Zn, Cu, H, Ag)

TABLE VI. Solution strategies of private school student groups during open inquiry: Laboratory exercise 2

Group	x_1	x_2	x_3	Solving strategy	Relevance to exercise 1	Chemical equations
1	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Excellent	–
2	-	-	-	Reaction of X1, X2, X3 with all known solutions successively	Good	Correct
3	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Good	Correct
4	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Correct
5	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Very good	Correct
6	✓	✓	✓	Reaction of X1, X2, X3 with all known solutions successively	Excellent	Correct

Fig. 2 shows the number of private-school students (24 in total) who responded yes, no, or perhaps to the three closed-ended questions in the questionnaire.

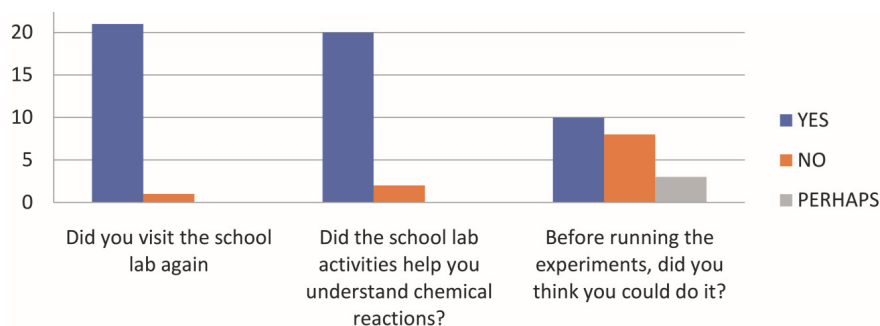


Fig. 2. Private school student responses to three closed-ended questionnaire questions ($N = 24$).

Indicative answers to the open-ended question: “Describe your experience in the school laboratory” (Supplementary material). We can group them into three main categories.

1) *The pedagogical environment during the implementation.* The students described their experience as positive, really nice, enjoyable, unforgettable, unique, an interesting way to learn.

2) *The effectiveness.* The students reported satisfactory collaboration, increased interest in the topic, and improved understanding of chemical reactions.

3) *The confidence gained.* All students expressed enthusiasm in completing the activities, even those who had some inhibitions at the beginning of the process.

From the results of completing the worksheets, it appears that all groups that participated in the study, made a serious effort both in performing the experiments and in drawing conclusions (Tables I, II, IV and V). Only one group from the public school (3rd group of class A2) faced serious difficulty, in fact they failed to complete the worksheets (Table III).

Although there was a huge difference in the percentage of students who had participated in an exercise in the school laboratory between the public 5.9 % (Fig. 1) and the private school 95.5 % (Fig. 2) all students did not face any difficulty in performing the experiments. The wording of the observations was generally correct (Tables I and IV) except for reactions 1 and 2 for which the well-known misconceptions that iron rusts and copper moulds were seen.

In the answers to the questions on the first worksheet there was a clear difference between the public school and the private school. The percentage of correct answers was 51.9 % for the public sector (Tables I and II) and 80 % for the private sector (Tables IV and V). It appeared that private school students were better prepared and more likely to complete chemical reactions correctly. About half of the errors in completing the chemical equations were due to incorrect coefficients, and the other half were due to incorrectly written chemical formulas. The mistakes were mainly due to gaps in students' theoretical knowledge from previously taught units, such as writing chemical formulas and understanding oxidation numbers. These findings align with those of Rosenthal and Sanger, as well as Kelly, who noted that students often struggle with incorrectly writing chemical formulas.^{30,31}

A large percentage of students did not complete the tenth reaction equation, the neutralization of HCl with NaOH, even though it was the simplest one (Tables I and II). This was most likely due to the design of the handout, as students may not have realized that they were required to complete it. Unfortunately, this issue had not been identified during the trial at the pilot school, so we were unable to adjust the worksheet format in advance. Moreover, since we adhered to our original plan that teachers would not intervene during the activity, it was not possible to correct the problem in the school laboratory.

All groups except the one mentioned above tried to develop a solution strategy in the free discovery stage. The vast majority 81.2 % in the public (Table III) and 100 % in the private sector (Table IV) chose to successively add all the known solutions to the three unknown solutions and record the results. Based on these,

they answered the second worksheet correctly. There was a relatedness of correct answers between the two worksheets as shown in the table (Tables III and V). We do not underestimate the possibility of students copying from each other in completing the second worksheet since it is only three answers, the distance of the lab benches is very short, but the worksheets show the strategy chosen and the effort put in.

A very large percentage of students – 98 % in the public sector (Fig. 1) and 90 % in the private sector (Fig. 2), reported that this process helped them better understand chemical reactions. However, 31 % of public school students (Fig. 1) and 38 % of private school students (Fig. 2) initially had reservations about their ability to succeed. Despite this, nearly all students actively participated and enthusiastically embraced this innovative teaching approach, as reflected in their responses to the 5th question of the questionnaire.

