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The first evidence of lumiphorbol as a metabolite of the *Euphorbia* species

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Abstract: *Euphorbia nicaeensis* All. belongs to the flowering plant family Euphorbiaceae. Our previous research has shown that the latex of this species is a rich source of jatrophone-type diterpenes. Due to the exceptional biological activities exhibited by these compounds, the research was extended to the root of this plant species. From the roots of *E. nicaeensis*, collected in Deliblato Sand (Serbia), a diterpene from an extremely rare class of lumiphorbol was isolated for the first time from *Euphorbia* species. The structure of the isolated compound was determined by spectroscopic techniques (1D- and 2D-NMR), as well as HRESIMS data.

Keywords: *Euphorbia nicaeensis*; Euphorbiaceae; diterpenes; lumiphorbol.

INTRODUCTION

Species of the *Euphorbia* genus are well known for their impressive structural diversity and adaptability to different environments.¹ These plants produce a wide range of secondary metabolites, among which diterpenes stand out due to their bioactivity and potential application in modern medicine.² Diterpenes from *Euphorbia* species are structurally diverse, including compounds such as phorbol esters, ingenane derivatives, jatrophone diterpenoids and many others.^{3–5} These metabolites often have protective roles in plants, such as deterring herbivores and protecting themselves from pathogens.^{5–8} However, their importance goes beyond the approved protective role in plants, as studies have shown promising pharmacological properties of these metabolites. A well-known group of diterpenes derived from *Euphorbia* are phorbol esters. These compounds are known for their

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ability to activate protein kinase C (PKC), an enzyme involved in cellular signaling pathways that regulate various cellular functions, including proliferation, differentiation and apoptosis.⁹ Although phorbol esters are associated with tumour-promoting activity, their mechanism of action has been crucial in cancer research, helping to develop therapeutic strategies targeting PKC.⁹ On the other hand, many phorbol derivatives have been shown not to have tumour-promoting effects, but rather exhibit anticancer activity against certain human cancer cell lines.^{10–12} Additionally, tiglane derivatives have demonstrated strong inhibitory activity against HIV-1 and HIV-2.¹³ Ingenane derivatives play a significant role in modern medicine. Ingenol-mebutate, derived from *Euphorbia peplus*, was approved for the topical treatment of actinic keratosis, a precursor of skin cancer.¹⁴ Its mechanism involves inducing rapid cell death in abnormal cells while minimizing damage to healthy tissue.¹⁴ However, it was withdrawn from use in the European Union in 2019 due to safety concerns.¹⁵ Research indicated a potential link between long-term use of ingenol-mebutate and an increased risk of skin cancer, including basal cell carcinoma and squamous cell carcinoma, and that the drug's risks outweigh its benefits.¹⁵ The most specific and rare class of diterpenes isolated from the *Euphorbia* genus are lumiphorbols, which are considered phorbol derivatives. It has been experimentally proven that lumiphorbols are derived from tiglane esters, where rings A and B are *cis* connected. The [2+2] cycloaddition, involving the double bonds at positions C1(2) and C6(7), occurs in the presence of light with a wavelength of 254 nm under laboratory conditions, while the mechanism of their formation in plants, particularly in the root, which is not exposed to UV radiation, remains unknown. A notable example is lumiphorbol triacetate, a cage derivative of 4 α -phorbol, which was first described by E. Hecker and his colleagues in 1968.¹⁶ In their study, Hecker *et al.* detailed the synthesis of lumiphorbol triacetate *via* ultraviolet (UV) irradiation of 4 α -phorbol-12,13,20-triacetate. This process induces an intramolecular cycloaddition, resulting in the unique cage-like structure of lumiphorbol.¹⁷ Due to its extremely specific structure, the biological activity of lumiphorbol derivatives was also investigated. For example, the ability of lumiphorbol-12,13,20-triacetate to activate PKC was evaluated. This compound was found to activate PKC by 93 % at high concentrations, while no activation was observed at standard concentration.¹⁸

Our previous study,¹⁹ where the subject of investigation was the latex of *E. nicaeensis*, showed that the species is a rich source of jatrophone derivatives that showed a strong inhibition of P-gp, so after the initial investigation, the research was extended to the root of the species.

EXPERIMENTAL

Apparatus and reagents

The optical rotations were measured on an Autopol IV (Rudolph Research Analytical) polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. All NMR data were

acquired on Bruker Avance III 500 NMR spectrometer (500 MHz for ^1H - and 125 MHz for ^{13}C -NMR, in CDCl_3 , with TMS as internal standard). The NMR spectra were analysed using TopSpin 3.6.2 software. The high-resolution LC/ESI positive TOF mass spectra were measured on a HPLC instrument (Agilent 1200 Series) coupled with a 6210 Time-of-Flight LC/MS system (Agilent Technologies). NP-HPLC-DAD: Agilent Technologies 1260 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax RX-Sil (250 mm $\times$ 9.4 mm; 5 μm) column. RP-HPLC-DAD: Agilent Technologies 1100 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax XDB-C18 column (250 mm $\times$ 9.4 mm; 5 μm). Dry-column flash chromatography (DCFC, Shusterman *et al.*, 1997) was performed on silica gel (ICN Silica 12–26 60 Å, Merck). Silica gel 60 F254 precoated aluminium sheets (0.25 mm, Merck) for TLC control were used. The TLC plates were visualized under a UV lamp at 254 nm and detected by spraying with solution of cerium molybdate in sulphuric acid, followed by heating. All solvents used for HPLC were HPLC grade, while all used solvents for DFCC and TLC were at least of analytical grade.

Plant material

The roots of *Euphorbia nicaeensis* were collected at Deliblato Sands (Serbia), collection site at latitude: 44°56'57" N and longitude: 21°11'13" E, in May 2018. The plant was identified by Professor Petar Marin, University of Belgrade – Faculty of Biology, Institute of Botany. Voucher specimen (No. 16855) has been deposited at the Herbarium of Botanical Garden “Jevremovac”, University of Belgrade, Belgrade (Serbia).

Isolation and purification

The process of isolating lumiphorbol involved the preparation of a root extract through continuous extraction with a 96 % aqueous ethanol solution. 152 g of ground *E. nicaeensis* roots were packed into a Soxhlet extraction flask and 250 g of 96 % ethanol was added for continuous extraction with heating for 2 h, and then left overnight in the solvent. After removal of the solvent by evaporation on a rotary vacuum evaporator, the obtained extract (25 g) was fractionated by DCFC using silica gel as the stationary phase and a mixture of petroleum ether and acetone in varying volume ratios as the mobile phase. Fraction F3 (2.1 g), obtained by elution with 15 % acetone in petroleum ether, was further fractionated by DCFC on silica gel using a mixture of petroleum ether and acetone in different ratios (98:2, 97.5:2.5, 95:5, 90:10, 80:20) as the mobile phase. The collected fractions were monitored by TLC, and similar fractions were combined, resulting in thirteen fractions (1–13). Fraction F3/10 (367.5 mg) was subjected to separation by NP-HPLC on silica gel (Zorbax Rx-SIL column, 250 mm $\times$ 9.4 mm, 5 μm) with *n*-hexane and acetone (95:5, isocratic mode, flow 3 mL/min, 25 °C, 227 nm, stop time 30 min, post time 1 min), yielding six subfractions, F3/10/I to F3/10/VI. The final separation of fraction F3/10/VI was carried out on RP-HPLC using a Zorbax XDB C18 column (250 mm $\times$ 9.4 mm, 5 μm) and a mixture of water and acetonitrile (ACN) in gradient mode as the mobile phase (50–80 % ACN (0–10 min), 80–90 % ACN (10–15 min), 90–100 % ACN (15–21 min), flow 4 mL/min, 25 °C, 227 nm, stop time 21 min, post time 2 min). As a result of this purification, lumiphorbol (0.7 mg) was isolated. The NMR spectra and HRMS data are available in Supplementary material to this paper.

RESULTS AND DISCUSSION

Luminicaenin A (**1**) was isolated as a colourless amorphous substance. The ion $[\text{M}+\text{H}]^+$ at m/z 479.2424 in HRESIMS indicated the molecular formula $\text{C}_{29}\text{H}_{34}\text{O}_6$, (Calcd. for $\text{C}_{29}\text{H}_{35}\text{O}_6$, 479.2428), corresponding to 13 degrees of

unsaturation. NMR spectra (Table I) revealed the functional groups, such as a 3-keto group (δ_{C} 218.1 ppm), a tertiary OH (δ_{H} *ca.* 5.2 ppm, *brs*; δ_{C} 82.6 ppm, C-9), and two ester residues, 12-benzoate (δ_{H} 8.03 ppm, *d*, 8, 2H, δ_{H} 7.46 ppm, *t*, 8 2H and δ_{H} 7.58 ppm, *t*, 1H; δ_{C} 166.2 ppm and δ_{C} 130.3, 129.9, 128.6 and 133.3 ppm) and 13-acetate (δ_{H} 2.12 ppm, *s*, 3H; δ_{C} 173.0 and 21.2 ppm). When the saturations originating from the mentioned functional groups are subtracted from the total number of saturations, six more unsaturations remain in the skeleton, which must be distributed through six rings given that in the NMR spectra there are no signals originating from carbon-carbon double bonds. The clue for a cage structure containing six rings was mostly obtained from HMBC, COSY and NOESY correlations shown in Fig. 1, as well as by the comparison of ^1H -NMR spectral data with those of the related lumiphorbols obtained by the intramolecular [2+2] photocycloaddition of 4 α -phorbol derivatives.¹⁷

TABLE I. ^1H - and ^{13}C -NMR data of **1** (500 MHz for ^1H , and 125 MHz for ^{13}C , CDCl_3 , TMS)

Compound	^1H (δ / ppm, <i>J</i> / Hz)	^{13}C (δ / ppm)
1	2.30, <i>t</i> , 6	43.4
2	—	42.9
3	—	218.1
4	2.43, <i>brs</i>	46.48
5α	3.24, <i>d</i> , 12	38.7
5β	1.59, <i>brd</i> , 13	—
6	—	46.50
7	2.75, <i>t</i> , 5	52.2
8	1.76, <i>dd</i> , 10, 5	55.0
9	—	82.6
10	2.72, <i>t</i> , 5	44.2
11	1.67, <i>qui</i> , 7	51.5
12	5.78, <i>d</i> , 8	81.2
13	—	68.2
14	1.49, <i>d</i> , 10	30.3
15	—	28.5
16	1.20, <i>s</i>	24.1
17	1.39, <i>s</i>	18.0
18	1.07, <i>d</i>	14.4
19	1.12, <i>s</i>	7.2
20	1.02, <i>s</i>	23.3
12-OR	—	—
1'	—	166.2
2'	8.03, <i>d</i> , 8	130.3
3'	7.46, <i>t</i> , 8	129.9
4'	7.58, <i>t</i> , 8	128.6
5'	—	133.3
13-OR	—	—
1''	—	173.0
2''	2.12, <i>s</i>	21.2

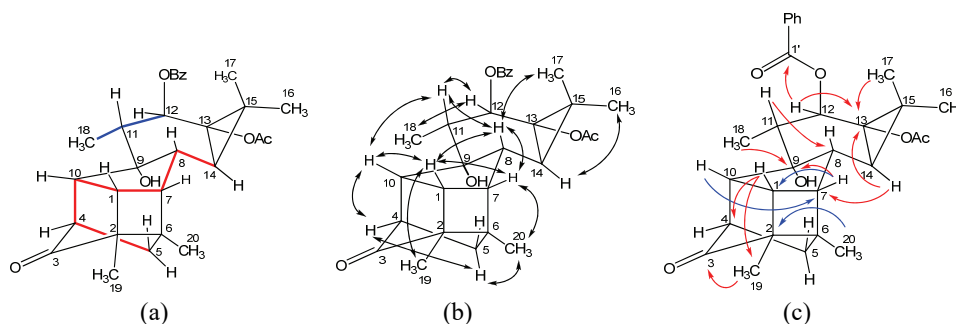


Fig. 1. 2D correlations of **1**; a) COSY correlations; spin system A (red), spin system B (blue), b) NOESY correlations and c) HMBC (H→C) correlations (the correlations occurring across four-membered ring marked blue).

The COSY spectrum of **1** has shown two independent spin systems A and B. The first one (A) included H-1, H-4, H-5, H-7, H-8, H-10 and H-14, while the second (B) included protons H-11, H-12 and H-18. The COSY correlation H-1/H-7 indicated connectivity C(1)–C(7), which is also supported by the HMBC correlation H-7/C-1. At the same time, the HMBC correlations H3-20/C-2 and H-10/C-7 (Fig. 1c, marked blue) were in accordance with the C(1)–C(7) connectivity in the four-membered ring. The NOEs (Fig. 1b) also supported the proposed cage structure of **1**. The structure of **1** was interpreted as 4,20-deoxy-4 α -lumiphorbol-12-benzoate-13-acetate.

Unlike the tiglane core, which is commonly found in Euphorbiaceae, lumiphorbol derivatives are extremely rare. To the best of our knowledge, only one diterpene with the lumiphorbol structure, *i.e.*, 12-*O*-palmitoyl-4-deoxy-16-hydroxylumiphorbol-13-acetate isolated from *Aleuritis fordii* (Euphorbiaceae) from Japan has been described in the literature so far.²⁰ Otherwise, lumiphorbol derivatives reported in the literature are the products of the intramolecular [2+2] photocycloaddition reaction of 4 α -phorbols carried out by irradiating with UV light ($\lambda = 254$ nm).^{16,17}

CONCLUSION

This study identified the roots of *E. nicaeensis* as a source of an extremely rare lumiphorbol diterpene skeleton. Considering all the facts presented, it can be concluded that lumiphorbols represent a significant class of compounds, with unique structural features and still unexplored biological activities due to their rarity, which indicates the need for further chemical and pharmacological research.

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/13212>, or from the corresponding author on request.

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ИЗВОД

ПРВИ ДОКАЗ ЛУМИФОРБОЛА КАО МЕТАБОЛИТА ВРСТЕ РОДА *Euphorbia*

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Euphorbia nicaeensis All. је цветница из породице Euphorbiaceae. Наше претходно истраживање је показало да је латекс ове врсте богат извор јатрофанских дитерпена. Због изузетних биолошких активности које ови дитерпени показују, истраживање је проширено на корен врсте у потрази за новим дитерпенима. Из корена *E. nicaeensis*, прикупљеног у Делиблатској пешчари, изолован је по први пут из врста рода *Euphorbia* дитерпен који припада изузетно реткој класи лумифорбола. Структура изолованог једињења одређена је применом спектроскопских техника (1D- и 2D-NMR), као и масене спектрометрије високог разлагања (HRESIMS).

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SUPPLEMENTARY MATERIAL TO
**The first evidence of lumiphorbol as a metabolite of the
Euphorbia species**

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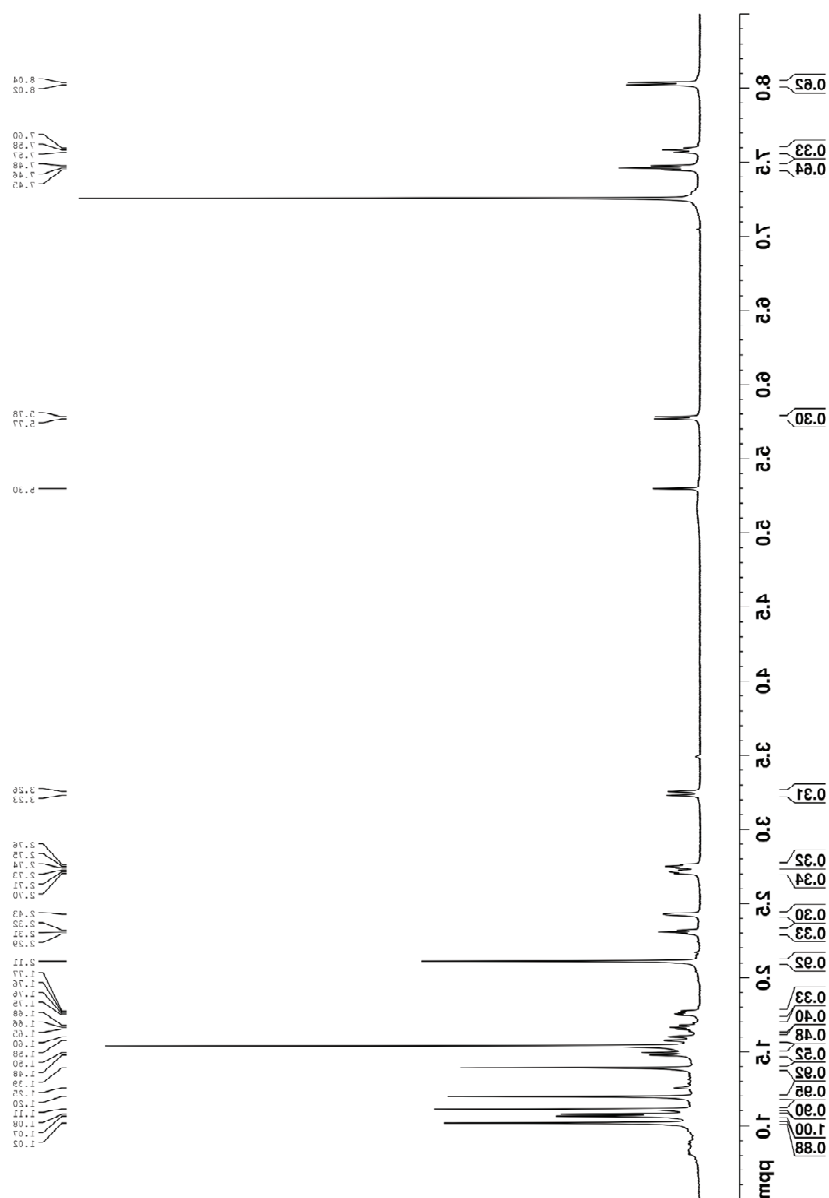
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Figure S1: ^1H -NMR (500 MHz, CDCl_3) spectrum of compound **1**

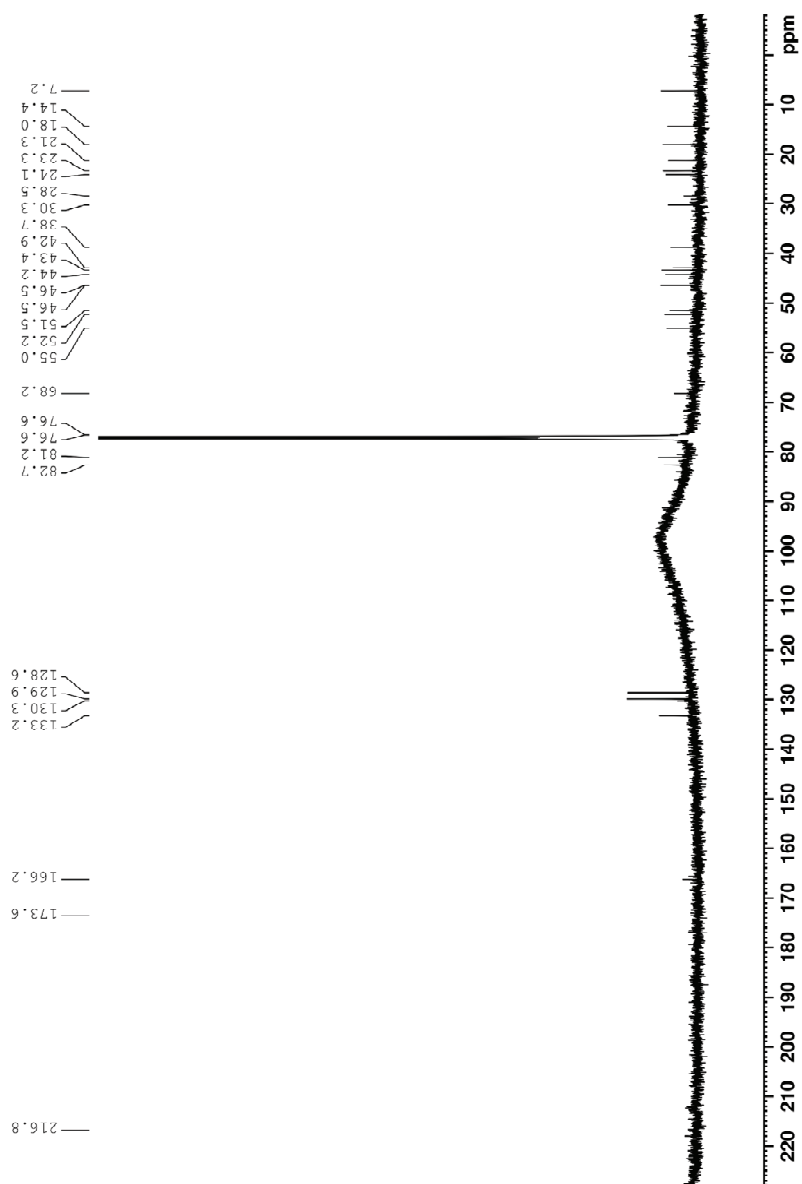
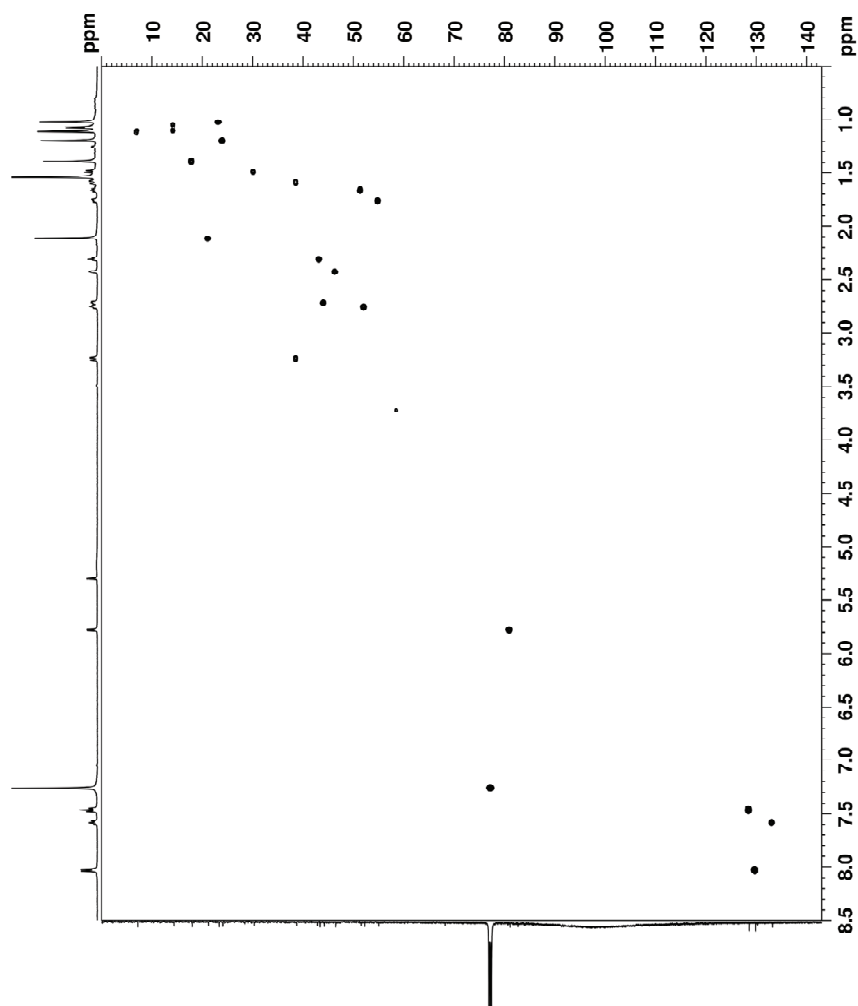


Figure S2: ¹³C-NMR (500 MHz, CDCl₃) spectrum of compound 1

Figure S3: HSQC (500 MHz, CDCl₃) spectrum of compound 1

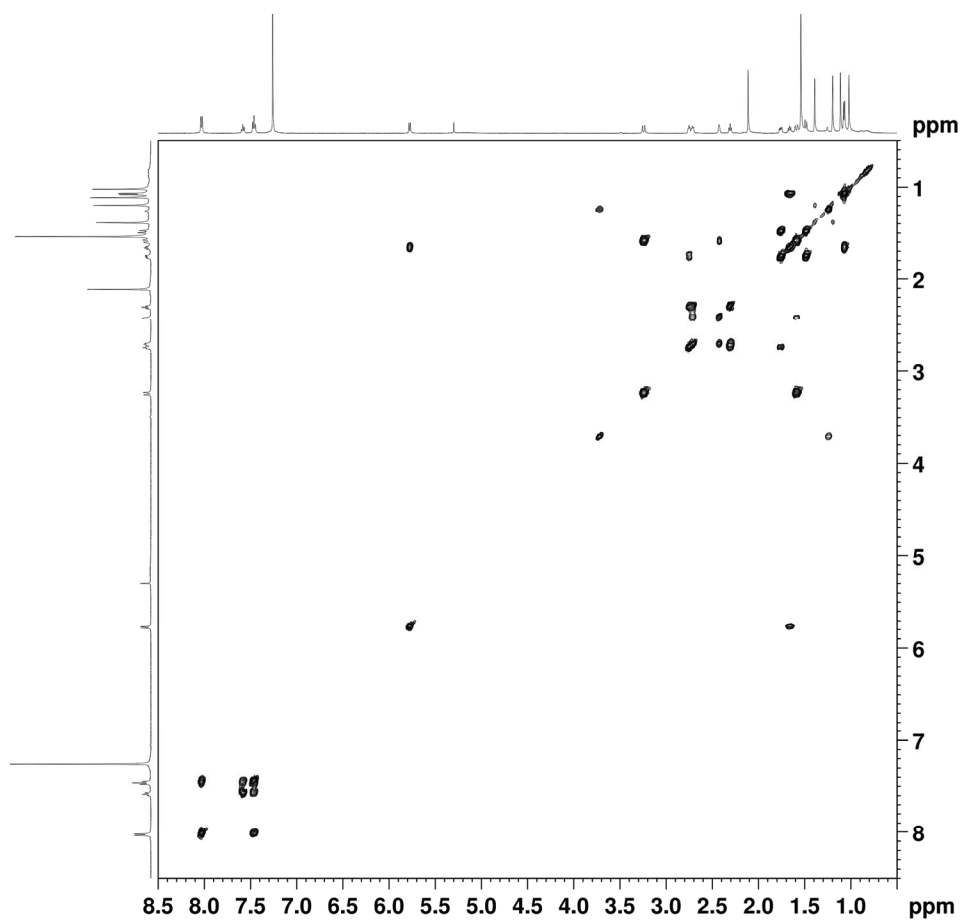


Figure S4: COSY (500 MHz, CDCl_3) spectrum of compound 1

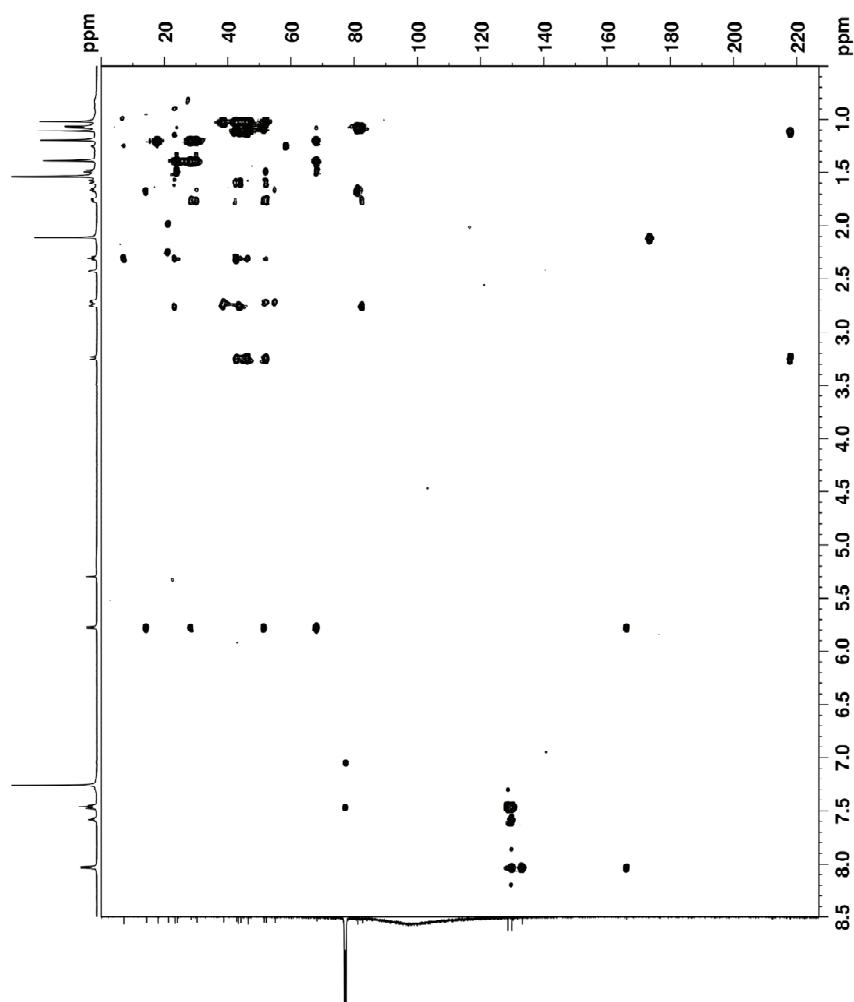


Figure S5: HMBC (500 MHz, CDCl_3) spectrum of compound 1

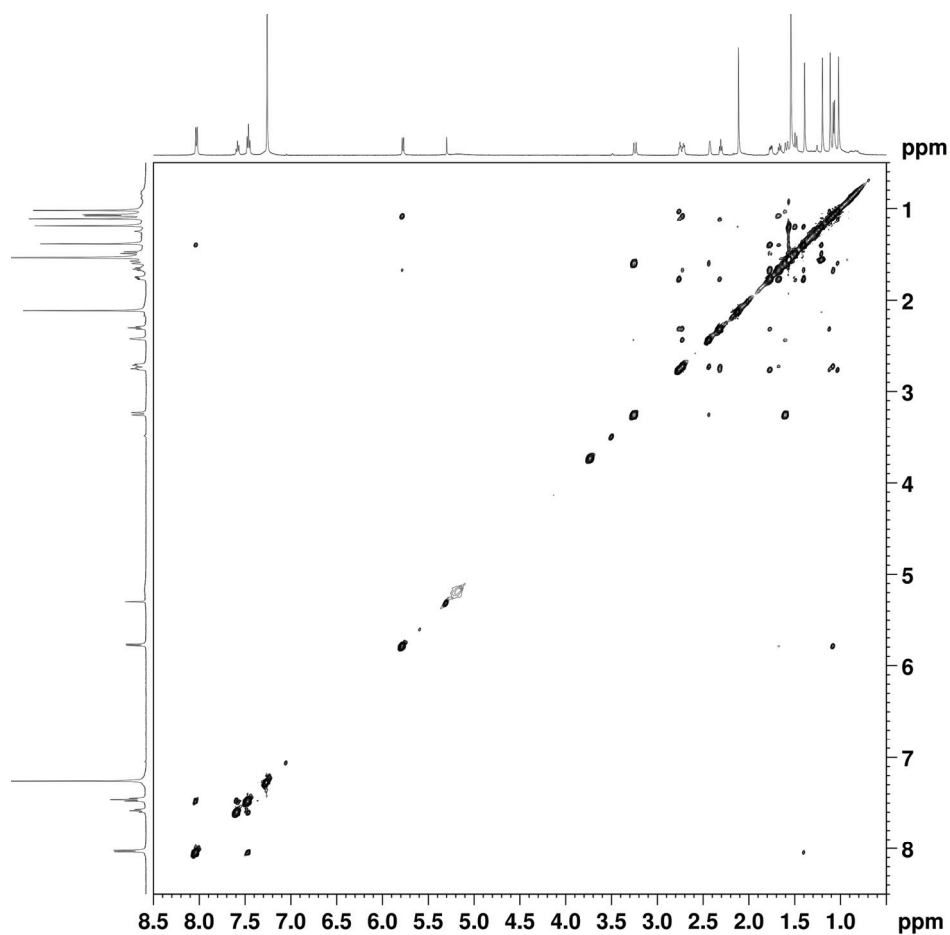


Figure S6: NOESY (500 MHz, CDCl₃) spectrum of compound 1

Compound Table

Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Diff (ppm)
Cpd 1: C ₂₉ H ₃₄ O ₆	0.379	478.2357	12615	C ₂₉ H ₃₄ O ₆	478.2355	0.25

Compound Label	m/z	RT	Algorithm	Mass
Cpd 1: C ₂₉ H ₃₄ O ₆	501.2248	0.379	Find By Formula	478.2357

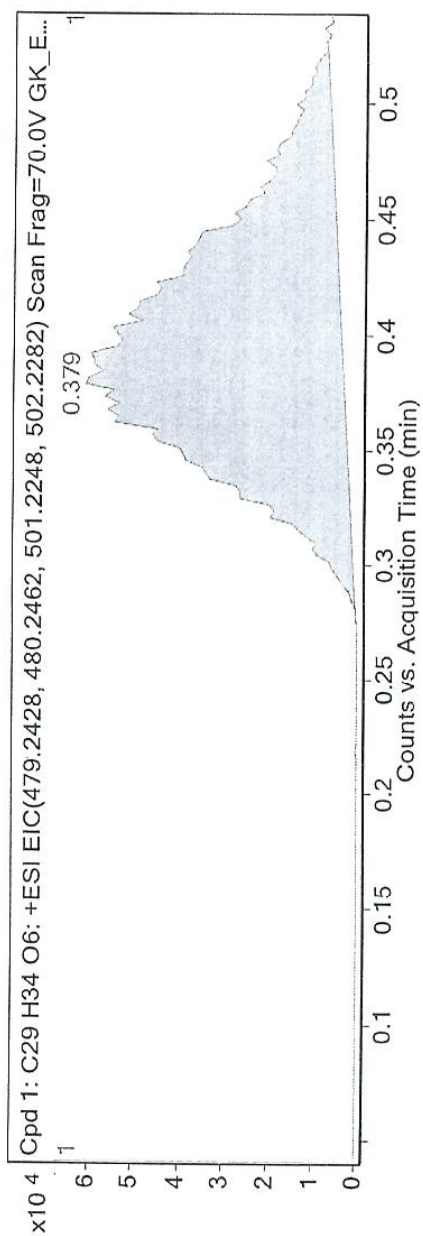


Figure S7: HRMS data of compound 1.



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Chemical characterization and differentiation of Montenegrin plum spirits obtained by two techniques of traditional batch distillation

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Abstract: Volatile compounds of plum spirit have a decisive influence on its quality and are closely related to the method of production. In this paper, minor and major volatile of plum spirits produced in small artisanal distilleries of Montenegro, obtained by two batch distillation techniques traditionally used (single and double distillation in alembic of the same construction) were analysed by GC-FID and GC-FID–MS methods. All plum spirits contained all 8 major volatile compounds analysed by GC-FID method, but out of a total of 138 minor aromatic compounds detected, only 32 are common to all samples. Wide ranges of concentrations of most volatile compounds indicated the great heterogeneity of plum spirit production methods in Montenegro, included, among others, two different distillation techniques. The principal component analysis have shown that differentiation of plum spirits obtained by single or double batch distillation is not possible using the all compounds analysed by GC-FID or GC-FID–MS, as well as compounds belonging the same chemical class, but rather based on the content of the volatile compounds typical for batch distillation tail fraction.

Keywords: GC-FID–MS; volatile compounds; alembic; single distillation; double distillation; PCA.

INTRODUCTION

In the last five decades, studies aimed to characterize and determinate quality and authenticity of plum spirits collected from the market and from small artisanal producers is carried out periodically in plum-producing European countries. Besides other chemical methods of analysis (including those from classical volumetric and UV/Vis spectrophotometric methods to synchronous fluorescence spec-

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troscopy and stable isotope ratio analyses), GC-FID and GC-MS methods were widely used for more detailed characterization of plum spirit.^{1–7} Volatile fingerprinting based on GC-MS analysis is an excellent method for plum spirits authentication, which can be very useful for distinguishing among the plum spirits that were obtained from different plum cultivars⁸ and by different production methods⁹ or originated from different regions.¹⁰

Plum is the most grown fruit species in Montenegro, primarily around the Lim River (Lim Valley – Upper Polimlje).¹¹ Almost the entire annual plum crop is processed into plum spirit, exclusively in small household artisan distilleries. Distilleries use traditional method of plum spirit production, with its small variations depending on the knowledge of the distiller, the equipment of the distillery and the preferences of final consumers. Traditional Montenegrin plum spirits obtained by classic single distillation are characterized by pronounced acidic taste and by low ethanol concentrations (< 30 vol. %). However, contemporary consumers are usually interested in consumption of plum spirits that are free from any sensory quality defects, including sharp, acidic, often unpleasant odour and taste. As consumer preferences change, manufacturers must periodically adjust characteristics of their products. Some authors have suggested that producers must adapt distillation operating recipes to meet consumer preferences.¹² This led to the modification of the traditional, classic method of single distillation in order to produce plum spirits with an alcohol content of over 40 vol. % and a reduced acid content, but only to the degree necessary to maintain the pleasing freshness and acidity that distinguish Montenegrin plum spirits. Anyway, the aim of plum spirit producer is to preserve traditional character of product, by using traditional raw materials and traditional production methods with minimal modification in order to preserve the plum fruits aroma and enable fulfilled requirements of law regulation governing the content of certain toxic compounds (*e.g.*, methanol) and, at the same time, avoidance the occurrence of sensory defects of product.

Batch distillation in alembic is considered as a most important step in the production of high quality spirit drinks (*e.g.*, cognac).^{13,14} Double distillation in the alembic is necessary to obtain a high-quality product because it allows for stronger purification and obtaining cleaner, less impressive, but sufficiently aromatic distillates. However, a unified plum spirit distillation technique does not exist in the north of Montenegro. Just like in some other producing areas of other Balkan countries (Serbia, Bosnia and Herzegovina and Croatia), Montenegrin plum spirits can be obtained by single or double distillation techniques in copper pot still, called alembic.

Single distillation (S) technique in the simple alembic of Charentais type (with a capacity of about 100 L), directly yields consumable plum spirits with 42–47 vol. % of ethanol. This distillation technique is carried out as follows. The boiler of traditional alembic (useful volume of about 100 L) is filled with about 70 to 80 kg

of fermented plum mash and about 20 L of water or tail fraction stored from the previous year. During the distillation, about 0.5–0.7 L of the head fraction is discarded, and the heart fraction (between 5 and 10 L, with an ethanol content between 42 and 47 vol. %) and the tail fraction (about 15–20 L with ethanol content between 10 and 15 vol. %) are collected separately. The next batch in the alembic is also filled with 70–80 kg of fermented mash and with tail fraction obtained from the previous distillation batch. Distillate fractions were separated as in the previous distillation. Thus, the head fraction is always discarded, the middle fraction with ethanol content about 42–47 vol. % is used for direct consuming, and the tail fraction is recycled (returned) to the next batch.

Double distillation (D) technique is carried out in the same type of Charentais type alembic. In the first distillation (without separating fractions), the first distillate with an ethanol content between 23 and 29 vol. % is obtained from the fermented plum mash. For the second distillation (redistillation), the first distillate is poured into the boiler of the same pot still. During the second distillation, the first fraction (head) is separated in the amount of 0.5–0.7 % calculated on the volume of the first distillate poured into the boiler of alembic. The cut-off point between the middle fraction (heart) and the tail fraction varies by manufacturer. The middle fraction is collected with ethanol content between 45 and 68 vol. %. The tail fraction is not recirculated to the next distillation batch. Middle fractions with less than 50 vol. % are consumed directly, and those with more than 50 vo. % must be diluted with deionized water to an ethanol concentration below 50 vol. %.

According to Spaho,¹⁵ plum spirit obtained by single stage distillation in alembic is very aromatic and contains higher concentration of congeners (acetic acid, esters, aldehydes, higher alcohols) than plum spirit produced by double distillation in same type of alembic. Some of these congeners are not desirable, and in high concentrations can give the unpleasant sensory character of plum spirits. However, some consumers in Montenegro emphasize that plum spirits obtained by single distillation have a more fruity smell and a softer taste than those obtained by double distillation. In other words, although the science and art of distillation favour plum spirits obtained by double distillation in alembic, producers must sometimes take into account the preference of certain consumers who like the taste and smell of distillates which were obtained by the single distillation in alembic.

In this study, we aimed to characterize the aromatic profile of Montenegrin plum spirits based on GC-FID and GC-FID-MS analysis, with a special emphasis on finding chemical markers that would allow distinguishing plum spirit samples obtained by the single and double distillation techniques in the traditional alembic, considering that both techniques are equally represented in distilleries in Montenegro.

EXPERIMENTAL

Plum spirits samples

Twelve plum spirits (6 produced by single distillation and 6 produced by double distillation) were collected in the spring of 2021 from small, artisanal producers of the Upper Polimlje region in the north of Montenegro. Characteristics of collected samples are shown in Table I. All plum spirits were produced from spontaneously fermented mashes of locally plum varieties in fermenters of 500–2000 L in volume. The fermented mashes were usually stored for more than a month after the fermentation is completed, even up to 6 months. Single and double distillation techniques were carried out in alembics, with the separation of fractions, in the manner specified in the Introduction. Three-member expert panel (using the 20-point Buxbaum method⁷) founded that all analysed plum spirits were of good sensory quality (sensory ratings range 17.55–18.30), with a dominant, more or less pronounced, fruity note, without defects in odour and taste (Table I). The plum spirits had a fresh, pleasant full taste, which was somewhat rounder and more pronouncedly acidic in the case of single-distilled plum spirits. Plum spirits obtained by single distillation usually had a higher content of total acids than those obtained by the double distillation. The plum spirits obtained by two distillation techniques are consumed with ethanol content ranging between 42 and 50 vol. %.

TABLE I. Plum spirit samples obtained by single and double distillation techniques; S1–S6 – samples obtained by single distillation, D1–D6 – samples obtained by double distillation; cultivars: P – Požegača, C – Čačanska Rodna, S – Stanley, SC – Čačanska Rodna+Stanley, T – Turgulja; stones: W – mash with stones, WO – mash without stones; mash storage: duration of fermented mash storage, days

Plum spirit code	Ethanol content in consumable plum spirit, vol. %	Ethanol content in heart fraction of distillate, vol. %	Additional information on samples (cultivar/ /stones/mash storage)	Total acids mg L ⁻¹	Sensory quality, points
Single distillation technique (S)					
S1	47.0	47.0	P/W/60	1104.0	17.90
S2	45.0	45.0	P/W/180	1732.8	18.25
S3	43.0	43.0	C/W/30	1216.8	17.70
S4	46.0	46.0	C/W/45	1944.0	18.30
S5	44.0	44.0	S/WO/60	820.8	18.20
S6	42.0	42.0	SC/W/60	1920.0	17.55
Double distillation technique (D)					
D1	49.0	63.0	T/WO/70	529.0	17.85
D2	50.0	55.0	P/W/55	676.8	17.80
D3	48.0	60.0	P/W/10	912.0	17.65
D4	45.0	45.0	C/W/180	1533.6	18.05
D5	45.0	53.0	C/W/30	496.8	17.85
D6	45.0	68.0	S/W/70	223.2	18.05

GC-FID and GC-FID–MS analysis of volatile compounds in plum spirits

The sample preparation and GC-FID analysis of major volatile compounds (acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2/3-methyl-1-butanol and 1-hexanol) and GC-FID-MS analysis of minor volatile compounds in plum spirits were carried out by the methods briefly described in the Supplementary material.⁹

Statistical analysis

Descriptive statistics, correlation analysis, and principal component analysis (PCA) were performed using Statistica 7 Software (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

According to the high sensory score, all analysed plum spirits, regardless of the distillation technique applied, are considered to be the spirits of good sensory quality, without defects, with a more or less expressed fruity aroma, medium to full body and pleasant taste. In general, plum spirits obtained by single distillation had a slightly higher content of total acids and a more pronounced freshness and fruity character of odour and taste than the plum spirits obtained by double distillation.