CONCLUSION

All students who participated in the research were involved in studying chemical reactions in the school laboratory. Most performed the reactions accurately and made correct observations regarding the chemical phenomena. Errors in completing the chemical equations were primarily due to theoretical gaps from previously taught units.

With appropriate theoretical and laboratory preparation, most students successfully developed a strategy for solving the unknown problem presented to them in the school laboratory. Through this teaching method, students not only connected theory with practice but also followed the steps of the scientific method during the experiment. Additionally, they developed a responsible attitude by adhering to safety rules and collaborating with their classmates.

All the students were enthusiastically engaged in this learning process, and a significant number of them stated in the questionnaire that it was an unprecedented experience and that this is how chemistry courses should be taught.

We conclude that students who have the appropriate theoretical and laboratory preparation can engage in research during chemistry instruction, tackle an unknown problem in the school laboratory, and devise a strategy to solve it. Through this process, students can overcome the stereotype that science is distant from their everyday experience, viewing it as a set of unknown symbols written on the blackboard, disconnected from reality. This teaching intervention enables students to develop a more positive attitude toward chemistry and chemical reactions, gain a deeper understanding of the importance of chemistry in everyday life, and become better equipped to conduct laboratory inquiries.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: <https://www.shd-pub.org.rs/index.php/JSCS/article/view/12609>, or from the corresponding author on request.

ИЗВОД

НАСТАВА О ХЕМИЈСКИМ РЕАКЦИЈАМА ЗА УЧЕНИКЕ ВИШИХ РАЗРЕДА СРЕДЊИХ ШКОЛА: ЛАБОРАТОРИЈСКИ ПРИСТУП

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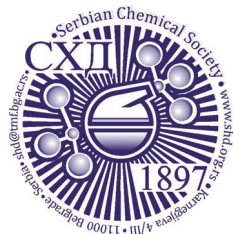
Ова студија истражује успешност ученика виших разреда средњих школа, после примене лабораторијског курса о хемијским реакцијама, у оквиру новог наставног програма хемије у Грчкој. Наставна интервенција је најпре спроведена као пилот програм са двадесетједним учеником, а након евалуације прелиминарних резултата, укључено је још 76 ученика који су чинили коначан узорак. Наши налази показују да је већина ученика који су похађали овај лабораторијски курс активно учествовала у проучавању хемијских реакција, без обзира на њихов претходни успех у редовној настави хемије. Општи школски успех ученика играо је улогу једино у поступку писања једначина хемијских реакција. Већина ученика је развила стратегије решавања током фазе отвореног истраживања. Сви ученици су са одушевљењем прихватили примењену наставну стратегију током интервенције и позитивно су одговорили на питање да ли сада боље разумеју хемијске реакције.

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SUPPLEMENTARY MATERIAL TO
**Teaching chemical reactions to upper secondary school students:
A laboratory approach**

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CHEMICALS AND LABORATORY EQUIPMENT FOR THE FIFTH TEACHING
SESSION

- a) 8 dropper vials of the solutions $\text{Pb}(\text{NO}_3)_2$, AgNO_3 , KI , Na_2CO_3 , NaCl , FeCl_3 , NaOH $C=1$ mol/L, HCl $C=1$ mol/L), 2 Cu wires, 1 Fe fastener
- b) 2 test tube racks with 10 test tubes, protective goggles and gloves

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TABLE S-I. First worksheet, guided inquiry

	Chemical reaction	Observations
SIMPLE REPLACEMENT		
1	1 mL $\text{CuSO}_4(\text{aq}) + \text{Fe}(\text{s}) \rightarrow$	
2	1 mL $\text{AgNO}_3(\text{aq}) + \text{Cu}(\text{s}) \rightarrow$	
3	1 mL $\text{HCl}(\text{aq}) + \text{Cu}(\text{s}) \rightarrow$	
4	1 mL $\text{HCl}(\text{aq}) + \text{Zn}(\text{s}) \rightarrow$	
DOUBLE REPLACEMENT		
5	1 mL $\text{NaCl}(\text{aq}) + 10\text{-}15 \text{ drops } \text{AgNO}_3(\text{aq}) \rightarrow$	
6	1 mL $\text{KI}(\text{aq}) + 10\text{-}15 \text{ drops } \text{AgNO}_3(\text{aq}) \rightarrow$	
7	1 mL $\text{KI}(\text{aq}) + 10\text{-}15 \text{ drops } \text{Pb}(\text{NO}_3)_2(\text{aq}) \rightarrow$	
8	1 mL $\text{FeCl}_3(\text{aq}) + 10\text{-}15 \text{ drops } \text{NaOH}(\text{aq}) \rightarrow$	
9	1 mL $\text{Na}_2\text{CO}_3(\text{aq}) + 10\text{-}15 \text{ drops } \text{HCl}(\text{aq}) \rightarrow$	
NEUTRALIZATION		
10	[1 mL $\text{NaOH}(\text{aq}) + 2\text{-}3 \text{ drops of phenolphthalein}$] (solution color:.....) + + $\text{HCl}(\text{aq})$, carefully, until the indicator changes color (color of solution.....)	