Volatile compounds in plum spirits

Eight major volatile compounds were quantified in plum spirits by GC-FID method (Table II). In Tables S-I–S-XII (shown in Supplementary material to this paper), concentration ranges of 138 minor volatile compounds (10 alcohols, 9 aldehydes, 2 ketones, 3 lactones, 10 acetals, 12 acids, 59 esters, 2 benzenoids, 15 terpenoids, 5 sesquiterpenoids, 4 isoprenoids, 7 hydrocarbons) analyzed by GC-FID-MS method are shown. In all samples, regardless of distillation techniques, following volatile compounds were found: all 8 major volatile compounds (acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2/3-methyl-1-butanol and 1-hexanol) and only 32 minor volatile compounds – 4 alcohols (1-hexanol, benzyl alcohol, 1-octanol and 2-phenylethanol), 3 aldehydes (nonanal, furfural and benzaldehyde), 5 acids (3-methylbutanoic acid, hexanoic acid, 2-hydroxy-4-methylpentanoic acid, octanoic acid and decanoic acid), 3 acetals (1,1-diethoxy-3-methylbutane, 1,1,3-triethoxypropane and 1,1-diethoxyhexane), 15 esters (ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexadecanoate, ethyl 2-methylbutanoate, 3-methylbutyl acetate, 2-phenylethyl acetate, ethyl lactate, ethyl benzoate, ethyl (*E*)-cinnamate, ethyl (*Z*)-2-butenate and diethyl succinate), and 2 terpenoids ((*E*)-linalool oxide (furanoid) and linalool). These compounds form the basic aromatic pattern of plum spirits from Upper Polimlje. Other minor volatile compounds analysed were not found in all plum spirit samples. Vyviurska *et al.* found a very similar aromatic profile in plum spirits obtained from 25 different plum cultivars.⁸ The monovarietal plum spirits in the study of mentioned authors contained between 95 and 195 identified volatile compounds, depending on the cultivar; the common compounds for all samples were 4 major volatile compounds (1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 1-hexanol) and 27 minor volatile compounds (9 alcohols, 2 aldehydes, 2 acids, 13 esters and 1 terpenoid). In French Mirabelle brandies, 175 volatile compounds were quantified, but only 105 were

found in all samples analysed, although the Mirabelle brandy production has been standardized in terms of cultivar and production methods.¹

TABLE II. Major volatile compounds contents (mg L⁻¹ a.a., except the methanol content (g L⁻¹ a.a.) in plum spirits

Compound	RT min	Single distillation (n = 6)			Double distillation (n= 6)		
		Min	Max	Mean±SD	Min	Max	Mean±SD
Acetaldehyde	1.124	125.44	226.37	188.76±40.79	91.11	263.88	153.32±65.47
Ethyl acetate	1.738	1101.12	2907.50	1623.75±654.31	658.71	3136.54	1558.03±890.52
Methanol	1.825	4.27	8.95	6.82±1.62	4.13	8.49	6.69±1.55
1-Propanol	3.686	1137.93	1693.50	1350.89±196.48	208.86	1735.89	890.90±532.20
2-Methyl-1-propanol	5.483	225.38	340.09	294.04±38.05	186.49	414.89	325.38±88.52
1-Butanol	7.725	30.28	528.39	170.03±190.67	49.40	201.50	88.97±61.32
2/3-Methyl-1-butanol	11.329	828.18	1090.25	975.84±120.26	777.72	1709.25	1145.79±415.73
1-Hexanol	17.795	13.47	115.60	43.37±39.70	12.38	55.98	23.65±16.06

Major volatile compounds

According to the content, major volatile compounds (Table II) form the basic body of all plum spirits analysed, besides ethanol and water. The contents of the analysed compounds were in the ranges that are characteristic for plum spirits originated from Southeast Europe.¹⁶ Some of these compounds (acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2/3-methyl-1-butanol) are mostly formed by microbiological activity during alcoholic fermentation and storage of fermented mash before distillation. The others are formed by enzymatic degradation of fruit ingredients during fruit processing and fermentation – methanol is generated by pectin methylesterase activity on pectic substances of fruit, whereas 1-hexanol is formed from linoleic acid through lipoxygenase pathway.¹⁷ Since the concentration of most major volatile compounds of the first distillate were obtained by the distillation of fermented plum mash, except total acids, cannot be significantly reduced by using even traditional double distillation,^{14,18,19} manufacturers should pay great attention to the selection of plum variety, method of primary processing and alcoholic fermentation of plum mash and its storage until distillation, as well as the cut-off points between the heart fraction and the tail fraction during double distillation.^{20–22} The heterogeneity in these pre-distillation steps in the production of Montenegrin plum spirits caused the contents of certain major compounds were higher in plum spirits obtained by double distillation than in those obtained by single distillation.

The analysed plum spirits had no sensory defects, *e.g.*, ester-like tone, tail-like tone, head-like tone, impure odour, which, according to Scholten and Kacprowski,²³ most commonly occur in fruit spirits. The contents of above-mentioned major compounds were lower than contents that these authors considered to be thresholds for the appearance of sensory defects. Due to its potential toxicity, max-

imal concentration (12 g L^{-1} a.a.) of methanol is limited by the Regulation EC.²⁴ All samples contained significantly lower methanol than prescribed.

Minor volatile compounds

As it is mentioned previously, 138 minor volatile compounds were quantified in Montenegrin plum spirits (Tables S-I–S-XII). Previous research showed that number of quantified minor volatile compounds in plum spirits varied between 89 and 195, depending on the country of origin, the plum cultivar, the plum spirit production method, and the GC–MS techniques used.^{1,3,4,7–9} Wide ranges of concentrations of minor compounds in analysed plum spirits obtained by the same distillation technique indicate the great heterogeneity of the production method of plum spirit from Upper Polimlje in Montenegro. In other words, in addition to the distillation technique, the composition of plum spirit can be strongly influenced by other factors, as the locality,¹⁰ variety^{8,9,20} and pre-distillation steps.²¹

It is interesting that ethyl pentanoate, ethyl heptanoate, ethyl salicylate, methyl decanoate, ethyl phenyllactate, ethyl linoleate, (*Z*)-linalool oxide (furanoid), α -terpineol, linalool acetate were found only in all plum spirits obtained exclusively by the single distillation technique. On the other hand, γ -decalactone, γ -dodecalactone, 1,1-diethoxy-nonane, 2-methyl-propanoic acid, 2-methylbutyl acetate, ethyl phenylacetate, ethyl tetradecanoate, 4-vinylanisole were found in all 6 plum spirits produced by double distillation technique. These results confirm the findings of other authors,^{13,14} that some constituents of the distillate are formed during distillation, while others are broken down, which in the case of Montenegrin plum spirits could probably be connected with the applied batch distillation technique. In other words, during distillation, especially in alembic, numerous reactions occur due to high temperatures (hydrolysis, esterification, acetalization, Maillard reactions and thermal degradation of pentoses), resulting in the formation of numerous compounds (furfural, esters, aldehydes, acetals, terpenoids and norisoprenoids). These reactions are especially intense during the first distillation – the distillation of the fermented raw material.

The attributes of minor volatile compounds can be pleasant or unpleasant, depending not only on the type of compound, but also on its concentration and odour threshold. The majority of the minor compounds, which belong to the classes of alcohols, aldehydes, lactones, acetals, esters, terpenoids and isoprenoids, are characterized by different nuances of a pleasant fruity and flowery smell.²⁵ Benzaldehyde is a characteristic ingredient of stone fruit spirits and has a typical bitter almonds odour (the fruit stone odour).¹⁷ Esters of long-chain fatty acids (saturated and unsaturated) have a so-called stearin-like smell.¹⁷ According Ledau-phin *et al.*,²⁰ 4-vinylanisole gives to calvados a tone that can be described as perspiration like, delicatessen tone. Small amounts of some phenols or anisoles can contribute to pleasant fruit characteristic aroma, but the exceeded sensorial thresh-

hold can lead to unpleasant taste and aroma (cork defect or leather odour), and some of them can give phenolic or “Brett” character of alcoholic drinks.²⁶ Some volatile compounds can negatively affect the sensory characteristics of plum spirit. For instance, the majority of volatile acids have extremely unpleasant smells: of stable, sweat, marsh, *etc.*²⁰

Pearson's correlation analysis (correlations between common volatile compounds)

For further characterization of Montenegrin plum spirits, it would be interesting to determine the correlation between the contents of the individual volatile compounds. A significant correlation was found between the content of some of 38 volatile compounds (6 major and 32 minor volatile compounds) found in all 12 samples (Table S-XIII of the Supplementary material). Based on correlations, the contents of some volatile compounds can be used to predict the contents of others. However, on the basis of these correlation coefficients, it was not possible to determine the difference between plum spirits obtained by single or double distillation.

Principal component analysis (PCA)

The principal component analysis based on the results of the GC-FID analysis of 8 major (Fig. 1) and GC-MS analysis of 32 minor (Fig. 2) compounds common to all samples, indicated that clustering of plum spirits obtained by various distillation techniques in alembic is not possible.

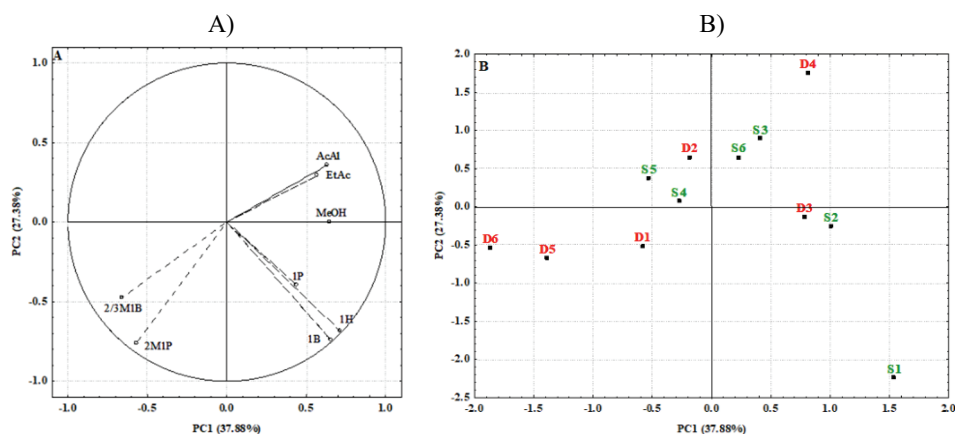


Fig. 1. Principal component analysis (PCA) calculated on 8 major volatile compounds of plum spirits; A) variable loadings, acetaldehyde (AcAl), ethyl acetate (EtAc), methanol (MeOH), 1-propanol (1P), 1-hexanol (1H), 1-butanol (1B), 2-methyl-1-propanol (2M1P), 2/3-methyl-1-butanol (2/3M1B); B) sample scores, plum spirits obtained by single distillation (S1–S6), plum spirits obtained by double distillation (D1–D6).

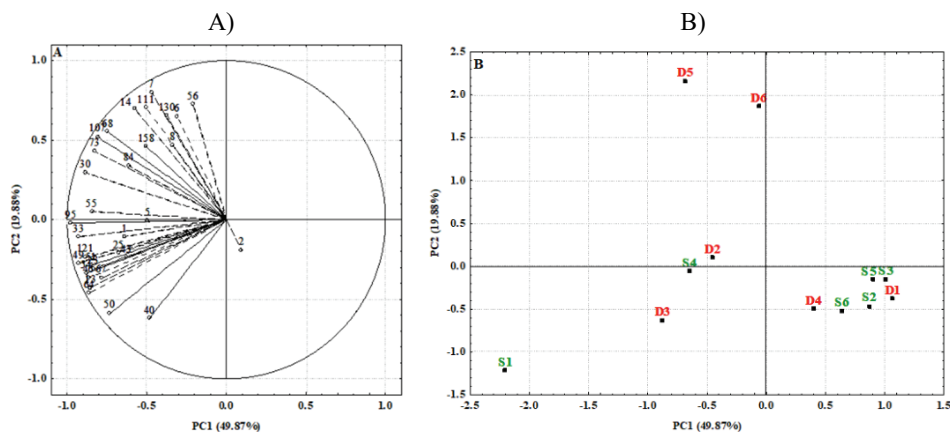


Fig. 2. Principal component analysis (PCA) calculated on 32 minor volatile compounds common for all plum spirit samples; A) variable loadings, alcohols (13, 40, 46, 56), aldehydes (5, 25, 55), acetals (23, 49, 51), acids (6, 30, 43, 68, 107), esters (1, 2, 7, 8, 14, 33, 64, 67, 73, 84, 95, 111, 121, 130, 158), terpenoids (50, 54); B) sample scores, plum spirits obtained by single distillation (S1–S6), plum spirits obtained by double distillation (D1–D6).

Minor volatile compounds belonging to the same chemical class (shown in Tables S-I–S-XII) have different origins and/or different dynamics of distillation, which has already been mentioned above. Therefore, PCAs based on contents of all 138 minor compounds or content of compounds that belong to the individual classes of chemical compounds (alcohols, carbonyl compounds, lactones, acetals, esters, terpenoids, sesquiterpenoids, isoprenoids, benzenoids and hydrocarbons, respectively) did not indicate a good discrimination between samples obtained by two different distillation technique in alembic (results not shown). The results of PCAs showed that the composition of plum spirit is more decisively influenced by pre-distillation steps (such as the choice of plum variety, the method of preparing fruits for fermentation or the fermented mash storage time) than the applied distillation technique itself. This also indicates that the same plum variety and the same pre-distillation steps should be used in future research, in order to more clearly observe the differences in the composition of plum spirits obtained by single or double distillation techniques. But, since the production methods of Montenegrin plum spirits are very heterogeneous, especially in terms of pre-distillation steps, chemical markers should be found that, regardless of these differences, could indicate whether the plum spirits were produced by single or double distillation.

During the distillation in alembic, the dynamics of distillation of most volatile compounds are similar in both the first and second distillations.¹⁴ Some volatile compounds pass into the distillate at the beginning of the distillation and some later, so the first group is considered as characteristic compounds for the head

fraction and others for the heart or tail fractions. By the single distillation technique still used in Montenegro, the tail fraction separated during the first distillation is returned to the next batch of distillation together with the mash, so it could be expected that the plum spirit obtained in this way should contain an increased amount of compounds typical of the tail fraction compared to plum spirit obtained by the double distillation technique in alembic. The previous studies have shown that the compounds typical of the tail fraction during distillation in alembics are octanoic acid, decanoic acid, dodecanoic acid, 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, 2-phenylethanol, furfural, 2-phenylethyl acetate, ethyl lactate and diethyl succinate.^{14,27} These 11 compounds may be interesting for differentiation of the plum spirits produced by a single distillation (absence of adequate separation of heart and tail fractions) and of the plum spirits produced by double distillation (the tail fraction is separated, but the distillation cut points between heart fraction and tail fraction in the second distillation are different). In this case, the PCA showed that the first two principal compounds (Fig. 3) explain 62.11 % of the total variance; PC1 (40.83 %) and PC2 (21.28 %).

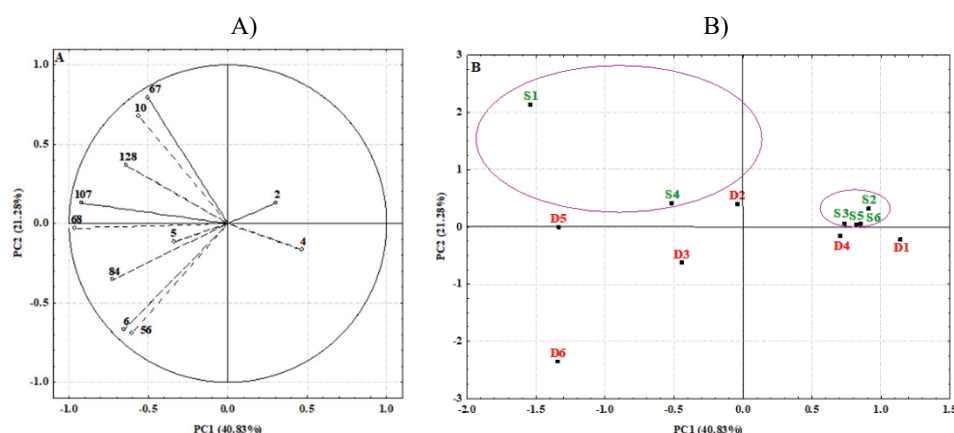


Fig. 3. Principal component analysis (PCA) calculated on 11 minor volatile compounds that are characteristic for tail fraction; A) variable loadings, ethyl lactate (2), 2-methyl-propanoic acid (4), furfural (5), 3-methyl-butanoic acid (6), 2-methyl-butanoic acid (10), 2-phenylethanol (56), diethyl succinate (67), octanoic acid (68), 2-phenylethyl acetate (84), decanoic acid (107), dodecanoic acid (128); B) sample scores, plum spirits obtained by single distillation (S1–S6), plum spirits obtained by double distillation (D1–D6).

Plum spirits obtained by a single distillation (S2, S3, S5, S6, as well as S1 and S4) were scattered in the positive region PC2. In these plum spirits, the contents of ethyl lactate, as well as those of diethyl succinate, 2-methylbutanoic acid, dodecanoic acid, and decanoic acid, were higher than in some plum spirits obtained by double distillation. The samples D4, D2 and D5, obtained by the double distillation, also contained high concentrations of certain tail compounds, which can be

explained by the late cut off point between heart and tail fractions in the second distillation. Namely, the ethanol content in the heart fraction in production of these three double distilled plum spirits was less than 60 vol. % , *i.e.*, 45, 55 and 53 vol. %, respectively, indicating that the compounds typical of the tail fraction were transferred, to a certain extent, also to the heart fraction. On the other hand, Spaho *et al.*²² have shown that the contents of a typical tail component, ethyl lactate, are not statistically different in heart fractions if, during the second distillation, the distillation cuts are done so that the ethanol content in the heart is greater than 60 vol. %. Although the recycling of the tail fraction used in the single distillation technique increases the contents of the so-called tail compounds, their contents in the final plum spirit will depend not only on their distillation dynamics but also on their origin. Thus, the most interesting are the compounds of plum spirit that are mainly formed by bacteria during the fermentation and storage of the fermented mash (*e.g.*, ethyl lactate). This component, depending on the used pre-distillation steps, can be much more present in some mashes, so that even the double distillation technique cannot remove them to a significant extent. In other words, plum spirits obtained from bacterially infected mashes distilled by double distillation technique may contain more of these compounds than plum spirits from mashes that were not bacterially infected and were distilled by single distillation technique.

CONCLUSION

There is a great heterogeneity in the plum spirits production methods in the north of Montenegro, included the two techniques of alembic batch distillation (single and double distillation). GC-FID and GC-FID-MS analysis defined for the first time the profile of volatile compounds in good-quality plum spirits from the north of Montenegro, which can serve as a basis for their authentication. The principal component analysis, based on the content of specific chemical markers, such as compounds typical of distillation tail fraction, can be used to define the manner of production of plum spirits more closely and more efficient control of the Montenegrin plum spirits quality in the future.

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/13040>, or from the corresponding author on request.

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ИЗВОД
ХЕМИЈСКА КАРАКТЕРИЗАЦИЈА И ДИФЕРЕНЦИЈАЦИЈА ЦРНОГОРСКИХ
ШЉИВОВИЦА ДОБИЈЕНИХ ДВЕМА ТЕХНИКАМА ТРАДИЦИОНАЛНЕ
ШАРЖНЕ ДЕСТИЛАЦИЈЕ

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Испарљиве компоненте шљивовице одлучујуће утичу на њен квалитет и блиско су повезане са начином производње. У овом раду, GC-FID и GC-FID–MS методама су анализирани шљивовице доброг сензорног квалитета произведене у малим занатским дестилеријама Црне Горе, добијене двема традиционално коришћеним техникама шаржне дестилације (једноструком и двоструком дестилацијом у аламбику исте конструкције). Све шљивовице су садржале свих 8 најзаступљенијих испарљивих компонената (анализираних GC-FID методом), али само 32 од 138 мање заступљених испарљивих компонената (анализираних GC-FID–MS методом). Широки распони концентрација већине испарљивих једињења указивали су на велику хетерогеност метода производње шљивовице у Црној Гори, укључујући, између осталог, две различите технике дестилације. Анализа главних компонената (PCA) је показала да није могућа диференцијација шљивовица, добијених једноструком или двоструком шаржном дестилацијом, базирана на свим компонентама анализираних методама GC-FID или GC-FID–MS, као и компонентама које припадају појединим хемијским класама, већ само на основу садржаја испарљивих компонената типичних за паточну фракцију при шаржној дестилацији.

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SUPPLEMENTARY MATERIAL TO
**Chemical characterization and differentiation of Montenegrin
plum spirits obtained by two techniques of traditional batch
distillation**

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EXPERIMENTAL DETAILS

Sample preparation

The sample preparation for determination of main volatiles (acetaldehyde, ethyl-acetate, methanol, 1-propanol, i-butanol, 1-butanol, isoamyl alcohol, and 1-hexanol) in the plum brandies by GC-FID was carried out by adding 3 μ L of 4-methyl-1-pentanol as an internal standard to 5 mL of brandy. A mixture of authentic standards is made by adding 3 μ L of each compound to 5 mL of 40% ethanol.¹

The extraction of minor aroma compounds for GC-FID–MS analysis was started by diluting an aliquot of 100 mL of plum beverage with 100 mL distilled water, followed by adding of 15 mL of dichloromethane, 1 mL of internal standard (methyl ester of 10-undecenoic acid, 1.8 mg/mL in dichloromethane), and continuously extracted on vortex for 3 min. The dichloromethane extract was dried over anhydrous magnesium sulphate, and concentrated under nitrogen flow to final volume of 1.5 mL.¹

GC-FID analysis of major volatile compounds

GC-FID analysis of major volatile components (acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2/3-methyl-1-butanol, and 1-hexanol) was carried out by the method described by Ivanović et al.¹ Briefly, a gas chromatograph Agilent 7890A (Agilent Technologies, Santa Clara, CA, USA) equipped with polar HP-INNOWax capillary column (30 m $\times$ 0.32 mm, 0.25 μ m film thickness, Agilent Technology) was used. Temperature program: 10 min at 40 $^{\circ}$ C; 5 $^{\circ}$ C/min to 90 $^{\circ}$ C; 50 $^{\circ}$ C/min to 240 $^{\circ}$ C; 10 min at 240 $^{\circ}$ C. The injector temperature was 220 $^{\circ}$ C, and the detector temperature was 300 $^{\circ}$ C. Carrier gas was helium (108.5 kPa). For quantitative evaluation, 4-methyl-1-pentanol was used as an internal standard (IS). Thus, an ethanol solution containing 5 g/L 4-methyl-1-pentanol was added to 10mL of each sample. The analyses were performed in split mode 15:1. The injection volume

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was 1 μ L. Identification of major volatile compounds was performed based on standard compounds.

GC-FID-MS analysis of minor volatile compounds

GC-FID-MS analysis of minor volatile compounds was carried out by the method described by Ivanović *et al.*¹ Briefly, Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5975C mass selective detector (MSD) and a FID connected by capillary flow technology through a two-way splitter was used. A non-polar HP-5MSI capillary column (30 m $\times$ 0.25 mm, 0.25 μ m film thicknesses Agilent Technology) was used. Temperature program: linearly increasing temperature at a rate of 3 $^{\circ}$ C/min in the range of 60 $^{\circ}$ C to 270 $^{\circ}$ C; 20 $^{\circ}$ C/min to 310 $^{\circ}$ C; 8 min at 310 $^{\circ}$ C. Helium was used as a carrier, auxiliary, and make up gas; inlet pressure was constant at 135.8 kPa (flow 1.0 mL/min at 210 $^{\circ}$ C), auxiliary pressure was 26.2 kPa, and FID make up flow was 25 mL/min. FID temperature was 300 $^{\circ}$ C. Split ratio was 5:1, and the injection volume was 1 μ L for all analyses. The mass spectra were obtained by electron ionization with 70 eV at 200 $^{\circ}$ C. The quadrupole temperature was set to 150 $^{\circ}$ C, and MS range was 40–550 amu. The transfer line temperature was 315 $^{\circ}$ C. Minor volatile compounds identification was based on the linear RI relative to n-alkanes C8–C32 and their comparison with different reference spectra (Wiley 7, NIST 17 and RT locked Adams 4 databases) followed by Automated Mass spectral Deconvolution and Identification System (Amdis 32, ver 2.73) and NIST search, ver. 2.3.

The results of analysis of major and minor volatile compounds are presented in tables, separately for plum spirits obtained by single distillation, and separately for plum spirits obtained by double distillation. For each group of samples, the results are given in the form of minimum (Min) and maximum (Max) values of the concentration of a given compound in plum spirits of each group, as well as its concentration mean values $\pm$ standard deviation (Mean $\pm$ SD) for each group of the samples. In the tables (from Table S-I to Table S-XII) shown in Supplementary, values given by bold numbers designated names and concentrations of compounds that were found in all plum spirit samples (produced by both distillation techniques). The representative GC-FID and GC-MS chromatograms are given in the Figure S-1 and Figure S-2.

ADDITIONAL RESULTS AND DISCUSSION

*Minor volatile components*TABLE S-I. Alcohols in Montenegrin plum spirits (mg L⁻¹)

No. ^a	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
11	3-hexenol	855	4.169	nd	0.128	0.046±0.054	nd	0.039	0.008±0.016
13	1-hexanol	866	4.378	0.857	24.267	6.202±9.007	1.083	11.723	4.043±3.876
27	1-heptanol	967	6.818	nd	1.983	0.534±0.746	nd	1.249	0.397±0.434
29	1-octen-3-ol	977	7.128	nd	0.108	0.025±0.044	nd	0.212	0.046±0.085
37	3-ethyl-4-methylpentan-1-ol	1021	8.518	nd	0.228	0.067±0.088	nd	0.299	0.072±0.115
40	benzyl alcohol	1034	9.005	2.311	9.804	6.377±2.974	1.186	6.526	3.700±2.018
46	1-octanol	1071	10.324	0.015	1.921	0.548±0.731	0.160	1.279	0.429±0.426
56	2-phenylethanol	1114	12.086	1.388	5.459	2.954±1.467	0.374	16.453	5.795±5.593
61	(Z)-3-nonen-1-ol	1154	13.776	nd	0.987	0.185±0.396	nd	0.776	0.204±0.294
88	1-decanol	1274	19.008	nd	0.184	0.036±0.074	nd	0.318	0.053±0.130

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-II. Aldehydes in Montenegrin plum spirits (mg L⁻¹)

No. ^a	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
5	furfural	833	3.790	0.115	0.750	0.390±0.240	0.005	1.986	0.877±0.745
18	heptanal	904	5.065	nd	0.470	0.096±0.188	nd	0.478	0.125±0.190
24	(E)-2-heptenal	956	6.509	nd	nd	nd	nd	0.078	0.024±0.038
25	benzaldehyde	962	6.689	0.447	17.376	7.132±6.670	0.573	23.789	12.863±9.319
34	octanal	1004	7.904	nd	0.164	0.035±0.066	nd	0.073	0.044±0.034
41	2-phenylacetaldehyde	1044	9.381	nd	nd	nd	nd	0.065	0.017±0.028
55	nonanal	1105	11.698	0.117	3.036	0.737±1.132	0.018	2.956	1.300±1.101
85	(E)-2-decenal	1263	18.534	nd	nd	nd	nd	0.143	0.059±0.058
98	(E,E)-2,4-decadienal	1319	21.010	nd	nd	nd	nd	0.010	0.002±0.004

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-III. Ketones in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
58	3-Nonen-2-one	1140	13.185	nd	0.224	0.038±0.092	nd	0.085	0.015±0.034
99	5-Undecen-4-one	1321	21.128	nd	0.231	0.038±0.094	nd	0.035	0.006±0.014

TABLE S-IV. Lactones in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
105	γ -Nonalactone	1365	23.027	nd	0.182	0.044±0.073	nd	0.067	0.021±0.031
122	γ -Decalactone	1469	27.492	nd	0.420	0.111±0.174	0.083	0.405	0.191±0.116
136	γ -Dodecalactone	1680	35.963	nd	0.410	0.085±0.162	0.012	0.377	0.192±0.126

TABLE S-V. Acetals in Montenegrin plum spirits (mg L⁻¹)

No. ^a	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
12	1,1-diethoxy-2-methylpropane	857	4.206	nd	nd	nd	nd	0.047	0.014±0.020
19	2,2-diethoxy-ethanol	910	5.278	nd	0.083	0.026±0.038	nd	0.052	0.024±0.021
23	1,1-diethoxy-3-methylbutane	951	6.381	0.005	0.222	0.062±0.081	0.009	0.191	0.084±0.082
28	1-(1-ethoxyethoxy)-pentane	971	6.942	nd	0.112	0.033±0.052	nd	0.417	0.120±0.156
32	1,1-diethoxy-pentane	999	7.708	nd	0.083	0.015±0.033	nd	0.106	0.030±0.042
49	1,1,3-triethoxy-propane	1078	10.654	0.020	0.229	0.068±0.084	0.013	0.163	0.065±0.056
51	1,1-diethoxy-hexane	1094	11.251	0.013	0.594	0.131±0.228	0.006	0.496	0.144±0.181
69	1,1-diethoxy-heptane	1191	15.368	nd	nd	nd	nd	0.153	0.025±0.062
102	(2,2-diethoxyethyl)-benzene	1328	21.367	nd	0.004	0.001±0.002	nd	0.064	0.011±0.026
109	1,1-diethoxy-nonane	1386	24.018	nd	0.591	0.146±0.222	0.002	0.600	0.226±0.236

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-VI. Acids in Montenegrin plum spirits (mg L⁻¹)

No. ^a	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
4	2-methyl- propanoic acid	832	3.744	nd	0.080	0.045±0.027	0.044	0.337	0.108±0.116
6	3-methyl- butanoic acid	837	3.856	0.001	0.277	0.133±0.091	0.096	0.577	0.278±0.168
10	2-methyl- butanoic acid	852	4.134	nd	0.282	0.070±0.111	nd	0.096	0.032±0.042
30	hexanoic acid	979	7.355	0.151	2.279	0.668±0.818	0.188	1.751	0.883±0.568
43	2-hydroxy- 4-methyl- pentanoic acid	1057	9.869	0.014	0.436	0.211±0.168	0.085	0.485	0.267±0.169
68	octanoic acid	1158	15.170	0.146	3.031	1.044±1.143	0.167	3.109	1.425±1.307
89	nonanoic acid	1278	19.221	nd	1.427	0.277±0.567	nd	0.774	0.378±0.316
107	decanoic acid	1379	23.702	0.598	5.343	2.066±2.134	0.873	7.407	3.168±2.358
128	dodecanoic acid	1567	31.598	nd	1.342	0.480±0.628	nd	2.659	0.491±1.064
155	hexadecanoic acid	1961	46.059	nd	0.327	0.098±0.138	nd	0.987	0.293±0.387
160	linoleic acid	2136	51.572	nd	0.164	0.044±0.071	nd	0.271	0.078±0.109
161	oleic acid	2142	51.746	nd	0.133	0.032±0.055	nd	0.087	0.031±0.039

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-VII. Esters in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
1	Ethyl butanoate	801	3.345	0.148	4.002	1.226±1.604	0.114	0.741	0.487±0.249
2	Ethyl lactate	820	3.506	6.449	24.246	15.195±7.075	7.874	42.199	20.480±13.018
7	Ethyl (Z)-2-butenate	841	3.940	0.001	0.174	0.054±0.077	0.005	0.393	0.144±0.156
8	Ethyl 2- methylbutanoate	847	4.039	0.017	0.197	0.094±0.075	0.005	0.232	0.090±0.084
9	Ethyl 3-methylbutanoate	850	4.090	nd	0.398	0.132±0.154	nd	0.110	0.026±0.043
14	3-Methylbutyl acetate	875	4.520	0.151	0.800	0.436±0.301	0.133	1.371	0.753±0.510
15	2-Methylbutyl acetate	877	4.554	nd	0.289	0.110±0.113	0.076	0.323	0.197±0.099
17	Ethyl pentanoate	903	5.048	0.010	0.088	0.051±0.032	nd	0.062	0.022±0.024
26	Ethyl 2-hydroxy-3- methylbutanoate	964	6.776	nd	0.340	0.123±0.134	nd	0.089	0.020±0.036
33	Ethyl hexanoate	1001	7.762	0.237	3.213	1.030±1.195	0.136	1.294	0.710±0.432
36	Hexyl acetate	1014	8.247	nd	0.094	0.016±0.038	nd	0.048	0.009±0.019
42	Ethyl 2-furoate	1054	9.729	nd	0.050	0.010±0.020	nd	0.024	0.006±0.010
45	Isoamyl lactate	1069	10.234	nd	0.079	0.015±0.032	nd	0.333	0.112±0.136
48	2,3-Butandiol diacetate	1074	10.464	nd	0.257	0.043±0.105	nd	0.097	0.018±0.039
52	Methyl benzoate	1098	11.349	nd	0.174	0.051±0.066	nd	0.073	0.025±0.034
53	Ethyl heptanoate	1101	11.463	0.003	0.113	0.031±0.044	nd	0.020	0.008±0.008

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
57	Methyl octanoate	1126	15.556	nd	0.106	0.025±0.042	nd	0.040	0.016±0.016
63	Benzyl acetate	1165	14.251	nd	0.320	0.099±0.121	nd	0.437	0.200±0.184
64	Ethyl benzoate	1171	14.552	1.017	15.634	5.949±5.620	1.311	9.923	5.056±3.436
67	Diethyl succinate	1182	14.998	0.392	5.289	1.593±1.892	0.122	1.970	0.894±0.649
72	Methyl salicylate	1196	15.570	nd	0.636	0.170±0.234	nd	1.288	0.271±0.503
73	Ethyl octanoate	1199	15.696	0.898	4.318	2.132±1.562	0.287	4.763	2.314±1.582
80	Hexyl	1238	17.448	nd	0.085	0.015±0.034	nd	0.052	0.017±0.027
	2-methylbutanoate								
81	Hexyl	1238	17.446	nd	nd	nd	nd	0.030	0.005±0.012
	3-methylbutanoate								
82	Ethyl	1246	17.812	nd	0.079	0.026±0.033	0.003	0.140	0.049±0.049
	phenylacetate								
84	2-Phenylethyl acetate	1258	18.332	0.007	0.271	0.067±0.101	0.012	0.423	0.239±0.139
86	Diethyl malate	1270	18.819	nd	0.280	0.047±0.114	nd	nd	nd
87	Ethyl salicylate	1272	18.935	0.054	2.335	0.557±0.881	nd	1.062	0.293±0.406
95	Ethyl nonanoate	1299	20.114	0.052	0.694	0.229±0.241	0.009	0.433	0.268±0.162
96	Nonanyl acetate	1313	20.774	nd	nd	nd	nd	0.048	0.017±0.021
101	Methyl decanoate	1326	21.369	0.003	0.017	0.009±0.005	nd	0.180	0.058±0.068
103	Ethyl phenyllactate	1352	22.437	0.010	0.421	0.134±0.157	nd	0.183	0.059±0.070
108	Ethyl	1381	23.741	nd	0.016	0.003±0.006	nd	0.568	0.219±0.238
	(Z)-cinnamate								
110	Ethyl	1389	24.147	nd	0.095	0.030±0.034	nd	0.142	0.038±0.058
	(E)-4-decenoate								
111	Ethyl decanoate	1397	24.485	0.803	2.870	1.734±0.910	0.288	8.304	3.033±2.980
117	2-Methylbutyl benzoate	1438	26.234	nd	0.038	0.014±0.013	nd	0.058	0.021±0.024
119	3-Methylbutyl octanoate	1448	26.610	nd	0.010	0.002±0.004	nd	0.172	0.039±0.068
121	Ethyl (E)-cinnamate	1467	27.396	0.028	1.132	0.440±0.532	0.012	0.779	0.364±0.306
125	Methyl dodecanoate	1526	29.864	nd	nd	nd	nd	0.055	0.009±0.022
126	Ethyl 3-hydroxy-tridecanoate	1536	30.278	nd	0.030	0.006±0.012	nd	nd	nd
130	Ethyl dodecanoate	1595	32.691	0.186	0.596	0.328±0.190	0.085	3.735	1.041±1.404
131	Phenylethyl hexanoate	1644	34.570	nd	nd	nd	nd	0.072	0.016±0.029
132	2-Methylbutyl decanoate	1646	34.653	nd	nd	nd	nd	0.187	0.031±0.076
133	3-Methylbutyl decanoate	1649	34.783	nd	nd	nd	nd	0.050	0.008±0.021
137	Diethyl azelate	1689	36.344	nd	0.060	0.020±0.028	nd	0.061	0.032±0.025
146	Ethyl tetradecadienoate	1758	38.887	nd	nd	nd	nd	0.090	0.015±0.037
147	Methyl pentadecanoate	1759	38.999	nd	0.008	0.001±0.003	nd	0.078	0.025±0.036
148	Ethyl <i>p</i> -methoxycinnamate	1762	39.004	nd	0.117	0.019±0.048	nd	nd	nd
149	Ethyl 9-tetradecenoate	1763	39.093	nd	0.002	0.000±0.001	nd	0.245	0.088±0.096
150	Ethyl 9-tetradecenoate (isomer)	1771	39.441	nd	0.027	0.005±0.011	nd	0.093	0.034±0.037

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
151	Ethyl tetradecanoate	1794	40.270	nd	0.075	0.029±0.032	0.009	0.502	0.189±0.190
153	Phenylethyl octanoate	1850	42.155	nd	0.021	0.004±0.009	nd	0.107	0.028±0.046
156	Ethyl 9-hexadecenoate	1974	46.492	nd	0.056	0.016±0.023	nd	0.192	0.084±0.076
158	Ethyl hexadecanoate	1994	47.203	0.043	0.452	0.191±0.200	0.018	1.995	0.760±0.807
159	Methyl linoleate	2097	50.443	nd	nd	nd	nd	0.091	0.025±0.040
163	Ethyl linoleate	2169	52.532	0.043	0.450	0.183±0.170	nd	2.965	0.986±1.196
164	Ethyl oleate	2175	52.719	nd	0.232	0.070±0.109	nd	0.766	0.223±0.300
165	Ethyl linolenate	2172	52.742	nd	0.053	0.026±0.021	nd	0.950	0.165±0.385
167	Ethyl octadecanoate	2201	53.518	nd	nd	nd	nd	0.108	0.046±0.053

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-VIII. Benzenoids in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
16	styrene	896	4.897	nd	0.089	0.035±0.042	nd	0.136	0.058±0.051
60	4-vinylanisole	1153	13.721	nd	0.067	0.018±0.029	0.006	0.467	0.253±0.175

TABLE S-IX. Terpenoids in Montenegrin plum spirits (mg L⁻¹)

No. ^a	compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
22	<i>α</i> -pinene	934	5.917	nd	nd	nd	nd	0.038	0.006±0.016
31	(<i>E</i>)- dehydroxylinalool oxide	994	7.589	nd	nd	nd	nd	0.032	0.005±0.013
35	(<i>Z</i>)- dehydroxylinalool oxide	1009	8.084	nd	0.115	0.019±0.047	nd	0.069	0.018±0.029
38	<i>o</i> -cymene	1023	8.575	nd	nd	nd	nd	0.143	0.038±0.062
39	limonene	1029	8.823	nd	0.024	0.004±0.010	nd	0.674	0.194±0.269
47	(<i>Z</i>)-linalool oxide (furanoid)	1073	10.451	0.054	1.577	0.431±0.571	nd	0.679	0.181±0.249
50	(<i>E</i>)-linalool oxide (furanoid)	1089	11.067	0.059	2.070	0.584±0.754	0.030	0.853	0.367±0.307
54	linalool	1102	11.513	0.074	1.403	0.426±0.517	0.055	1.287	0.427±0.446
59	(<i>Z</i>)- <i>β</i> -terpineol	1145	13.404	nd	nd	nd	nd	0.045	0.007±0.018
65	(<i>Z</i>)-linalool oxide (pyranoid)	1173	14.671	nd	0.184	0.064±0.078	nd	0.296	0.067±0.120
66	terpinen-4-ol	1178	14.826	nd	0.339	0.066±0.136	nd	0.293	0.067±0.119
70	<i>α</i> -terpineol	1192	15.405	0.031	1.044	0.401±0.471	nd	0.923	0.428±0.383
74	<i>p</i> -menthen-1-ene-9- al	1217	16.505	nd	nd	nd	nd	0.092	0.016±0.037
75	(<i>E</i>)-carveol	1222	16.740	nd	nd	nd	nd	0.046	0.011±0.018
83	linalool acetate	1256	18.215	0.006	0.668	0.240±0.281	nd	0.618	0.197±0.229

^avalues given in bold were found in all plum spirit samples (produced by both distillation techniques);

TABLE S-X. Sesquiterpenoids in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
114	longifolene	1408	24.883	nd	nd	nd	nd	0.100	0.017±0.041
124	(<i>E,E</i>)- <i>α</i> -farnesene	1511	29.209	nd	nd	nd	nd	0.178	0.030±0.073
127	(<i>E</i>)-nerolidol	1566	31.529	nd	0.118	0.034±0.054	nd	0.286	0.077±0.108
134	<i>α</i> -bisabolool oxide b	1656	35.091	nd	0.081	0.030±0.038	nd	0.052	0.011±0.021
138	(<i>Z,E</i>)-2,6-farnesol	1723	37.614	nd	0.091	0.035±0.033	nd	0.251	0.067±0.096

TABLE S-XI. Isoprenoids in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
90	vitispirane	1280	19.311	nd	0.045	0.009±0.018	nd	0.061	0.010±0.025
118	dihydro- <i>β</i> -ionol	1447	26.573	nd	0.079	0.022±0.032	nd	0.040	0.008±0.016
123	(<i>E</i>)- <i>β</i> -ionone	1487	28.286	nd	0.020	0.003±0.008	nd	nd	nd
181	squalene	2828	70.246	nd	0.096	0.017±0.038	nd	0.014	0.005±0.006

TABLE S-XII. Hydrocarbons in Montenegrin plum spirits (mg L⁻¹)

No.	Compound	RI	RT (min)	Single distillation (n=6)			Double distillation (n=6)		
				Min	Max	Mean±SD	Min	Max	Mean±SD
168	tricosane	2301	56.534	nd	0.043	0.007±0.018	nd	0.024	0.004±0.010
169	tetracosane	2401	59.365	nd	0.030	0.005±0.012	nd	0.029	0.005±0.012
173	pentacosane	2507	62.004	nd	0.039	0.010±0.016	nd	0.057	0.016±0.025
174	hexacosane	2607	64.543	nd	0.031	0.015±0.013	nd	0.059	0.027±0.021
177	heptacosane	2706	67.071	nd	0.094	0.016±0.038	nd	0.074	0.027±0.030
179	octacosane	2801	69.442	nd	nd	nd	nd	0.033	0.005±0.013
182	nonacosane	2902	71.842	nd	0.501	0.084±0.204	nd	0.099	0.022±0.038

Just about half out of all minor compounds identified in traditional home made plum spirits from Romania and Serbia,^{2,3} were also common to the analyzed Montenegrin plum spirits. In other words, there are significantly different GC-MS aromatic profiles of plum spirits from different regions of the Balkans.

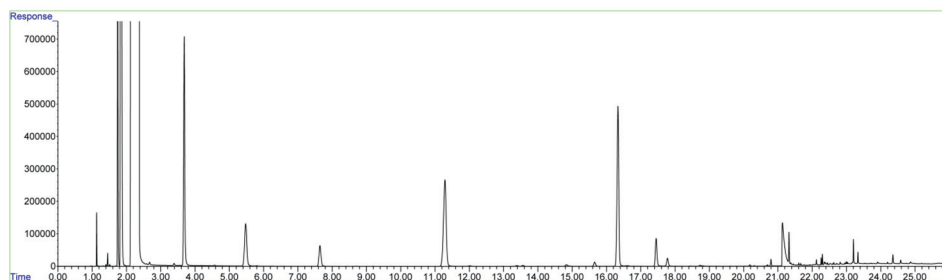


Figure S-1. GC-FID chromatogram of Montenegrin plum spirit sample

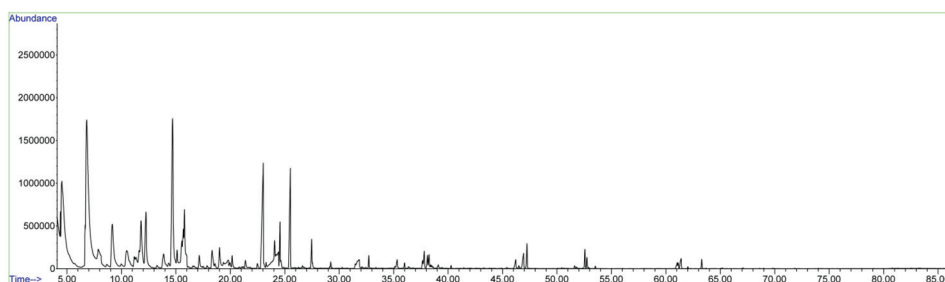


Figure S-2. GC-MS chromatogram of Montenegrin plum spirit sample

Pearson's correlation analysis (Correlations between common volatile compounds)

Although methanol and acetaldehyde were found in all samples, these compounds did not correlate significantly (results not shown) with other compounds typical of all plum spirits. In most cases (Table S-XIII), a significant positive Pearson's correlation was established between the content of 38 volatile components common for all the plum spirits analyzed. Significant negative values

of correlation coefficient were observed only in a few cases: ethyl acetate and 3-methylbutanoic acid ($r = -0.61$), 1-propanol and 2-phenylethanol ($r = -0.77$), 1-propanol and 2-phenylethyl acetate ($r = -0.62$). It is obvious that the compounds with negative correlation have different distillation dynamics (some of them are distilled mainly in the head fraction, and others in the tail fraction.).

Values of some correlation coefficients were as follow: ethyl hexanoate and ethyl octanoate ($r = 0.79$), ethyl octanoate and ethyl decanoate ($r = 0.82$), ethyl dodecanoate and ethyl octanoate ($r = 0.68$), 2/3-methyl-1-butanol and 3-methylbutyl acetate ($r = 0.85$), 2-phenylethanol and 2-phenylethyl acetate ($r = 0.67$). The obtained values of the mentioned correlation coefficients were similar but slightly higher than those, for the same components, found in various fruit brandies and other distilled beverages.^{4,5}

It is interesting that octanoic acid, as one of the typical ingredients generated during alcoholic fermentation as a yeast metabolite, correlated significantly with other typical yeast metabolites, such as 2-methyl-1-propanol, 2/3-methyl-1-butanol, 2-phenylethanol, 3-methylbutanoic acid, hexanoic acid, decanoic acid, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 3-methylbutyl acetate, 2-phenylethyl acetate. A similar pattern in terms of correlations was found for other components that are formed primarily during alcoholic fermentation of the plum mash. On the other hand, linalool originating from plum fruit correlated significantly mainly with plum spirit components also originating from fruits, providing the typical plum aroma, such as 1-hexanol, 1-octanol, nonanal, benzaldehyde, ethyl nonanoate, ethyl benzoate, ethyl (*E*)-cinnamate, and (*E*)-linalool oxide (furanoid).

Statistically significant correlation was not found between the concentrations of analysed components and the sensory evaluation scores of the plum spirits (results not shown). According Brandes *et al.*,⁶ it is relatively easy to establish a correlation between the content of the components that adversely affect sensory characteristics of fruit spirits, such as free fatty acids (they give a tail-like character of the distillates), but it is difficult to determine the correlation between sensory evaluation score and components considered to contribute positively to the aroma of fruit spirits. As we have already noted under the Materials and methods, the analysed plum spirit samples were of good quality, without sensory defects, free from extraneous and unpleasant odour and taste.

TABLE S-XIII. Correlation matrix of 38 (6 major and 32 minor) volatile components quantified in all plum spirit samples

C/C	EA	1P	2M1P	1B	2/3M1B	1H	A13	A40	A46	A56	Ad55	Ad5	Ad25	Ac6	Ac30	Ac43	Ac68	Ac107
EA	1	/	/	/	/	/	/	/	/	/	/	/	/	-0.61	/	/	/	/
1P	/	1	/	/	/	/	/	/	/	-0.77	/	/	/	/	/	/	/	/
2M1P	/	/	1	/	0.74	/	/	/	/	/	/	/	/	0.66	/	/	0.65	/
1B	/	/	/	1	/	0.99	0.90	/	0.82	/	0.58	/	/	/	/	/	/	/
2/3M1B	/	/	0.74	/	1	/	/	/	/	0.78	/	/	/	0.68	/	/	0.79	0.70
1H	/	/	/	0.99	/	1	0.89	/	0.82	/	0.59	/	/	/	/	/	/	/
A13	/	/	/	0.90	/	0.89	1	0.59	0.96	/	0.78	/	/	/	0.69	/	/	/
A40	/	/	/	/	/	/	0.59	1	0.58	/	/	/	/	/	/	0.70	/	/
A46	/	/	/	0.82	/	0.82	0.96	0.58	1	/	0.79	/	/	/	0.65	/	/	/
A56	/	-0.77	/	/	0.78	/	/	/	/	1	/	/	/	/	/	/	0.62	/
Ad55	/	/	/	0.58	/	0.59	0.78	/	0.79	/	1	0.59	0.62	/	0.75	/	0.68	0.62
Ad5	/	/	/	/	/	/	/	/	/	/	0.59	1	0.79	/	/	/	/	/
Ad25	/	/	/	/	/	/	/	/	/	/	0.62	0.79	1	/	/	0.78	/	/
Ac6	-0.61	/	0.66	/	0.68	/	/	/	/	0.82	/	/	/	1	/	/	0.68	/
Ac30	/	/	/	/	/	/	0.69	/	0.65	/	0.66	/	/	/	1	/	0.89	0.89
Ac43	/	/	/	/	/	/	/	0.70	/	/	/	/	0.78	/	/	1	/	/
Ac68	/	/	0.65	/	0.79	/	/	/	/	0.62	0.68	/	/	0.68	0.89	/	1	0.91
Ac107	/	/	/	/	0.70	/	/	/	/	/	0.62	/	/	/	0.89	/	0.91	1
At23	/	/	/	0.62	/	0.65	0.80	/	0.76	/	0.80	0.70	0.82	/	0.63	0.64	/	/
At49	/	/	/	0.77	/	0.78	0.94	/	0.94	/	0.87	/	0.64	/	0.72	/	/	0.59
At51	/	/	/	0.79	/	0.80	0.93	/	0.93	/	0.92	/	0.59	/	0.66	/	/	/
E1	/	/	/	/	/	/	/	0.71	0.59	/	/	/	/	/	/	/	/	/
E33	/	/	/	0.76	/	0.75	0.89	0.59	0.88	/	0.70	/	/	/	0.86	/	0.74	0.74
E73	/	/	/	/	0.60	/	/	/	/	/	/	/	/	/	0.89	/	0.86	0.94
E95	/	/	/	0.67	/	0.69	0.86	/	0.84	/	0.87	/	0.66	/	0.89	0.59	0.70	0.77
E111	/	/	/	/	0.65	/	/	/	/	/	/	/	/	/	0.70	/	0.68	0.84
E130	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.58	/	/	0.75
E158	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.71
E8	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.58
E14	/	/	/	/	0.85	/	/	/	/	0.80	/	/	/	0.69	0.71	/	0.86	0.82
E84	/	-0.62	/	/	/	/	/	/	/	0.67	0.79	/	0.64	0.65	0.61	/	0.61	/
E2	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.68	/	/
E64	/	/	/	0.67	/	0.70	0.89	0.79	0.89	/	0.66	/	0.76	/	0.59	0.77	/	/
E121	/	/	/	/	/	/	0.77	0.71	0.82	/	0.60	/	0.68	/	0.62	0.70	/	0.63
E7	/	/	/	/	0.88	/	/	/	/	0.87	/	/	/	0.80	0.67	/	0.85	0.75
E67	/	/	/	0.79	/	0.76	0.86	0.71	0.75	/	/	/	/	/	0.73	0.65	/	/
T50	/	/	/	0.91	/	0.89	0.96	0.68	0.92	/	0.60	/	/	/	/	/	/	/
T54	/	/	/	0.73	/	0.76	0.92	/	0.96	/	0.83	/	0.64	/	0.62	/	/	/

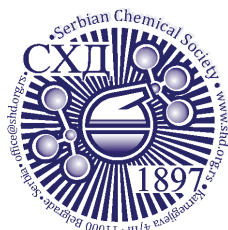
TABLE S-XIII. Continuing

C/C	At23	At49	At51	E1	E33	E73	E95	E111	E130	E158	E8	E14	E84	E2	E64	E121	E7	E67	T50	T54
EA	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
1P	/	/	/	/	/	/	/	/	/	/	/	/	-0.62	/	/	/	/	/	/	/
2M1P	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
1B	0.62	0.77	0.79	/	0.76	/	0.67	/	/	/	/	/	/	/	0.67	/	/	0.79	0.91	0.73
2/3M1B	/	/	/	/	/	0.60	/	0.65	/	/	/	0.85	/	/	/	/	0.88	/	/	/
1H	0.65	0.78	0.80	/	0.75	/	0.69	/	/	/	/	/	/	/	0.70	/	/	0.76	0.89	0.76
A13	0.80	0.94	0.93	/	0.89	/	0.86	/	/	/	/	/	/	/	0.89	0.77	/	0.86	0.96	0.92
A40	/	/	/	0.71	0.59	/	/	/	/	/	/	/	/	/	0.79	0.71	/	0.71	0.68	/
A46	0.76	0.94	0.93	0.59	0.88	/	0.84	/	/	/	/	/	/	/	0.89	0.82	/	0.85	0.92	0.96
A56	/	/	/	/	/	/	/	/	/	/	/	0.80	0.67	/	/	/	0.87	/	/	/
Ad55	0.80	0.87	0.92	/	0.70	/	0.87	/	/	/	/	/	0.79	/	0.66	0.60	/	/	0.60	0.83
Ad5	0.70	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Ad25	0.82	0.64	0.59	/	/	/	0.66	/	/	/	/	/	0.64	/	0.76	0.68	/	/	/	0.64
Ac6	/	/	/	/	/	/	/	/	/	/	/	0.69	0.65	/	/	/	0.80	/	/	/
Ac30	0.63	0.72	0.66	/	0.86	0.89	0.89	0.70	0.58	/	/	0.71	0.61	/	0.59	0.62	0.67	0.73	/	0.62
Ac43	0.64	/	/	/	/	/	0.59	/	/	/	/	/	/	0.68	0.77	0.70	/	0.65	/	/
Ac68	/	/	/	/	0.74	0.86	0.70	0.68	/	/	/	0.86	0.61	/	/	/	0.85	/	/	/
Ac107	/	0.59	/	/	0.74	0.94	0.77	0.84	0.75	0.71	0.58	0.82	/	/	/	0.63	0.75	/	/	/
At23	1	0.90	0.86	/	0.69	/	0.87	/	/	/	/	/	/	/	0.85	0.78	/	0.71	0.70	0.84
At49	0.90	1	0.96	0.58	0.88	0.60	0.92	/	/	/	/	/	/	/	0.89	0.86	/	0.78	0.84	0.95
At51	0.86	0.96	1	/	0.79	/	0.87	/	/	/	/	/	/	/	0.81	0.71	/	0.66	0.82	0.95
E1	/	0.58	/	1	0.74	0.66	/	/	/	/	/	/	/	/	0.64	0.83	/	0.61	/	/
E33	0.69	0.88	0.79	0.74	1	0.79	0.89	/	/	/	/	/	/	/	0.82	0.84	/	0.89	0.82	0.81
E73	/	0.60	/	0.66	0.79	1	0.78	0.82	0.68	0.65	0.64	0.76	/	/	/	0.71	0.74	0.61	/	/
E95	0.87	0.92	0.87	/	0.89	0.78	1	/	/	/	/	/	0.62	/	0.83	0.82	/	0.79	0.72	0.87
E111	/	/	/	/	/	0.82	/	1	0.96	0.89	0.74	0.67	/	/	/	/	0.77	/	/	/
E130	/	/	/	/	/	0.68	/	0.96	1	0.92	0.66	/	/	/	/	/	/	/	/	/
E158	/	/	/	/	/	0.65	/	0.89	0.92	1	0.65	/	/	/	/	/	/	/	/	/
E8	/	/	/	/	/	0.64	/	0.74	0.66	0.65	1	/	/	/	/	/	/	/	/	/
E14	/	/	/	/	/	0.76	/	0.67	/	/	/	1	0.70	/	/	/	0.88	/	/	/
E84	/	/	/	/	/	/	0.62	/	/	/	/	0.70	1	/	/	/	0.64	/	/	/
E2	/	/	/	/	/	/	/	/	/	/	/	/	/	1	/	/	/	/	/	/
E64	0.85	0.89	0.81	0.64	0.82	/	0.83	/	/	/	/	/	/	/	1	0.91	/	0.82	0.87	0.89
E121	0.78	0.86	0.71	0.83	0.84	0.71	0.82	/	/	/	/	/	/	/	0.91	1	/	0.75	0.71	0.85
E7	/	/	/	/	/	0.74	/	0.67	/	/	/	0.88	0.64	/	/	/	1	/	/	/
E67	0.71	0.78	0.66	0.61	0.89	0.61	0.79	/	/	/	/	/	/	/	0.82	0.75	/	1	0.86	0.68
T50	0.70	0.84	0.82	/	0.82	/	0.72	/	/	/	/	/	/	/	0.87	0.71	/	0.86	1	0.84
T54	0.84	0.95	0.95	/	0.81	/	0.87	/	/	/	/	/	/	/	0.89	0.85	/	0.68	0.84	1

/ - $P > 0.05^{NS}$ (r values < 0.58 not shown); $P < 0.05^*$ (r values 0.58-0.71); $P < 0.01^{**}$ (r values 0.72-0.85); $P < 0.001^{***}$ (r values 0.86-0.99); Major volatile components: **EA**-ethyl acetate; **1P**-1-propanol; **2M1P**-2-methyl-1-propanol; **1B**-1-butanol; **2/3M1B**-2/3-methyl-1-butanol; **1H**-1-hexanol; Minor volatile components: **A-Alcohols** (**A13**-1-hexanol; **A40**-benzyl alcohol; **A46**-1-octanol; **A56**-2-phenylethanol); **Ad-Aldehydes** (**Ad55**-nonanal; **Ad5**-furfural; **Ad25**-benzaldehyde); **Ac-Acids** (**Ac6**-3-methylbutanoic acid; **Ac30**-hexanoic acid; **Ac43**-2-hydroxy-4-methylpentanoic acid; **Ac68**-octanoic acid; **Ac107**-decanoic acid); **At-Acetals** (**At23**-1,1-diethoxy-3-methylbutane; **At49**-1,1,3-triethoxypropane; **At51**-1,1-diethoxyhexane); **E-Esters** (**E1**-ethyl butanoate; **E33**-ethyl hexanoate; **E73**-ethyl octanoate; **E95**-ethyl nonanoate; **E111**-ethyl decanoate; **E130**-ethyl dodecanoate; **E158**-Ethyl hexadecanoate; **E8**-ethyl 2-methylbutanoate; **E14**-3-methylbutyl acetate; **E84**-2-phenylethyl acetate; **E2**-ethyl lactate; **E64**-ethyl benzoate; **E121**-ethyl-(*E*)-cinnamate; **E7**-ethyl-2-(*Z*)-butenoate; **E67**-diethyl succinate; **T-Terpenoids** (**T50**-(*E*)-linalool oxide (furanoid); **T54**-linalool);

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***In silico* molecular docking and ADMET prediction of *Ginkgo biloba* biflavonoids as dual inhibitors of human HMG-CoA reductase and α -amylase**

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Abstract: There is an increasing interest in investigating phytochemicals as inhibitors of HMG-CoA reductase (HMGR) and α -amylase enzymes. Inhibition of these enzymes helps manage hypercholesterolemia and diabetes by reducing cholesterol synthesis and blood sugar levels, respectively. In this study, computational techniques *via* molecular docking and ADMET prediction were used to determine the potential of five *Ginkgo biloba* biflavonoids (amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin) as dual inhibitors of HMGR and α -amylase. Amentoflavone (–42.26 kJ/mol) and bilobetin (–41.00 kJ/mol) exhibited stronger binding affinities to HMGR compared to the reference drug atorvastatin (–38.91 kJ/mol). For α -amylase, amentoflavone (–48.12 kJ/mol), bilobetin (–47.28 kJ/mol) and ginkgetin (–46.44 kJ/mol) exhibited stronger binding affinities compared to the reference drug acarbose (–43.93 kJ/mol). These docking results indicate that amentoflavone and bilobetin have the potential to act as dual inhibitors of these two enzymes. ADMET analysis showed that bilobetin demonstrated favorable oral bioavailability and drug-likeness, adhering to Lipinski's rule of five. Despite exhibiting low gastrointestinal absorption, it was predicted to be neither mutagenic nor hepatotoxic. Therefore, bilobetin is a promising candidate for dual antidiabetic and antihypercholesterolemic applications. Further *in vitro* and *in vivo* studies are recommended to confirm these promising results.

Keywords: bilobetin; hypercholesterolemia; diabetes; binding affinity; pharmacokinetic properties; computer-aided drug discovery.

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INTRODUCTION

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase (HMGR) and α -amylase are two key human metabolic enzymes involved in chronic diseases such as hypercholesterolemia and diabetes. HMGR is the key regulatory enzyme in the mevalonate pathway, facilitating the conversion of HMG-CoA to mevalonate, which is an essential step in the synthesis of cholesterol.¹ Inhibition of this enzyme has proven to be an effective strategy for lowering cholesterol levels and helps prevent atherosclerosis, heart attack, and coronary heart diseases.^{2,3} Statins (*e.g.*, atorvastatin) are a class of cholesterol-lowering drugs that inhibit HMGR. However, the prolonged use of statins is associated with various negative effects, commonly referred to as “statin-associated adverse effects”. These effects include skeletal muscle disorders such as rhabdomyolysis and myopathy, liver dysfunction and an increased risk of diabetes.^{4,5} α -Amylase, on the other hand, is a digestive enzyme primarily produced in the pancreas and salivary glands, responsible for the digestion of carbohydrates in humans.⁶ Pancreatic α -amylase breaks down α -1,4 glycosidic bonds in complex carbohydrates like starch, converting them into oligosaccharides and disaccharides. These are then further degraded by glucosidases into glucose units that can be absorbed by the small intestine.^{7,8} The rapid digestion of starch can lead to elevated postprandial blood glucose levels (hyperglycemia) in diabetic patients.⁹ Postprandial hyperglycemia (PPHG) contributes to the development of type 2 diabetes mellitus and has been identified by epidemiological studies as an independent risk factor for cardiovascular disease.^{10,11} Inhibiting the enzymatic action of α -amylase can help reduce PPHG and lower the risk of developing diabetes.¹² Acarbose, an inhibitor of α -amylase, is a medication used in the management of diabetes. While effective in controlling PPHG in many patients, its long-term administration is often associated with gastrointestinal adverse effects.¹³ Given the adverse effects associated with current HMGR and α -amylase inhibitors, there is a need to explore alternative treatments that are both effective and have fewer side effects for managing hypercholesterolemia and diabetes.

Medicinal plants are increasingly being studied as potential sources of therapeutic and bioactive compounds for inhibiting HMGR and α -amylase.^{14,15} They offer several benefits, like lesser side effects, safer profiles, effectiveness in treating diseases, widespread accessibility, and low cost.¹⁶ One promising medicinal plant is *Ginkgo biloba*, commonly known as ginkgo or maidenhair tree. With a long history in traditional Chinese medicine, both its leaves and seeds have been utilized for centuries to treat a variety of diseases. Ginkgo has been used ethnomedicinally across different cultures worldwide, including in Asia, America, Europe and Australia, for treating respiratory and cardiovascular diseases, nervous system disorders, urinary problems and memory improvement.¹⁷ Currently,

ginkgo leaf extracts are primarily available as herbal supplements for improving memory.

The therapeutic and bioactive characteristics of *G. biloba* are thought to be associated with its flavonoids and terpene trilactones.¹⁷ Biflavonoids, which are dimers of flavonoids, are important phytochemical constituents of *G. biloba*. The most predominant biflavonoids in *G. biloba* are sciadopitysin, ginkgetin, bilobetin, isoginkgetin and amentoflavone. These compounds have been reported to possess various biological activities, including antibacterial, antifungal, antiviral, antioxidant, anticancer and anti-inflammatory activities.¹⁸ Although *G. biloba* biflavonoids are recognized as compounds with potent health-promoting properties, their specific potential role in the treatment of cardiovascular and metabolic diseases, such as hypercholesterolemia and diabetes, remains largely understudied.

The traditional drug discovery is a complex, expensive, and lengthy process. On average, the traditional drug development pipeline requires around 12 years and 2.7 billion USD to bring a new drug from initial research to market.¹⁹ In recent years, *in silico* or computer-aided drug discovery (CADD) approaches have been widely adopted to improve the efficiency of drug development.²⁰ These techniques enable researchers to virtually screen thousands of compounds, identify potential drug candidates at an early stage, and minimize the reliance on expensive and time-intensive laboratory experiments. Molecular docking, a key component of CADD, predicts the binding orientation and affinity of small molecules to target proteins or enzymes, which are crucial for determining drug efficacy.²¹ Additionally, ADMET prediction, another component of CADD, estimates a drug's pharmacokinetic and toxicological properties, helping to prioritize compounds with favorable drug-like characteristics.

Currently, there is a lack of comprehensive *in silico* study investigating the potential of *G. biloba* biflavonoids as dual inhibitors of HMG-CoA reductase and α -amylase. To address this gap, the molecular docking was used in this work to evaluate the binding affinities and interactions of five ginkgo biflavonoids (amentoflavone, bilobetin, ginkgetin, isoginkgetin and sciadopitysin) with these two enzyme targets. Additionally, ADMET predictions were performed to evaluate the drug-likeness and potential pharmacokinetic properties of these compounds.

EXPERIMENTAL

Preparation of target proteins

The 3D crystal structures of the human HMG-CoA reductase (HMGR) enzyme (PDB ID: 1HWK)²² in complex with atorvastatin, and the human pancreatic α -amylase enzyme (PDB ID: 2QV4)²³ in complex with acarbose (Fig. 1), were downloaded in PDB format from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (<https://www.rcsb.org/>). These structures have resolutions of 2.22 and 1.97 Å, respectively. Because HMGR is a symmetric tetramer, only chains A and B were used in the docking sim-

ulation.²⁴ In contrast, for α -amylase, which consists of a single protein chain, only chain A was used. The PDB files for both proteins were prepared using Discovery Studio Visualizer (DSV) software version 21.1.0.20298, with all bound substances, such as co-crystallized ligands, cofactors and water molecules, removed from their structures. AutoDockTools²⁵ software version 1.5.6 was then used to further prepare the proteins by adding polar hydrogens and Kollman charges. Finally, the proteins were saved in PDBQT format, the required format for docking.

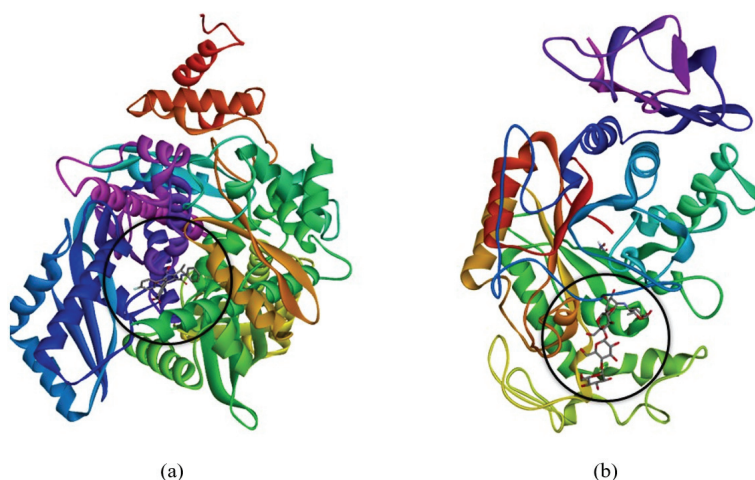


Fig. 1. 3D Crystal structures of human enzymes: a) HMGR (PDB ID: 1HWK) with co-crystallized atorvastatin (encircled in black) and b) α -amylase (PDB ID: 2QV4) with co-crystallized acarbose (encircled in black) bound at the active site.

Preparation of ligands

The five most common ginkgo biflavonoids¹⁸ were selected as ligands to target both HMGR and α -amylase, with their structural formulas shown in Fig. 2. The 3D structures of these ligands, in SDF format, were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Their structures were then optimized using Avogadro²⁶ software version 1.2.0, applying the universal force field and steepest descent algorithm. The ligands were further prepared using AutoDockTools, with the number of torsions set to default. Finally, the ligands were saved in PDBQT format, the required format for docking.

Molecular docking

AutoDock Vina²⁷ software version 1.1.2 was used for the rigid protein-flexible ligand molecular docking simulations. The search space for ligand binding was restricted to a grid box covering the binding sites of the co-crystallized ligands,²⁴ atorvastatin and acarbose. The spacing between grid points was set to 1 Å, with the grid coordinates provided in Table I. AutoDock Vina generated several docking conformations for each ligand, each with a specific binding affinity. The conformation with the most negative binding affinity for each ligand was recorded for further analysis. Protein-ligand interactions were then visualized and analysed using DSV.²⁴

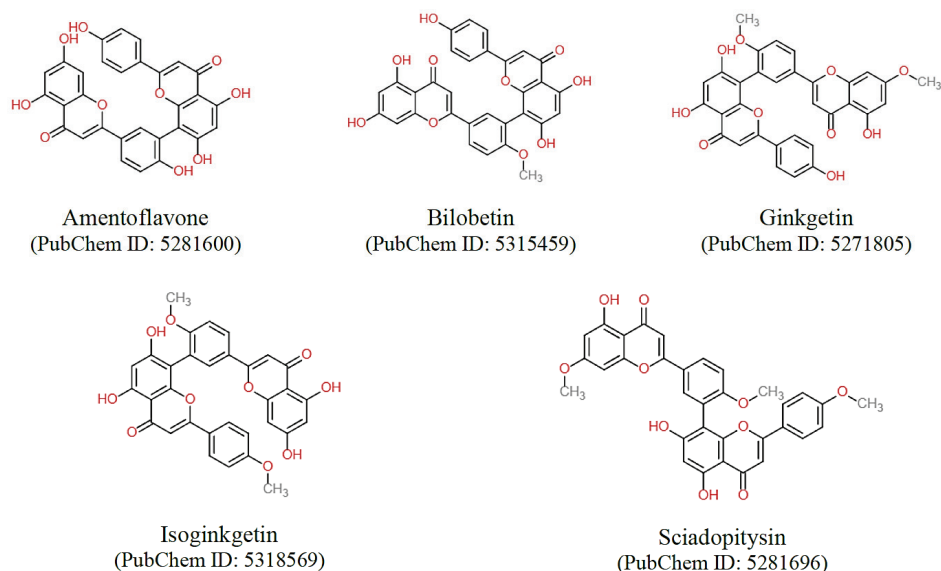


Fig. 2. Structural formulas of the studied biflavonoids.

TABLE I. Grid coordinates for the docking simulation targeting HMGR and α -amylase

Grid	HMGR			α -Amylase		
	<i>x</i>	<i>y</i>	<i>z</i>	<i>x</i>	<i>y</i>	<i>z</i>
Center	2.894171	-9.553439	-12.241561	12.384745	48.136073	26.209218
Size	18	22	24	25	25	30

ADMET prediction

The drug-likeness and pharmacokinetic properties of the studied biflavonoids, including absorption, distribution, metabolism, excretion, and toxicity (ADMET), were predicted using the SwissADME²⁸ (<http://www.swissadme.ch/>) and pkCSM²⁹ (<http://biosig.lab.uq.edu.au/pkcsm/prediction>) online computational tools.

RESULTS AND DISCUSSION

Validation of docking protocol

AutoDock Vina's accuracy as a molecular docking tool was evaluated by redocking atorvastatin and acarbose into the active sites of HMGR and α -amylase, respectively, and comparing their orientations to the native crystal structures using root-mean-square deviation (*RMSD*). The obtained *RMSD* values of the superimposed structures (Fig. 3) were 0.8061 Å for atorvastatin and 1.2590 Å for acarbose, both below the 2.0 Å threshold,^{24,30} confirming the reliability of AutoDock Vina for docking the five bioflavonoids, into the active site of these target proteins.

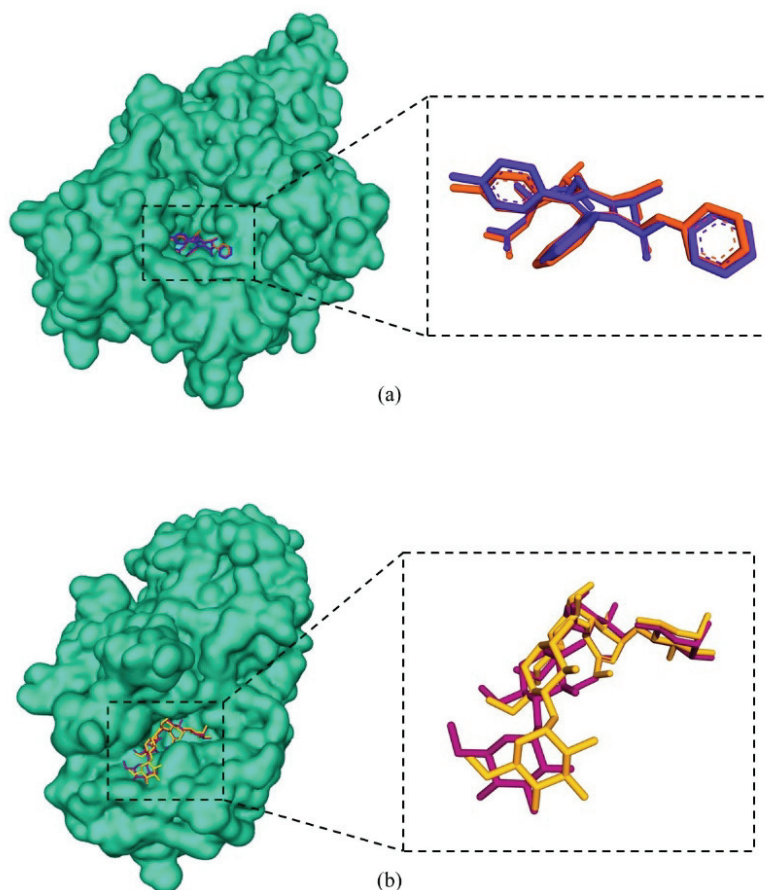


Fig. 3. Superimposed 3D structures of: a) the co-crystallized (orange) and docked (blue) orientations of atorvastatin within the active site of HMGR and b) the co-crystallized (dark fuchsia) and docked (yellow) orientations of acarbose within the active site of α -amylase.

Molecular docking results

The efficacy of a drug depends on its binding affinity and interactions with the target protein.²¹ Binding affinity measures how strongly a ligand interacts with a target protein³¹ and is typically expressed in energy units, kJ mol^{-1} . In this study, molecular docking was employed to determine the binding affinities of five ginkgo biflavonoids towards HMGR and α -amylase. As presented in Table II, the binding affinities of the studied biflavonoids ranged from -34.73 to -42.26 kJ mol^{-1} for HMGR and from -42.26 to -48.12 kJ mol^{-1} for α -amylase. The negative sign indicates that the binding interaction between the biflavonoid ligands and the target proteins is spontaneous and energetically favourable, allowing the ligands to naturally bind to the target proteins without external energy

input. Higher negative values also suggest stronger affinities and more stable binding.^{32,33}

TABLE II. Binding affinity values (kJ mol⁻¹) of the five *Ginkgo biloba* biflavonoids towards human enzymes HMGR and α -amylase

Ligand name	Protein	
	HMGR	α -Amylase
Amentoflavone	-42.26	-48.12
Bilobetin	-41.00	-47.28
Ginkgetin	-37.24	-46.44
Isoginkgetin	-38.07	-42.68
Sciadopitysin	-34.73	-42.26
Atorvastatin ^a	-38.91	—
Acarbose ^b	—	-43.93

^aReference HMGR inhibitor; ^breference α -amylase inhibitor

The docking results showed that two biflavonoids, amentoflavone (-42.26 kJ/mol) and bilobetin (-41.00 kJ/mol), exhibited stronger binding affinities to HMGR compared to the reference drug, atorvastatin (-38.91 kJ/mol). For α -amylase, three biflavonoids—amentoflavone (-48.12 kJ/mol), bilobetin (-47.28 kJ/mol), and ginkgetin (-46.44 kJ/mol) showed stronger binding affinities than the reference drug, acarbose (-43.93 kJ/mol). A ligand with a highly negative binding affinity is generally considered to have good inhibitory potential, as strong binding to the enzyme's active site usually correlates with the effective inhibition of the enzyme's activity by preventing it from performing its normal function. These docking results indicate that amentoflavone and bilobetin have the potential to act as dual inhibitors of HMGR and α -amylase, potentially offering better inhibitory activity than the standard drugs. Since HMGR is responsible for cholesterol synthesis and α -amylase is involved in the metabolism of carbohydrates into simple sugars, the strong binding of these biflavonoids to both enzymes could potentially inhibit their activities, leading to reduced cholesterol and blood sugar levels. Therefore, amentoflavone and bilobetin may possess both antihypercholesterolemic and antidiabetic properties.

The molecular interactions of the top two ligands with dual inhibitory potentials, amentoflavone and bilobetin, with HMGR and α -amylase, are presented in Fig. 4. The amino acids involved in the protein-ligand interactions are summarized in Tables III and IV. The docking study predicted the existence of hydrogen bonds, hydrophobic and electrostatic intermolecular noncovalent interactions between the biflavonoids and HMGR and α -amylase. These interactions are crucial for stabilizing the protein–ligand complexes.³⁴

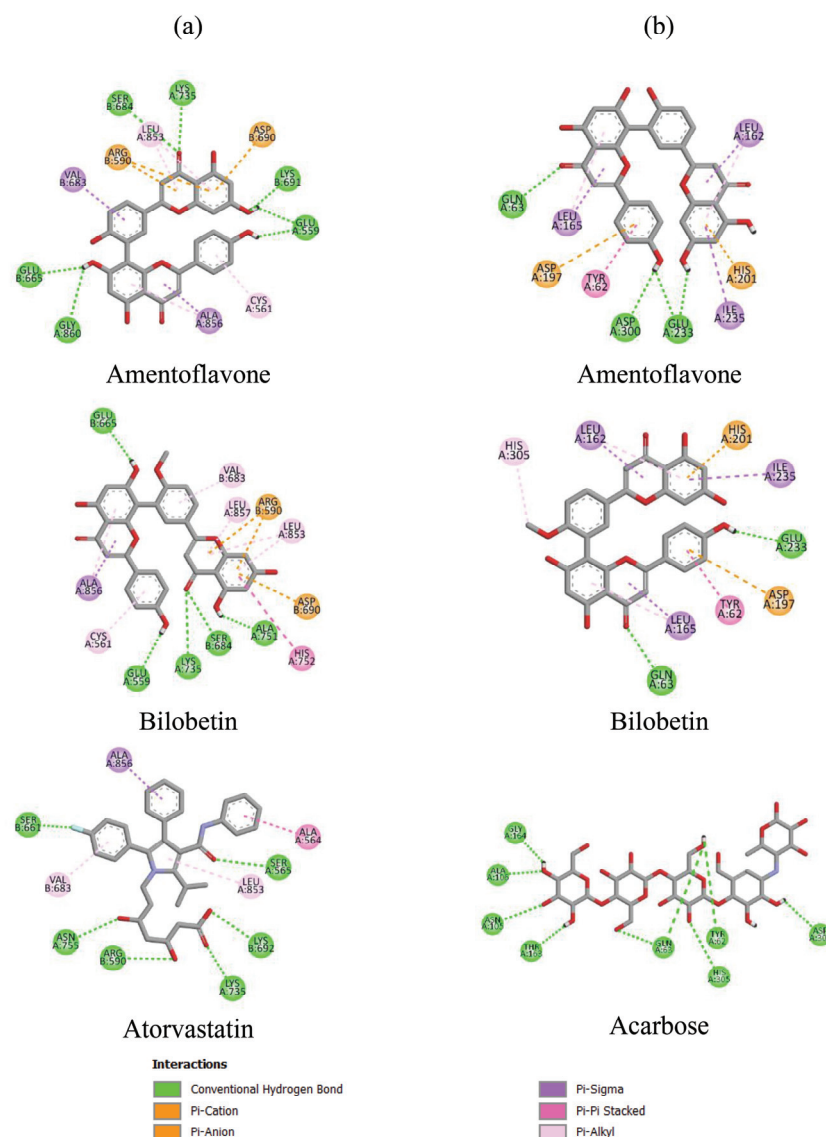


Fig. 4. 2D interaction diagrams of amentoflavone, bilobetin and atorvastatin in complex with: a) HMGR and b) α -amylase. π - σ , π - π stacked, and π -alkyl interactions are classified as hydrophobic interactions, while π -cation and π -anion interactions are classified as electrostatic interactions.

The analysis of the HMGR-amentoflavone complex (Fig. 4a) revealed that amentoflavone formed multiple hydrogen bonds with key residues Lys735A, Ser684B, Lys691B, Glu559A, Gly860A and Glu665B. It also established the hydrophobic interactions with Ala856A, Val683B, Cys561A and Leu853A, and

electrostatic interactions with Arg590B and Asp690B. Similarly, bilobetin demonstrated a stable binding profile, forming hydrogen bonds with Lys735A, Ser684B, Glu559A, Ala751A and Glu665B amino acids of HMGR. Its binding was further stabilized by hydrophobic interactions involving Ala856A, His752A, Cys561A, Val683B, Leu853A and Leu857A, as well as the electrostatic interactions with Arg590B and Asp690B.

TABLE III. Molecular interactions of the top two biflavonoids with HMGR amino acids

Ligand name	HMGR amino acids involved in interaction		
	Hydrogen bonds	Hydrophobic interactions	Electrostatic interactions
Amentoflavone	Lys735A, Ser684B, Lys691B, Glu559A, Gly860A, Glu665B	Ala856A, Val683B, Cys561A, Leu853A	Arg590B, Asp690B
Bilobetin	Lys735A, Ser684B, Glu559A, Ala751A, Glu665B	Ala856A, His752A, Cys561A, Val683B, Leu853A, Leu857A	Arg590B, Asp690B
Atorvastatin ^a	Ser565A, Lys735A, Asn755A, Arg590B, Ser661B, Lys692B	Ala856A, Ala564A, Leu853A, Val683B	None

^aReference HMGR inhibitor

TABLE IV. Molecular interactions of the top two biflavonoids with α -amylase amino acids

Ligand name	α -Amylase amino acids involved in interaction		
	Hydrogen bonds	Hydrophobic interactions	Electrostatic interactions
Amentoflavone	Gln63, Glu233, Asp300	Leu162, Leu165, Ile235, Tyr62	His201, Asp197
Bilobetin	Gln63, Glu233	Leu162, Leu165, Ile235, Tyr62, His305	His201, Asp197
Acarbose ^a	Gln63, Asn105, Ala106, His305, Thr163, Gly164, Tyr62, Asp300	None	None

^aReference HMGR inhibitor

In comparison, atorvastatin, the standard drug, also interacted with HMGR through hydrogen bonds with Lys735A, Ser565A, Asn755A, Arg590B, Ser661B and Lys692B. Its binding was stabilized by hydrophobic interactions with Ala856A, Ala564A, Leu853A and Val683B. However, unlike amentoflavone and bilobetin, atorvastatin lacked electrostatic interactions (Table III), which may account for its relatively weaker binding affinity compared to the two biflavonoids.

Interestingly, amentoflavone and bilobetin share key hydrogen bonds with Lys735A and hydrophobic interactions with Ala856A and Val683B, similar to atorvastatin. As a competitive inhibitor of HMGR, atorvastatin competes with the

enzyme's natural substrate (HMG-CoA) for binding at the active site, thereby inhibiting the enzyme's ability to catalyse the conversion of HMG-CoA into mevalonate, a crucial step in cholesterol biosynthesis. These findings suggest that amentoflavone and bilobetin may inhibit HMGR through a similar mechanism.

On the other hand, analysis of the α -amylase-amentoflavone complex (Fig. 4b) revealed that amentoflavone formed hydrogen bonds with key residues Gln63, Glu233 and Asp300, while also establishing hydrophobic interactions with Leu162, Leu165, Ile235 and Tyr62. Additionally, it formed electrostatic interactions with His201 and Asp197. Bilobetin similarly demonstrated strong binding to α -amylase, forming hydrogen bonds with Gln63 and Glu233. It also formed hydrophobic interactions with Leu162, Leu165, Ile235, Tyr62 and His305, along with electrostatic interactions with His201 and Asp197.

Acarbose, the standard antidiabetic drug, primarily interacted with α -amylase through a network of hydrogen bonds involving residues Gln63, Asn105, Ala106, His305, Thr163, Gly164, Tyr62 and Asp300. Notably, acarbose lacked the hydrophobic and electrostatic interactions observed with both amentoflavone and bilobetin (Table IV). The stronger binding affinity of amentoflavone and bilobetin to α -amylase, compared to acarbose, may be attributed to their hydrophobic and electrostatic interactions. This suggests that these two biflavonoids might inhibit α -amylase better than acarbose.

Comparing these interactions, it is evident that both amentoflavone and bilobetin share key hydrogen bonding with the Gln63 and Tyr62 amino acids, similar to acarbose. Acarbose also functions through competitive inhibition by binding to the active site of α -amylase, thereby inhibiting the catalytic breakdown of carbohydrates into simpler sugars. The similarity in interactions suggests that these two biflavonoids may inhibit α -amylase through a mechanism of action similar to that of acarbose.

Drug-likeness and ADMET profiles

The Lipinski's rule of five (Ro5), also known as Pfizer's rule of five, is a widely recognized set of guidelines used in the pharmaceutical industry to evaluate the drug-likeness and oral bioavailability of compounds during the early stages of drug development. The rule³⁵ states that an orally active drug should generally not violate more than one of the following criteria: hydrogen bond donors < 5, hydrogen bond acceptors < 10, molecular weight < 500 g/mol and octanol–water partition coefficient ($Mlog P$) < 5. Table V presents the drug-like properties of the top two ligands with dual inhibitory potentials, amentoflavone and bilobetin. Bilobetin violates only the molecular weight criterion, whereas amentoflavone violates both the molecular weight and hydrogen bond donor criteria. These results suggest that bilobetin is more likely to be an orally active drug in humans compared to amentoflavone. Furthermore, the calculated bioavail-

ability scores indicate that bilobetin has a 55 % probability of being bioavailable, while amentoflavone has only a 17 % probability. Therefore, bilobetin is a more promising drug candidate with better drug-like properties than amentoflavone.

TABLE V. Drug-like properties of the top two biflavonoids predicted by SwissADME

Property	Amentoflavone	Bilobetin
Molecular weight, g/mol	538.5	552.5
<i>Mlog P</i>	0.25	0.44
Hydrogen bond acceptors	10	10
Hydrogen bond donors	6	5
Lipinski's rule violations	2	1
Bioavailability score	0.17	0.55

TABLE VI. ADMET (admission, distribution, metabolism, excretion, toxicity) profiles of the top two biflavonoids

Parameter	Pharmacokinetic property	Amentoflavone	Bilobetin
Absorption ^a	Gastrointestinal absorption	Low	Low
	P-glycoprotein substrate	No	No
Distribution ^a	Blood–brain barrier permeability	No	No
Metabolism ^a	CYP1A2 inhibitor	No	No
	CYP2C19 inhibitor	No	No
	CYP2C9 inhibitor	No	Yes
	CYP2D6 inhibitor	No	No
	CYP3A4 inhibitor	No	No
Excretion ^b	Total clearance, mL min ⁻¹ kg ⁻¹	3.05	3.72
Toxicity ^b	AMES toxicity (mutagenicity)	No	No
	Hepatotoxicity	No	No

^aPredicted by SwissADME; ^bpredicted by pkCSM

Table VI presents the pharmacokinetic properties and ADMET profiles of amentoflavone and bilobetin. Both compounds exhibit low gastrointestinal (GI) absorption which may limit their effectiveness as orally administered drugs. However, both are not substrates for P-glycoprotein (P-gp), suggesting they may not be actively transported out of cells by this transporter,³⁶ potentially enhancing their bioavailability. Moreover, both biflavonoids do not penetrate the blood–brain barrier (BBB), indicating that they are unlikely to cause central nervous system side effects. Since the targets of these compounds, HMGR and α -amylase, are not located in the central nervous system, BBB penetration is not necessary for their therapeutic effects.³⁷ Cytochrome P450 (CYP450) enzymes play crucial roles in drug metabolism and detoxification. They oxidize drugs and other xenobiotics in the body for excretion. There are several CYP450 isoforms, including CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 which are involved in biotransformation of drugs. Inhibition of these CYP450 isoforms can affect drug metabolism and can lead to toxicity due to bioaccumulation.^{29,37} The

results showed that amentoflavone is a non-inhibitor of all these CYP450 isoforms, while bilobetin is a non-inhibitor of most of them, except for CYP2D6. This suggests that both compounds are metabolized efficiently and are less likely to interfere with the body's normal drug-metabolizing processes, reducing the risk of adverse drug interactions. The excretion parameter reveals that bilobetin has a slightly higher total clearance ($3.72 \text{ mL min}^{-1} \text{ kg}^{-1}$) compared to amentoflavone ($3.05 \text{ mL min}^{-1} \text{ kg}^{-1}$). These values are important for determining dosing rates to achieve steady-state concentrations.²⁹ Importantly, bilobetin and amentoflavone exhibit no mutagenic or hepatotoxic properties, suggesting favourable safety profiles. This lack of toxicity is a significant advantage for their therapeutic use, as it indicates a lower risk of side effects during treatment. Given the adverse effects associated with current HMGR and α -amylase inhibitors, amentoflavone and bilobetin present promising alternatives for the management of hypercholesterolemia and diabetes. Their combination of effectiveness and low toxicity positions them as the potential candidates for further development as safer therapeutic agents.

CONCLUSION

This study reports for the first time that both amentoflavone and bilobetin possess dual inhibitory activity against HMG-CoA reductase (HMGR) and α -amylase, as it is revealed by molecular docking, with binding affinities superior to those of the reference antihypercholesterolemic and antidiabetic drugs, atorvastatin and acarbose, respectively. These findings suggest that both compounds have the ability to inhibit the key enzymes involved in cholesterol biosynthesis and carbohydrate metabolism. Pharmacokinetic predictions indicate that bilobetin is a more promising drug candidate than amentoflavone, due to its better compliance with Lipinski's rule of five and a higher probability of oral bioavailability. Although bilobetin shows low gastrointestinal absorption, it is predicted to be non-mutagenic and non-hepatotoxic, supporting its favourable safety profile. Therefore, bilobetin is a potential candidate for the treatment of hypercholesterolemia and diabetes, which could be more effective and safer to use. Further *in vitro* and *in vivo* studies are recommended to confirm these promising results.