EXERCISES:

- Write, in the table above (next to the dots), the chemical equations of the reactions that "take place", showing the precipitate or gas, where it is produced.
- Write the colours of the following precipitates:
 - AgCl , b) AgI ,
 - PbI_2 , d) $\text{Fe}(\text{OH})_3$
- Based on the experiments you did, rank the elements Cu, Zn, H, Ag, in increasing order of activity, justifying your answer
.....

CHEMICALS AND LABORATORY EQUIPMENT FOR THE SEVENTH TEACHING SESSION

- 4 dropper vials of known solutions $\text{Pb}(\text{NO}_3)_2$, KI, Na_2CO_3 , HCl, 3 dropper vials of the unknown solutions (X_1 : Na_2CO_3 , X_2 : HCl, X_3 : KI)
- 2 test tube holders with 12 test tubes, protective goggles and gloves

TABLE S-II. Second worksheet, open inquiry

1. Research Question: Design an experiment to detect the anions present in each of the "unknown" solutions X1, X2, X3. Write down the steps/procedure you will follow, justifying your choices. List the instruments and substances you will use in each step.
2. Carry out the experiment you designed. Write the experimental results you used to find the contents of each vial.
3. Write the chemical equations of the reactions that took place.
4. Complete the table:

Solution	X1	X2	X3
Anion which it contains			

INDICATIVE ANSWERS TO THE OPEN-ENDED QUESTION

Public school:

1. The experiments in the school laboratory left me with positive feelings, because in addition to being easier to understand, they were also more fun.
2. It was really nice, and I would like to do it again in the future.
3. It was an enjoyable way to learn Chemistry and do experiments, resulting in better remembering of what we were taught.
4. It was a nice experience, because it was the first time, I entered a workshop, and I even participated.
5. When some reactions changed colour, I was surprised, and it was really cool.
6. I had quite a good time in the laboratory, we did experiments and learned new things.
7. It was very good; it helped me understand Chemistry much more and to be able to have a hands-on experience.
8. I thought it would be somewhat difficult because it was the first time I entered a laboratory, but in general the teacher's instructions and the cooperation with the group made things easier.
9. At first, I had my usual anxiety but nothing out of the ordinary. Then it became very easy, although a bit boring because I was the one who wrote all the reactions.
10. It was something new for me and I was quite wary but, in the end, it wasn't too difficult, and I really enjoyed it.
11. The experience was even better because we did the tasks in groups, so if someone was "stuck" or didn't know something, they helped the other and when we completed the experiments correctly, we felt satisfaction.
12. It was undoubtedly an unforgettable experience. If I had the chance, I would do it again without hesitation. I was able to understand the course better and was given the opportunity to interact with my classmates.

13. The experience was unique. Many schools do not offer such things. What we all experienced was fantastic.
14. I found it quite a good experience both with my group and with the teacher who helped us understand. We spent our time pleasantly and creatively.
15. It was a good experience that I have never experienced before, and I enjoyed it. I learned more about chemical reactions.
16. It has helped me understand chemical reactions more and I am now more interested in them as a subject.
17. When I entered the laboratory, I also felt like one of those who work in a laboratory. I really liked it, and I would also like to do a job like that.
18. I really liked it. And it was a very nice experience, since I also understood the reactions.
19. I would also like to enter the laboratory again next year and do experiments again and have teachers doing a demonstration again.
20. It was very nice. It helped us understand the reactions and we also had fun at the same time.
21. It's amazing to do experiments. I was very happy to be able to do them with the other girls.
22. It was perfect. It was the first time we did a lesson in a lab, and it will be unforgettable. Experiments helped me understand Chemistry better.

Private school:

1. I really liked it, and I would like to go to the labs more often, since it helps our understanding.
2. Exciting, as we were given the opportunity to do various experiments with lab equipment.
3. It was a very nice experience, since I did things that I probably won't do again.
4. Very positive experience, much more interesting, experiments with evidence, practical study. They helped me learn more about the reactions.
5. Nice experience with unexpected results.
6. It was an interesting way to learn about chemical reactions.
7. Pleasant and interactive, nice cooperation with classmates.
8. It was a pleasant and interesting teaching session that I believe gave us knowledge that will stay with us.
9. Nice experience, impressive reactions, organized.