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ИЗВОД

IN SILICO МОЛЕКУЛСКИ ДОКИНГ И АДМЕТ ПРЕДВИЂАЊЕ БИФЛАВАНОИДА ИЗ
GINKGO BILOBA КАО ДУАЛНИХ ИНХИБИТОРА ХУМАНЕ НМГ-СОА РЕДУКТАЗЕ И
 α -АМИЛАЗЕ

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Повећава се занимање за фитохемикалије као инхибиторе ензима НМГ-СоА редуктазе (НМГР) и α -амилазе. Инхибиција ових ензима помаже у третирању хиперхолестеролемије и дијабетеса редукујући синтезу холестерола, односно ниво шећера у крви. У овој студији су коришћене рачунарске технике за АДМЕТ предвиђање да би се одредио потенцијал пет *Ginkgo biloba* бифлаваноида (аментофлавона, билобетина, гинкгетина, изогинкгетина и сајадоптисина) као дуалних инхибитора НМГР и α -амилазе. Аментофлавон (–42,26 kJ/mol) и билобетин (–41,00 kJ/mol) испољили су јачи афинитет везивања за НМГР, у поређењу са референтним леком аторвастатином (–38,91 kJ/mol). За α -амилазу, аментофлавон (–48,12 kJ/mol), билобетин (–47,28 kJ/mol), и гинкгетин (–46,44 kJ/mol) испољен је јачи афинитет везивања у поређењу са референтним леком акарбозом (–43,93 kJ/mol). Резултати доковања указују да аментофлавон и билобетин имају потенцијала да делују као дуални инхибитори за ова два езима. АДМЕТ анализа је показала да је билобетин испољио повољну оралну биодоступност и сличност са лековима, због поштовања правила петице Липинског. Упркос испољавању ниске гастроинтестиналне апсорпције, показало се да није ни мутаген ни хепатотоксичан. Према наведеном, билобетин је обећавајући кандидат за дуалну антидијабетску и антихиперхолестеролемијску примену. Препоручене су даље *in vitro* и *in vivo* студије за потврђивање ових обећавајућих резултата.

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Molecular dynamics simulation of uranium nitride oxidation

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Abstract: A molecular dynamic simulation of the uranium mononitride (UN) oxidation in an Ar–O medium in the temperature range of 373–2073 K is performed. The study is performed for UN particles with a crystalline and amorphous structure at an oxygen concentration in the gas mixture of 22.5 mol. %. The most efficient oxidation for an amorphous particle occurs at lower temperatures than that for a crystalline particle. Unlike crystalline fragments, amorphous particles undergo more severe fragmentation when they bind to oxygen. Fragmentation of UN particles is one of the main factors regulating the oxidation of finely dispersed media. The oxidation of a UN particle begins from its surface and in the case of an amorphous particle occurs faster than when the particle is crystalline. The process of particle fragmentation is facilitated by the penetration of oxygen atoms inside the particle. An increase in the oxygen concentration in the gas mixture stimulates the oxidation process. The structural changes in the system are investigated by constructing the partial radial distribution functions. The many-body U–N interactions prevent nitrogen escaping into the gaseous environment.

Keywords: amorphous and crystalline fragments; argon; calculation; oxygen; structure.

INTRODUCTION

The pyrochemical technology for reprocessing spent nuclear fuel (SNF) includes several stages, among which a high-temperature processing (HTP) of SNF is at the beginning of the entire technological chain and is the main stage. The HTP stage includes several steps. At the first step, the SNF pellets are dispersed and separated from the fuel rod cladding using the high-temperature nitriding procedure.¹ At the second stage of HTP, the components of nitride SNF

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are oxidized in an atmosphere of dry oxygen and argon with the formation of oxides of fissile materials and part of the fission products. The conversion of UN to UO_2 is one of the preparatory operations before conducting spent nuclear fuel recovery procedures. The need to convert spent nitride nuclear fuel into oxides arises from the fact that the direct reduction of UN during the electrolytic process in molten salt is incomplete, and UO_2 is more suitable for pyrochemical reprocessing.

High thermal conductivity and melting point values at high densities make uranium mononitride (UN) one of the best fuel components for the fourth-generation nuclear reactors.² The advantages of nitride fuel over oxide fuel allow the fuel to operate at higher burnups. The increased formation of interstitial N atoms and U vacancies leading to a hyperstoichiometry of nitride fuel at elevated temperatures does not have a significant effect on its thermal and mechanical properties.³ At the same time, fission gas atoms have low solubility in the fuel matrices. The release of these gases leads to swelling of the fuel and deterioration of its thermal conductivity. The energy barrier to the diffusion of released gases decreases when moving from pure UN to the disordered $\text{U}_{0.5}\text{Pu}_{0.5}\text{N}$.⁴ Moreover, the energy barrier for Kr diffusion (0.30–1.25 eV) turns out to be lower than that for Xe diffusion (0.50–1.75 eV) due to the smaller size of the Kr atom.

The oxidation of real powdered UN with a surface area of $0.2\text{--}0.4\text{ m}^2\text{ g}^{-1}$ in the temperature range of 503–543 K resulted in the formation of an amorphous product with the composition of $\text{UO}_3\text{N}_{0.2\text{--}0.4}$, which releases nitrogen slowly and recrystallizes into $\alpha\text{-UO}_3$.⁵ At the same time, X-ray studies showed that U_2N_3 and UO_2 are formed as intermediate oxidation products. It is known that UO_2 can be reduced to metallic uranium with a yield of up to 99.7 %.⁶ However, this result cannot be obtained using the electrolytic method if the reduction of the spent nitride fuel is carried out.

Based on density functional theory (DFT) calculations, it was shown that in the pressure range of 0–1000 kbar, uranium is capable of reacting with nitrogen to form various U–N compounds in the form of semiconductors and metals.⁷ A new tetragonal phase for UN_2 , more stable than the known phases for this compound, was found. In addition, the new stoichiometry of U_3N_5 was shown to be stable at atmospheric pressure. At pressures above 500 kbar, not only the phases with a high nitrogen content (UN_4 , UN_3), but also the phase with a high uranium content (U_2N), which initially has an orthorhombic structure, turned out to be stable. The pressure-induced phase transition of U_2N to the monoclinic phase occurs at a pressure of 980 kbar. Both the orthorhombic and monoclinic U_2N structures consist of one-dimensional zigzag U-chains, which play an important role in stabilizing U-rich compounds. All phase transitions (with the exception of the transition to UN_2) are first-order transitions and occur with a change in volume.

The chemical bond in the uranium dinitride (UN_2) molecule is not studied properly. This is mostly due to the complexity of the electronic structure of the uranium atom, the quantum mechanical calculation which requires taking into account both electronic correlation and relativistic effects. Particularly large discrepancies are observed in the calculations of excitation energies when different theoretical approaches are used.^{8–10}

The valence shells of early-middle actinides (5f6d7sp from Pa to Pu) are energetically close to each other, and the orbital radii regularly increase with the principal quantum number.¹¹ The formation of multiple 5f, 6d, 7sp bonds with partner atomic orbitals (AO) is characteristic of these actinides. The different occupancy of the 5f AO in these elements is due to the strong electron repulsion between the 5f, 6d and 6p shells, which are located in close radial regions.

Studying the electronic structure and bonds in individual ions allows us to better understand the formation of bonds between the same elements in a bulk material. The uranyl ion (UO_2^{2+}) predominantly forms complexes with oxygen-containing ligands that have donor properties with respect to the central cation UO_2^{2+} . The stability and electronic structure determine the stability of the bonds in the complex. The U–O bond length in UO_2^{2+} is smaller (1.704 Å) than the U–N bond length in UN_2 (1.736 Å).¹² The linear form of each of these structures is the most stable. The transition to the bent form of the uranyl or UN_2 molecule causes the destabilization of the U 6p_{3/2} orbitals, which is more pronounced for UO_2^{2+} . Although the study was performed for “naked” molecules, the results obtained can also be applied to complexes with very weak ligand fields.

The geometric structure of NUO^+ appears to be linear with possible additional N_2 ligands in the second coordination sphere of the type $[\text{NUO} \cdot (\text{N}_2)_5]^+$.¹³ The dynamics of this complex is characterized by high N–U stretching modes and relatively low U–O modes. Stabilization of the complex occurs due to $\text{N} \equiv \text{U}$ and $\text{U} \equiv \text{O}$ triple bonds. In NUO^+ , the interaction between more covalent N–U bonds with more ionic U–O bonds is observed, with the terminal atoms effectively competing for U-5f, 6d and 7sp atomic orbitals. Here, the charge transfers from U to N and O occur in amounts of 0.4 and 1.2e, respectively.¹³

The aim of the present work is to study the stability of crystalline and amorphous UN particles immersed in a gas mixture of argon and oxygen, as well as to determine the optimal conditions for the oxidation of these particles, and to identify the influence of particle structure on their oxidation.

MOLECULAR DYNAMICS MODEL

The oxidation of UN was studied using two types of models. In one of them, crystalline UN particles, and in the other, amorphous UN particles were placed in a gaseous mixture of argon and oxygen. Consideration of particles with regular (crystalline) and irregular (amorphous) structures surrounded by a gaseous Ar–O medium was executed to study the influence of the particles structure on the process of their oxidation. The details of preparation pro-

cedure of the simulated systems “UN crystalline/amorphous particle in the Ar–O gaseous medium” are presented in the Supplementary material to this paper.

Taking into account that there are no experimental data on the reflection of gas atoms from the wall, in our model, the Maxwellian reflection boundary condition was used as the boundary condition, acting on all walls of the rectangular cell with the accommodation coefficient equal to 0.5. Modelling at the predominantly high temperatures of the gas medium with such conditions led to the formation of random gas flows that acted on the solid UN particle. As a result, the UN particle acquired both translational and rotational motions. Such behaviour of the particle in the argon–oxygen mixture was largely represented by the behaviour of a real particle in a gas flow and increased its adsorption capacity.

The principal part of the calculations was executed in the NVT ensemble at different temperatures from the range of $373 \leq T \leq 2073$ K (for every temperature, a separate calculation was executed). The thermostat relaxation time was $\tau_T = 4$ fs. The model contained 3172 atoms: 1772 Ar, 400 O, 500 U and 500 N. The density of the system was 0.48 g cm^{-3} . The pressure in the gas medium did not exceed 500 bar. The total calculation time for the systems with amorphous/crystalline UN particle for every temperature was up to 1 ns with the time step for integrating the motion equations of $\Delta t = 0.1$ fs. The Verlet scheme was used in the calculations for integrating the equations of motion.¹⁴

The U–U, U–N and U–O interactions were described by many-body EAM potential, the concept of which is based on the “embedded atom” strong-binding model.^{15,16} The interaction of crystalline or amorphous particle with components of gaseous medium as well as the interactions between gaseous components (Ar–O, Ar–U, Ar–N, Ar–Ar, O–O, N–O) were described using the Lennard–Jones potential with parameters taken from.¹⁷ These data are summarized in Table I.

TABLE I. Parameters of the Lennard–Jones potential¹⁷

Bond	ε / eV	$\sigma / \text{\AA}$
Ar–O	0.0103	3.385
Ar–U	0.00314	3.192
Ar–N	0.00929	3.537
N–O	0.00929	3.512
Ar–Ar	0.0104	3.41
O–O	0.0103	3.36

During the simulation, it was revealed that due to the use of the EAM potential, N atoms are strongly bound to U atoms. As a result, nitrogen atoms do not break away from uranium atoms even at the highest temperatures at which the calculations were performed. At the same time, the O atoms, also interacting with the U atoms according to the appropriately selected EAM potential, actively bonded with the uranium atoms. To demonstrate the stabilizing effect of many-particle interactions at the final stage of simulation (when the duration of the calculation was 220 ps), the EAM interaction between the U and N atoms was replaced by a potential built on the basis of the Morse potential function.¹⁸ The description of the potential details as well as potential parameters are presented in the Supplementary material.

DFT calculations showed that in the process of removing oxygen from UO_2 , the reduction process is enhanced by the appearance of electrical conductivity in the recovered material. More precisely, electrical conductivity appears when the composition U_2O_3 is reached, which initiates direct reduction of this oxide.¹⁹ To establish how strongly the concentration of oxy-

gen in the gaseous medium affects the degree of oxidation of UN particles, another series of calculations were carried at $T = 973$ K, with adding 100 (oxygen concentration 26.7 mol. %), 200 (30.4 mol. %), 300 (33.8 mol. %) and 400 oxygen particles (maximum oxygen concentration of 36.8 mol. %). When the oxygen concentration in a Ar–O gas mixture was 36.8 %, the formation of the U_2O_3 compound became possible.

Additional oxygen was added into the system sequentially through the upper edge of the MD cell, one particle every 0.2 ps. When the required number of oxygen atoms was added, a relaxation MD calculation was performed for 100 ps to equilibrate the system. Then, the main calculation was carried out with the new number of O atoms in the system.

The content of bonds in the system was monitored using a special algorithm we developed, which allowed us to determine which U atoms formed a U–O bond. The algorithm was realized as follows. First of all, it analysed coordinates of all atoms of the system at equal time intervals (5 ps). Second, it was assumed that a bond between the U and O atoms was formed if the distance between these atoms became less than 3.3 Å. This parameter of connectivity between these atoms was determined while studying the stability of UO_{2-x} hypostoichiometric compounds.²⁰ Third, the operation of the algorithm was based on storing a list of distances where $r \leq 3.3$ Å, along with the numbers of atoms between which the selected distances were formed. The formed bonds were determined by processing the entire list based on the stored numbers of the selected atoms. The minimum and maximum (just 3.3 Å) distances in the formed bonds were also determined. Finally, the number of O atoms forming bonds with U atoms was visually represented in relative units or percentages relative to the total number of O atoms in the system.

All calculations were performed with the use of the open-source program code for MD simulation LAMMPS (large-scale atomic/molecular massively parallel simulator)²¹ on a URAN cluster-type hybrid computer at the N. N. Krasovskii Institute of Mathematics and Mechanics UB RAS with a peak performance of 216 Tflop/s and 1864 CPU.

RESULTS AND DISCUSSION

Fig. 1a shows the configuration of the system with an amorphous UN fragment in the Ar–O gas mixture obtained at a temperature of 923 K, at time of 470 ps. In the model, fragmentation of the UN nanoparticle occurs. Many small fragments separated from the particle, although its larger part was also preserved. The UN particle has lost its initial spherical appearance, and the surface of its remaining part has acquired a “pockmarked” shape. While Ar atoms are uniformly distributed throughout the volume of the MD cell, the O atoms preferentially tend to occupy positions near UN fragments and, especially, near the larger particle fragment. However, at this initial stage of oxidation, the detachment of N atoms from the UN fragments is not observed.

Fig. 1b and 1c provide information about the state of a microheterogeneous system containing an amorphous (Fig. 1b) and a crystalline (Fig. 1c) UN fragment at a temperature of 1023 K and a time of $t = 260$ ps. Argon atoms are not shown in the figure to clarify the fragmentation details. Both the amorphous and crystalline UN particles are subjected to fragmentation, with a tendency to retain a larger UN fragment. The larger remaining particle and the resulting clusters are surrounded by oxygen. There is noticeably more unbound oxygen, and, conver-

sely, there are fewer small UN clusters in the presence of crystalline formations in the system. Consequently, the processes of fragmentation and oxidation in a system with an amorphous particle occur more actively than in the presence of a crystalline particle of the same size.

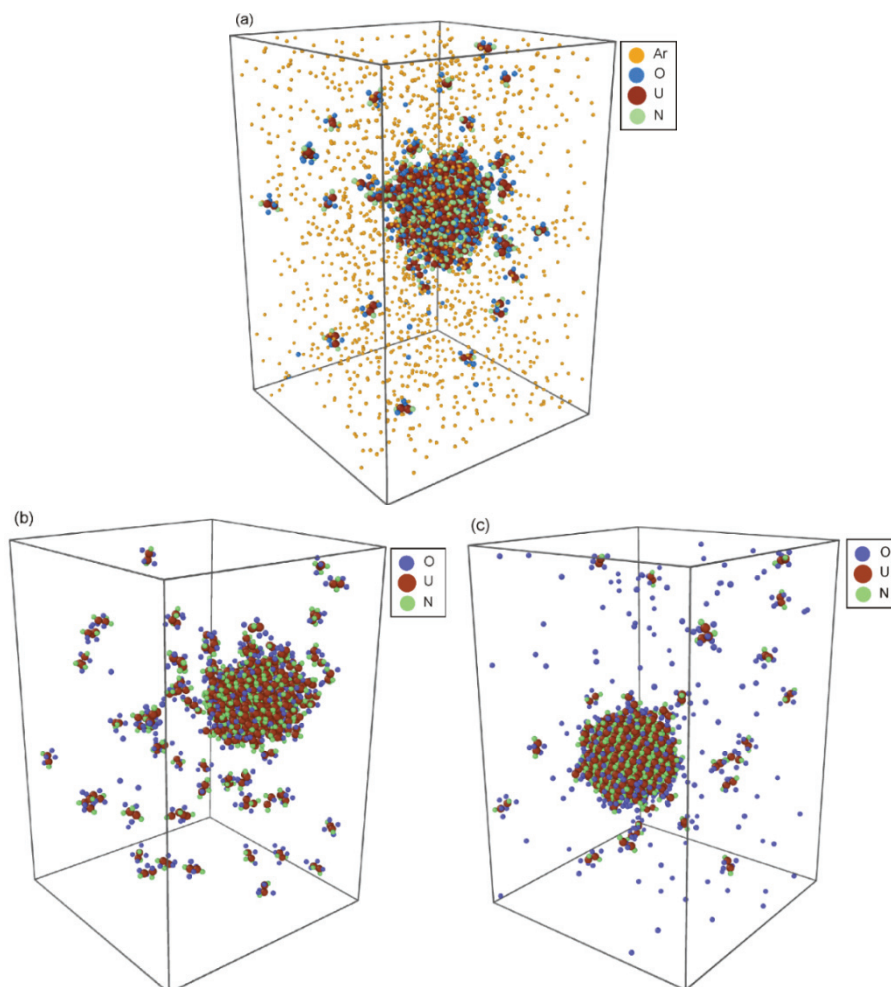


Fig. 1. Configurations of the system “UN fragments in a gaseous mixture of Ar+O”: a) the amorphous particle configuration at a temperature of 923 K corresponding to the time moment of $t = 470$ ps; configurations of: b) amorphous and c) crystalline UN particles at $T = 1023$ K and $t = 260$ ps; Ar atoms are not shown here for the visibility.

The difference in the structures of amorphous and crystalline particles can be seen in the form of partial radial distribution functions $g_{U-N}(r)$ characterizing the placement of N atoms around U atoms for these particles. The $g_{U-N}(r)$ functions

for crystalline particles have many pronounced peaks, and the number of these peaks decreases as the temperature of the system rises (Fig. 2). In contrast, the $g_{\text{U-N}}(r)$ functions for amorphous particles are characterized by only three obvious peaks.

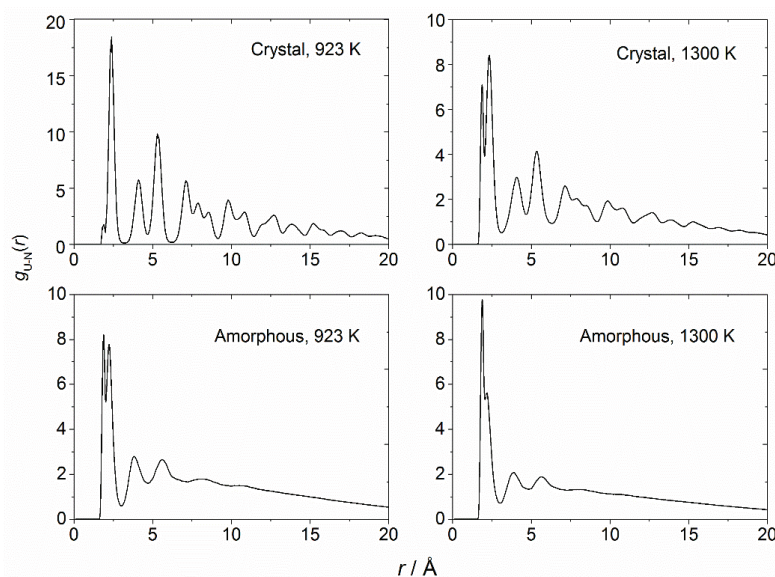


Fig. 2. Partial radial distribution functions $g_{\text{U-N}}(r)$ obtained by oxidation of crystalline and amorphous UN fragments in a gas mixture of Ar with 22.5 mol. % oxygen at different temperatures.

The intensity of the last two peaks decreases with the increase of temperature. The first, most intensive peak of the $g_{\text{U-N}}(r)$ function can be split or have a shoulder in both the amorphous and crystalline particles. For the crystalline particles, the intensity of the first peak of the $g_{\text{U-N}}(r)$ function decreases also with the increase of temperature, because the probability of finding N atoms around U atoms at nearest neighbour distances decreases. In the case of amorphous particles, the opposite trend may be observed, with the main peak of the $g_{\text{U-N}}(r)$ function splitting.

The time sequence of the formation of U–O bonds in systems with UN nanoparticles every 5 ps during the final part of the MD calculation at temperatures of 823 and 1273 K, is presented in Fig. 3. The initial interval (not shown in the figure) of the appearance of U–O bonds has a duration of 0.5 million time steps (50 ps). This initial interval is characterized by the rapid fragmentation of amorphous particles.

The left side of the figure presents the results of the formation of U–O bonds over time for the initially crystalline UN particles, and the right side gives the

information for the amorphous UN particles. The horizontal dashed line and the number above indicate the time-averaged number of U–O bonds (n_b) appearing in each case. It can be seen that the n_b value for the crystalline nanoparticle at both temperatures is lower than that for the amorphous particle. Moreover, with the increase of temperature, the n_b value rises noticeably for the crystalline fragment, whereas in the case of oxygen adsorption onto a fragment with an amorphous structure, the opposite effect can be observed.

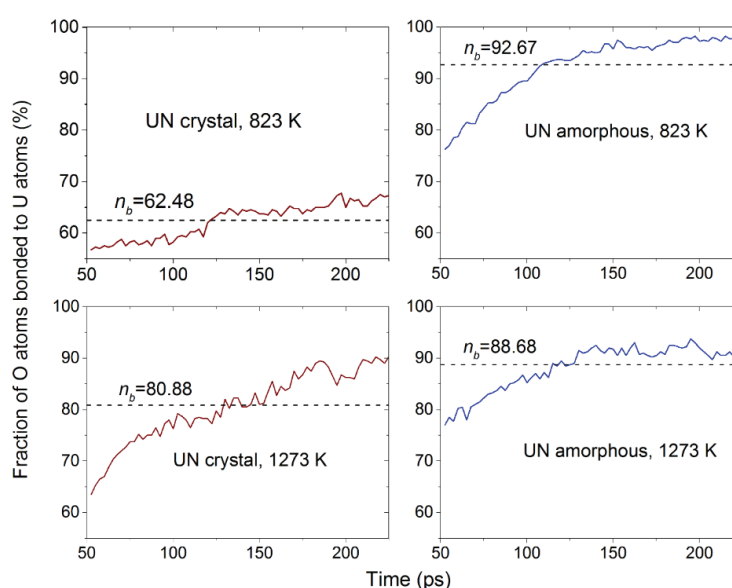


Fig. 3. Fraction of O atoms bonded to U atoms for initially crystalline and amorphous UN particles at different temperatures; the horizontal dashed line and the number above indicate the n_b time-averaged number of U–O bonds appearing in each case.

Fig. 4 shows the average number of attachments of O atoms to U atoms (or n_b) obtained after every MD run and presented as a percentage of the total number of oxygen atoms in the system. As can be seen from the figure, there is a significant difference in the oxygen adsorption on crystalline and amorphous particles. A relatively small amount of oxygen atoms, varying from 62 to 65 % of the total amount of oxygen (400 atoms) present in the system, is adsorbed onto the surface of the crystalline particle up to a temperature of 923 K. Next, there is a trend of a slight increase in the amount of adsorbed oxygen when the temperature reaches 1123 K, where the proportion of deposited oxygen is 69 %.

A subsequent rise in temperature causes an increase in the fraction of deposited oxygen atoms up to 82.7 % at a temperature of 1300 K. Then, up to a temperature of 1673 K, a high level of adsorbed oxygen is maintained, with its fraction fluctuating by around 6 %. A further increase in temperature to 2073 K

causes an almost linear reduction in the fraction of oxygen attached by the UN particle, so that the percentage of oxygen at this temperature becomes even lower than at 1123 K.

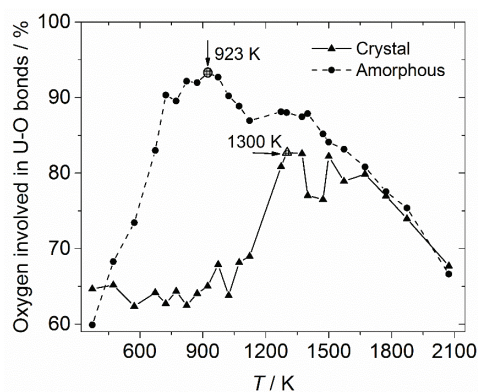


Fig. 4. The fraction of O atoms forming bonds with the U atoms of the crystalline and amorphous UN particles in relation to the number of all oxygen atoms in the system.

The fraction of oxygen attached to the amorphous UN particle immediately rises quickly when the temperature reaches 723 K (from an initial temperature of 373 K). Further, the growth rate of oxygen added to the amorphous particle slows down until it reaches a maximum at 923 K. After that, there is a decline in the amount of oxygen attached by the amorphous particle, leading to its local minimum at a temperature of 1123 K. The following increase in the fraction of adsorbed oxygen occurs up to 1400 K, and then a trend toward a rapid decrease (approximated by a linear decline) in attached oxygen appears. Moreover, an increase in temperature, starting from 1673 K, leads to similar values of the fraction of oxygen attached to the amorphous and crystalline particles. Uranium mononitride does not maintain its composition upon heating to its melting point T_m . The decomposition of UN begins much earlier, prior to reaching T_m . Uranium mononitride begins to decompose already at 1673 K. At this temperature, the nitrogen pressure caused by the decomposition of UN is only 10^{-3} Pa.²² However, it is not the departure of N atoms from the nanoparticle that is important; rather, it is the loosening of its surface that leads to the softening of the surface vibrational modes. For particles smaller than 5 nm, the softening occurs at lower temperatures (by several hundred degrees) compared to macroscopic objects. The softening of vibrations leads to an increase in adsorption capacity per unit surface area.²³ Additionally, the surface of the particles becomes rough, further enhancing their adsorption capacity. These two factors begin to dominate at temperatures above 1673 K, while, conversely, the difference in the structure of the particles is levelled out. The identical behaviour in the adsorption characteristic of nanoparticles with different structures can be explained by changes in the factors influencing the adsorption of oxygen.

Thus, in a system with approx. the same quantity of crystalline and amorphous particles, the most productive temperature range for oxidation is 923–973 K.

The results of the study of additionally added oxygen effect into the system are presented in Fig. 5. The change in the fraction of bound oxygen for particles of both types showed that, in the case of a crystalline particle, each subsequent $n_b(T)$ dependence (obtained with a large number of oxygen atoms) is lower than the previous similar dependence. This indicates that the rate of U–O bonds formation in the system lags behind the number of sites prepared for oxygen adsorption. This may be due to the lack of available sites for oxygen inside the crystalline particle, as well as the lack of such sites on the surface of the particle and the resulting fragments.

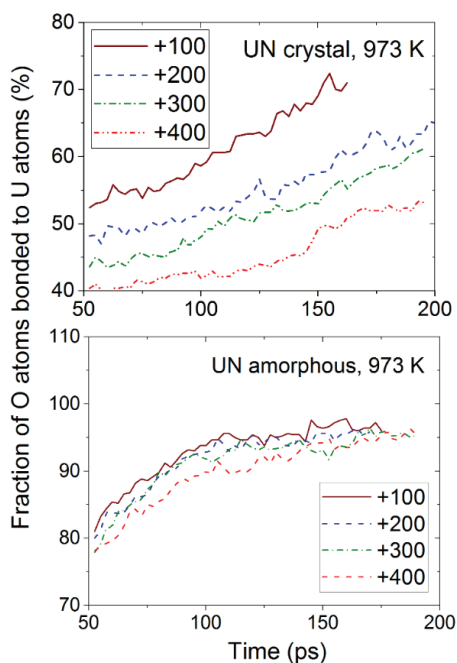


Fig. 5. Fraction of O atoms bonded to U atoms for initially crystalline and amorphous UN particle after sequential addition of oxygen to the system at different temperatures; numbers with a “+” sign indicate the number of added oxygen atoms.

A different result is observed for the amorphous particle. Here, enough potential sites for oxygen capture can be located both outside all the existing fragments and inside the not yet destroyed part of the particle. Therefore, each subsequent dependence of $n_b(T)$ for the amorphous particle follows a path quite close to the previous dependence (relating to a system with 100 fewer oxygen atoms).

The structure of a nanoparticle affects the packing of the attached oxygen. Fig. 6 shows the U–O partial radial distribution functions for two temperatures corresponding to the maxima of the n_b distributions for crystalline and amorphous particles. At 923 K, the $g_{U-O}(r)$ function for a crystalline particle has a

large number of peaks, indicating a predominantly regular arrangement of oxygen atoms both around and within the particle. A similar function for an amorphous particle is characterized by only two intensive peaks, with the third peak being diffuse and weakly expressed. The connection between the structures of UN fragments and their oxygen environment and filling is also evident here.

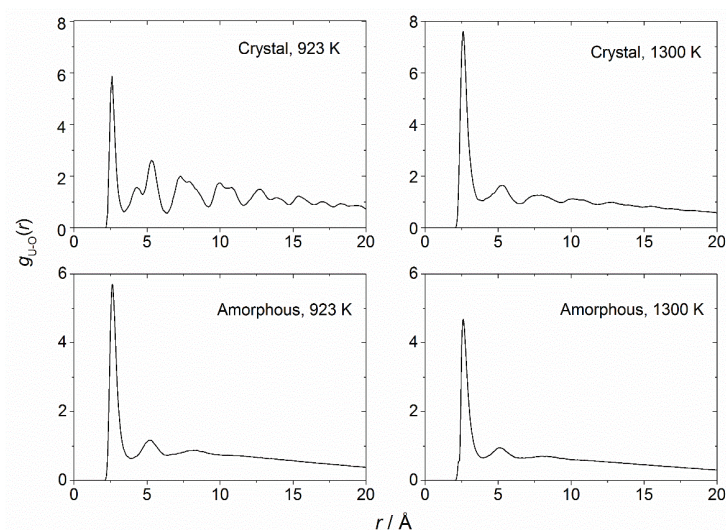


Fig. 6. Partial radial distribution functions $g_{U-O}(r)$ obtained by oxidation of crystalline and amorphous UN fragments in a gas mixture of Ar with 22.5 mol. % oxygen at different temperatures.

After heating the system to a temperature of 1300 K, the structure of the crystalline particle undergoes significant distortions, as a result of which the number of peaks in the $g_{U-O}(r)$ function is reduced, and the remaining peaks become blurred. In the corresponding function for the amorphous particle at 1300 K, only two peaks are detected, and the third peak, still present at a temperature of 923 K, becomes faintly noticeable. Thus, with the increase of temperature, there is a more noticeable disorder of the oxygen subsystem in the system with the crystalline UN particle. However, the oxygen subsystem in the system with the amorphous UN particle is also subject to structural disorder.

To visually summarize the results of the study, Fig. 7a shows the configuration of the system “amorphous fragments in a gas mixture Ar–O” close to the initial state (corresponding to a time of 20 ps) and the final configuration obtained in calculations with the U–N pair interaction potential and corresponding to a time moment of 685 ps.

The final demonstration run for the studied system with an amorphous particle at a temperature of 923 K showed that all UN fragments quickly lose nit-

rogen, which in its free form goes into a gas mixture containing argon and the remaining oxygen not attached to uranium. Such a process can actually take place due to the intense release of energy in the resulting exothermic reaction (1):

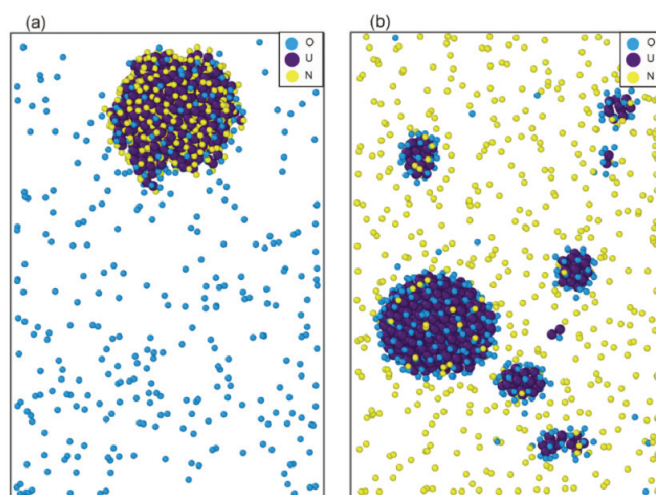
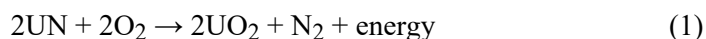


Fig. 7. *zx*-projections of the configuration of systems “amorphous fragments in a gas mixture Ar–O” at a temperature of 923 K: a) UN fragment corresponding to a time moment of 20 ps; b) UO_x fragments at 685 ps.

Changing the U–N interaction to a pair interaction results in the larger UN fragment quickly breaking up into small clusters. All small clusters containing 1–3 U atoms, within a few picoseconds after changing the U–N interaction, quickly lose N atoms and acquire a small number of O atoms. Then these clusters spontaneously begin to assemble into larger $(\text{UO}_x)_n$ clusters that acquire a convex, close to spherical shape. Such clusters of different sizes are surrounded by Ar and N atoms. A small number of O atoms also remain in the gas mixture. As it can be seen from the figure, the initial UN particle is finally transformed into $(\text{UO}_x)_n$ fragments that are the final product of the model oxidation process.

The present calculations were performed with an initial oxygen content of 22.5 mol. % in the oxygen–argon mixture. The studies performed show that the completeness of the conversion of UN into uranium oxides depends on several interrelated factors. Firstly, for the progression of oxidation, the state of the particles that are exposed to oxygen is important. In particular, the structure of the particles and the state of their surface are important. It has been shown that intense oxidation of amorphous particles occurs at a lower temperature than for crystalline particles of the same size. This is facilitated by the easier fragmentation of amorphous particles compared to crystalline particles in a gaseous Ar–O

medium. The fragmentation facilitates the access of oxygen to the oxidized material. This is the rate of fragmentation of particles into smaller fragments that is mainly the limiting factor in the oxidation of UN powder.

However, the completeness of oxidation is determined not only by the increase in surface area when the integrity of the UN particle is damaged, but also by the possibility of access of oxygen atoms inside it. This possibility opens up when the U–N bonds turn out to be weaker than the U–O bonds, and the N atoms leave their positions, allowing the O atoms to form bonds with uranium. Since the rate of crushing of powder particles acts as a limiting factor for its oxidation, the completeness of this process directly depends on the size of the powder particles, as observed in the experiment.²⁴

To investigate how the oxygen concentration in the Ar–O gas mixture affects the oxidation process of UN particles, similar calculations were performed in the temperature range of 923–1300 K with an oxygen concentration of 8.6 mol. % and the same amount of Ar, U and N atoms. At this lower oxygen concentration in the system, only slight fragmentation of the amorphous particle was observed, while the UN crystal retained its integrity throughout the entire calculation.

During the first 100 ps, when the effect of fragmentation of the UN particle on its oxidation is not so significant, the fraction of O atoms associated with the U atoms of the amorphous particle was ~6 % greater than for the crystalline particle. During this period, at an initial molar oxygen concentration of 22.5 mol. %, the number of formed U–O bonds for crystalline and amorphous particles exceeded these characteristics at an initial oxygen concentration of 8.6 mol. % by 2.7 and 2.9 times, respectively. It is evident that an increase in the oxygen concentration in the system significantly accelerates the process of initial oxidation.

Thus, for oxidation to occur more efficiently, it is necessary that the oxygen concentration in the gaseous mixture be as high as allowed by the design features of the experimental setup, and that the UN powder be fine and amorphous. In this case, care should be taken to ensure that the coagulation of the fine particles is inhibited. According to the results of the present work, it is advisable to execute the oxidation of UN powder at temperatures of 923–973 K, when the amorphous fraction dominates in the composition of the powder. To prevent the powder from being carried out of the chamber in which it is blown with an Ar–O gas mixture, a fine-mesh steel mesh can be used.

CONCLUSION

This work shows that the high-temperature processing operation (in the Ar–O atmosphere) should be a single-stage process. It involves the oxidation of nitride powder from spent nuclear fuel in an atmosphere of dry oxygen and argon, converting the nitrides of fissile materials and fission products into oxides.

The process of transforming nitrides into oxides in a Ar–O gaseous mixture is largely determined by the structure of the initial nitride material. Thus, at pressures that do not lead to structural phase transitions in the UN phase (not much different from atmospheric pressure), the amorphous powder is actively oxidized at lower temperatures than the crystalline fine fraction. In order to significantly activate the oxidation of the crystalline fraction, it is necessary to increase the temperature of the system.

The fragmentation of the amorphous UN particle that occurs at lower temperatures leads to an increase in its surface area, which contributes to an increase in the intensity of oxidation. Thus, it is possible to lower the temperature at which the oxidation procedure is performed by preliminary conversion of crystalline UN particles into an amorphous state.

Realistic systems exchange mass, momentum, and/or energy with the environment, *i.e.*, they have open boundaries. The limited size of MD models, even with the use of periodic boundary conditions that expand the system, makes the comparisons with experimental results difficult. Solving this problem is the main task of MD modelling in the future.

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/13065>, or from the corresponding author on request.

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ИЗВОД

МОЛЕКУЛСКО ДИНАМИЧКА СИМУЛАЦИЈА ОКСИДАЦИЈЕ УРАНИЈУМ-НИТРИДА

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Извршена је молекулско динамичка симулација оксидације уранијум-мононитрида (UN) у Ar–O средини у температурском опсегу од 373–2073 K. Истраживање је урађено за UN честице кристалне и аморфне структуре при концентрацији кисеоника од 22,5 mol. % у гасној смеси. Најефикаснија оксидација аморфних честица се дешава на нижим температурама него за кристалне честице. За разлику од кристалних фрагмената, аморфне честице подлежу значајнијој фрагментацији када се везују за кисеоник. Фрагментација UN честица је један од главних фактора који доводе до њихове оксидације. Оксидација UN честице почиње од њене површине и у случају аморфних честица дешава се брже него када су честице кристалне. Процес фрагментације честице је олакшан продирањем атома кисеоника унутар честице. Пораст концентрације кисеоника у гасној смеси подстиче процес оксидације. Структурне промене у систему су испи-

тиване помоћу парцијалне радијалне функције расподеле. Вишечестичне U–N интеракције спречавају да азот пређе у гасовито стање.

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SUPPLEMENTARY MATERIAL TO

Molecular dynamics simulation of uranium nitride oxidation

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PREPARATION OF THE SIMULATED SYSTEMS

An amorphous particle was created in a preliminary molecular dynamics calculation by melting a crystal at a temperature of 3000 K and then keeping it at this temperature for 200 ps (2 million time steps). The process of the amorphous UN structure preparation is shown in Fig. S1. After the particle acquired an irregular structure, it was uniformly and rapidly cooled over a time interval of 100 ps until a temperature of 300 K was reached.

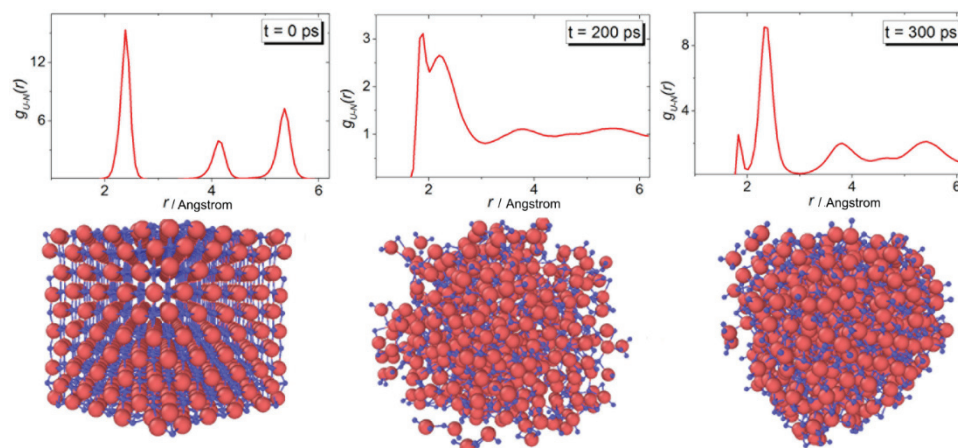


Fig. S-1. The process of the amorphous UN structure preparation, the insets above show the partial radial distribution functions $g_{U-N}(r)$ corresponding to each of the above states; uranium atoms are marked in red, nitrogen atoms are in blue.

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The preparation of the simulated systems “UN crystalline/amorphous particle in the Ar-O gaseous medium” was started from the creation of the argon-gaseous medium. The process of its preparation was carried out in the NPT ensemble as follows: 1) initially, an argon crystal was melted at a fixed barostat pressure of 1 bar, converting it into a gaseous state at $T = 1273$ K for 400 ps; 2) at the second stage, oxygen was gradually introduced into the gaseous argon mixture, by adding one atom every 0.2 ps (2000 time steps) until the desired oxygen concentration was reached; 3) finally, the mixed argon-oxygen gas system was equilibrated for 400 ps. The barostat relaxation time was $\tau_T = 10$ fs.

The previously obtained and equilibrated argon-oxygen medium was further used to introduce an amorphous or crystalline UN particle into it. For that, the volume of the simulation cell was increased by moving its upper face upward. This face was raised until an amorphous particle (its volume was greater than the volume of the crystalline particle) could be placed between it and the previously obtained surface of the gas system. The new position of the upper face of the MD cell was fixed, and the UN particle (crystalline or amorphous) was placed in the upper part of the MD cell. The lower part of the cell was occupied by the gaseous Ar-O mixture.

A microheterophase system “UN crystalline/amorphous particle in a gaseous Ar-O mixture” was created in the MD calculation using Maxwellian boundary conditions. Already at the beginning of this calculation, Ar and O atoms occupied the free volume formed at the top of the MD cell. Due to the use of Maxwellian boundary conditions, the UN particle immersed in the gaseous medium performed random movements over the cell.

MORSE POTENTIAL FUNCTION

In the demonstration calculation, the EAM potential describing U-N interactions in the system was replaced by the pair potential. Pair potential built on the basis of the Morse potential function¹⁴ has a form:

$$\Phi = \frac{z_i z_j e^2}{r_{ij}} + (b_i + b_j) \exp \left[\frac{a_i + a_j - r_{ij}}{b_i + b_j} \right] + D_{ij} \{ \exp[-2\beta_{ij}(r_{ij} - r_0)] - 2 \exp[-\beta_{ij}(r_{ij} - r_0)] \} \quad (\text{S1})$$

here e ($= 1$) is the elementary electrical charge, r_{ij} is the distance between i and j ions, other potential parameters are presented in Table S-I.

TABLE S-I. Parameters of the potential represented by equation (S1).

Ion	z	a , Å	b , Å	Bond	D_{ij} , eV	β_{ij} , Å ⁻¹	r_0 , Å
N ³⁻	-1.450	1.797	0.080	N ³⁻ –U ³⁺	7.00	1.25	2.364
U ³⁺	+1.450	1.228	0.080				

The form of potential (S1) is presented in Fig. S2.

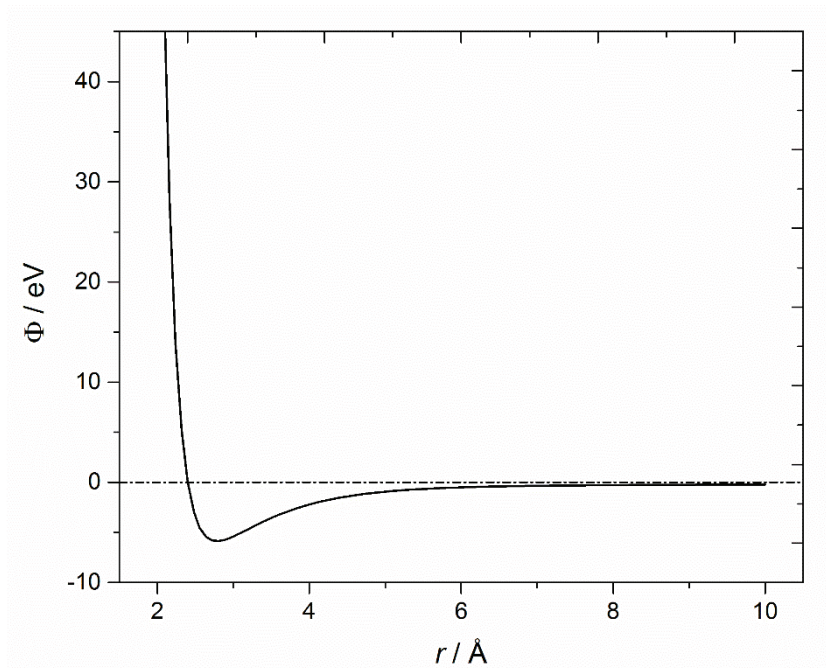


Fig. S-2. The U-N interaction potential represented by formula (S1).

The replacement of the potential describing the U-N interaction was performed to simulate a system with an amorphous particle that had already split into fragments, the largest of which had an irregular shape and tended to undergo further segmentation. The newly used (pair) potential had been previously employed to simulate the crystalline UN phase in the temperature range of 300-2000 K at a pressure of 1 bar. Our calculation with this pair potential at a temperature of 923 K is presented in the demonstration movie (external link): (https://watercluster.ucoz.ru/publ/2024/supplementary/supplementary_for_journal_of_the_serbian_chemical_society/190-1-0-222).



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Different electrode modification protocols for evaluating the water-splitting properties of a P(V)-metalloporphyrin

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Abstract: Water electrolysis is currently a notable research domain, having the identification of highly active, stable, and low-cost electrocatalysts as one of its most important pursuits. Herein, an A_4 P(V)-centered metalloporphyrin – (5,10,15,20-tetraphenylporphinato) dichlorophosphorus (V) chloride – was evaluated in terms of its electrocatalytic water-splitting activity in acidic, neutral and alkaline media. The experiments were performed on electrodes modified with the porphyrin complex using different protocols, and the most electrocatalytically active sample was the one obtained by applying a catalyst ink containing the metalloporphyrin and carbon black on glassy carbon. The best results were observed for the strongly alkaline medium (1 mol L^{-1} KOH), in which the electrode exhibited a hydrogen evolution reaction overpotential of 0.77 V and a Tafel slope of 0.135 V dec^{-1} . Its stability was outlined by chronoamperometry and Raman spectroscopy. The results supplement the available data regarding the properties and applicative potential of metalloporphyrins and outline the implications of using different electrode manufacturing procedures.

Keywords: electrocatalyst; aggregate; hydrogen evolution reaction; oxygen evolution reaction; macrocycle.

INTRODUCTION

The ongoing struggles to replace fossil energy sources with renewable ones, to manage the increasing energy demand, the expanding global economy and the fast-growing population have led researchers to consider hydrogen as the auspicious energy carrier of the future.¹ Because the energy infrastructure is currently reliant to a great extent on finite and non-renewable energy sources a global energy crisis can only be delayed but not avoided. Furthermore, it is known that the use of fossil fuels has a detrimental effect on the environment.² The problems surrounding them have led to a concerted effort, by researchers, to

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study the potential of alternative energy sources and to find a suitable energy carrier.³ The scientific literature outlines hydrogen as an environmentally friendly energy carrier with highly desirable properties.⁴ Hydrogen can be generated *via* several methods but only some of them are non-polluting in the sense that they rely on renewable energy sources (water, biomass, wind power and solar power).⁵ Since the goal is to obtain hydrogen in ways that avoid further environmental pollution the focus is on the eco-friendly approaches. Water-splitting, a method for hydrogen generation *via* the direct decomposition of water into its elements is considered, by researchers, to be the most promising way for replacing the fossil fuel-based energy infrastructure.⁶ During water electrolysis pure hydrogen and oxygen are obtained from the splitting of water molecules using electric current.⁷ Electrocatalysts play an essential role since they decrease the kinetic energy barrier between the two half-cell reactions – the hydrogen and the oxygen evolution reactions (HER and OER) – involved in the process.⁸ Currently, the benchmark electrocatalysts are based on noble metals that cannot be used for large-scale hydrogen production because they are costly and scarce.⁹ Many alternatives have been proposed that are cheaper, less scarce or even abundant, and exhibit fairly high catalytic efficiency and stability.¹⁰ However, none of them have replaced the noble metal-based electrocatalysts.

Porphyrins are a class of aromatic compounds that have been shown to exhibit water-splitting catalytic activity.^{11–13} They share a common feature known as the porphyrin macrocycle, they can be substituted peripherally with a wide variety of functional groups, and the center of the macrocycle can be metalated with almost every known metal ion – leading to the formation of metalloporphyrins. Changes to the chemical structure of porphyrins result in changes to their properties which can turn them into candidates for different types of applications. Furthermore, single molecules can associate with neighboring ones to form stable and well-defined aggregates with features that are often different from those of their constituents.^{14,15}

The electrocatalytic water-splitting properties of metalloporphyrins have been investigated at least since 1985.¹⁶ Recent publications indicate the persistent interest in the applicative potential of metalated porphyrin derivatives for the water-splitting field.^{11,17}

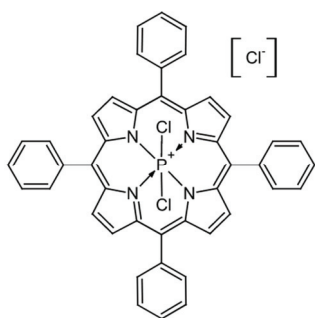
This paper presents the study of the water-splitting properties of a symmetrically substituted A₄ P(V)-centered metalloporphyrin, namely (5,10,15,20-tetraphenylporphinato) dichlorophosphorus (V) chloride, in solutions covering a wide pH range. Identified publications involving this metalated porphyrin do not contain investigations of its HER and OER electrocatalytic properties.^{18–22} The experimental results obtained during the testing of electrodes modified with the porphyrin complex by employing different protocols supplement the currently available data regarding the characteristics and applicative potential of metallo-

porphyrins and outline the consequences of using diverse procedures to manufacture electrodes.

EXPERIMENTAL

Materials and reagents

The porphyrin complex – (5,10,15,20-tetraphenylporphinato) dichlorophosphorus (V) chloride – was purchased from Porphyrin Systems (Halstenbek, Germany) and used as received. The chemical structure of the porphyrin complex is shown in Scheme 1.



Scheme 1. The chemical structure of (5,10,15,20-tetraphenylporphinato) dichlorophosphorus (V) chloride.

Dichloromethane (DCM), tetrahydrofuran (THF), acetonitrile (CH_3CN), *N,N*-dimethylformamide (DMF) and Nafion[®] 117 solution were acquired from Sigma Aldrich. Ethanol 99.5 % (EtOH) and acetone were obtained from Honeywell (Charlotte, NC, USA) and Chimreactiv (Bucharest, Romania). Benzonitrile (PhCN), dimethylsulfoxide (DMSO), KOH, KCl and H_2SO_4 98 % were purchased from Merck (Darmstadt, Germany). All reagents were used as procured. GC (glassy carbon) tablets were received from Andreescu Labor & Soft SRL (Bucharest, Romania) and Carbon Black – Vulcan XC 72 from Fuel Cell Store (Bryan, TX, USA). Double-distilled water was used to prepare all aqueous solutions.

Electrode modification protocols

The first protocol employed to manufacture the metalloporphyrin-based electrodes consists of the drop-casting on the surface of GC tablets of solutions obtained by dissolving the porphyrin complex in organic solvents having different polarity values. Solvent polarity decreases in the following order: $\text{DMSO} > \text{DMF} > \text{CH}_3\text{CN} > \text{PhCN} > \text{EtOH} > \text{THF} > \text{DCM}$.^{23,24} The drop-casted volume was 10 μL and the drying of the covered GC substrates was performed at 40 °C. The resulting electrodes were modified with one metalloporphyrin layer. Samples with two and three layers were also prepared by repeating the mentioned procedure. Table I presents the codes used to identify the electrodes.

Electrode manufacturing was also performed by following a different protocol, based on a previously reported study.²⁵ Catalyst inks containing the porphyrin complex as such or mixed with Carbon Black resulted after a 30 min ultrasonication treatment. The surface of the GC tablets was covered by drop-casting a catalyst ink volume of 10 μL . This stage was followed by the drying of the modified substrates at 40 °C which led to the obtaining of the modified electrodes. Table II shows the codes for identifying the samples and the ink compositions.

TABLE I. The codes for identifying the electrodes modified utilizing the first protocol

Electrode code	Solvent	Porphyrin layers	Electrode code	Solvent	Porphyrin layers
GC _{P1} -DMSO-1	DMSO	1	GC _{P1} -EtOH-1	EtOH	1
GC _{P1} -DMSO-2		2	GC _{P1} -EtOH-2		2
GC _{P1} -DMSO-3		3	GC _{P1} -EtOH-3		3
GC _{P1} -DMF-1	DMF	1	GC _{P1} -THF-1	THF	1
GC _{P1} -DMF-2		2	GC _{P1} -THF-2		2
GC _{P1} -DMF-3		3	GC _{P1} -THF-3		3
GC _{P1} -CH ₃ CN-1	CH ₃ CN	1	GC _{P1} -DCM-1	DCM	1
GC _{P1} -CH ₃ CN-2		2	GC _{P1} -DCM-2		2
GC _{P1} -CH ₃ CN-3		3	GC _{P1} -DCM-3		3
GC _{P1} -PhCN-1	PhCN	1			
GC _{P1} -PhCN-2		2			
GC _{P1} -PhCN-3		3			

TABLE II. The codes for identifying the electrodes modified utilizing the second protocol

Electrode code	Porphyrin amount	Carbon black	Nafion solution	Double-distilled water
	mg	mg	μL	μL
GC _{P1}	10	-	50	450
GC _{P1} -CB	5	5	50	450

Electrochemical study

Three electrodes were connected to a Voltalab potentiostat model PGZ 402 from Radiometer Analytical (Lyon, France) and were subsequently inserted into a glass cell. One of the electrodes – a Pt plate with a geometric surface (S_{geom}) of 0.8 cm^2 – was used as the counter electrode. The reference electrode was the Ag/AgCl (sat. KCl) electrode. Each metalloporphyrin-modified electrode served as the working electrode. It was placed inside a polyamide support that ensured a constant S_{geom} of 0.28 cm^2 . Acidic ($0.1 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$), neutral ($0.1 \text{ mol L}^{-1} \text{ KCl}$) and strongly alkaline ($1 \text{ mol L}^{-1} \text{ KOH}$) electrolyte solutions were prepared in order to cover a wide pH range. Before each HER experiment the electrolyte solutions were thoroughly deaerated with high-purity nitrogen. The OER and HER linear sweep voltammograms (LSVs) were *IR*-corrected and recorded at a scan rate (ν) of 5 mV/s .²⁶ Unless stated otherwise, the electrochemical parameters j and E are the geometric current density and the electrochemical potential expressed vs. the reversible hydrogen electrode (RHE). The E values were represented in terms of the RHE with Eq. (S-1) of the Supplementary material to this paper. Eqs. (S-2) and (S-3) were used to calculate the O_2 and H_2 evolution overpotentials (η_{OER} and η_{HER}), while Eq. (S-4) is the Tafel equation.²⁷⁻²⁹

Analysis by SEM and Raman spectroscopy

SEM analysis was carried out with a tabletop scanning electron microscope, CUBE II model, from EmCrafts Co. Ltd. (Kwangju, South Korea). A MultiView-2000 system from Nanonics Imaging Ltd. (Jerusalem, Israel) equipped with a Shamrock 500i spectrograph from Andor (Essex, UK) was used to record the Raman spectra.

RESULTS AND DISCUSSION

Tests on the electrodes manufactured using the first protocol

The LSVs recorded in 0.1 mol L⁻¹ H₂SO₄ solution during the OER tests performed on electrodes obtained with the first modification protocol evidence their low electrocatalytic activity, characterized by high overpotentials and low current densities (Fig. S-1 of the Supplementary material). Figs. S-1a, S-1b and S-1c present the results obtained for the electrodes modified with one, two and three metalloporphyrin layers. Relative to the unmodified GC sample (GC₀) only GC_{P1}-DMF-1 and GC_{P1}-EtOH-1 exhibit a slightly higher activity. In the neutral electrolyte solution (Fig. S-2) several of the modified electrodes display higher OER activity compared with GC₀, but under these mild conditions the overpotentials are high and the current densities low. The anodic LSVs recorded in the strong alkaline solution can be seen in Fig. S-3. The lowest η_{OER} values were determined for GC_{P1}-DMSO-1 (Fig. S-3a). At $j = 10 \text{ mA cm}^{-2}$, a benchmark value at which the overpotential is often specified, $\eta_{\text{OER}} = 1.15 \text{ V}$.³⁰

The cathodic polarization curves obtained during the HER experiments performed on the electrodes modified with the first protocol in the acidic, neutral, and alkaline media are shown in Fig. S-4, Fig. S-5 and Fig. 1. The lowest η_{HER} value in 0.1 mol L⁻¹ H₂SO₄ solution belongs to GC_{P1}-EtOH-3 (1.54 V at $j = -10 \text{ mA cm}^{-2}$) – Fig. S-4c. In 0.1 mol L⁻¹ KCl solution GC_{P1}-CH₃CN-1 (Fig. S-5a) and GC_{P1}-DMF-2 (Fig. S-5b) have the lowest η_{HER} value at $j = -10 \text{ mA cm}^{-2}$, of $\sim 1.38 \text{ V}$. The best results are observed in the strongly alkaline medium for the electrodes modified with the porphyrin complex drop-casted from DMF in one layer (Fig. 1a) and from DMSO in one (Fig. 1a), two (Fig. 1b) and three layers (Fig. 1c). A η_{HER} value of $\sim 0.90 \text{ V}$ was determined in all these cases and it reveals the limits of the modification protocol used to manufacture the tested electrodes.

It was pointed out in the Introduction section that porphyrin molecules can self-assemble into aggregates. To see if this is also the case with the studied porphyrin complex, a modified electrode, GC_{P1}-DMSO-1, was selected for SEM characterization. The micrographs recorded in two different areas on the surface of the sample are rendered in Fig. 2. Both images (Fig. 2a and Fig. 2b) show that the metalloporphyrin molecules associated as irregular aggregates with dimensions in the micrometric domain. These structures are sporadic and their distribution is inhomogeneous. It is known that inhomogeneities act as active site generators during electrocatalytic processes.²⁸

Tests on the electrodes manufactured using the second protocol

The anodic LSVs recorded on the metalloporphyrin-modified electrodes manufactured using the second protocol described in the Experimental are shown in Fig. S-6. In the acidic environment (Fig. S-6a) GC_{P1}-CB and GC_{P1} display low

OER activity and features corresponding to oxidation processes are present on the polarization curves. In the neutral and strongly alkaline solutions (Figs. S-6b and S-6c) the η_{OER} values determined for GC_{CB} are lower than for the other electrodes, indicating that the presence of the porphyrin derivative negatively affects the OER activity of the metalloporphyrin-modified electrodes.

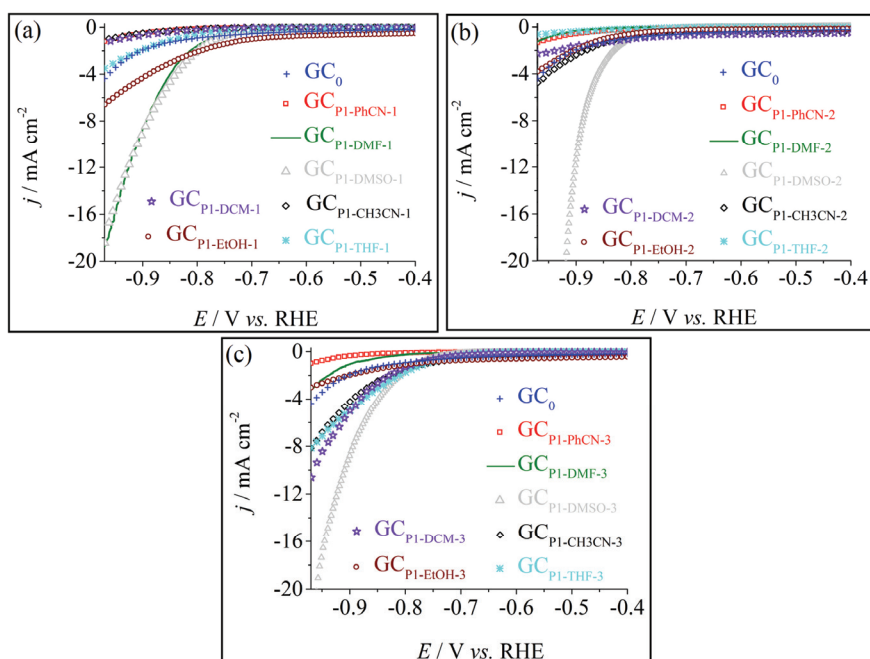


Fig. 1. Cathodic polarization curves recorded on GC_0 and on: a) $\text{GC}_{\text{P1-PhCN-1}}$, $\text{GC}_{\text{P1-DMF-1}}$, $\text{GC}_{\text{P1-DMSO-1}}$, $\text{GC}_{\text{P1-CH3CN-1}}$, $\text{GC}_{\text{P1-THF-1}}$, $\text{GC}_{\text{P1-DCM-1}}$, $\text{GC}_{\text{P1-EtOH-1}}$; b) $\text{GC}_{\text{P1-PhCN-2}}$, $\text{GC}_{\text{P1-DMF-2}}$, $\text{GC}_{\text{P1-DMSO-2}}$, $\text{GC}_{\text{P1-CH3CN-2}}$, $\text{GC}_{\text{P1-THF-2}}$, $\text{GC}_{\text{P1-DCM-2}}$, $\text{GC}_{\text{P1-EtOH-2}}$; c) $\text{GC}_{\text{P1-PhCN-3}}$, $\text{GC}_{\text{P1-DMF-3}}$, $\text{GC}_{\text{P1-DMSO-3}}$, $\text{GC}_{\text{P1-CH3CN-3}}$, $\text{GC}_{\text{P1-THF-3}}$, $\text{GC}_{\text{P1-DCM-3}}$, $\text{GC}_{\text{P1-EtOH-3}}$. Electrolyte solution: $1 \text{ mol L}^{-1} \text{ KOH}$. $\nu = 5 \text{ mV s}^{-1}$.

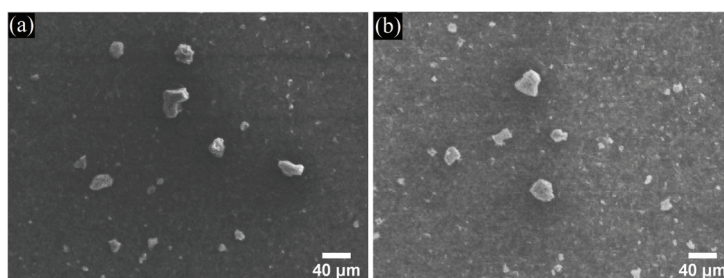


Fig. 2. SEM images recorded in two different areas on the surface of the $\text{GC}_{\text{P1-DMSO-1}}$ electrode. a) The micrograph obtained for the first area selected for analysis. b) The micrograph obtained for the second area selected for analysis.

Fig. 3 shows the cathodic LSVs obtained during the HER experiments. In the 0.1 mol L⁻¹ H₂SO₄ and 0.1 mol L⁻¹ KCl solutions the highest electrocatalytic activity is displayed by GC_{CB} (Fig. 3a and b), but in the strongly alkaline medium the lowest overpotential values belong to GC_{P1} and GC_{P1-CB} (Fig. 3c). The η_{HER} value determined for GC_{P1-CB} at $j = -10 \text{ mA cm}^{-2}$ is 0.77 V and it outlines this electrode as having the highest HER electrocatalytic activity.

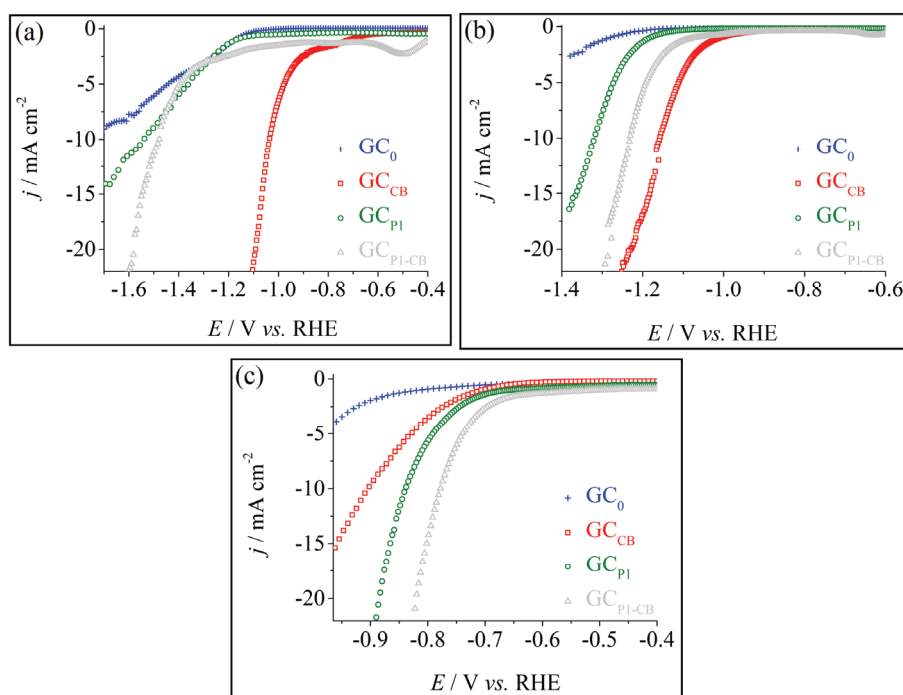


Fig. 3. Cathodic polarization curves recorded on GC₀, GC_{CB}, GC_{P1} and GC_{P1-CB} in the following electrolyte solutions: a) 0.1 mol L⁻¹ H₂SO₄, b) 0.1 mol L⁻¹ KCl and c) 1 mol L⁻¹ KOH. $\nu = 5 \text{ mV s}^{-1}$.

The electrochemical properties of GC_{P1-CB} were further evaluated and the results are presented in Fig. 4. Fig. 4a shows the plot of the capacitive current density (j_{dl}) vs. the scan rate for the specified electrode, while the Tafel plot and the chronoamperogram recorded during its electrochemical stability testing are rendered in Fig. 4b and c.

The j_{dl} values required to represent the plot from Fig. 4a were obtained by carrying out cyclic voltammetry experiments in 0.1 mol L⁻¹ KCl solution at ν values of 10, 20, 30, 40 and 50 mV s⁻¹ in an E range with no Faradic currents. The recorded data was subsequently used in Eq. (S-5) from the Supplementary material.³¹ The linear increase observed in Fig. 4a correlates with the charging and discharging of the Helmholtz double-layer.³² The absolute value of the plot's

slope is the electrode's electric double layer capacitance (C_{dl}) deriving from the stored charge in the double-layer at the electrode/electrolyte solution interface.³¹ This parameter is important for the supercapacitors domain in which a higher value is preferred to a lower one.³³ Table III presents the C_{dl} value determined for GC_{P1}-CB together with the R^2 , the roughness factor (R_f) and the electrochemically active surface area ($EASA$) values. The R_f value is equal to the ratio between the real surface of the electrode and S_{geom} and was calculated by dividing C_{dl} to $60 \mu\text{F cm}^{-2}$, which is the specific capacitance of a smooth oxide surface.³⁴ The $EASA$ was calculated with Eq. (S-6).³¹

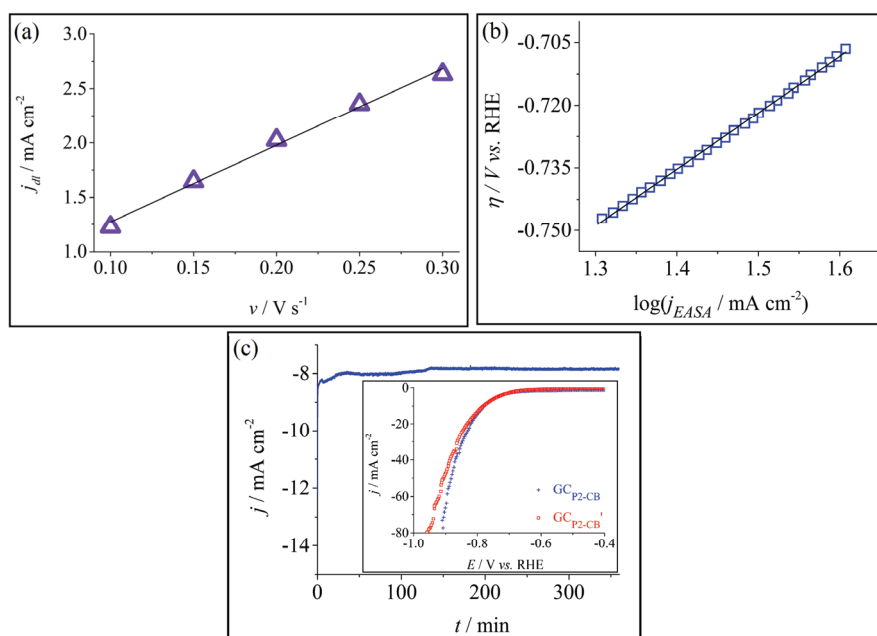


Fig.4. a) The j_{dl} - v graphical representation for GC_{P1}-CB in 0.1 mol L^{-1} KCl solution. b) The Tafel plot for GC_{P1}-CB in 1 mol L^{-1} KOH solution. The current density (j_{EASA}) values from the plot are $EASA$ -normalized. c) Chronoamperogram obtained on GC_{P1}-CB in 1 mol L^{-1} KOH solution and inset with the LSVs obtained on the same electrode before (GC_{P1}-CB) and after (GC_{P1}-CB') the electrochemical stability test (1 mol L^{-1} KOH solution, $v = 5 \text{ mV s}^{-1}$).

TABLE III. The C_{dl} , R^2 , R_f and $EASA$ values obtained for the GC_{P1}-CB electrode

Electrode	$C_{dl} / \text{mF cm}^{-2}$	R^2	R_f	$EASA / \text{cm}^2$
GC _{P1} -CB	7.05	0.9915	117.5	32.9

There are two reasons why the $EASA$ was estimated. Firstly, it is known that the value of this parameter is directly proportional to the number of catalytically active centers participating in an electrocatalytic process, including the water-splitting.³⁵ Secondly, it was used to represent the Tafel plot from Fig. 4b which

is required to determine the Tafel slope – a parameter relevant to an electrode's water-splitting activity, whose lower value indicates faster reaction kinetics.¹⁵ The Tafel slope was found to be 0.135 V dec^{-1} ($R^2 = 0.9993$). The high pH value of the electrolyte solution leads to the covering of a high degree of the electrode's surface with H_2 bubbles. These in turn affect the value of the slope by making it higher than 0.12 V dec^{-1} .³⁶ A slope between 0.04 and 0.12 V dec^{-1} points to the HER occurring *via* a Volmer–Heyrovsky mechanism and to the rate of the discharge step being consistent with that of the desorption step.³⁷ However, under alkaline conditions when the value exceeds the upper limit of the specified interval a Volmer-limited mechanism is usually involved, with the Volmer step being faster than the Heyrovsky step.³⁶

The electrochemical stability test performed on GCp1-CB lasted 6 h and the chronoamperogram obtained by maintaining a constant E value of -0.77 V vs. RHE is presented in Fig. 4c. It can be seen that the current density steadily evolves towards a constant value. At $t = 135 \text{ min}$ this value is $\sim -7.8 \text{ mA cm}^{-2}$. The electrode is relatively stable throughout the experiment. The inset in Fig. 4c presents the LSVs recorded on the electrode before and after the test. The two curves overlap almost perfectly at the current density of -10 mA cm^{-2} . This result is a positive indication of the sample's stability which is further evidenced using Raman spectroscopy. Fig. S-7 shows the Raman spectra recorded on GCp1-CB before and after the chronoamperometric experiment. The signals identified on the spectrum obtained before the test are also present on the one obtained afterwards, indicating that the electrode did not undergo notable structural changes.

Discussion regarding the HER properties of GCp1-CB

The P(V)-centered porphyrin-based electrode manufactured using the second protocol described in the Experimental section and denoted GCp1-CB was shown to possess the highest HER catalytic activity. As a metalloporphyrin, its catalytic center is probably situated in the center of the metalated macrocycle, namely at the M-N_4 site.^{38,39} The $EASA$ value obtained for the electrode is high compared to its geometric surface ($EASA = 32.9 \text{ cm}^2$ and $S_{\text{geom}} = 0.28 \text{ cm}^2$), which points to the presence of inhomogeneities and to the sample benefiting from a relatively large number of active sites. Charge transport effects also play an important role in the electrocatalytic behavior of metalloporphyrins. Aside from the charge transport occurring between adjacent molecules through non-covalent π – π interactions, there is also the one taking place at the interface between the molecules and the GC support.³⁸ The P(V)-metalloporphyrin has a phosphorous metal atom located in the center of its macrocycle. This atom is hexacoordinated and the macrocycle possesses a distorted octahedral geometry. The P atom is less electronegative compared with the C atoms of the GC and induces an electronegative doping effect on the substrate's surface. The electron-donating and electron-with-

drawing effects of the functional moieties, that porphyrin derivatives are substituted with, constitute another factor influencing their water-splitting properties. Electron-withdrawing substituents in particular decrease the macrocycle's electron density leading to a decrease in the HER overpotential.³⁹ The phenyl groups of the porphyrin complex exhibit only a weak electron-withdrawing inductive effect which in turn has a weak impact on the overpotential. The pH of the electrolyte solution should not be overlooked either since its significant effect on an electrode's water-splitting performance is well known.^{40,41} As specified, the strongly alkaline environment in which GC_{P1-CB} is immersed affects the value of the Tafel slope and is responsible for slower HER kinetics.

What can be concluded about the water-splitting activity of the GC_{P1-CB} electrode is that even though a relatively large number of catalytically active sites are probably present on its surface, other factors – such as an electronegative doping effect, a weak electron-withdrawing effect and the effect of the electrolyte solution's pH – negatively affect this property causing the decrease of the specimen's HER catalytic performance. The latter observation is reason to think the electrode will probably not end up being used in large-scale applications. Table S-I from the Supplementary material compares the HER activity of GC_{P1-CB} with that of other electrodes modified with free-base or metalated porphyrin derivatives. Its Tafel slope value is lower than most of the values mentioned in the table. While this is an advantage, its η_{HER} value is among the highest. The result points to the limitations of the utilized electrode modification strategy. Drop-casting-based protocols are low-cost and simple, but for improved water-splitting properties more sophisticated approaches could be required.

CONCLUSION

The water-splitting electrocatalytic properties of a symmetrically substituted A₄ P(V)-metalated porphyrin were evaluated in acidic, neutral and strongly alkaline electrolyte solutions. Metalloporphyrin-based electrodes were obtained by employing different electrode modification protocols. The results show that the specimen with the highest electrocatalytic activity is the one manufactured by drop-casting a catalyst ink containing the P(V)-porphyrin and Carbon Black on GC substrate. In 1 mol L⁻¹ electrolyte solution, it displays a HER overpotential of 0.77 V (at $j = -10 \text{ mA cm}^{-2}$) and a Tafel slope of 0.135 V dec⁻¹. It is relatively stable in the strongly alkaline medium but the high value of the overpotential prevents it from being used for commercial applications. Manufacturing protocols that utilize substrate modification techniques more complex than the simple drop-casting method will be assessed in upcoming investigations.

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/13072>, or from the corresponding author on request.

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ИЗВОД

РАЗЛИЧITI ПРОТОКОЛИ МОДИФИКАЦИЈЕ ЕЛЕКТРОДЕ P(V)-МЕТАЛ-ПОРФИРИНИМА ЗА ИСПИТИВАЊЕ АКТИВНОСТИ ЗА РАЗЛАГАЊЕ ВОДЕ

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Електролиза воде је тренутно значајна област истраживања, при чему је један од најважнијих задатака идентификација високо активних, стабилних и јефтиних електрокатализатора. У овом раду је испитан A_4 P(V)-центрирани металопорфирин (5,10,15,20-тетрафенилпорфинато) дихлорофосфор (V)-хлорид, у смислу његове електрокаталитичке активности за разлагање воде у киселој, неутралној и алкалној средини. Експерименти су урађени на електродама које су модификоване порфиринским комплексом коришћењем различитих протокола, при чему се као електрокаталитички најактивнији показао узорак добијен наношењем суспензије металопорфирина и угља развијене површине на стакласти угљеник. Најбољи резултати су добијени у јако алкалном раствору (1 mol L^{-1} KOH), у којем је електрода показала пренапетост реакције издвајања водоника од 0,77 V и Тафелов нагиб од $0,135 \text{ V dek}^{-1}$. Стабилност ове електроде је утврђена хроноамперометријом и Рамановом спектроскопијом. Резултати приказани у раду представљају допринос постојећим подацима о својствима и могућој примени металопорфирина те указују на утицај протокола припреме електроде на добијене резултате.

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SUPPLEMENTARY MATERIAL TO
**Different electrode modification protocols for evaluating the
water-splitting properties of a P(V)-metalloporphyrin**

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EQUATIONS

$$E_{RHE} = E_{Ag/AgCl(sat. KCl)} + 0.059 \text{ pH} + E^{\circ}_{Ag/AgCl(sat. KCl)} \quad (S-1)$$

$$\eta_{OER} = E_{RHE} - 1.23 \quad (S-2)$$

$$\eta_{HER} = |E_{RHE}| \quad (S-3)$$

$$\eta = b \times \log(j) + a \quad (S-4)$$

$$j_{dl} = \frac{j_a + j_c}{2} \quad (S-5)$$

$$EASA = \frac{C_{dl} \times S_{geom}}{C_s} \quad (S-6)$$

where E_{RHE} / V is the converted potential vs. RHE; $E_{Ag/AgCl(sat. KCl)}$ / V is the measured potential vs. the Ag/AgCl(sat. KCl) reference electrode; $E^{\circ}_{Ag/AgCl(sat. KCl)} = 0.197$ V; η_{OER} / V is the O₂ evolution overpotential; η_{HER} / V is the H₂ evolution overpotential; η / V is the overpotential; j / A cm⁻² is the current density; b / V dec⁻¹ is the Tafel slope; j_{dl} / A cm⁻² is the capacitive current density; j_a / A cm⁻² is the absolute value of the anodic j corresponding to a given scan rate value, at an electrochemical potential value where there are only double-layer adsorption and desorption features; j_c / A cm⁻² is the absolute value of the cathodic j corresponding to a given scan rate value, at an electrochemical potential value where there are only double-layer adsorption and desorption features; $EASA$ / cm² is the electrochemically active surface area; C_{dl} / F cm⁻² is the electric double-layer capacitance; S_{geom} / cm² is the geometric surface of the electrode and C_s / F cm⁻² is the specific capacitance.¹⁻⁴

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ELECTROCHEMISTRY DATA

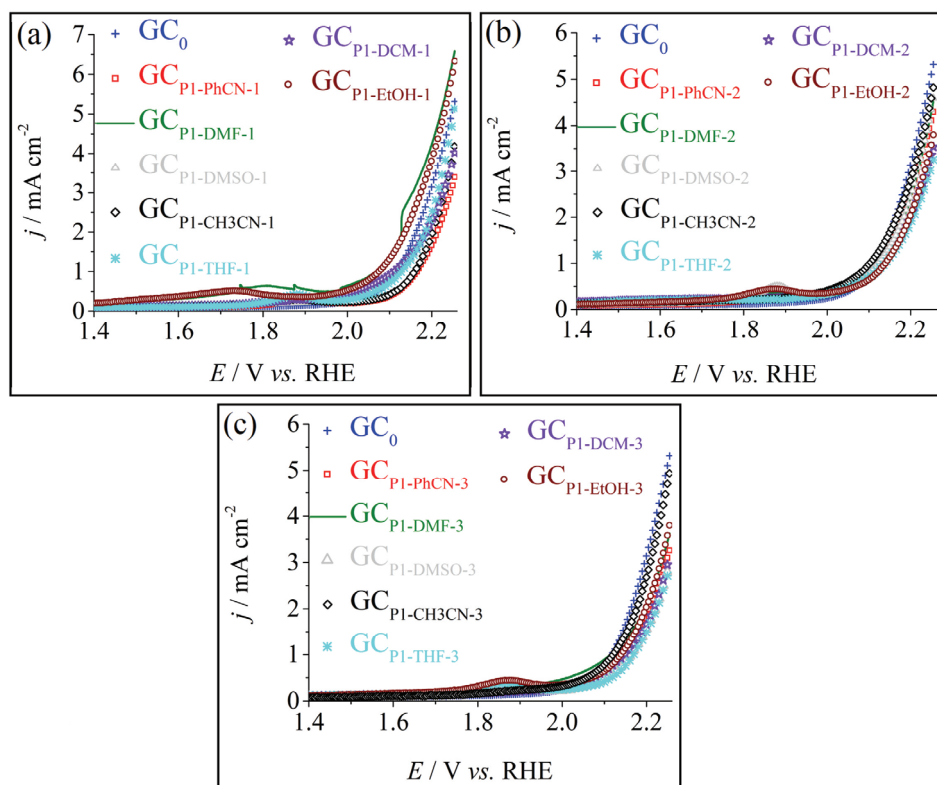


Fig. S-1. Anodic polarization curves recorded on GC_0 and on (a) $GC_{P1-PhCN-1}$, $GC_{P1-DMF-1}$, $GC_{P1-DMSO-1}$, GC_{P1-CH_3CN-1} , $GC_{P1-THF-1}$, $GC_{P1-DCM-1}$ and $GC_{P1-EtOH-1}$; (b) $GC_{P1-PhCN-2}$, $GC_{P1-DMF-2}$, $GC_{P1-DMSO-2}$, GC_{P1-CH_3CN-2} , $GC_{P1-THF-2}$, $GC_{P1-DCM-2}$ and $GC_{P1-EtOH-2}$ and (c) on $GC_{P1-PhCN-3}$, $GC_{P1-DMF-3}$, $GC_{P1-DMSO-3}$, GC_{P1-CH_3CN-3} , $GC_{P1-THF-3}$, $GC_{P1-DCM-3}$ and $GC_{P1-EtOH-3}$. Electrolyte solution: 0.1 mol L^{-1} H_2SO_4 . $v = 5 \text{ mV s}^{-1}$.

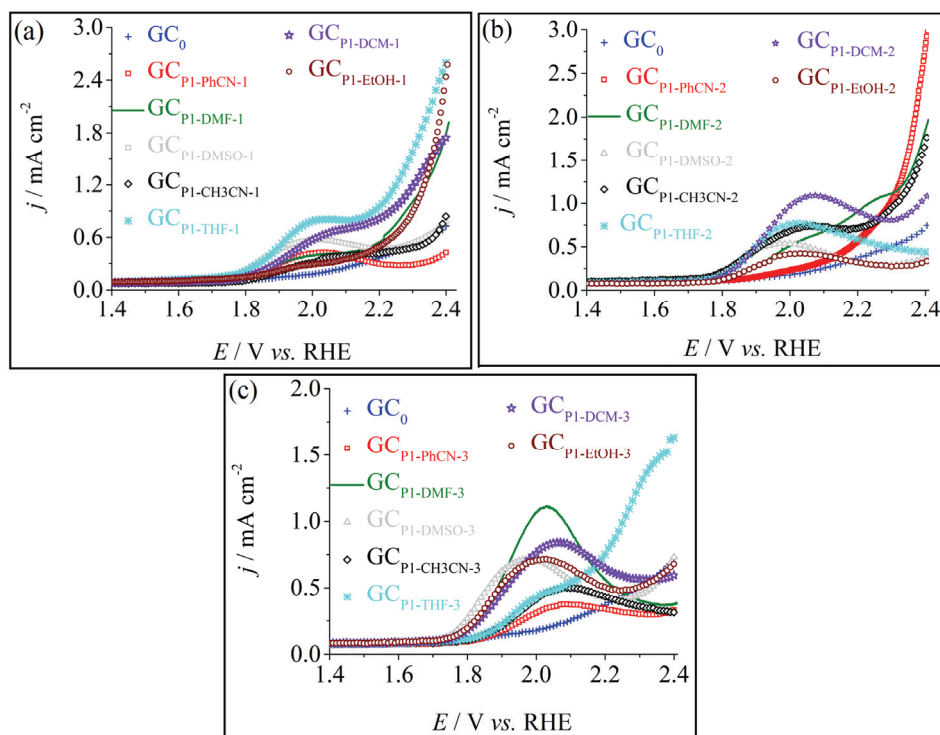


Fig. S-2. Anodic polarization curves recorded on GC_0 and on (a) $GC_{P1-PhCN-1}$, $GC_{P1-DMF-1}$, $GC_{P1-DMSO-1}$, $GC_{P1-CH3CN-1}$, $GC_{P1-THF-1}$, $GC_{P1-DCM-1}$ and $GC_{P1-EtOH-1}$; (b) $GC_{P1-PhCN-2}$, $GC_{P1-DMF-2}$, $GC_{P1-DMSO-2}$, $GC_{P1-CH3CN-2}$, $GC_{P1-THF-2}$, $GC_{P1-DCM-2}$ and $GC_{P1-EtOH-2}$ and (c) on $GC_{P1-PhCN-3}$, $GC_{P1-DMF-3}$, $GC_{P1-DMSO-3}$, $GC_{P1-CH3CN-3}$, $GC_{P1-THF-3}$, $GC_{P1-DCM-3}$ and $GC_{P1-EtOH-3}$. Electrolyte solution: 0.1 mol L^{-1} KCl. $v = 5 \text{ mV s}^{-1}$.

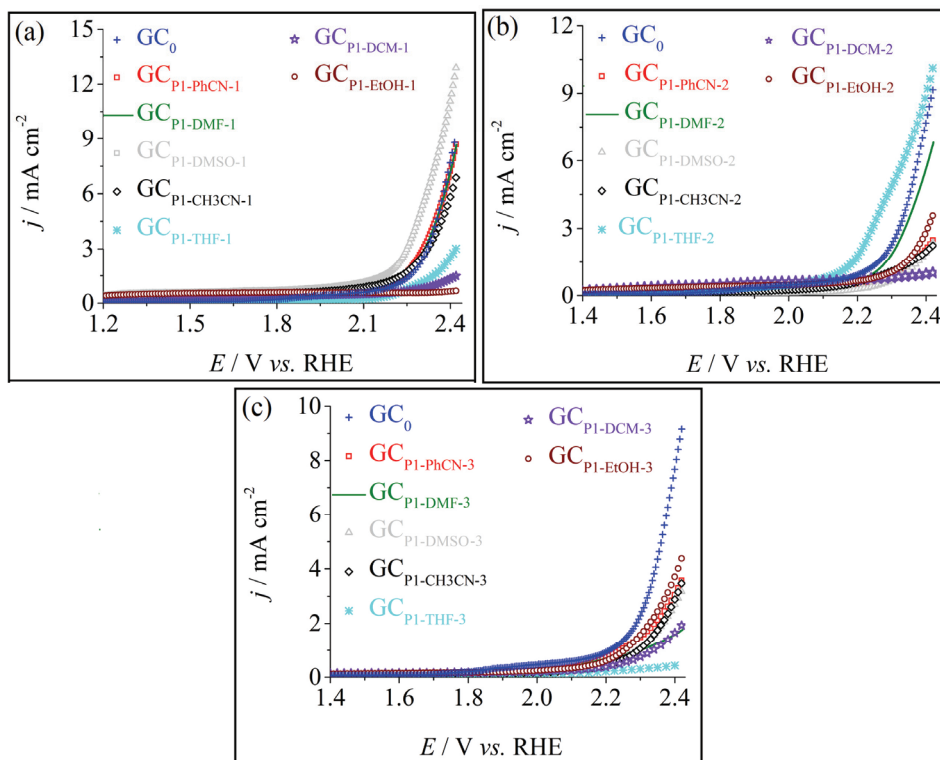


Fig. S-3. Anodic polarization curves recorded on GC₀ and on (a) GC_{P1-PhCN-1}, GC_{P1-DMF-1}, GC_{P1-DMSO-1}, GC_{P1-CH3CN-1}, GC_{P1-THF-1}, GC_{P1-DCM-1} and GC_{P1-EtOH-1}; (b) GC_{P1-PhCN-2}, GC_{P1-DMF-2}, GC_{P1-DMSO-2}, GC_{P1-CH3CN-2}, GC_{P1-THF-2}, GC_{P1-DCM-2} and GC_{P1-EtOH-2} and (c) on GC_{P1-PhCN-3}, GC_{P1-DMF-3}, GC_{P1-DMSO-3}, GC_{P1-CH3CN-3}, GC_{P1-THF-3}, GC_{P1-DCM-3} and GC_{P1-EtOH-3}. Electrolyte solution: 1 mol L⁻¹ KOH. $\nu = 5 \text{ mV s}^{-1}$.

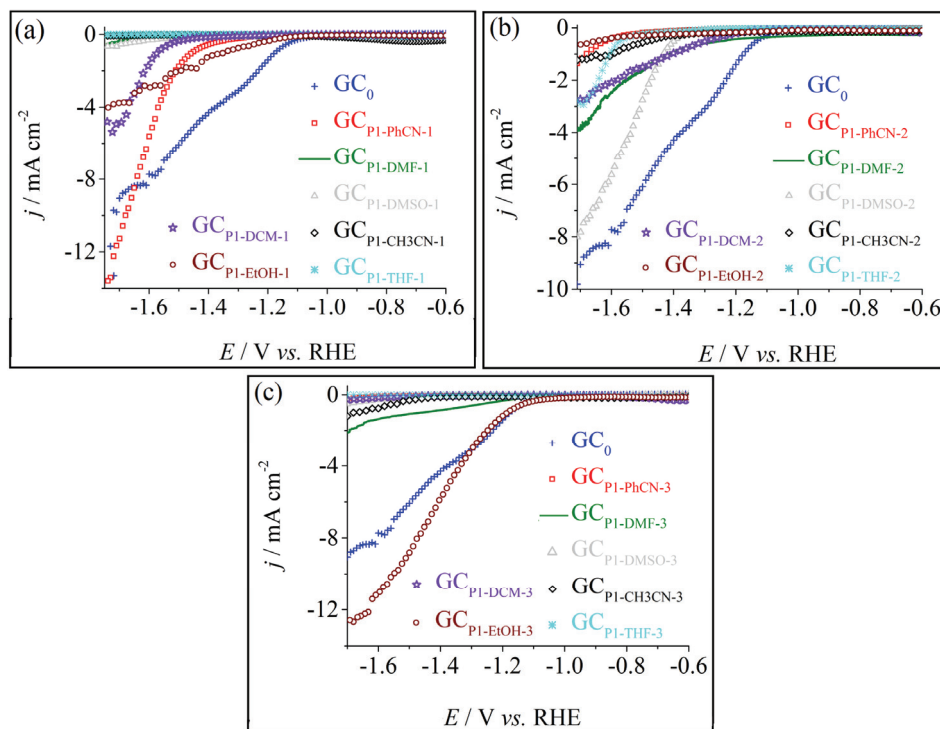


Fig. S-4. Cathodic polarization curves recorded on GC_0 and on (a) $\text{GC}_{\text{P1-PhCN-1}}$, $\text{GC}_{\text{P1-DMF-1}}$, $\text{GC}_{\text{P1-DMSO-1}}$, $\text{GC}_{\text{P1-CH}_3\text{CN-1}}$, $\text{GC}_{\text{P1-THF-1}}$, $\text{GC}_{\text{P1-DCM-1}}$ and $\text{GC}_{\text{P1-EtOH-1}}$; (b) $\text{GC}_{\text{P1-PhCN-2}}$, $\text{GC}_{\text{P1-DMF-2}}$, $\text{GC}_{\text{P1-DMSO-2}}$, $\text{GC}_{\text{P1-CH}_3\text{CN-2}}$, $\text{GC}_{\text{P1-THF-2}}$, $\text{GC}_{\text{P1-DCM-2}}$ and $\text{GC}_{\text{P1-EtOH-2}}$ and (c) on $\text{GC}_{\text{P1-PhCN-3}}$, $\text{GC}_{\text{P1-DMF-3}}$, $\text{GC}_{\text{P1-DMSO-3}}$, $\text{GC}_{\text{P1-CH}_3\text{CN-3}}$, $\text{GC}_{\text{P1-THF-3}}$, $\text{GC}_{\text{P1-DCM-3}}$ and $\text{GC}_{\text{P1-EtOH-3}}$. Electrolyte solution: $0.1 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$. $\nu = 5 \text{ mV s}^{-1}$.

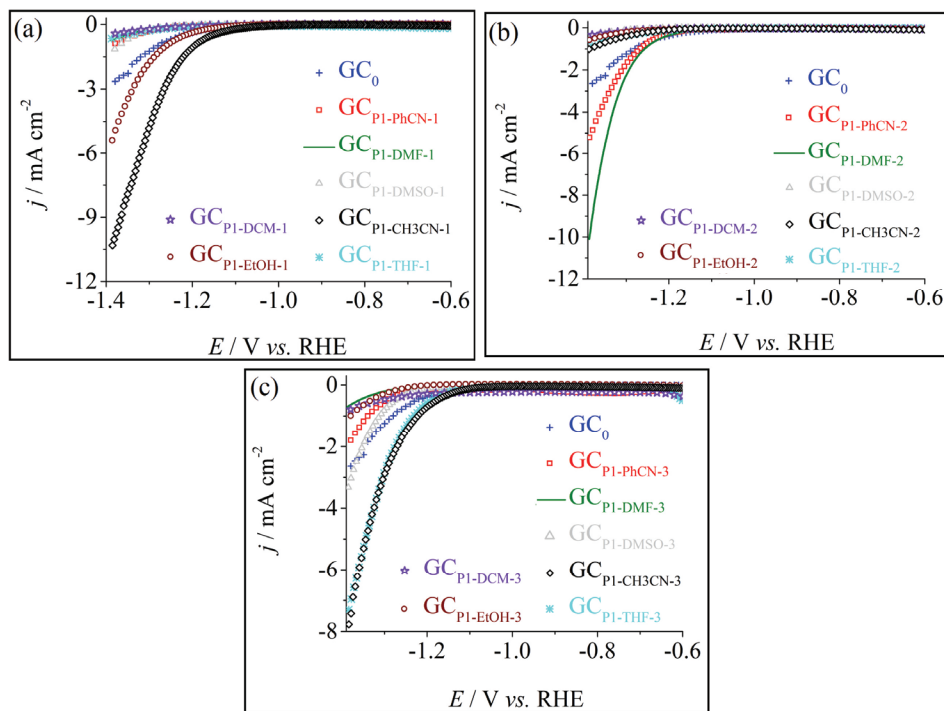


Fig. S-5. Cathodic polarization curves recorded on GC_0 and on (a) $\text{GC}_{\text{P1-PhCN-1}}$, $\text{GC}_{\text{P1-DMF-1}}$, $\text{GC}_{\text{P1-DMSO-1}}$, $\text{GC}_{\text{P1-CH3CN-1}}$, $\text{GC}_{\text{P1-THF-1}}$, $\text{GC}_{\text{P1-DCM-1}}$ and $\text{GC}_{\text{P1-EtOH-1}}$; (b) $\text{GC}_{\text{P1-PhCN-2}}$, $\text{GC}_{\text{P1-DMF-2}}$, $\text{GC}_{\text{P1-DMSO-2}}$, $\text{GC}_{\text{P1-CH3CN-2}}$, $\text{GC}_{\text{P1-THF-2}}$, $\text{GC}_{\text{P1-DCM-2}}$ and $\text{GC}_{\text{P1-EtOH-2}}$ and (c) on $\text{GC}_{\text{P1-PhCN-3}}$, $\text{GC}_{\text{P1-DMF-3}}$, $\text{GC}_{\text{P1-DMSO-3}}$, $\text{GC}_{\text{P1-CH3CN-3}}$, $\text{GC}_{\text{P1-THF-3}}$, $\text{GC}_{\text{P1-DCM-3}}$ and $\text{GC}_{\text{P1-EtOH-3}}$. Electrolyte solution: $0.1 \text{ mol L}^{-1} \text{ KCl}$. $v = 5 \text{ mV s}^{-1}$.

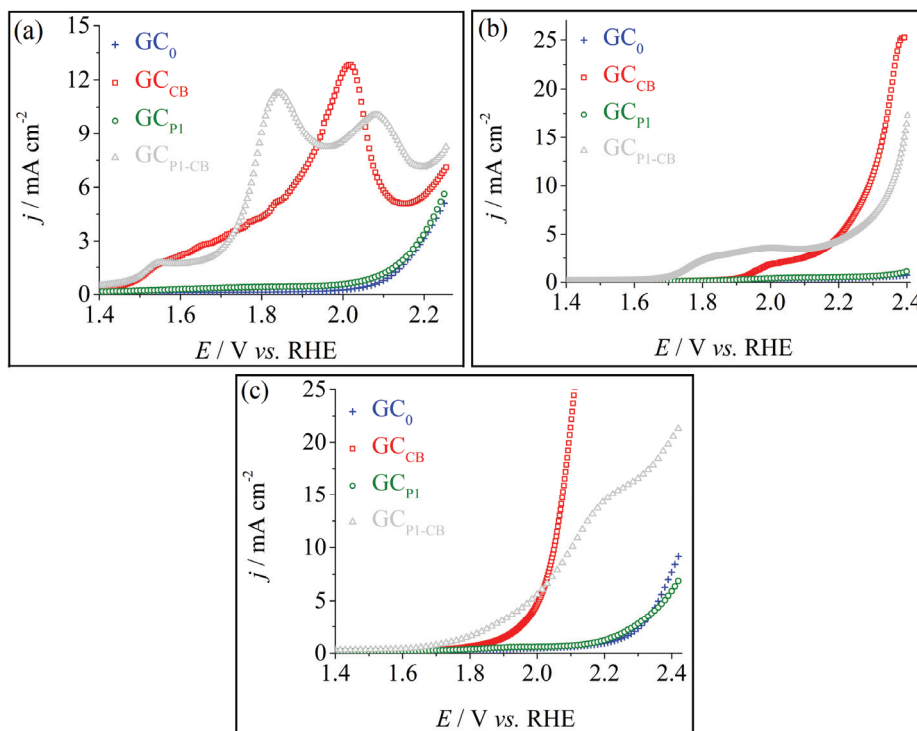


Fig. S-6. Anodic polarization curves recorded on GC₀, GC_{CB}, GC_{P1} and GC_{P1-CB} in the following electrolyte solutions: (a) 0.1 mol L⁻¹ H₂SO₄, (b) 0.1 mol L⁻¹ KCl and (c) 1 mol L⁻¹ KOH. $\nu = 5 \text{ mV s}^{-1}$.

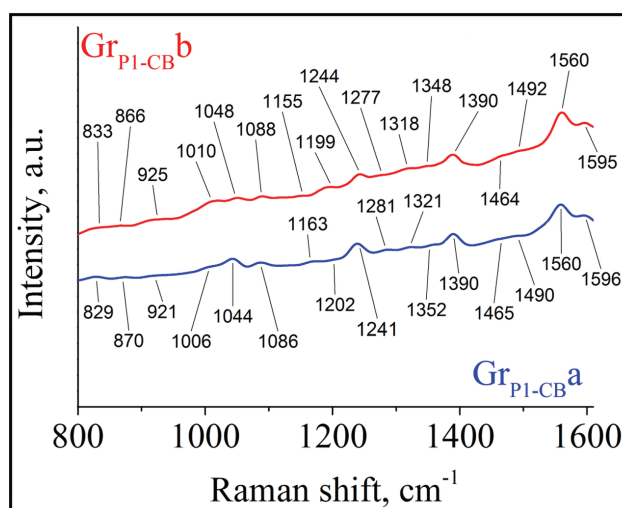


Fig. S-7. Raman spectra recorded on GC_{P1-CB} before a cathodic stability experiment performed in 1 mol L⁻¹ KOH solution (GC_{P1-CB}^a) and after the experiment (GC_{P1-CB}^b).

TABLES

TABLE S-I. The HER activity of GC_{P1-CB} and of other porphyrin-based electrodes

Catalyst @substrate	Environment	η_{HER} / mV at $j = -10 \text{ mA cm}^{-2}$	Tafel slope, mV dec ⁻¹	Ref.
Zn-TPP/G @Cu foil ^a	0.5 mol L ⁻¹ H ₂ SO ₄	~ 480 ^b	-	5
Zn-TAPP/G @Cu foil ^c	0.5 mol L ⁻¹ H ₂ SO ₄	~ 480 ^b	-	5
Zn-TPyP/G @Cu foil ^d	0.5 mol L ⁻¹ H ₂ SO ₄	~ 560 ^b	-	5
ZnTAPP-NA @GC ^e	1 mol L ⁻¹ KOH	546	121	6
CoTAPP-NA @GC ^f	1 mol L ⁻¹ KOH	470	110	6
CoTPP-SD @CFP ^g	1 mol L ⁻¹ KOH	475	-	7
CoCOP @CFP ^h	1 mol L ⁻¹ KOH	310	161	7
CoTCPP @FTO/Ag ⁱ	0.5 mol L ⁻¹ H ₂ SO ₄	666	264	8
CoTCPP polymer @FTO/Ag ^j	0.5 mol L ⁻¹ H ₂ SO ₄	475	197	8
CoTMPyP/ERGO @GC ^k	0.1 mol L ⁻¹ KOH	347 ^l	99	9
CoTMPyP/ERGO @GC	1 mol L ⁻¹ KOH	315 ^l	96	9
Co-2DP @Ti foil ^m	1 mol L ⁻¹ KOH	367 ^l	126	10
CoP-2ph-CMP-800 @GC ⁿ	1 mol L ⁻¹ KOH	360	121	11
CoP-3ph-CMP-800 @GC ^o	1 mol L ⁻¹ KOH	380	-	11
CoP-4ph-CMP-800 @GC ^p	1 mol L ⁻¹ KOH	440	-	11
G _{ZnP} -DMF-1 ^q	1 mol L ⁻¹ KOH	520	150	2
Porphylar-based ink @carbon paper ^r	1 mol L ⁻¹ PBS	~ 770 ^s	227	12
G _{CB-PZn} ^t	0.1 mol L ⁻¹ KCl	1020	249	3
Fe-porphyrin polymer @carbon paper ^u	1 mol L ⁻¹ KOH	678	363	13
Co-porphyrin polymer @carbon paper ^v	1 mol L ⁻¹ KOH	437	195	13
Ni-porphyrin polymer @carbon paper ^w	1 mol L ⁻¹ KOH	644	345	13

Cu-porphyrin polymer @carbon paper ^x	1 mol L ⁻¹ KOH	436	236	13
Pt-TAPP/G @Cu foil ^y	0.5 mol L ⁻¹ H ₂ SO ₄	~ 550	-	5
2H-TAPP/G @Cu foil ^z	0.5 mol L ⁻¹ H ₂ SO ₄	600 ^{aa}	-	5
Ni-TAPP/G @Cu foil ^{ab}	0.5 mol L ⁻¹ H ₂ SO ₄	600 ^s	-	5
GP2-DMF ^{ac}	0.5 mol L ⁻¹ H ₂ SO ₄	108	205	14
GP4-NiPh-THF ^{ad}	1 mol L ⁻¹ KOH	430	140	15
[ERGO/CoTMPyP] ₇ /PDDA/4-ABA@GC ^{ae}	0.1 mol L ⁻¹ KOH	474 ¹	116	16
GCPI-CB	1 mol L ⁻¹ KOH	770	135	This work

^a Zn-TTP = 5,10,15,20-tetraphenyl-21H,23H-porphine on single-layer graphene; ^b at -3 mA cm⁻²; ^c Zn-TAPP = 5,10,15,20-tetrakis(4-aminophenyl)-21H,23H-porphine on single-layer graphene; ^d Zn-TPyP = 5,10,15,20-tetrakis(4-pyridyl)-21H,23H-porphine on single-layer graphene; ^e ZnTAPP-NA = Zn(II) 5,10,15,20-tetra(4-aminophenyl)-21H,23H-porphyrin - ferrocene-1,1'-dicarbaldehyde; ^f CoTAPP-NA = Co(II) 5,10,15,20-tetra(4-aminophenyl)-21H,23H-porphyrin - ferrocene-1,1'-dicarbaldehyde; ^g CoTPP-SD@CFP = Co(II) 5,10,15,20-tetrakis(4-aminophenyl)porphyrin - salicylaldehyde@carbon fibre paper; ^h CoCOP = Co(II) 5,10,15,20-tetrakis(4-aminophenyl)porphyrin-based covalent organic polymer; ⁱ CoTCPP = Co(II) meso-tetra(4-carboxyphenyl)porphyrin; ^j CoTCPP polymer = crystalline Co(II) meso-tetra(4-carboxyphenyl)porphyrin-based polymeric system; ^k CoTMPyP/ERGO = tetrakis(N-methylpyridyl)porphyrinato cobalt / electrochemically reduced graphene oxide; ^l at -1 mA cm⁻²; ^m Co-2DP = multilayer 2D polymer based on Co(II) 5,10,15,20-tetrakis(4-aminophenyl)-21H,23H-porphyrin and 2,5-dihydroxyterephthalaldehyde; ⁿ CoP-2ph-CMP-800, ^o CoP-3ph-CMP-800 and ^p CoP-4ph-CMP-800 = conjugated mesoporous polymer based on Co-porphyrins and pyrolyzed at 800 °C; [ERGO/CoTMPyP]₇/PDDA/4-ABA@GC = multilayer films containing tetrakis(N-methylpyridyl)porphyrinato cobalt, on treated glassy carbon electrode; ^q G_{ZnP-DMF-1} = Zn(II) 5,10,15,20-tetrakis(4-pyridyl)-porphyrin drop-casted from DMF in one layer on graphite; ^r Porphylar = organic polymer obtained from the condensation of terephthaloyl chloride and 5,10,15,20-tetrakis(4-aminophenyl)porphyrin; ^s at -7 mA cm⁻²; ^t G_{CB-PZn} = Zn(II) 5-(4-pyridyl)-10,15,20-tris(4-phenoxyphenyl)-porphyrin and Carbon Black drop-casted as catalyst ink on graphite; ^{u,v,w,x} Fe-porphyrin polymer, Co-porphyrin polymer, Ni-porphyrin polymer, Cu-porphyrin polymer = organic polymers obtained from the polymerization reaction of poly(*p*-phenylene terephthalamide) with 5,10,15,20-tetrakis(4-aminophenyl)porphyrin metalated with Fe, Co, Ni and Cu; ^y Pt-TAPP/G = Pt(II) 5,10,15,20-tetrakis-(4-aminophenyl)-21H,23H-porphine on single-layer graphene; ^z 2H-TAPP/G = 5,10,15,20-tetrakis-(4-aminophenyl)-21H,23H-porphine on single-layer graphene; ^{aa} at -9 mA cm⁻²; ^{ab} Ni-TAPP/G = Ni(II) 5,10,15,20-tetrakis-(4-aminophenyl)-21H,23H-porphine on single-layer graphene; ^{ac} GP2-DMF = Pt(II) 5-(3-hydroxyphenyl)-10,15,20-tris(3-methoxyphenyl)-porphyrin drop-casted on graphite substrate from N,N-dimethylformamide; ^{ad} GP4-NiPh-THF = graphite substrate modified with suspension of nickel phosphite in solution of 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin dissolved in tetrahydrofuran; ^{ae} [ERGO@CoTMPyP]₇/PDDA/4-ABA@GC = multilayer films containing tetrakis(N-methylpyridyl)porphyrinato cobalt, on treated glassy carbon electrode.

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An effective and facile approach for the determination of bioactive components of *Rheum ribes* in the Kurdish state of Iraq and Siirt region in Turkey

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Abstract: Developing a streamlined and accessible method for identifying the bioactive components of *Rheum ribes* (rhubarb) holds significant promise in unlocking its therapeutic potential and advancing research in natural medicine. In this study, the bioactive components of rhubarb such as total phenolics and flavonoids as well as the antioxidant activity of its methanolic extract were determined. Total phenolic content was between 84.02 and 387.53 mg/L gallic acid equivalent (GAE) in extracts. Total flavonoid contents determined by the aluminium chloride colorimetric method ranged from 69.98 to 935.75 mg L⁻¹ of routine equivalents (RE) in the extracts. The antioxidant activities were determined using ferric reducing the antioxidant potential (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods. In the FRAP assay, the highest antioxidant activity (*IC*₅₀) was found as 25.18±0.04 mg L⁻¹ extract. In the DPPH method, the maximum percentage inhibition was found as 88.11 %. Iron chelating activities of the samples were above 70 %. The chemical compound contents of the extracts were determined by LC–MS/MS. In this step, a total of 25 phenolic and flavonoid compounds in extracts were analysed qualitatively and quantitatively. Malic acid (15.72±0.53 mg kg⁻¹) and rutin (76.93±0.03 mg kg⁻¹) in the extract were identified as the major phytochemicals compounds. The results of the study confirm that rhubarb have potential biological activities and can be introduced as an important sources of natural antioxidants.

Keywords: rhubarb; phenolic compounds; antioxidant activities; flavonoids.

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INTRODUCTION

Rhubarb, comprising approximately sixteen plants within the Polygonaceae family,¹ is harvested in Turkey and Iraq and serves both as a culinary delight and a natural remedy.² It mostly grows in the spring season under the snow of the high mountains in the north and centre of Asia.

Not only are rhubarb samples consumed as food, but they are also valued for their medicinal properties, making them a versatile resource for human use. In addition to its medical importance, rhubarb is used in cuisine in desserts such as rhubarb crumble, as well as in jams, jellies and sauces, since its tart flavour became famous. Rhubarb is basically a vegetable but it is often thought to be a fruit.³ Some of their leaves are toxic, but the stalks are used in foods for their tart flavour. Rhubarb stalks are either cooked or eaten raw, and raw stalks can also be dipped in sugar to eliminate sourness.⁴

They provide rich sources of natural antioxidants. The components of rhubarb are separated and predicted to have antiulcer, antioxidant, antidiabetic, antimicrobial, wound healing, nephroprotective, anticancer and hepatic protective activities.⁵ They also include tocopherols, vitamin C, carotenoids and phenolic compounds. Since phenolic compounds oxidize and combine with proteins and other components, they can protect plants against tissue injuries. Phenolic compounds in plants may serve as defence systems against herbivores. Products of photosynthesis may also produce high levels of oxygen, free radicals and reactive oxygen species (ROS). The rhubarb plants have a myriad of antioxidant compounds to survive.⁶

Many of these compounds have basic similar molecular and have at least one aromatic ring and a hydroxyl group. They also include phenolic acids, flavonoids and gallate esters (hydrolysable tannins). Flavonoids are also recognized as a cause of wound healing potential of rhubarb since it is identified to decrease per oxidation of lipids not merely via averting or decelerating the beginning of cell necrosis but also via secularity improvement. Because of flavonoids and tannins' astringent and antimicrobial properties, which seem accountable for the contraction wound and epithelialization escalation rate, tannins and flavonoids promote the process of wound healing.⁷

Some rhubarb samples have large somewhat triangular-shaped leaves with long fresh petioles. Through its medical advantages can be used for the treatment of acidity in the stomach and to address constipation.^{8,9} Additionally, it also kills intestinal worms and helps to purify the human liver.⁸ They are used as a purgative, anti-inflammatory agent and herbal medicines for the treatment of constipation and cancer.¹⁰ Some beneficial effects of rhubarb are also on Parkinson's disease, immune system and acute respiratory syndrome. *Rheum ribes* has been clinically used as a laxative agent,¹¹ antibacterial, isolation and identification of about 200 chemical compounds for at least 2000 years. It is identified that environmental and

genetic factors and their interactions involve the pharmaceutically important secondary metabolites in medical plants.¹² Besides, Rhubarb is one of the most well-known and widely used traditional Chinese medicines for the treatment of constipation, inflammation and cancer. It derives from the roots and rhizomes of *Rheum officinale* recorded in the Chinese Pharmacopoeia.¹³

Phenolic compounds in plants are essential for human diets and are of considerable interest due to their antioxidant properties. These compounds have an aromatic ring bearing one or more hydroxyl groups and their structures may range from a simple phenolic molecule to a complex high-molecular-weight polymer. The antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. The major effective constituents of rhubarb samples are phenolic compounds, sennosides and anthraquinone glycosides. Rhubarbs can lower sugar and lipid levels in human blood and could be used to treat hyper lipids, obesity and diabetes.¹⁴

Antioxidants may protect the cells against the effects of free radicals and molecules produced when the body is exposed to tobacco smoke and radiation. Free radicals may play a role in heart disease, cancer and other diseases. Antioxidants come up frequently in discussions about good health and preventing diseases. These powerful antioxidants mostly come from fresh fruits and vegetables. Aqueous and methanol extracts of the roots of Rhubarb emodin have been indicated to have anticancer and antioxidant potential.¹⁵ One of the primary factors for the development and progression of many life-threatening diseases and disorders like cancer, atherosclerosis and diabetes is oxidative stress. Presently, in modern pharmacopoeia around 25 % of drugs originate from plants (phytomedicines) and several others are synthetic analogues built on the prototype compounds separated from plants. In Chinese folk medicine, *R. emodin* is used in the treatment of cancer, ulcers and liver treatments.¹⁶ The rhubarb's anticancer influence is accredited to the aloe-emodin which not merely subdued the spread but also encouraged apoptosis of two human cancer cell lines.¹⁷

The aim of this study is to determine the antioxidant activity (DPPH % inhibition), the total phenolic content and total flavonoid concentrations, FRAP antioxidant capacities and iron chelating capacity of extracts of rhubarb samples. Besides, a comprehensive method was developed, optimised and validated to determine 37 phytochemicals in rhubarb samples using liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS).

These include coumarin, hesperidin, *p*-coumaric acid, gallic acid, caffeic acid, vanillic acid, salicylic acid, quinic acid, *p*-hydroxybenzoic acid, ferulic acid, chlorogenic acid, rosmarinic acid, protocatechuic acid, cinnamic acid, sinapinic acid, fumaric acid, malic acid, syringic acid, naringenin, rutin, quercetin, quercitrin, isoquercitrin and nicotiflorin.^{18,19} The developed method went thorough

validation, encompassing assessments of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), as well as determination of limits of detection and quantification (*LOD*, *LOQ*). The evaluation of measurement uncertainty (*U* % at 95 % confidence level with $k = 2$) was also conducted as part of the validation process. Furthermore, the developed and validated LC–MS/MS method was employed for phytochemical screening of methanol-chloroform extracts from the samples. Also, the presence of specific phenolic acids, including organic acids such as quinic, malic, fumaric, chlorogenic and vanillic acids, alongside flavonoids such as rutin, hesperidin and isoquercitrin, the total phenolic and flavonoid contents, as well as the antioxidant potential were analysed in rhubarb samples.

EXPERIMENTAL

Collection and preparation of samples

The *Rheum ribes* samples were collected from seven different locations in Siirt (Pervari and Şirvan) and Northern Iraq (Gara, Korajar, Sor, Rash and Qalandar). The samples taken were air-dried completely at laboratory temperature in the shaded area and then powdered until dust particles were obtained with a mixer. Powdered plant samples were placed in glass jars and stored at laboratory temperature. The extracts were prepared for total phenolic matter content, DPPH radical scavenging activity analysis, total flavonoid content and FRAP analysis of *R. ribes* samples using the amounts specified in the literature.^{20–22} For the analysis of phenolic compounds by LC–MS/MS, *R. ribes* samples were subjected to the Soxhlet extraction method proposed by Teğin *et al.*²³ For this purpose, 10 g of dried and powdered *R. ribes* samples were Soxhlet extracted with 160 mL 80:20 methanol–water mixture. After extraction, the solvents obtained were evaporated under vacuum at 35 °C by a rotary evaporator until dry extracts were obtained. Dry filtrates were diluted to 1000 mg/L and filtrated with a 0.2 µm syringe filter before LC–MS/MS analysis.^{19,23} To determine the phenolic content composition by LC–MS/MS, the method given by Yılmaz *et al.*¹⁸

LC–MS/MS device and chromatographic conditions

Qualitative and quantitative analysis of phytochemicals were performed by using an LC–MS/MS system equipped with a Shimadzu – Nexera ultrahigh performance liquid chromatography (UHPLC) device and Shimadzu LCMS 8040 triple quadrupole mass spectrometer (MS). The liquid chromatography system used consists of the LC-30 AD gradient pump, the DGU-20A3R degasser, the CTO-10ASvp column oven and the SIL-30AC autosampler. Chromatographic separation was performed on an RP-C18 Inertsil ODS-4 (100 mm×2.1 mm, 2 µm) analytical column. During the analysis, the column furnace temperature was set at 35 °C. In the elution gradient, ultrapure water for mobile phase A and acetonitrile for mobile phase B were used. In addition, 10 mmol/L ammonium formate and 0.1 % formic acid were added to the water phase A to facilitate better chromatographic separation and ionization. After several attempts to achieve optimal separation of analyses, the most suitable UHPLC gradient profile was obtained with a gradient profile of 5–20 % B (0–10 min), 20 % B (10–22 min), 20–50 % B (22–36 min) 95 % B (36–40 min) and 5 % B (40–50 min). The mobile phase flow rate was set at 0.25 mL/min and the injection volume was about 4 µL.¹⁹

The triple quadrupole mass spectrometer is equipped with an electrospray ionization (ESI) source operating in both a negative and a positive mode. The LC–ESI-MS/MS data were collected and processed by the registered Lab Solutions software (Shimadzu, Kyoto, Japan). The

quantitative analysis of the compounds was carried out using multiple reaction monitoring (MRM) mode and the parent ions were combined with one or two product ions (the first one was used for qualitative purposes and the second one for quantitative purposes). Other parameters optimized in the mass spectrometer were: interface temperature, 350 °C; DL temperature, 250 °C; heat block temperature, 400 °C; nebulizer gas flow (N₂), 3 L/min; drying gas flow (N₂) 15 L/min.²⁴

LC–MS/MS method validation studies

In the presented study, a comprehensive LC–MS/MS method was optimized and validated for the qualification and quantification of 37 phytochemicals in Rhubarb samples. The performance characteristics of the method were determined by using standard solutions and wavelengths recommended by the manufacturer for detection. Furthermore, to increase the credibility of the results by compensating for the matrix effects and analyte losses during the sample preparation and analyses, quercetin D3, rutin D3 and ferulic acid D3 were used as the deuterated internal standards for flavonoid compounds, respectively. Within this context, the developed method was fully validated in terms of linearity, accuracy (recovery), inter-day and intra-day precision (repeatability), limits of detection and quantification (*LOD/LOQ*) and relative standard uncertainty (U % at 95 % confidence level ($k = 2$)). Parameters related to the LC–MS/MS method validation studies are given in Table S-I and Fig S-1 of the Supplementary material to this paper.^{24,25}

Linearity

The linearity was evaluated using an external standard calibration curve with eight concentration levels for each analyte was analyzed in triplicate. The calibration curves were constructed as a plot of the ratio of the concentration of the analyte to the concentration of the internal standard (IS, x) versus the ratio of the area of the analyte to IS (y).

Precision (repeatability) and accuracy (recovery)

Precision and accuracy studies for the developed method were carried out by standard addition to a selected extract of a species. Intra-day (within-day) precision (repeatability) was evaluated by analysing six replicates of fortified samples within a single day. On the other hand, to conduct an inter-day precision assay six replicates of fortified samples were examined per day for three consecutive days. As a consequence of the intra-day and inter-day studies, percent relative standard deviation (*RSD* / %) and recovery values were used to assess the precision and accuracy (Table S-I). Eq.(1) was used to calculate the recovery:

$$\text{Recovery (\%)} = 100(DC - OC)/SC \quad (1)$$

where *DC*, *OC* and *SC* signify detected, original and spiked concentrations, respectively.

Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection (*LOD*) and quantification (*LOQ*) data for each phytochemical analyte used were determined by serial dilution of standard solutions and analysing them under the described LC–MS/MS conditions until the detection of the lowest concentration signalled by the standards (*S/N* ratio 3:1). After determining the lowest detectable concentrations for each analyte, ten internal standard solution mixtures (ISs included) at these concentrations were prepared and injected to the LC–MS/MS system. Calculation of *LOD* and *LOQ* data were carried out using Eqs. (2) and (3), Table S-I:

$$LOD = \text{Mean} + 3SD \quad (2)$$

$$LOQ = \text{Mean} + 10SD \quad (3)$$

where *SD* indicates the standard deviation.

Preparation of plant samples for extraction

After the samples collected were dried at the laboratory environment temperature in the shaded area, they were pulverized. Powdered samples were placed in glass jars and stored at laboratory temperature. A 4 g of the powdered sample was placed in a beaker and 40 mL of 80 vol. % methanol was added. After the mixture was sonicated for 2 min on the Wiggin Hauser homogenizer and 5 min on the Sonopuls HD 2070, it was left overnight on the incubated shaker Standard & Cooled – MD13. The extract was then filtered through ordinary filter paper. After the extracts were dried at 38 °C in an oven, the stock concentrations were formed by adding 80 vol. % methanol so that the concentration of the solid part remaining in the bottom of the tube was 10 mg/mL.

Determination of phenolic content

The total phenolic contents of samples were determined according to the Folin–Ciocalteu reactivity and Gallic acid standard.^{27,28} From the extract solution, 0.1 mL of Folin–Ciocalteu reactant was added to the flask and then thoroughly shaken. After 3 min, 1 mL of 6 % Na₂CO₃ solution was added and the mixture was allowed to stand for 1 h with intermittent agitation. Absorbance was measured at 760 nm with a Uvmini-1240 spectrophotometer. The same procedure was repeated in the gallic acid solutions. The calibration graphs of the analytes obtained are given in Fig. S-2 of the Supplementary material.

Determination of total flavonoid contents in samples

Total flavonoid contents in samples were determined using the aluminium chloride colorimetric method.^{30,31} The 0.5 mL of the extract solution was mixed with 2 mL of distilled water and 150 µL of 5 % sodium nitrate. After 6 min, 150 µL of 10 % aluminium chloride and 2 mL of 1 mol/L sodium hydroxide were added and left at room temperature for 15 min. The absorbance values of the sample solutions were measured at 510 nm with a Shimadzu UV–Vis Uvmini-1240 spectrophotometer. The routine was used as a standard. The calibration graphs of the analytes obtained are given in Fig. S-3 of the Supplementary material.

Preparation of plant samples for DPPH analysis

For DPPH analysis,³² 4 mL of 0.01 mM DPPH solution prepared in 80 % methanol was added to 1 mL of extract and the mixture was absorbed at 517 nm wavelength with Shimadzu UV–Vis Uvmini-1240 spectrophotometer after waiting 15 min in the dark. The control solution was 1 mL of solvent and 4 mL of DPPH mixture.^{18,33} DPPH activities were calculated using Eq. (4):

$$\text{DPPH activity (\% incubation)} = 100 \frac{A_C - A_1}{A_C} \quad (4)$$

where A_C is the control absorbance and A_1 is the sample absorbance.

FRAP analysis

FRAP analysis was performed by adding 3 mL of FRAP reagent into 100 µL of sample diluted appropriately.³⁴ The incubation was allowed to proceed for 6 min at room temperature in the dark and then the absorbance was measured at 593 nm with a Shimadzu UV–Vis Uvmini-1240 spectrophotometer. FRAP solution was 10 mL acetate buffer + 10 mL FeCl₃·6H₂O + 1 mL TPTZ solution. Acetate buffer (300 mmol/L at pH 3.6) solution was prepared using 3.1 g of CH₃COONa in 16 mL of acetic acid/L distilled water. HCl (40 mmol/L) solution was prepared by 400 µL of HCl (32 %)/100 mL of purified water. TPTZ (10 mmol) solution was pre-

pared by dissolving 31.2 mg TPTZ in 10 mL of 40 mmol/L HCl. FeCl_3 solution was prepared by dissolving 54.1 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mL of pure water.

Preparation of plant samples for DPPH analysis

For DPPH analysis,³² 4 mL of 0.01 mM DPPH solution in 80 % methanol was added to 1 mL of extract and the mixture was absorbed at 517 nm wavelength with Shimadzu UV-Vis Uvmini-1240 spectrophotometer after waiting 15 min in the dark. The control solution was 1 mL of solvent and 4 mL of DPPH mixture.^{18,33} DPPH activities were calculated using Eq. (4).

RESULTS AND DISCUSSION

Determination of phenolic content

As shown in Fig. S-2 of the Supplementary material, the linear relation between the concentration of phenolic compound and the absorbance ($R^2 = 0.9996$) was illustrated.²⁹ The mean and standard deviation values of phenolic acids and flavonoids found in samples taken from seven regions are given in Table I. The major phenolic is gallic acid. Mean values of it were found as 835, 1079, 719, 796, 410, 2026 and 973 mg/kg in Pervari, Shirvan, Sor, Rash, Korajar, Gara and Qalandar, respectively. In addition, the mean values of quinic acids were found as 881, 1362, 1842, 542, 1487, 363 and 968 mg/kg in Pervari, Shirvan, Sor, Rash, Korajar, Gara and Qalandar, respectively.

TABLE I. Determinations of phenolic acids and flavonoid compounds (mean $\pm$ s, $\mu\text{g kg}^{-1}$); nd.: not detected

Analyte	Pervari	Şirvan	Sor	Rash
Coumarin	8.330 $\pm$ 0.002	5.530 $\pm$ 0.001	9.890 $\pm$ 0.002	7.750 $\pm$ 0.002
Hesperidin	24.510 $\pm$ 0.006	2.660 $\pm$ 0.001	23.640 $\pm$ 0.006	6.070 $\pm$ 0.002
<i>p</i> -Coumaric acid	2.660 $\pm$ 0.001	1.760 $\pm$ 0.001	4.040 $\pm$ 0.002	7.660 $\pm$ 0.004
Gallic acid	834.74 $\pm$ 0.24	1079 $\pm$ 0.3	719.24 $\pm$ 0.20	796.100 $\pm$ 0.225
Caffeic acid	0.900 $\pm$ 0.001	0.14 $\pm$ 0.00	0.13 $\pm$ 0.00	0.14 $\pm$ 0.00
Vanillic acid	8.850 $\pm$ 0.004	12.200 $\pm$ 0.006	6.500 $\pm$ 0.003	10.800 $\pm$ 0.005
Salicylic acid	nd.	nd.	1.43 $\pm$ 0.00	0.23 $\pm$ 0.00
Quinic acid	881.30 $\pm$ 0.07	1361.86 $\pm$ 0.11	1841.99 $\pm$ 0.15	541.46 $\pm$ 0.04
<i>p</i> -Hydroxybenzoic acid	4.600 $\pm$ 0.001	1.250 $\pm$ 0.000	1.400 $\pm$ 0.000	nd.
<i>trans</i> -Ferulic acid	1.160 $\pm$ 0.001	2.920 $\pm$ 0.001	0.950 $\pm$ 0.001	1.06 $\pm$ 0.00
Chlorogenic acid	0.020 $\pm$ 0.000	nd.	0.020 $\pm$ 0.003	nd.
Rosmarinic acid	73.59 $\pm$ 0.05	nd.	2.38 $\pm$ 0.05	nd.
Protocatechuic acid	2.700 $\pm$ 0.001	3.530 $\pm$ 0.001	3.290 $\pm$ 0.001	3.580 $\pm$ 0.001
Cinnamic acid	71.84 $\pm$ 0.01	54.27 $\pm$ 0.01	118.75 $\pm$ 0.01	125.48 $\pm$ 0.02
Sinapinic acid	nd.	nd.	nd.	nd.
Fumaric acid	3.50 $\pm$ 0.00	2.21 $\pm$ 0.00	2.64 $\pm$ 0.00	1.82 $\pm$ 0.00
Malic acid	3311.7 $\pm$ 3.7	5438.7 $\pm$ 0.4	8495.8 $\pm$ 0.6	3882 $\pm$ 1
Syringic acid	nd.	nd.	5.86 $\pm$ 0.14	3.780 $\pm$ 0.001
Naringenin	2.480 $\pm$ 0.001	nd.	nd.	1.120 $\pm$ 0.001
Rutin	240.65 $\pm$ 0.04	380.15 $\pm$ 0.04	677.46 $\pm$ 0.06	926.10 $\pm$ 0.11
Quercetin	45.03 $\pm$ 0.02	47.31 $\pm$ 0.02	87.08 $\pm$ 0.02	145.45 $\pm$ 0.04
Quercitrin	nd.	nd.	nd.	nd.

TABLE I. Continued

Analyte	Pervari	Şirvan	Sor	Rash
Isoquercitrin	3.100±0.000	5.96±0.00	8.130±0.001	22.060±0.001
Cosmosiin	1.780±0.001	N.D.	0.190±0.001	nd.
Nicotiflorin	8.780±0.002	15.270±0.002	21.330±0.004	nd.
	Karajar	Gara	Qalandar	
Coumarin	6.030±0.001	4.950±0.001	9.310±0.002	
Hesperidin	1.910±0.001	nd.	nd.	
<i>p</i> -Coumaric acid	36.87±0.02	96.96±0.05	21.40±0.01	
Gallic acid	410.480±0.116	2025.970±0.571	972.680±0.274	
Caffeic acid	1.560±0.001	4.850±0.002	1.480±0.001	
Vanillic acid	10.130±0.005	12.270±0.006	127.280±0.065	
Salicylic acid	nd.	nd.	1.31±0.01	
Quinic acid	1487.37±0.12	363.41±0.03	967.81±0.07	
<i>p</i> -Hydroxybenzoic acid	nd.	1.07±0.00	nd.	
trans-Ferulic acid	33.270±0.001	73.91±0.02	71.17±0.04	
Chlorogenic acid	nd.	nd.	nd.	
Rosmarinic acid	nd.	nd.	nd.	
Protocatechuic acid	3.760±0.001	5.950±0.002	6.730±0.002	
Cinnamic acid	65.66±0.02	66.80±0.01	90.40±0.01	
Sinapinic acid	nd.	nd.	3.65±0.01	
Fumaric acid	2.79±0.00	2.72±0.00	1.69±0.00	
Malic acid	10552.5±0.4	4656.31±1.19	15721.9±0.5	
Syringic acid	2.590±0.001	6.750±0.001	3.020±0.002	
Naringenin	N.D.	96.72±2.00	2.06±0.05	
Rutin	185.18±0.15	76927.45±0.03	545.45±12.23	
Quercetin	nd.	nd.	nd.	
Quercitrin	nd.	5.20±0.01	nd.	
Isoquercitrin	1.980±0.003	1886.910±0.001	6.360±0.251	
Cosmosiin	nd.	nd.	nd.	
Nicotiflorin	nd.	17.010±1.004	nd.	

DPPH and FRAP analyses

The DPPH activities are presented in Table II, along with the FRAP analysis results of samples collected from seven regions. Additionally, the percentage of iron chelating activity observed in the samples is also provided in Table II.

Analysis of method validation parameters

In the linearity analysis, the developed analytical method was found to be linear for all compounds between the ranges of tested concentrations with acceptable correlation coefficients ($R^2 > 0.90$). The calibration curve equations and the coefficients of determination (R^2) are presented in Table S-I.

Intraday and inter-day repeatabilities were lower than 2.13 and 2.51 %, respectively. On the other hand, intra-day and inter-day recoveries were between 99.5–100.8 and 99.2–100.7 %, respectively. The results of the study demonstrate

that the accuracy and precision evaluation of the LC–MS/MS method was quite satisfactory for routine monitoring purposes.²⁴ Relative standard uncertainties (U^{95}) of the analytes were also determined by the accuracy (recovery) and the precision (repeatability) studies according to Eurachem's guide.²⁶

TABLE II. Antioxidant activity, total phenolic compounds as gallic acid equivalent, total flavonoid analysis, FRAP antioxidant analysis as FeSO_4 equivalent and iron chelating percentages

Sample location	DPPH inhibition, %	Total phenolic, mg gallic acid/L extract	Total flavonoid, mg routine/L extract	FRAP, mg FeSO_4 /L extract	Iron chelating, %
Gara	75.83±0.18	84.02±0.00	69.98±0.01	3.12±0.01	95.18±0.00
Korajar	85.89±0.01	128.42±0.01	246.46±0.01	7.30±0.04	90.18±0.01
Sor	87.29±0.01	387.53±0.02	935.75±0.02	25.18±0.04	73.88±0.01
Rash	87.45±0.00	358.77±0.02	683.86±0.02	23.85±0.06	71.22±0.00
Qalandar	87.45±0.01	278.03±0.02	659.73±0.01	17.76±0.01	81.36±0.01
Pervari	70.27±0.01	85.28±0.00	110.71±0.01	3.14±0.00	97.34±0.00
Şirvan	88.11±0.01	134.73±0.01	333.94±0.06	8.55±0.02	91.85±0.01

The developed LC–MS/MS method for the quantification of 25 phytochemicals was fully validated. Five different linearity ranges (25–1000, 100–5000, 250–10000, 1000–20000 and 5000–20000 $\mu\text{g/L}$) were used for the studied analytes in the method. The inter-day and intra-day *RSD* values of the analytes were smaller than 0.032 and 0.026, respectively.

The average and the standard deviation (*STD*) of the antioxidant activity (DPPH inhibition, %), average and *STD* between gallic acid equivalent, total flavonoid analysis, average and *STD* of FRAP antioxidant analysis and iron chelating average and *STD* of samples were determined and given in Table II. As seen in the results of sample analysis in Table II, the DPPH fractions of 1 mL extract prepared with methanol were given. Accordingly, the highest inhibition was indicated by the sample gathered from the Şirvan region (88.11 %) and the lowest inhibition was in the Pervari sample (70.27 %). There is a slight difference in the antioxidant activity between all the samples. It is also evident that rhubarb samples have high antioxidant activity.²⁵ In a study conducted by Öztürk *et al.*, the antioxidant activity of chloroform and methanol extracts of rhubarb roots and stems was investigated. The roots exhibited activity with 93.1 and 84.1 % inhibition of chloroform and methanol extracts, while the stem extracts showed 82.2 and 82.0 % inhibition, respectively.³⁵

Following the outcomes of the analysis, the total phenolic concentration in 1 mL extract was given in Table II. Total phenolic concentrations were given as gallic acid equivalents. Gallic acid was calculated according to the standard regression curve of the gallic acid equivalent (Fig. S-2). According to these results, the highest value showed the sample collected from the Sor region (387.6 mg/L extract) and the lowest value collected from the Gara region (84.02 mg/L extract).

This difference may be due to the growth of samples in different regions. The concentration results of the samples are plotted and given in Fig. 1. In a study conducted by Öztürk *et al.*, the highest phenolic amounts were found in the chloroform extract of the rhubarb root and the methanol extract of the stem, with 48 and 35 μg PEs/mg extract, respectively. The phenolic compounds are known as powerful chain-breaking antioxidants. In the study conducted by Eman *et al.*, the total phenolic content in the ethanol extract and water extract of Rhubarb roots was found to be 655.47 and 1115.04 mg/100g d.w.²⁹

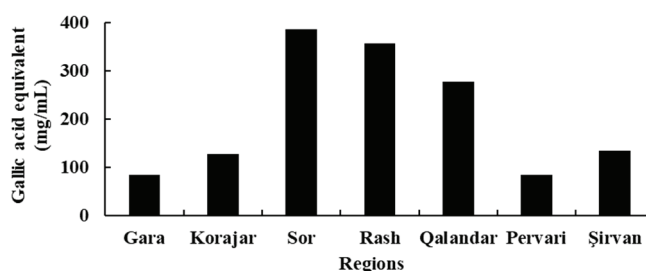


Fig. 1. Total phenolic concentrations as gallic acid equivalent.

The results of the total flavonoid concentrations found in 1 mL extract are given in Table II. Total flavonoid concentrations are given as routine equivalents. The routine equivalence calculation is based on the routine standard regression curve given. According to these results, the highest value showed the sample collected from the Sor region (935.8 mg/L extract) and the lowest value collected from the Gara region (69.98 $\mu\text{g}/\text{mL}$ extract). The results of the concentration of the samples are plotted and given in Fig. 2. In a study conducted by Öztürk *et al.*, the total amounts of flavonoids in the chloroform extract of rhubarb roots and stems were found to be highest in chloroform extracts, with 145 and 20 μg QEs/mg extract, respectively.³⁵

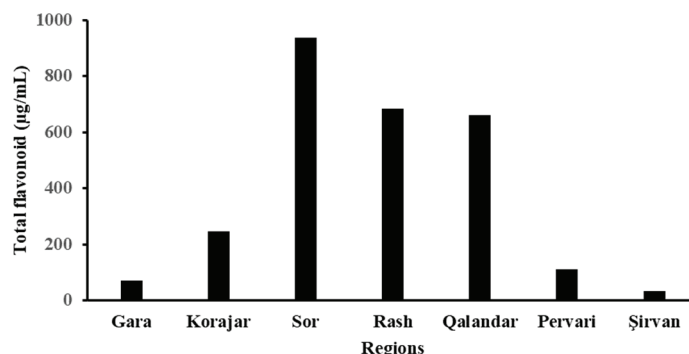


Fig. 2. Total flavonoid concentrations.

In various studies, the antioxidant activity of plant extracts (in the ethanolic extract) has been associated with their flavonoid content. In the study conducted by Eman *et al.*, the extracts richest in flavonoids were water and ethanol extracts.²⁹ The total amount of flavonoids in them was found to be 149.01 mg/100g d.w in the water extract and 687 mg/100g d.w in the ethanolic extract.²⁹

The results of FRAP antioxidant capacities (total antioxidant amount) obtained in samples with 1 mL extract, prepared in methanol, were given in Table I. FRAP antioxidant capacities are given as FeSO₄ equivalents. According to these results, the highest antioxidant effect was obtained from the Sor region (25.18 mg/mL extract) and the lowest antioxidant effect from the Gara region (3.14 mg/mL extract). Calculation of the FeSO₄ equivalent is based on the FRAP standard regression curve given in Fig S-3 of the Supplementary material. The concentration results of FRAP antioxidant capacities obtained from seven regions are plotted and given in Table II.

Iron chelating capacities of samples found in extracts prepared with methanol are given in Table I. According to the results, the highest iron chelating capacity (97.34 %) was collected from the Pervari region and the lowest iron chelating capacity (71.22 %) was collected from the Rash region.

Quantitative determinations of phytochemicals in samples by LC–MS/MS

The developed, optimized and validated LC–MS/MS method (Table S-I and Fig. S-1) was applied for the simultaneous determination of 37 phytochemicals in methanol extracts of rhubarb samples. Codes from 1 to 37 were used for the studied species. LC–MS/MS-total ion chromatogram (TIC) chromatograms of the analysed extracts for compounds were given in the supplementary file (Figs. S-4–S-10). The quantitative analysis data of sample extracts related to LC–MS/MS data are given in Table II. According to LC–MS/MS results, extracts were found to be rich in phenolic acid and flavonoid content.

Generally, according to the LC–MS/MS results, the phenolic acid contents of the studied extracts were richer than flavonoid contents. Specifically, quinic, malic, fumaric, chlorogenic, vanillic and caffeic acid amounts were noteworthy in the overall assessment of the quantification results. Additionally, several phenolic compounds such as vanillic acid, syringic acid and sinapic acid were detected to be in low amounts in some of the extracts of the studied species. Rutin, hesperidin, isoquercitrin and quercetin were the most abundant flavonoids in the studied extracts. On the other hand, contents of gallic, *p*-hydroxybenzoic, *p*-coumaric, cinnamic, sinapinic and rosmarinic acids, coumarin, quercitrin, quercetin were determined. Quercitrin was reported to have benefits in delaying skin aging in humans.³⁶ Although rutin (a flavonoid) detected at an extract concentration of 2404 µg/g has been reported to have potential bioactivities such as cardioprotective, neuroprotective and antioxidant activities, its potential for use as a thera-

peutic agent is limited due to its low bioavailability.³⁷ Nicotiflorin (a flavonoid) has been reported to have several bioactivities such as α -glucosidase inhibitory ($19.36 \pm 2.43 \mu\text{M}$ for KR),³⁸ *in vitro* antiglycation and hepatoprotective activities.³⁹ Flavonoid glycoside hesperidin (more than 2000 mg kg^{-1}) was reported to exhibit various pharmacological actions such as cardioprotective, antihyperlipidemic, antidiabetic and antihypertensive activities.⁴⁰ The phytochemical screening of hesperidin was carried out by LC–MS/MS method and several different species contained various amounts of hesperidin. Naringenin, a highly bioactive flavonoid aglycone, was reported to have several pharmacological effects such as antibacterial, antioxidant, anticancer and cardioprotective activities.^{41,42} Quinic acid (minimum $541 \mu\text{g kg}^{-1}$ and maximum $1841 \mu\text{g kg}^{-1}$) was found predominantly in all extracts of the studied samples. It was one of the most abundant phytochemicals in the studied plant species. The malic acid has the highest quantity in all extracts of samples. As for fumaric acid, its content in the extracts of samples may be notable. The protocatechuic acid content in all extracts is also noticeable. Chlorogenic acid content was detected in two samples (Pervari and Sor). The contents of vanillic acid (minimum $6.5 \mu\text{g kg}^{-1}$ and maximum $127 \mu\text{g kg}^{-1}$) in all extracts studied were found to be in significant amounts. Although all the extracts contained a certain amount of caffeic acid (minimum $0.13 \mu\text{g kg}^{-1}$ and maximum $1.56 \mu\text{g kg}^{-1}$), the amount of this phenolic acid is higher than the content of chlorogenic acid but lower than the contents of other phenolic acids. Furthermore, all the extracts contain vanillic acid, *tr*-ferulic acid, salicylic acid and *p*-coumaric acid in a certain range, not in a very high concentration. The salicylic acid in the extract was determined in three regions (Sor, Rash and Qalandar). In this study, salicylic acid was identified as a minor component in the quantitative analysis of methanol extracts of the samples by LC–MS/MS. Rosmarinic acid was a famous bioactive phenolic acid and was determined in extracts of the Pervari and Sor regions (73.6 and 2.38 mg kg^{-1} , respectively). It might be said that the rosmarinic acid contents of these species vary, depending on the growing conditions and extraction conditions. Gallic acid was highly abundant in methanol extracts of samples. Besides, several other extracts contained minor amounts of gallic acid. The high antioxidant activity of these species was mainly attributed to the quercetin content as the gallic acid content of the species was not examined. Therefore, it can be said that this high antioxidant potential of samples is related to its high gallic acid content rather than its quercetin content.

While the contents of rutin and isoquercitrin flavonoids in all extracts of samples were determined, nicotiflorin, hesperidin, quercetin and naringenin flavonoids were determined in some extracts of samples. Contents of quercetin in four extracts (Pervari, Şirvan, Sor and Rash) are notable. Also, the isoquercitrin content of extracts found in the Gara region was much higher than in other regions. As for nicotiflorin, its content was determined in four extracts of samples (Pervari,

Şirvan, Sor and Gara) and nicotiflorin content of extracts in Sor is higher than the others.

In the study conducted by Eman *et al.*, the amount of gallic acid in the ethanol and water extract of Rhubarb root was found to be 761 and 400 µg/100 g d.w, respectively.²⁹ Compared to this study, it was found to be higher only in Sor and Karajar regions. It was found to be less than the amount of gallic acid found in other regions. In terms of protocatechuic acid, it was found to be much higher than the values found in this study. In the study conducted by Eman *et al.*, it was observed that the amounts of protocatechuic acid, chlorogenic acid, caffeic acid, vanilic, hesperidin, quercetin and rosmarinic were much higher than the values found in this study.²⁹ However, as seen in Table II, the result of the rutin analysis of ethanol and water (7 µg/100 g d.w) extract performed by Eman *et al.* was found to be lower than the rutin results found in this study, as seen in Table II.²⁹

The optimized LC–MS/MS method was applied to qualify and quantify the 25 phytochemicals in rhubarb samples. A detailed analytical method validation procedure was conducted comprising the inter-day and the intra-day precision (repeatability), accuracy (recovery), the limits of detection and quantification (*LOD*, *LOQ*), linearity, and the relative standard uncertainty. This method provides a rapid, sensitive and accurate LC–MS/MS method for the simultaneous quantitative determination of 25 types of phenolic compounds in rhubarb samples. The phenolic compound contents varied significantly within the same species. The variations in the phenolic compound contents were primarily due to the geographic distribution and different altitudes where the plant was grown. It was determined that there were significant amounts of important phytochemicals such as gallic, quinic, cinnamic and malic acids, and rutin, hesperidin and ioquercitrin flavonoids in the extracts of the studied species. It has been found that the various parts of the species have very different biological effects and chemical content. The extracts from rhubarb samples were found to be more active, especially in terms of antioxidant capacity. According to the results of LC–MS/MS, it seems that the chemical contents are also different. The various parts of the plant to be studied must be pursued separately, as well as the plant chemistry studies should be pursued on different solvent and extraction varieties.

CONCLUSION

To conclude, the development of a straightforward and accessible methodology for characterizing the bioactive components of *Rheum ribes* has revealed its immense therapeutic potential and significance in the realm of natural medicine.

Through the determination of crucial parameters such as total phenolics, flavonoids, and antioxidant activity, this study sheds light on the richness of the bioactive compounds present in *R. ribes* extracts. The observed variation in phenolic and flavonoid contents underscores the diverse chemical composition of these

extracts across different samples. Moreover, the robust antioxidant activities exhibited by the extracts, as demonstrated by FRAP, DPPH and iron chelating assays, highlight their efficacy in scavenging free radicals and preventing oxidative damage. LC–MS/MS analysis further elucidated the chemical composition of the extracts, identifying key phenolic and flavonoid compounds, such as malic acid and rutin, as major contributors to their biological activities.

Overall, these findings present the potential of *R. ribes* extracts as valuable sources of natural antioxidants, paving the way for the further exploration of their therapeutic applications and integration into pharmaceutical and nutraceutical industries.

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/12981>, or from the corresponding author on request.

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ИЗВОД

ЕФИКАСАН И ЈЕДНОСТАВАН ПРИСТУП ОДРЕЂИВАЊА БИОАКТИВНИХ *Rheum ribes* У ПРОВИНЦИЈИ КУРДИСТАН У ИРАКУ И РЕГИОНУ СИИРТ У ТУРСКОЈ

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Развијање поједностављене и приступачне методе за идентификацију биоактивних компоненти *Rheum ribes* (рабарбаре) доприноси откривању његовог терапеутског потенцијала и унапређењу истраживања у природној медицини. Одређене су биоактивне компоненте рабарбаре, као што су укупни феноли и флавоноиди, као и антиоксидативна активност њеног метанолног екстракта. Укупни садржај фенола у екстрактима био је између 84,02 and 387,53 mg/L еквивалента галне киселине (GAE). Укупни садржај флавоноида у екстрактима, одређен колориметријском методом, је од 69,98 to 935,75 mg L⁻¹ еквивалената рутина (PE). Антиоксидативна активност је одређена применом метода антиоксидативног потенцијала који редукује гвожђе (FRAP) и 1,1-дифенил-2-пикрилхидразил (DPPH). Резултати FRAP методе су показали да је највећа антиоксидативна

активност (IC_{50}) $25,18 \pm 0,04 \text{ mg L}^{-1}$. У DPPH методи, максимални проценат инхибиције је 88,11 %. Активности хелирања гвожђа у узорцима биле су изнад 70 %. Садржај хемијских једињења у екстрактима је одређен помоћу LC–MS/MS. Квалитативно и квантитативно је анализирано укупно 25 фенолних и флавоноидних једињења у екстрактима. Као главна фитохемијска једињења у екстракту су јабучна киселина ($15,72 \pm 0,53 \text{ mg kg}^{-1}$) и рутин ($76,93 \pm 0,03 \text{ mg kg}^{-1}$). Резултати потврђују да рабарбара има потенцијалну биолошку активност и да се може да буде важан извор природних антиоксиданата.

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SUPPLEMENTARY MATERIAL TO
**An effective and facile approach for the determination of
bioactive components of *Rheum ribes* in the Kurdish state of
Iraq and Siirt region in Turkey**

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FIDAN², ORHAN ACAR³ and ERDAL YABALAK^{4**}

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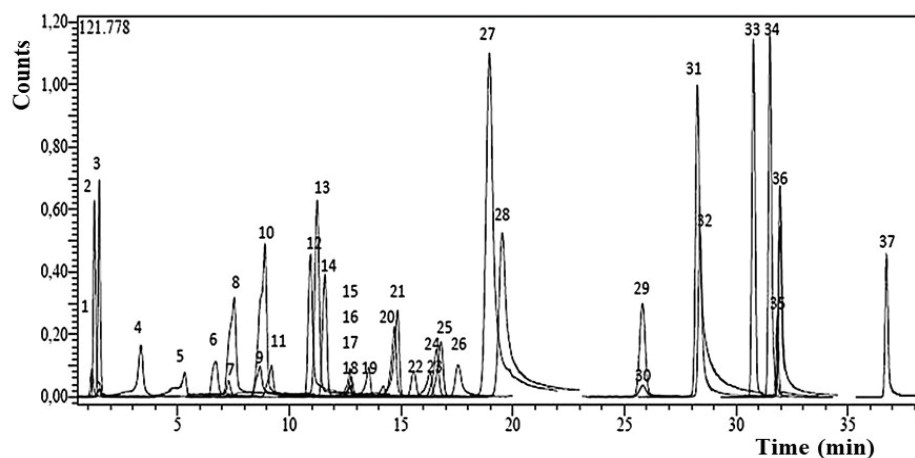


Fig. S-1. UHPLC-ESI-MS/MS chromatogram of standard mixg (1: Coumarin, 2: Hesperidin, 3: P-Coumaric acid, 4: O-Coumaric acid, 5: Gallic acid, 6: Caffeic acid, 7: Vanillic acid, 8: Salicylic acid, 9: Kainic acid, 10: 4-OH-benzoic acid, 11: Ferulic acid, 12: Chlorogenic acid, 13: Rosmarinic acid, 14: Protocatechic acid, 15: Cinnamic acid, 16: Sinapinic acid, 17: Fumaric acid, 18: Vanillin, 19: Pirokatekol, 20: Malic acid, 21: Syringic acid, 22: Hesperetin, 23: Naringenin, 24: Rutine, 25: Quercetin, 26: Quercitrin, 27: Apigenin, 28: Krisin, 29: Liquiritigenin, 30: Isocerythrin, 31: Apigettrin, 32: Rhoifolin, 33: Nicotiflorin, 34: Fisetin, 35: Luteolin, 36: Myricetin, 37: Kaempferol)^{1,2}

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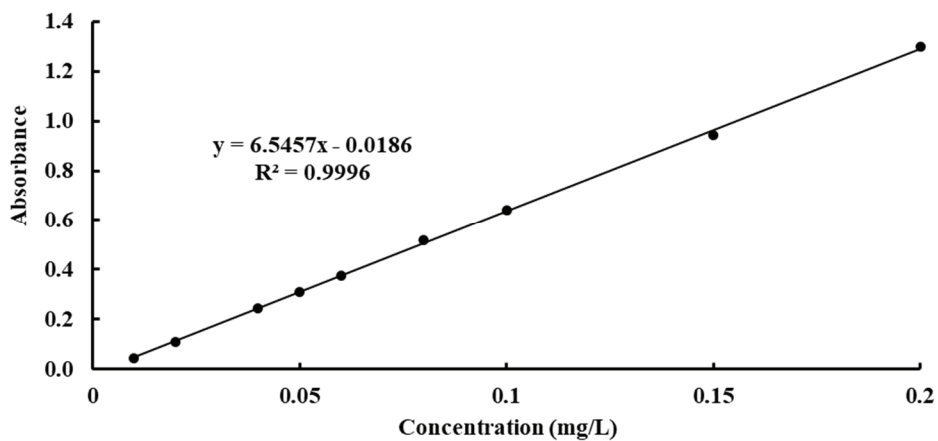


Fig. S-2. The relation between the concentration of phenolic and the absorbance (Gallic acid standard regression)

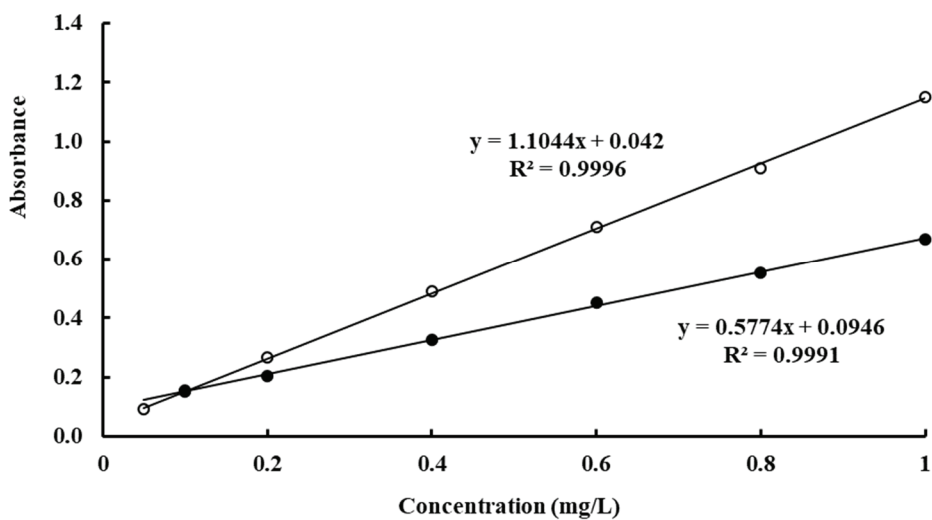


Fig. S-3. Standard regression of total flavonoid (Routine) (○-) and standard regression of FRAP (FeSO₄) (●-).

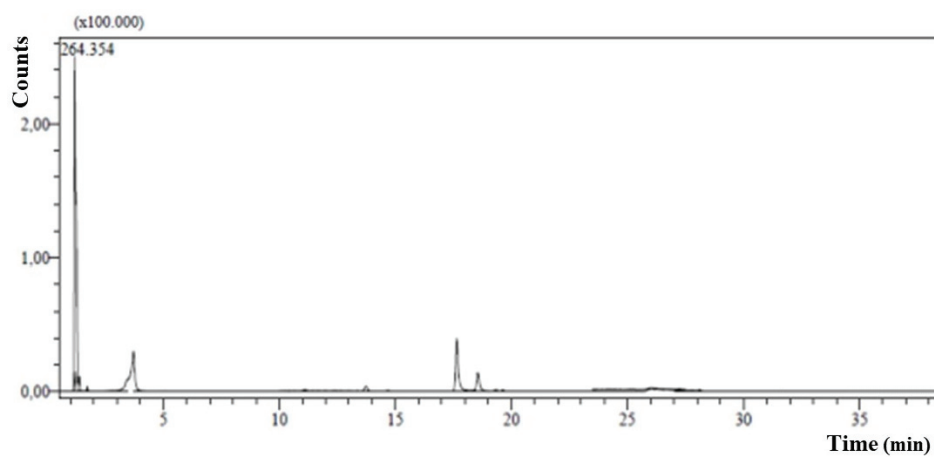


Fig. S-4. The chromatograms of Gara

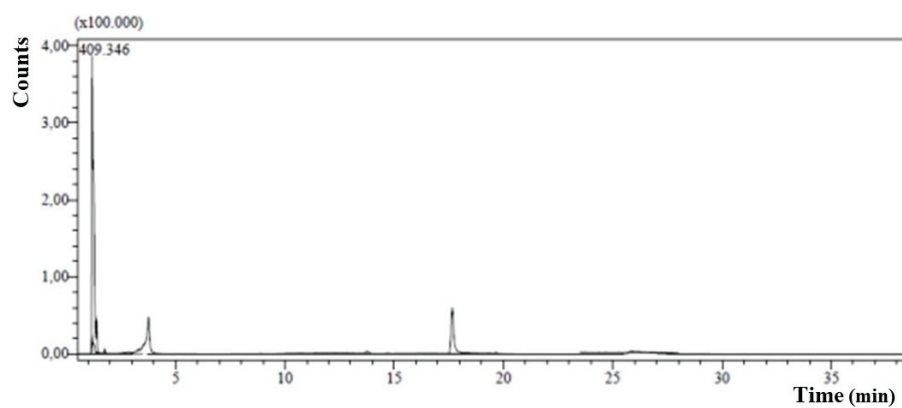


Fig. S-5. The chromatograms of Korajar

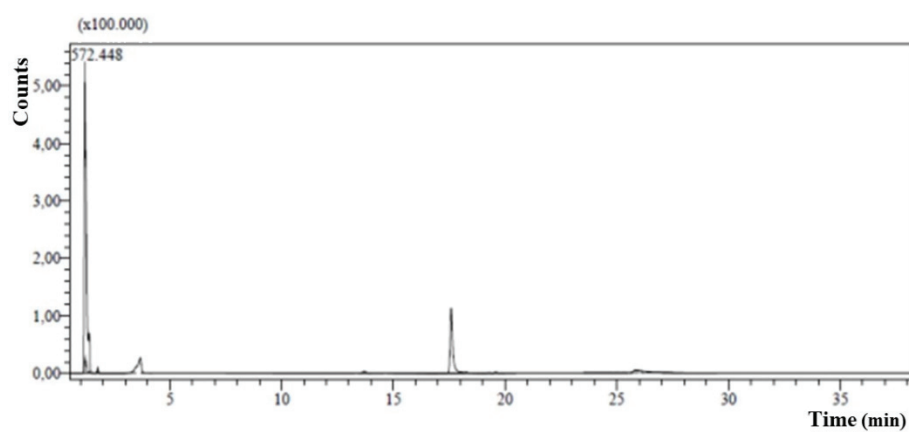


Fig. S-6. The chromatograms of Sor

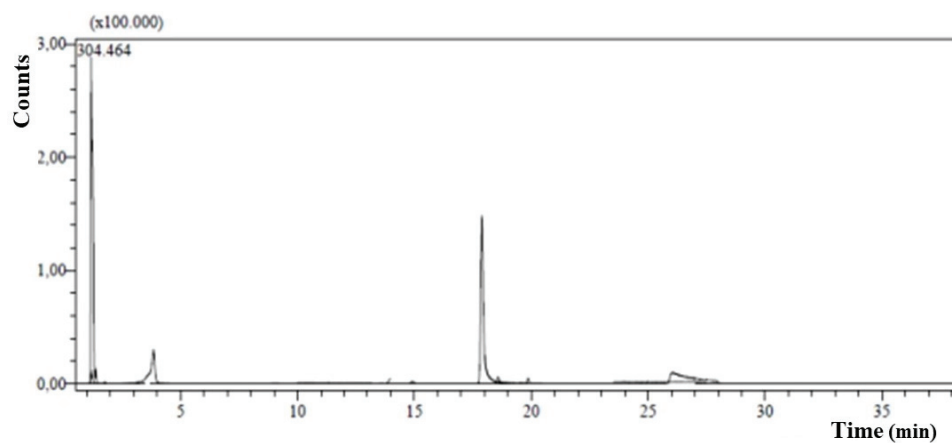


Fig. S-7. The chromatograms of Rash

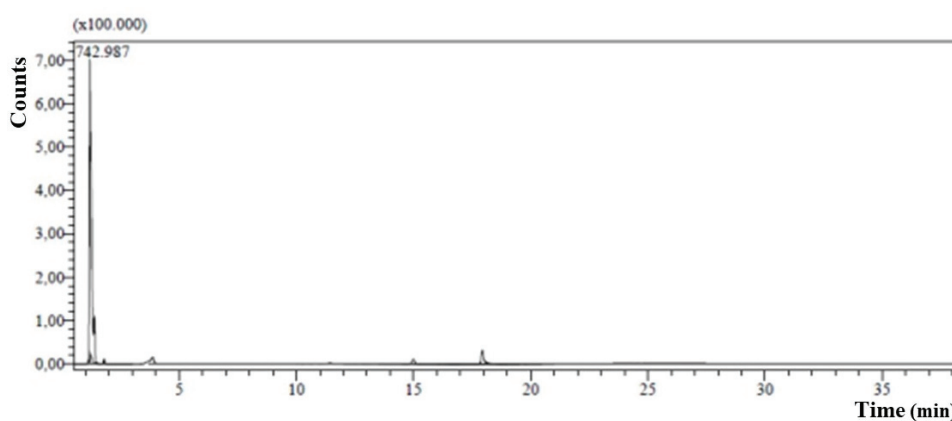


Fig. S-8. The chromatograms of Qalandar

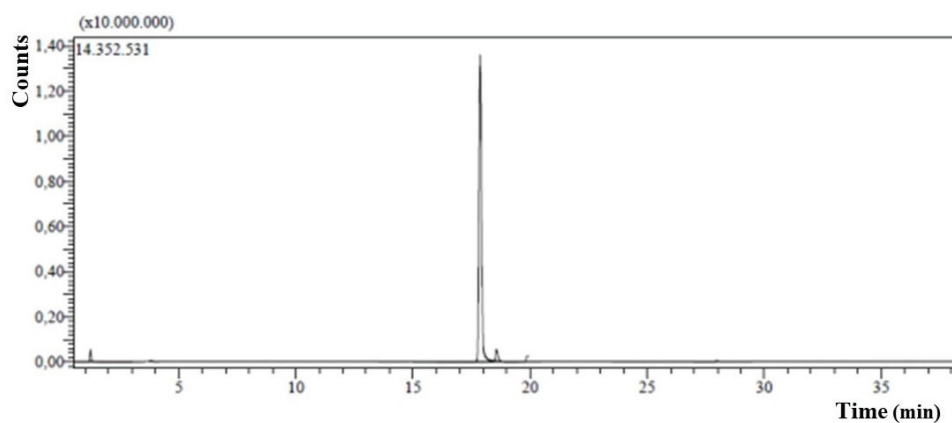


Fig. S-9. The chromatograms of Pervari

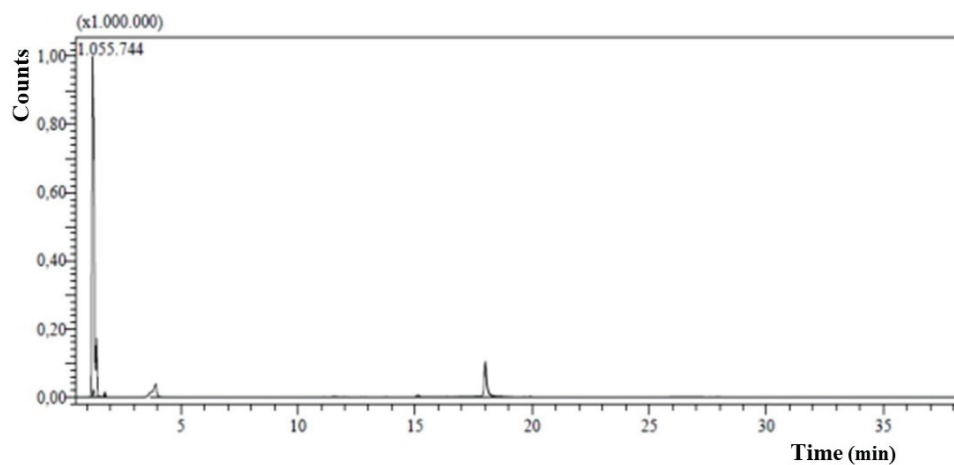


Fig. S-10. The chromatograms of Şirvan

TABLE S-I. Analytical parameters of the LC-MS / MS analysis method^{1,2}

Code no	Analytes	RT ^a	Mother ion (m/z) ^b	Fragment ions collision energy (eV)	Ion model	Calibration equation	R ^{2c}	RSD (%) ^d		Linearity range	LOD/LOQ ^e	Recovery (%)		U ^f
								Same day	Diff. days	(µg/L)	(µg/L)	Same day	Diff. days	
1	Quinic acid	1.13	190.95	85.3–93.3	Neg	$y = 41.06 \times + 10671$	0.996	0.00259	0.00274	250–10000	75.8/79.4	1.00288	0.98778	0.0082
2	Malic acid	1.23	133.00	115.2–71.3	Neg	$y = 316.95 \times - 42041$	0.999	0.00477	0.00527	250–10000	55.3/67.5	1.01266	0.99836	0.0113
3	Fumaric acid	1.48	115.00	71.4	Neg	$y = 64.99 \times - 11592$	0.997	0.00536	0.00460	100–5000	28.1/34.5	0.99748	0.99867	0.0124
4	Gallic acid	3.00	168.85	125.2–79.2	Neg	$y = 226.76 \times + 38152$	0.998	0.01601	0.01443	250–10000	95.5/106.9	1.00004	1.00454	0.0282
5	Protocatechuic acid	4.93	152.95	108.3	Neg	$y = 297.75 \times + 30590$	0.995	0.01236	0.01296	100–5000	28.2/31.4	0.99404	1.01070	0.0411
6	Pyrocatechol	6.48	109.00	108.35–91.3	Neg	$y = 30.61 \times + 14735$	0.996	0.01313	0.01339	1000–20000	261.1/278.4	0.99987	0.99936	0.0235
7	Chlorogenic acid	7.13	353.15	191.2	Neg	$y = 781.36 \times - 18697$	0.998	0.00058	0.00076	25–1000	6.2/8.1	1.00806	0.99965	0.0069
8	4-OH-benzoic acid	7.39	136.95	93.3–65.3	Neg	$y = 409.03 \times + 112079$	0.998	0.01284	0.01538	250–10000	33.2/38.1	0.99662	1.00058	0.0289
9	Vanillic acid	8.57	166.90	152.3–108.3	Neg	$y = 35.84 \times - 12097$	0.999	0.00528	0.00619	1000–20000	122.2/139.7	1.00093	1.04095	0.0508
10	Caffeic acid	8.80	178.95	135.2–134.3	Neg	$y = 3963.32 \times + 178156$	0.998	0.01454	0.01469	25–1000	18.4/22.4	1.00917	0.98826	0.0354
11	Syringic acid	9.02	196.95	182.2–167.3	Neg	$y = 42.33 \times - 52547$	0.996	0.01049	0.01345	1000–20000	212.5/233.3	0.99922	0.99977	0.0238
12	Vanillin	10.87	151.00	136.3–92.2	Neg	$y = 446.10 \times + 70934$	0.998	0.00696	0.00793	250–10000	44.3/53.1	0.99679	0.99611	0.0280
13	Salicylic acid	11.16	136.95	93.3–65.3	Neg	$y = 5286.26 \times + 309192$	0.989	0.01016	0.01242	25–1000	5.0/6.5	1.00989	0.99013	0.0329
14	p-Coumaric acid	11.53	162.95	119.3–93.3	Neg	$y = 3199.20 \times + 13002$	0.992	0.01820	0.01727	25–1000	7.3/9.1	1.00617	1.01224	0.0516
15	Rutin	12.61	609.05	300.1–271.1	Neg	$y = 561.91 \times - 16879$	0.997	0.00473	0.00624	25–1000	5.5/6.5	1.00994	0.98017	0.0159

16	Ferulic acid	12.62	192.95	178.3	Neg	$y = 80.45 \times -31782$	0.997	0.00708	0.00619	250–10000	36.6/42.0	0.99987	1.00289	0.0494
17	Sinapinic acid	12.66	222.95	208.3–149.2	Neg	$y = 141.96 \times -73294$	0.992	0.01446	0.01517	250–10000	78.7/86.1	1.00164	0.99962	0.0281
18	Hesperidin	12.67	610.90	303.1–465.1	Poz	$y = 1340.27 \times -43769$	0.998	0.00945	0.01126	25–1000	3.4/4.2	1.01733	1.01263	0.0262
19	Isoquercitrin	13.42	463.00	300.1–271.1	Neg	$y = 803.23 \times +4981$	0.999	0.00682	0.00515	25–1000	5.4/6.3	1.00594	1.00722	0.0133
20	Rosmarinic acid	14.54	359.00	161.2–197.2	Neg	$y = 909.67 \times -201692$	0.994	0.02014	0.01751	100–5000	6.6/8.8	0.99206	1.03431	0.0713
21	Nicotiflorin	14.68	593.05	285.1–255.2	Neg	$y = 498.38 \times +79274$	0.991	0.00737	0.00875	100–5000	22.4/25.5	1.02558	1.00970	0.0276
22	o-Coumaric acid	15.45	162.95	119.4–93.3	Neg	$y = 1219.34 \times -10915$	0.999	0.02730	0.02566	25–1000	24.4/31.1	0.98344	0.99061	0.0513
23	Rhoifolin	16.11	577.05	269.2–211.1	Neg	$y = 237.15 \times +11887$	0.999	0.00747	0.01528	100–5000	23.1/27.9	1.01046	1.01739	0.0941
24	Quercitrin	16.41	447.15	301.1–255.1	Neg	$y = 339.39 \times +38910$	0.999	0.01528	0.02320	100–5000	22.0/25.2	0.99726	1.00620	2.0079
25	Apigetrin	16.59	431.00	268.2–239.2	Neg	$y = 1775.55 \times +91121$	0.993	0.01797	0.01607	25–1000	5.4/6.1	1.01394	1.00419	0.0597
26	Coumarin	17.40	147.05	91.0–103.2	Poz	$y = 33.64 \times -89700$	0.994	0.01306	0.01239	1000–20000	208.4/228.4	0.99947	1.00081	0.0237
27	Myricetin	18.72	317.00	179.2–151.3	Neg	$y = 583.55 \times +205727$	0.999	0.00652	0.00711	250–10000	53.2/57.2	0.99982	1.00042	0.0126
28	Fisetin	19.30	284.95	135.2–121.3	Neg	$y = 547.46 \times +274791$	0.991	0.00557	0.00820	250–10000	54.4/61.4	0.99877	1.00031	0.0148
29	Cinnamic acid	25.61	147.00	103.15–77.3	Neg	$y = 9.06 \times -12403$	0.996	0.00648	0.00816	5000–20000	821.8/859.7	1.00051	0.99927	0.0143
30	Liquiritigenin	25.62	254.95	119.3–135.1	Neg	$y = 2384.96 \times +59141$	0.996	0.01849	0.01738	25–1000	5.5/6.6	1.00333	0.99957	0.0341
31	Quercetin	28.17	300.90	151.2–179.2	Neg	$y = 1198.48 \times +480562$	0.990	0.01589	0.01360	100–5000	23.3/28.9	0.98470	1.00103	0.0543
32	Luteolin	28.27	284.75	133.2–151.2	Neg	$y = 3272.65 \times +150557$	0.997	0.00575	0.00696	25–1000	5.4/6.5	1.00772	0.99524	0.0174
33	Naringenin	30.68	270.95	151.2–119.3	Neg	$y = 4315.1 \times +178410$	0.995	0.02054	0.02019	25–1000	5.4/6.4	0.99883	1.01002	0.0521
34	Apigenin	31.43	268.95	117.3–151.2	Neg	$y = 4548.36 \times +295252$	0.990	0.02304	0.02204	25–1000	5.4/6.3	1.01444	1.01331	0.0650
35	Hesperetin	31.76	300.95	164.2–136.2	Neg	$y = 876.67 \times +48916$	0.997	0.03209	0.02605	25–1000	5.6/6.9	0.98850	0.99435	0.0562
36	Kaempferol	31.88	284.75	255.1–117.3	Neg	$y = 26.29 \times +87558$	0.992	0.01436	0.01070	1000–20000	206.6/214.3	0.99971	0.99851	0.0209
37	Chrysin	36.65	252.95	143.3–119.4	Neg	$y = 2032.13 \times +95593$	0.993	0.00490	0.00630	25–1000	5.4/6.2	1.00338	1.00437	2.0083

^aRT: Retention time, ^bMaster ion (*m/z*): Molecular ions of standard compounds (*m/z* rate), ^cR²: Determination coefficient, ^dRSD: Relative standard deviation, ^eLOD/LOQ (µg/L): Detection limit/ Assignment limit, ^fU (%): 95% Relative standard uncertainty at confidence level (*k*=2)). ^{1,2}

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Thermal, morphological and surface properties of composite materials with perlite reinforcement

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Abstract: This paper deals with the preparation and the characterization of perlite/polyvinyl alcohol, perlite/polyvinylpyrrolidone, perlite/polymethyl methacrylate and perlite/polystyrene composites. The polymer composites were prepared by solvent casting technique with 5 wt. % of perlite filler. Perlite is a filler with unique properties, such as low density and thermal conductivity, thus the development of Polymer–Perlite composites may be of interest for producing lighter packaging with thermal insulation capability. The perlite/polymer nanocomposites were characterized using X-ray diffraction (XRD) analysis, thermogravimetric analysis (TGA), Fourier-transform infrared spectroscopy (FTIR), inverse gas chromatography (IGC), field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM). TGA measurements showed remarkable increases in the thermal stability of the polystyrene by the perlite loading as compared to the matrix. The FE-SEM image of the cryomilled sample shows that the perlite particles were embedded within a polystyrene matrix. This finding is consistent with the work of adhesion data obtained by IGC.

Keywords: polyvinyl alcohol; polyvinylpyrrolidone; polymethyl methacrylate; polystyrene; inverse gas chromatography.

INTRODUCTION

Perlite is a naturally occurring granular material and is mainly composed of SiO₂ and Al₂O₃. The lightweight, low cost and low thermal conductivity properties of perlite can enable it to be used as a filler in polymers. The incorporation of perlite into the polymer matrix can improve the mechanical, thermal

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and barrier properties of the final product. Sahraeian *et al.*¹ have studied the rheological, thermal and dynamic mechanical properties of low-density polyethylene/perlite nanocomposites. The results revealed that the dynamic mechanical properties and thermal stabilities of nanocomposites were improved by the addition of surface-treated nano-perlite. In another study, de Oliveira *et al.*² investigated the effect of expanded perlite content on the morphological, mechanical, thermal and rheological properties of polystyrene/expanded perlite composites. Doğan *et al.*³ prepared the chitosan/perlite nanocomposites using the solvent casting method. Perlite was dispersed homogeneously in the chitosan matrix. The thermal stability of chitosan after perlite incorporation was improved. They have also found that the hydrophilic properties of nanocomposites increased with the increasing perlite content. Tian and Tagaya,⁴ focused on the preparation of poly (vinyl alcohol) (PVA)/perlite nanocomposites. They have found that the heat resistance of PVA was enhanced by perlite addition. Raji *et al.*,⁵ used two perlite structures (raw or expanded) as filler in polypropylene-based composites. They have found that better dispersion and interfacial adhesion of the raw perlite in the polypropylene matrix was achieved compared to the expanded perlite. As mentioned above, there have been numerous studies to investigate perlite-filled polymer composites. However, to the best of our knowledge, the perlite-reinforced PVP-based composites have not been found in the literature.

Inverse gas chromatography (IGC) is a versatile, powerful, sensitive and relatively fast technique for the characterization of the physicochemical properties of materials. Due to its applicability in determining surface properties of solids in any form such as films, fibres and powders of both crystalline and amorphous structures, IGC became a popular technique for surface characterization, used extensively soon after its development. The properties that can be determined using the IGC technique include enthalpy and entropy of sorption, surface energy (dispersive and specific components), work of adhesion/co-adhesion, miscibility and solubility parameters, surface heterogeneity, glass transition temperature and Lewis acid–base parameters. One of the greatest advantages of this method is that no special sample preparation is required. In fact, IGC requires the minimum sample preparation when compared to other surface energy analyzing techniques. Therefore, various forms of solids and even semi-solids can be characterized quickly and efficiently. The knowledge of surface properties of solids has great technological importance for all interfacial phenomena, as it is a very useful parameter in studies of adsorption and wettability processes.^{6–10}

Polymer composites have been widely used in various areas of transportation vehicles, construction materials, packaging industry, electronics and sporting goods and consumer products. The synthesis and development of polymer/silicate composites have attracted considerable attention from both basic research and

commercial applications, as they often exhibit remarkable improvements in the mechanical properties, thermal stability, gas barrier properties, enhanced ionic conductivity, reduced flammability and biodegradation, *etc.*, as compared to the pure polymers or conventional micro- and macro-composites. These improvements in the properties are the result of the nanometer scale dispersion of silicate in the polymer matrix. The dispersion of clay layers in a monomer or polymer matrix can result in the formation of three types of composite materials. The first type is a intercalated, that can be formed if the interlayer distance increases but the layer morphology remains unchanged, an intercalated nanocomposite structure is obtained. The other type is intercalated-and-flocculated conceptually the same as intercalated nanocomposites. The last type is the exfoliated structure, which can only be obtained if the silicate layers totally disperse in the polymer matrix. The exfoliated polymer-silicate nanocomposites are especially desirable for improved properties because of the homogeneous dispersion of clay and huge interfacial area between polymer and silicate.¹¹⁻¹³

Polymers and polymer composites are used in a wide range of areas in daily life due to their superior properties such as being highly durable, lightweight, hygienic and flexible to process. Most polymer products do not decompose in nature and cause serious environmental pollution.

Due to the negative effects of today's traditional polymers on human and environmental health and their centuries-long recycling, biodegradable polymers that can decompose in nature are increasing their importance day by day. The features that make biopolymers superior to traditional polymers can be summarized as; easy decomposition, reducing dependence on fossil fuels, not leaving a toxic effect, being easier to recycle, requiring less energy in their production, being renewable and ecological. In this article, nanocomposite materials were prepared using polystyrene and polymethyl methacrylate polymers, which are used extensively in industrial areas and polyvinyl alcohol and polyvinyl pyrrolidone polymers, which have biodegradable (environmentally friendly) properties. The method of solvent casting used in the preparation of polymer nanocomposites is the most common method used in the preparation of homogeneously distributed nanocomposites. Thanks to the solvent casting method, it is easy to control the concentrations of polymer and inorganic components. Using this method, which does not require special equipment and has been widely preferred for years due to its easy applicability, homogeneously distributed silicate/polymer nanocomposites were obtained. Polymer nanocomposites containing 5 % filler (perlite) by weight were prepared using four different polymer matrices by the solvent casting method. Thus, the functionality, flexibility, coatability, wetting and adhesion, thermal and mechanical strength performances of the materials were improved by ensuring homogeneous distribution (exfoliated) of the nanofillers in the polymer matrices. The characterization of the structural, mor-

phological, thermal, surface and interface properties of the obtained nanocomposites was carried out using many analysis techniques (FTIR, XRD, SEM, TEM, TGA and IGC). In addition, it was aimed that the obtained nanocomposites would be an alternative to traditional nanocomposites in industrial applications due to their properties such as being lightweight, having a low carbon footprint and having high thermal and mechanical properties. Another aim of the study is to produce innovative polymer nanocomposites with superior properties, compared to traditional nanocomposites, as well as making a difference in the literature in many ways.

In this study, we investigated the use of perlite as reinforcement in various polymer matrix composites. For this work, we selected PVA, PVP, PMMA and PS. PVP and PVA are water-soluble, low cost and easily available synthetic polymers of great industrial importance. They have been commonly used in medical and pharmaceutical applications.¹⁴ PMMA and PS are well-known and widely used thermoplastic polymers for several industrial applications. The effects of incorporating perlite into PVA, PVP, PMMA and PS were investigated using FTIR, XRD, TGA, FE-SEM and TEM. Furthermore, surface free energy (γ_s), Lewis acidic parameter (K_A) and Lewis basic parameter (K_B) of the examined solids and the work of adhesion between perlite and polymers were determined by using IGC.

IGC Background

In the IGC study, the retention times of probes were measured at infinite dilution conditions, allowing us to determine the interactions between the probes and the solid where lateral probe-probe interactions are negligible.^{15,16} The first value determined is the net retention volume (V_N) of the probes:¹⁷

$$V_N = FJ(t_R - t_M)(T / T_A) \quad (1)$$

where F is the flow rate of the carrier gas, J is the compression correction factor, t_R is the probe retention time, t_M is the dead time which is determined with methane, T is the column temperature and T_A is the ambient temperature.

The surface free energy (γ_s) of a solid has two components, the dispersive component (γ_s^d) and the specific component (γ_s^{sp}). The dispersive component results from the relatively weak van der Waals dispersion forces (London forces, Debye forces and Keesom forces) and the specific component includes all of the polar forces (dipole forces and acid-base forces):

$$\gamma_s = \gamma_s^d + \gamma_s^{sp} \quad (2)$$

In this study, values were calculated according to the Schultz-Lavielle method:¹⁸

$$RT \ln V_N = 2Na\sqrt{\gamma_s^d}\sqrt{\gamma_L^d} + C \quad (3)$$

where R is the gas constant, N is Avogadro's number, a is the cross-sectional area of the probe, γ_L is the surface tension dispersive component of the probe and C is the constant.

The free energy of adsorption has dispersive and acid-base components. The specific component of the adsorption-free energy (ΔG^{sp}) is related to the electron donor/acceptor ability of the probe:¹⁹

$$\Delta G^{\text{sp}} = -RT \ln\left(\frac{V_{\text{Nsp}}}{V_{\text{Nref}}}\right) \quad (4)$$

where V_{Nsp} is the retention volume of a polar probe and V_{Nref} is the specific retention volume of n -alkanes with the same $\alpha(\gamma_L^{\text{d}})^{-1/2}$ value as the polar probes. The specific component of the adsorption (ΔH^{sp}) was determined from the temperature dependency of ΔG^{sp} :

$$\Delta G^{\text{sp}} = \Delta H^{\text{sp}} - T\Delta S^{\text{sp}} \quad (5)$$

where is ΔS^{sp} the entropy of adsorption corresponding to the specific interactions.

Gutmann and van Oss concepts are very common approaches to determining the acid-base character of solid surfaces.²⁰ According to the Gutmann approach, the specific interaction enthalpies between adsorbent and adsorbed correlate with acid-base properties:²¹

$$-\Delta H^{\text{sp}} = K_A DN + K_D AN^* \quad (6)$$

where K_A and K_D are the electron-accepting (acid) number and electron-donating (base) number of the examined solid surface, respectively. AN^* is the acceptor number and DN is the donor number of the polar probes.²²

On the other hand, the work of adhesion between the perlite and the polymers was calculated according to the following equations:⁶

$$W_a^{\text{tot}} = W_a^{\text{d}} + W_a^{\text{sp}} \quad (7)$$

$$W_a^{\text{d}} = 2\sqrt{\gamma_1^{\text{d}}\gamma_2^{\text{d}}} \quad (8)$$

$$W_a^{\text{sp}} = 2\left(\sqrt{\gamma_1^+ \gamma_2^-} + \sqrt{\gamma_1^- \gamma_2^+}\right) \quad (9)$$

where W_a^{tot} , W_a^{d} and W_a^{sp} are the total work of adhesion, dispersive component and specific component of work of adhesion, respectively. In Eq. (8), γ_1^{d} and γ_2^{d} are the dispersive components of the free surface energy of perlite and polymer, respectively. In Eq. (9), γ^+ and γ^- are the acidic and basic parameters of solids 1 (perlite) and 2 (polymer), respectively. For the calculation of γ^+ and γ^- , trichloromethane (TCM) and ethyl acetate (EA) were used:⁶

$$\gamma^+ = \Delta G_{EA}^{sp} / \left(4N^2 a_{EA}^2 \gamma_{EA}^- \right) \gamma_{EA}^- \quad (10)$$

$$\gamma^- = \Delta G_{TCM}^{sp} / \left(4N^2 a_{TCM}^2 \gamma_{TCM}^+ \right) \gamma_{TCM}^+ \quad (11)$$

The values of γ_{EA}^+ and γ_{TCM}^- were taken as 6.2 and 1.5 mJ/m², respectively.²³

EXPERIMENTAL

Materials

The crushed raw perlite sample was obtained from Cumaovası Perlite Processing Plants of Etibank (Izmir, Turkey). The chemical composition of Turkish perlite is reported earlier in literature and it consisted mainly of 71–75 % SiO₂, 12.5–18 % Al₂O₃, 2.9–4 % Na₂O, 4–5 % K₂O, 0.5–2 % CaO and 0.1–1.5 % Fe₂O₃.²⁴ The decantation and centrifugation process was used to remove dust and other water-soluble matter from perlite. About 50 g of perlite was mixed with 1 L of distilled water in a beaker. This mixture was then covered and left for 2 days. Then, the top and middle part of the mixture was drawn with a pipette and the remaining sediment was removed from the process as waste. Centrifugation (about 20 min at 8000 rpm) has been used to separate perlite from the suspension. The resulting perlite was diluted with 1 L of distilled water again. This process was repeated several times until there was no sediment visible on the bottom of the beaker. Subsequently, the residual perlite was dried for 24 h at 80 °C. Then it was milled into fine powder using a Retsch cryogenic grinder and sieved to obtain a particle size distribution between 60 and 100 µm. The specific surface area (by multipoint BET analysis) of purified perlite was 3.5 m² g⁻¹, perlite/PVA was 9.855 m² g⁻¹, perlite/PVP was 5.279 m² g⁻¹, perlite/PMMA was 8.025 m² g⁻¹ and perlite/PS was 11.48 m² g⁻¹ (Quantachrome, Autosorb-6). Therefore, the highest surface area belongs to the perlite/PS nanocomposite, which provides the highest performance properties. Poly(methyl methacrylate) (*M_w* ≈ 120,000), polystyrene (*M_w* ≈ 280,000), polyvinylpyrrolidone (*M_w* ≈ 1,300,000), poly(vinyl alcohol) (*M_w* ≈ from 85,000 to 124,000) and all other chemicals were purchased from Sigma–Aldrich.

Preparation of nanocomposites

Four different kinds of composites, perlite/PVA, perlite/PVP, perlite/PMMA and perlite/PS were prepared by the solvent casting technique. First, 0.5 g of perlite was added to 200 ml solvent and the mixture was stirred with magnetic stirring at 50 °C for 2 h. Then, 9.5 g of polymer was dissolved in 300 ml of the same solvent. The filler mixture was slowly added to the polymer solution. The polymer–filler mixture was stirred at 50 °C for 4 h. The mixture was submitted to sonication in an ice/water ultrasonic bath for 30 min at 35 kHz. At the end of the process, the dispersion was poured into glass Petri dishes and the solvent was evaporated at room temperature in a chemical hood. The solvent for perlite/PVA and perlite/PVP composites was distilled water while chloroform for perlite/PMMA and perlite/PS composites. The polymer films were ground to powder in a cryo-mill under constant cooling with liquid nitrogen (Retsch CryoMill, Germany) before the characterization step. The composite samples were named PVP-PER, PVA-PER, PS-PER and PMMA-PER. Each composite contained the 5 wt. % of perlite.

Characterization techniques

The composite samples and perlite were sieved to pass a 60 μm for XRD and TEM analysis and the 150–200 μm sieve fraction was used for FE-SEM, TGA and IGC analysis. The XRD analysis was carried out between 1 and 50° (2 θ) at a scanning speed of 0.02° min⁻¹ by a diffractometer (Rigaku Ultima-IV, Japan) with CuK α radiation (40kV/30mA). Thermogravimetric analysis (TGA) was conducted using a simultaneous thermal analyzer (Perkin Elmer STA 8000, USA) under a flowing N₂ atmosphere at a scan rate of 10 °C min⁻¹ from 25 to 725 °C. FT-IR (Fourier transform infrared spectroscopy) analysis was performed on Perkin Elmer Spectrum Two IR spectrometer (USA) using the KBr technique in the range 400–4000 cm⁻¹ with a resolution of 1 cm⁻¹.

Inverse gas chromatography (IGC) measurements were carried out using a gas chromatograph (Agilent 7890A, USA), equipped with a flame ionization detector. A series of *n*-alkanes (*n*-heptane, *n*-octane, *n*-nonane and *n*-decane) and polar solutes (tetrahydrofuran, diethyl ether, acetone, ethyl acetate, trichloromethane) were injected at infinite dilution conditions. The injection volume was 0.1 μL (liquid). Nitrogen was used as the carrier gas with a flow rate of 40 ml/min. Measurements were carried out at four different column temperatures (40, 50, 60 and 70 °C). The temperatures of the injector and detector were 150 and 200 °C, respectively. For each analysis, approximately 1 g of the examined material was packed into the stainless steel gas chromatography column (1.5 m long, 5.35 mm I.D.), closing both ends with glass wool. The columns were conditioned at 70 °C under the N₂ flow for 4 h. A non-adsorbing standard molecule (methane) was used to determine the dead time.

The detailed morphological characterization of the examined samples was performed by field emission scanning electron microscopy (FE-SEM, Regulus 8230, Hitachi, Japan) and transmission electron microscopy (TEM, HT7800, Hitachi, Japan). Before FE-SEM observation, samples were coated with a thin layer of gold using a sputter coater (EM ACE 600, Leica, Germany). TEM samples were prepared by placing a drop of a dilute suspension of nanocomposite powders on a carbon-coated copper TEM grid and allowing it to dry at room temperature.

RESULTS AND DISCUSSION

X-ray diffraction analysis

X-ray diffraction (XRD) analysis was used to reveal the structure of the polymers before and after the incorporation of perlite. As can be seen in Fig. 1, a broad peak appeared between 16 and 30° indicated that perlite was mainly amorphous.²⁵ PVA is known to be a crystalline polymer and pure PVA has three diffraction peaks at 2 θ 20, 23 and 41°.²⁶ On the other hand, the XRD pattern of pure PVP showed two broad peaks centered at about 2 θ 12 and 21°, giving a sign that it is an amorphous polymer.²⁷ The pure PMMA sample exhibited three broad XRD peaks with their intensity decreasing systematically. These broad diffraction peaks approximately at about 2 θ 14, 30 and 42° denoting the amorphous structure of the PMMA and they have also been observed in other works.^{28,29} PS exhibits two typical broad peaks at 2 θ 11 and 19°. Similarly, the absence of sharp crystalline peaks indicates an amorphous structure in the XRD pattern of the pure PS.³⁰

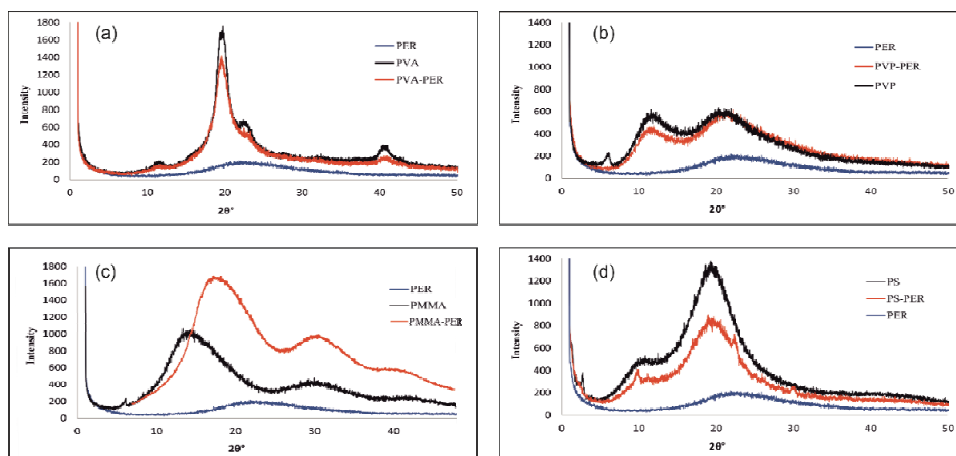


Fig. 1. X-ray diffraction patterns of perlite, neat polymers and perlite/polymer composites.

Thermogravimetric analysis

TGA is a standard method to study the overall thermal properties of polymers, polymer blends and polymer composites. The thermal behaviours of the perlite, neat polymers and perlite/polymer composites are presented in Fig. 2. The results obtained from these curves are summarized in Table I. The amount of char residue (percentages by weight at 600 °C), the temperatures of 5 % ($T_{0.05}$) and 50 % weight loss ($T_{0.5}$) are the main criteria indicating the thermal stability of the polymer composites. As shown in Fig. 2, the thermal stability of perlite is very high and the char residue was determined as 98.5 % at 600 °C. It is worth mentioning that the initial drop at low temperatures (50–150 °C) in the TGA graph of PVA and PVP indicates that the PVA and PVP contained physisorbed water.³¹

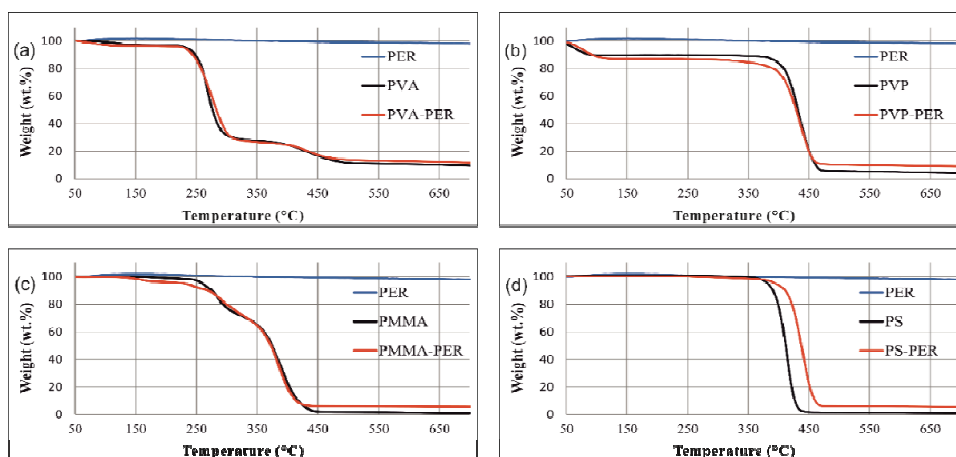


Fig. 2. TGA curves of perlite, neat polymers and perlite/polymer composites.

TABLE I. Thermal stability of perlite, neat polymers and perlite/polymer composites

Samples	$T_{0.05} / ^\circ\text{C}$	$T_{0.5} / ^\circ\text{C}$	Char at 600 $^\circ\text{C}$, wt. %
PVA	234	276	10.5
PVA-PER	229	283	12.6
PVP	62	432	4.8
PVP-PER	72	429	9.4
PMMA	260	374	1.4
PMMA-PER	231	373	5.7
PS	382	412	1.2
PS-PER	395	438	5.7

FTIR characterization

The FTIR spectra of pure materials and the composites were presented in Fig. 3. The spectrum of the perlite can be identified with the following major peak assignments: Si–O rocking vibration (460 cm^{-1}), symmetric and asymmetric stretching vibration of Si–O–Si (790 and 1060 cm^{-1}), stretching O–H vibrations which indicate the presence of isolated and surface Si–OH groups (broad band between 3190 – 3690 cm^{-1}).^{32–34} There wasn't a notable discrepancy between the FTIR spectrum of the PVA including perlite and the spectrum of pure PVA.

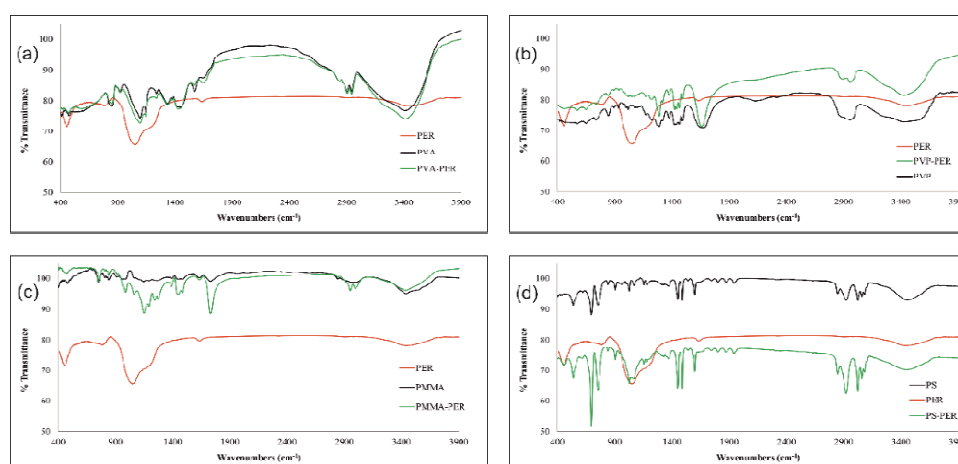


Fig. 3. FTIR curves of perlite, neat polymers and perlite/polymer composites.

Typical FTIR bands in PMMA are: 3000 and 2950 cm^{-1} C–H stretching vibrations of methyl group, 1730 cm^{-1} the C=O stretching vibrations of ester group, 1450 and 1300 cm^{-1} asymmetric and symmetric C–H deformation vibrations, 1165 cm^{-1} the stretching vibrations of ester group 990 , 850 and 750 cm^{-1} C–H vibrations out of the ring.³⁵ Some differences were observed in the FTIR spectrum after the perlite addition into the PMMA matrix. The most important was the increasing intensity of the band at about 1730 cm^{-1} related to the C=O

stretching vibrations of ester group. It implies that there is an interaction of perlite and C=O of ester in PMMA via a coordination bond involving the hydrogen bond of an oxygen atom with hydroxyl group of the surface of perlite and thus, complexation occurred.³⁶

Surface energetics of raw materials and composites

Inverse gas chromatography was applied to check the changes in surface energy as being result of composite manufacturing. Values of the dispersive component of the surface free energy and specific parameters describing the surface's ability to specific interactions are presented in Table II.

TABLE II. Values of dispersive component of the surface free energy (mJ/m^2), K_A , K_D and S_C of perlite, neat polymers and perlite/polymer composites

Material	$\gamma_s^d / \text{mJ m}^{-2}$				K_A	K_D	$S_C = K_D/K_A$
	40 °C	50 °C	60 °C	70 °C			
PER	—	—	34.2	30.9	0.13	0.17	1.3
PVA	41.9	40.9	40.3	39.9	0.06	0.21	3.5
PVA-PER	32.4	30.2	27.9	25.8	0.07	0.14	2.0
PVP	35.4	33.4	31.5	29.7	0.06	0.35	5.8
PVP-PER	24.5	23.9	22.1	21.1	0.06	0.29	4.8
PMMA	40.9	38.2	35.8	33.5	—	—	—
PMMA-PER	32.3	28.9	25.9	23.1	—	—	—
PS	33.7	30.8	27.8	25.1	0.13	0.53	4.0
PS-PER	39.9	37.7	36.7	35.6	0.11	0.41	3.8

The addition of the filler to the component most often caused the decrease of the dispersive component of surface-free energy. The resulting values were lower than those found for perlite alone. However, this was not the case for the PS-PER composite. The γ_s^d values found for this composite are the highest one – higher than for polymer alone and the filler. It might indicate the more active system when the ability to dispersive interaction is taken into account. Moreover, values of K_A and K_D parameters are also significantly higher for this composite. The tendency of PS-PER composite to act as an electron acceptor is slightly lower than this of PER or PS alone. The ability for electron donor interactions (K_D value) is lower than those of PS while is significantly increased in comparison to the K_D value for PER. However, the surface character of PS-PER remained the same in comparison to the polymer matrix.

Retention data collected during IGC experiments allowed to calculate also values of dispersive (W_a^d), specific (W_a^{sp}) component of work of adhesion between polymer and filler as well as total value of work of adhesion (W_a^{tot}), Table III. These parameters might be successfully used as a measure of the magnitude of adhesion between components in complex systems, *e.g.*, composites.^{6,7} The values of work of adhesion were determined at different temperatures due to

technical problems during examination of, *e.g.*, PER in the whole range of temperatures.

TABLE III. Values of dispersive, specific component of the work of adhesion and its total value (mJ/m^2) perlite/polymer composites

Material	W_a^d				W_a^{sp}		W_a^{tot}	
	40 °C	50 °C	60 °C	70 °C	60 °C	70 °C	60 °C	70 °C
PVA-PER	32.4	30.2	27.9	25.8	29.4	30.6	96.5	94.8
PVP-PER	24.5	23.9	22.1	21.1	27.3	30.3	80.0	80.4
PMMA-PER	32.3	28.9	25.9	23.1	—	—	60.9	55.6
PS-PER	39.9	37.7	36.7	35.6	31.6	32.5	95.5	92.3

Morphological characterization

The microstructure of the perlite was investigated by field emission scanning electron microscopy (Fig. 4). FE-SEM images shows that the perlite particles have an irregular glassy flake shape.

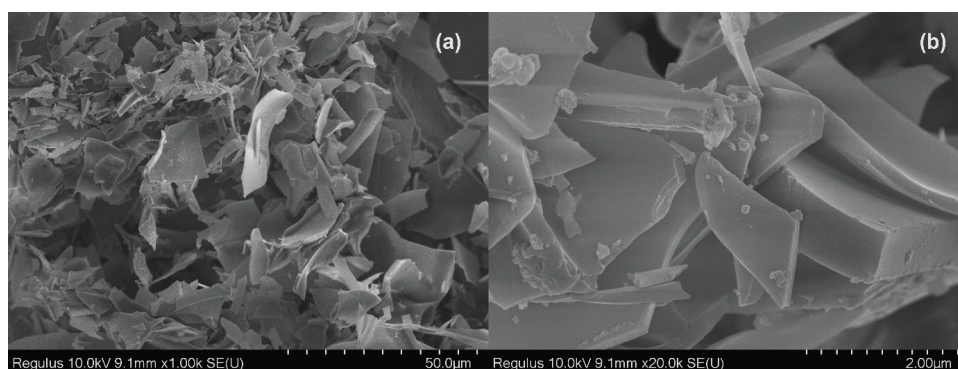


Fig. 4. FE-SEM images of perlite: a) at 1000 $\times$ magnification; b) at 2000 $\times$ magnification.

Fig. 5 shows the FE-SEM images of cryo-milled polymer composites. Cryo-milling works with liquid nitrogen medium below the ductile-brittle transition temperature of the polymers. Thus, the polymer is in a fragile state and grinding could be achieved in much shorter times without deformation compared to conventional mechanical milling techniques.³⁷ It can be observed that there are many fragments of perlite on the whole surface of the PVA matrix (Fig. 5a and b), however comparing PVA-PER (Fig. 5a) to other composites (Fig. 5c, e and g), it seemed that interfacial adhesion between the perlite and PVA matrix was poor. Fillers are pulled out of the PVA matrix during the cryo-milling. PVP-PER has a smooth fractured surface, typical of a brittle material, other composites have a rougher surface than PVP-PER.

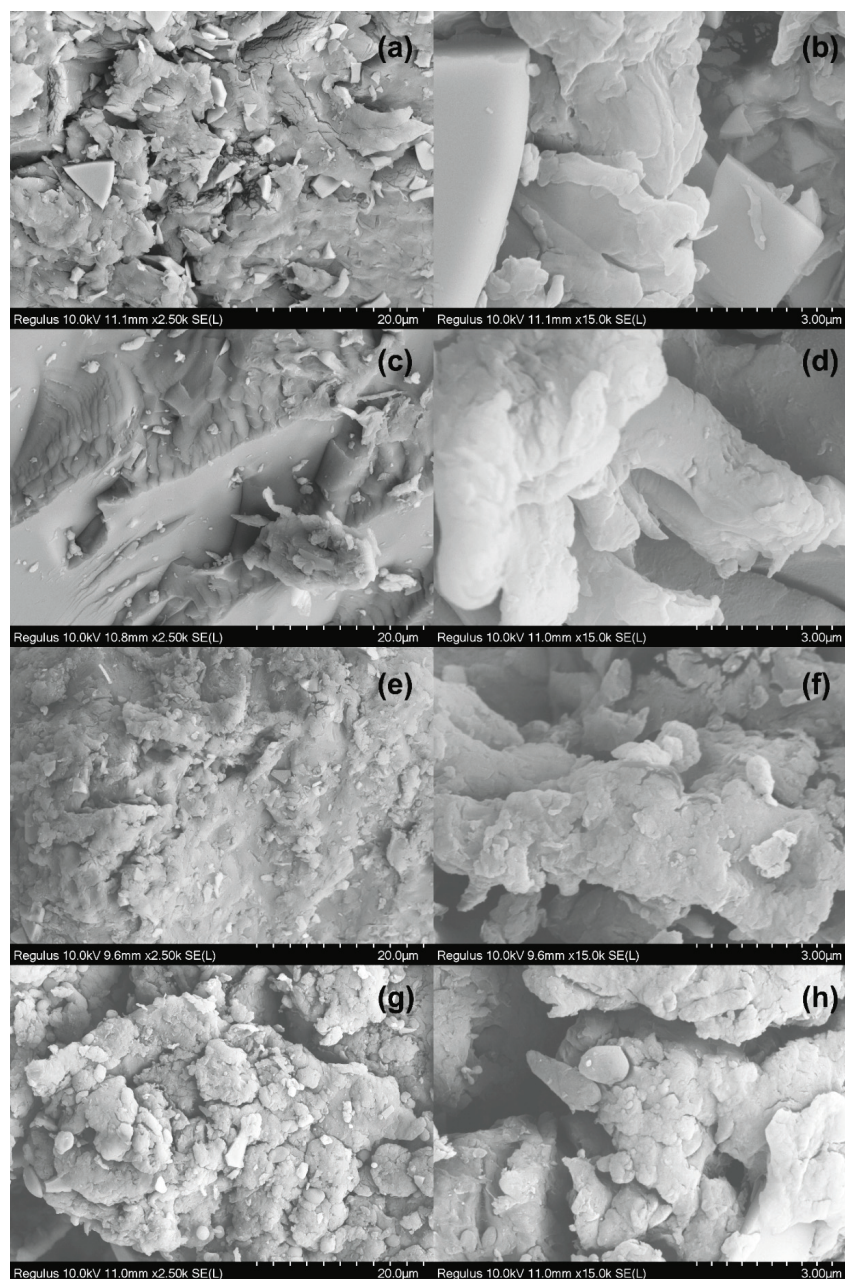


Fig. 5. FE-SEM images of PVA-PER (a and b), PVP-PER (c and d), PMMA-PER (e and f) and PS-PER (g and h).

As can be seen in Fig 5g and h, incorporation of perlite results in a more layered structure of the PS matrix, which indicates a strong interaction between

the filler and the polymer matrix.³⁸ This observation is confirmed by high values of work of adhesion between the components of this composite (Table III). It should be noted that these strong interactions between are attributed to specific interactions. The dispersive component of the work of adhesion is highest for PVA–PER system but it, as shown above, does not assure the stability of composite. However, higher values of W_a^{sp} found for PS–PER composite results in higher stability of the system although total work of adhesion W_a^{tot} is on similar level for these two systems.

TEM analysis was used for the examination of the dispersion of perlite in the polymers (Fig. 6). The examination of the below figures shows that there are no perlite agglomerates in the examined composites.

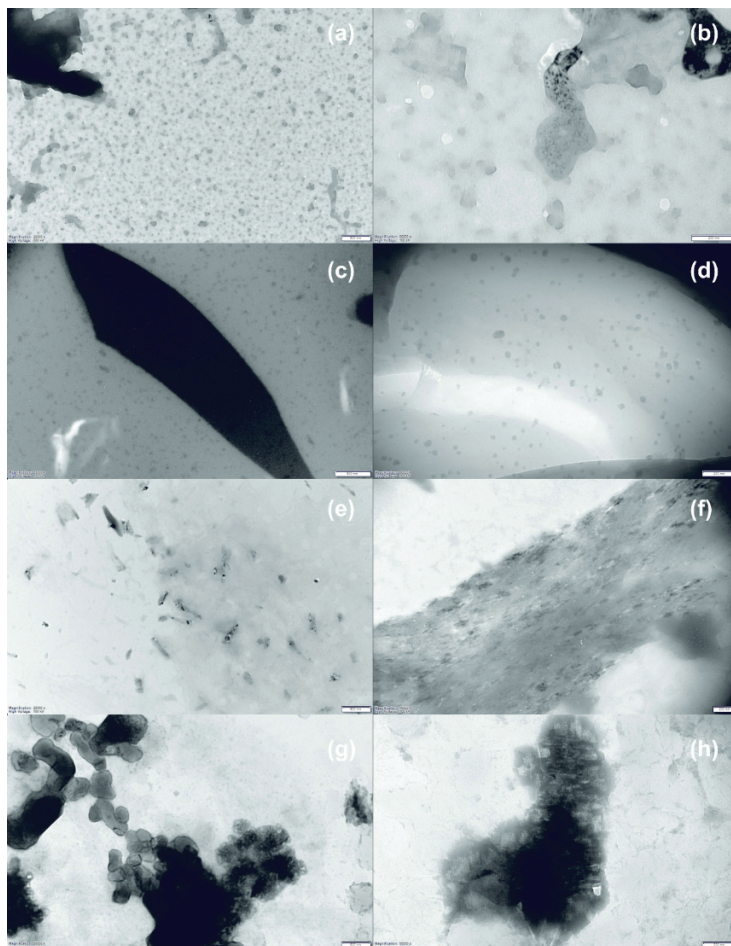


Fig. 6. TEM images for neat PVA–PER (a and b), PVP–PER (c and d), PMMA–PER (e and f) and PS–PER (g and h). Scale bars represent 500 nm (a, c, e and g) and 200 nm (b, d, f and h).

The intensities of the diffraction peaks of PVA–PER were slightly decreased compared to pure PVA. This was also seen in PVP–PER and PMMA–PER. However, the peak intensities of the PS–PER have been considerably decreased upon the addition of perlite. This change in diffraction intensity can be attributed to the amorphous nature increasing with the perlite incorporation into the polymer.

Within the experimental temperature range, PVA–PER, PVP–PER and PMMA–PER samples display similar degradation profiles, when compared to their corresponding pure polymer matrixes. This means that perlite did not significantly alter the degradation mechanism of PVA, PVP and PMMA in these conditions. As seen in Fig. 2, the best thermal result was obtained from PS–PER. From Table I, the $T_{0.5}$ value of pure PS is 412 °C, while that of PS–PER is 438 °C. PS and PS–PER shared a one-step thermal degradation process and the addition of perlite has shifted the decomposition curve of TGA to considerably higher temperature. This can be attributed to the good distribution of the perlite in the PS matrix.

Among all the studied polymers, the most significant change in the FTIR spectrum was seen in PS samples. According to the patterns, the intensity was changed by the incorporation of perlite in the case of PS composite due to the uniform distribution of perlite in the PS matrix³⁹. The IR spectra of the PS–PER and PMMA–PER exhibit a band at around 1070 cm^{-1} , corresponding to the asymmetric stretching vibrations of Si–O bonds in perlite.

The dispersive component of the work of adhesion decreases with the increase of the temperature of the IGC experiment. The highest values were found for PVA–PER and PS–PER systems while the lowest for the PVP–PER composite.

CONCLUSION

Perlite is a filler with unique properties, such as low density and thermal conductivity, thus the development of PS/Perlite composites may be interesting for producing lighter packaging with thermal insulation capability.² The incorporation of perlite results in a more layered structure of the PS matrix, which indicates a strong interaction between the filler and the polymer matrix. This observation is confirmed by high values of work of adhesion between the components of this composite. It should be noted that these strong interactions are attributed to specific interactions.

1. The distribution of perlite particles in polymer matrix is very important because the distribution of the filler (additive) has a significant effect on the final morphology and on the properties of polymer nanocomposites. It was determined by XRD analysis whether perlite was distributed homogeneously in the prepared nanocomposites. In general, the formation of intercalated nanocomposites causes

an increase in the basal space in the XRD pattern, while the formation of exfoliated nanocomposites causes the patterns between the layers to completely disappear and therefore the distinct peaks cannot be observed. In this study, no significant change was observed in the diffraction peaks of the XRD patterns of the nanocomposites prepared using 5 wt. % perlite. This result indicates that the perlite particles are brought together by providing a homogeneous distribution in the polymeric matrixes. The compatibility of this situation with many other studies has been confirmed by comparing with the XRD patterns available in the literature.

2. TEM and SEM images, which are complementary to XRD characterizations, are the most popular techniques used to determine the morphology of nanocomposites. In general, the microstructure features obtained from TEM, provide useful details to the overall picture that can be derived from XRD results and support the understanding of various issues involving nanocomposite formation. The distribution microstructure of intercalated and exfoliated perlite layers in this study was also investigated by SEM and TEM analyses. The homogeneous distribution of perlite in the obtained polymer matrix nanocomposites was confirmed by TEM analyses in addition to XRD characterizations. TEM images show that the perlite layers are well-structured and homogeneously distributed within the polymer matrices.

3. The morphologies of nanocomposites are evaluated using SEM and TEM analysis techniques. SEM images provide information about the reinforcement (filler) materials in the composites, the dispersion defects and the phase boundaries during bonding, surface the roughness and the cracked surfaces. In this study, SEM analysis was performed to observe the surface of the materials and the distribution of the filler (reinforcement) in the polymer matrix. It was noticed from the SEM images that the prepared nanocomposites exhibited a very good distribution.

4. FTIR spectrum of all samples were taken in order to determine structural characterization. As seen from the FTIR spectrum of the obtained nanocomposite, there is not much difference between the spectrum of the neat polymers. This situation supports the formation of homogeneously distributed nanocomposites as a complement to XRD, SEM, TEM characterizations.

5. TGA analyses were performed in order to determine the thermal characterizations of all samples. It was observed that perlite clay used as filler had high thermal stability. The combustion resistance of polymers is quite low, therefore the use of silicate particles as filler increases their thermal resistance. The thermal resistances of all nanocomposites obtained in this study increased slightly. However, the highest thermal resistance was observed for the PS-PER nanocomposite and this was achieved as a result of the TGA decomposition curve being shifted to a significantly higher temperature with the addition of perlite filler.

6. One of the most commonly measured parameters for the description of the energy situation on the surface of a solid is the surface energy. The surface energy can affect, for example, catalytic activity or the strength of particle–particle interaction. Due to the important role of surface energy in physicochemical interactions, surface energy measurement has attracted the attention of material and surface researchers. In this study, the highest surface energy value was obtained for PS–PER nanocomposite ($\gamma_s^d = 35.5 \text{ mJ/m}^2$, at 70°C). This value is even higher than the value obtained for PS–orgona montmorillonite nanocomposite ($\gamma_s^d = 28.9 \text{ mJ/m}^2$, at 70°C). This situation is quite positive for perlite filled nanocomposites prepared without using any organomodified filler in this study. Based on the obtained results, the synthesized PS–PER composite shows the best performance and is the most suitable for practical applications.

7. The acid or basic properties of solid surfaces are interesting aspects of a surface structure and important in the fields of ion exchange and heterogeneous catalysis. Acid/base catalysed reactions belong to the technologically most important classes of heterogeneous catalytic conversions. IGC may provide the determination of important parameters, including Lewis acid-base interaction parameters between the matrix and filler of composite materials. Determining the changes in this surface property will make a significant contribution to the development of polymer matrix nanocomposites which are used in many industrial sectors.

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ИЗВОД

ТЕРМАЛНЕ, МОРФОЛОШКЕ И ПОВРШИНСКЕ ОСОБИНЕ КОМПОЗИТНИХ МАТЕРИЈАЛА СА ПЕРЛИТНИМ ОЈАЧАЊИМА

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Овај рад се бави припремом и карактеризацијом композита које чине перлит/поливинил-алкохол, перлит/поливинилпириolidон, перлит/полиметил метакрилат и перлит/полистирол. Полимерни композити су припремљени техником ливења раствора са 5 теж. % перлита као пунила. Перлит је пунило са јединственим особинама, као што су ниска густина и топлотна проводљивост, па развој полимер-перлит композита може бити интересантан за производњу лакше амбалаже са способношћу топлотне изолације. Перлит/полимер нанокомпозити су карактерисани помоћу рендгенске дифракционе (XRD) анализе, термогравиметријске анализе (TGA), Фуријеове трансформационе инфрацрвене спектроскопије (FTIR), инверзне гасне хроматографије (IGC), скенирајуће електронске микроскопије (FE-SEM) и трансмисионе електронске микроскопије (ТЕМ). Мерења TGA су показала значајно повећање термалне стабилности полистирола услед присуства перлита у односу на матрицу. FE-SEM слика криомлевеног узорка показује да су перлитне честице

уграђене унутар полистиролне матрице, што је у складу са подацима о адхезији добијеним методом IGC.

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Modification of surface properties and photocatalytic performance of pure and oxygen-doped graphitic carbon nitride *via* DBD plasma treatment

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Abstract: Graphitic carbon nitride (CN) is a non-metallic semiconductor with applications in photocatalysis, including the photocatalytic reduction of Cr(VI) under visible irradiation. To improve its intrinsic properties, two modification strategies were applied: *i*) oxygen doping by co-calcination of urea with two different amounts of oxalic acid and *ii*) dielectric barrier discharge (DBD) plasma treatment. The plasma treatment was applied to pristine and previously oxygen-doped CNs. The properties of the photocatalysts were studied by XRD, FTIR, FESEM, EDS, PL and DRS analysis, as well as by determination of the number of acidic surface functional groups. Both modification methods increased the oxygen content: during oxygen doping, nitrogen was replaced by oxygen in the lattice, while during plasma treatment, oxygen-containing functional groups were introduced at the surface. Plasma treatment of oxygen-doped CNs facilitated surface functionalisation due to the open heptazine ring structure. Oxygen doping narrowed the band gap, which was further slightly reduced by subsequent plasma treatment, thereby lowering the oxidation and reduction potentials. Although the absorption of visible light was improved, the reduction of the band gap resulted in a reduced activity for the photocatalytic reduction of Cr(VI). The best results were obtained with plasma-treated pure CN, due to the increased content of oxygen-containing surface groups, which led to a slightly reduced recombination rate of charge carriers

Keywords: semiconductor; photocatalytic reduction; water treatment; Cr(VI).

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INTRODUCTION

Graphitic carbon nitride (CN) is a unique semiconductor-based photocatalyst, characterised by a graphite-like structure, which has been widely used in many applications such as solar cells or photocatalytic water-splitting, but also in environmental remediation, including photocatalytic reduction of toxic Cr(VI). Numerous advantages, such as a suitable band gap energy (~ 2.7 eV), high conduction band potential, *i.e.*, high reduction potential of electrons, non-toxicity and resistance to extreme pH conditions, have attracted much attention to CN.^{1,2} In addition, CN synthesis is typically achieved by facile direct thermal condensation of nitrogen-rich precursors such as urea, thiourea, melamine, cyanamide, or dicyandiamide. In particular, urea-derived CN has been reported to possess the most favourable properties for Cr(VI) reduction.³ Nevertheless, bulk CN faces challenges such as high recombination rates of photogenerated charge carriers and low visible light utilization, which limit its photocatalytic efficiency. Numerous modification strategies have been proposed to enhance its photocatalytic activity, such as elemental doping to alter the electronic structure and band gap width, forming heterojunctions with other semiconductors, carbon materials or depositing metal nanoparticles (NPs) on the CN surface to improve the separation of photogenerated electrons and holes.⁴ The choice of modification strongly depends on the desired application, so it is advantageous to tailor the photocatalyst to the operating conditions.

In addition to the aforementioned strategies involving the integration of other materials, another effective approach to optimizing CN for photocatalytic reduction involves the introduction of defects and functional group modifications.^{5,6} It is well known that surface properties, such as specific surface area, presence of surface functional groups, number of active sites, and surface charge, significantly influence the photocatalytic performance of materials, since photo-induced reactions take place at the surface of the photocatalyst.⁷ Functionalization of the CN surface with electron-withdrawing oxygen-containing functional groups, can alter the charge distribution within the CN. It has been reported that this modification enhances the separation and migration of photogenerated charge carriers, prevents their recombination and provides additional active sites on the CN surface.^{5,8,9} Furthermore, the introduction of these hydrophilic groups can improve the dispersibility of CN in water, thereby increasing the surface area exposed to Cr(VI) ions for adsorption and facilitating the interactions with radiation necessary for photocatalyst activation.^{10–12}

As one of the most promising physical methods, plasma surface modification has been widely used to improve the surface properties of carbon materials by introducing different functional groups and increasing the number of adsorption sites.¹³ The type of functional groups introduced depends on the atmosphere used in the plasma treatment. For example, to increase the amount of oxygen-con-

taining functional groups, an air atmosphere is usually used, which further simplifies and economises this method. In addition, plasma treatment of a photocatalyst can alter its electronic structure, increase its hydrophilicity and improve its dispersity, thereby increasing the surface area available for adsorption.¹⁴ In theory, a plasma consists of neutral species, positive ions and electrons, which form an oxidizing medium and can interact with materials to optimize their surface morphology or chemical properties, without destroying the fundamental structure.^{15,16} Dielectric barrier discharge (DBD) plasma belongs to the non-thermal plasma (NTP) discharge and has been widely used for material modification due to its advantages, such as the ability to operate at an atmospheric pressure, low gas temperature, it has uniform discharge and high electron density.^{17–19} Mao *et al.* used DBD, at room temperature and atmospheric pressure air without flow, to modify the surface properties of CN.⁷ The results showed that the plasma treatment didn't affect the crystal structure of CN, but the crystallinity was reduced. In addition, EDS analysis showed that there was a higher oxygen content and FTIR confirmed that the peak assigned to –OH groups was higher from the pure CN. This probably led to the reduced recombination of electrons and holes. The obtained photocatalyst was investigated for the photocatalytic degradation of Rhodamine B (RhB) under visible irradiation and its activity was improved compared to the pristine CN.

Plasma treatment can be applied to pure CN, although there is evidence that it may be more effective when applied to pre-treated CN with introduced defects such as dopants or vacancies. The introduction of these defects can lead to the opening of the CN heptazine ring, facilitating the attachment of new functional groups to the “exposed” ends.^{20,21} As CN naturally contains some oxygen, it is assumed that doping CN with additional oxygen to increase its content could promote ring opening and facilitate the introduction of carboxyl, carbonyl and hydroxyl groups. It has also been reported that oxygen-doped CN has a narrower band gap and a more porous structure than pure CN, which could further enhance its photocatalytic activity.^{22,23}

In this study, DBD plasma treatment was used to modify both pure CN, obtained by thermal polymerisation of urea, and oxygen-doped CN, obtained by co-calcination of urea with oxalic acid. While plasma treatment has previously been used for surface modification of CN, research on its application to oxygen-doped CN remains limited. Furthermore, the influence of the modifications of CN on the photocatalytic reduction of Cr(VI) hasn't been investigated. Therefore, the effects of DBD plasma treatment on the crystallinity, chemical composition, morphology, optical absorption, electronic properties and acidic surface group content, as well as on the Cr(VI) photocatalytic reduction of urea-derived and oxygen-doped CN, were investigated.

EXPERIMENTAL

Direct thermal polymerization of urea (Thermo Fisher Scientific, Germany (99.5%)) was used for the synthesis of bulk CN. An alumina crucible containing urea, covered with a lid, was heated in a muffle furnace in air atmosphere at a heating rate of 10 °C/min to 550 °C and held at this temperature for 4 h. The furnace was cooled to ambient temperature and the resulting yellow sample was ground to powder and collected for further use.

Oxygen-doped CN samples were prepared by the co-calcination method of urea with oxalic acid (OA, oxalic acid dihydrate, Zorka Pharma a. d., Šabac (99.5 %)). 10 g of urea was well ground with a certain amount of oxalic acid (2 or 5 g) in a mortar, transferred to an alumina crucible with a lid and kept in a muffle furnace for 4 h at 550 °C in air. When 2 g of oxalic acid was used, the heating rate was 10 °C/min (CN(2)), while when 5 g of oxalic acid was used, the heating rate was 5 °C/min (CN(5)). In the second case, the heating rate had to be lower because the higher amount of oxalic acid resulted in a rapid release of gases, causing the sample to decompose completely.²²

The plasma treated samples were obtained by dielectric barrier discharge (DBD) plasma at atmospheric pressure in air, with the configuration consisting of two plane-parallel electrodes, dimension (20.0×8.0) cm², covered by a 0.70 mm thick alumina layer. The sample was placed between the electrodes, where their distance was fixed with glass space holders in order to obtain a discharge gap of 3 mm and to prevent the sample from scattering outside the system. All samples were treated under the same conditions: the operating voltage was 16 kV at a frequency of 150 Hz and the treatment time was 5 min. The samples obtained were labeled as CN-pt, CN(2)-pt and CN(5)-pt.

The crystal structure and the surface chemical functional groups of the samples were characterized using an X-ray diffractometer (XRD) (Cu-K α 1,2 radiation, λ = 0.1540 nm, Rigaku SmartLab, Tokyo, Japan) at a room temperature, with 0.02° 2 θ steps and 10 °/min counting time, in the 5–40° 2 θ range and a Fourier transmission infrared spectrometer (FTIR), Thermo Scientific Nicolet iS10 in attenuated total reflection mode and in the wave number range of 400–4000 cm⁻¹, respectively. The morphology of the sample was characterised using field emission scanning electron microscopy, FESEM (Tescan Mira3 XMU) at a working voltage of 20 kV. To ensure the conductivity of the samples, they were previously sputter-coated with gold. An EDS analysis was performed on an INCAX-act LN2-free analytical silicon drift detector of characteristic X-rays with PentaFET® Precision and Aztec 4.3 software package (Oxford Instruments, UK) connected to a Tescan Mira3 XMU. Elemental mapping was carried out over three selected areas of dimension 50 μ m × 50 μ m. The UV–Vis diffuse reflectance spectra (DRS) of the samples were recorded on a Shimadzu 2600 UV–Vis spectrophotometer with integrated ISR-2600-Plus sphere and BaSO₄ as the standard reference. The Kubelka-Munk function given as $F(R)$ was used to calculate the absorption spectra from the reflectance data. Photoluminescence (PL) spectra were obtained using a Fluorolog-3 FL3-221 spectrofluorometer system (Horiba Jobin-Yvon) with a 450 W xenon lamp as the excitation source and an excitation wavelength of 370 nm.

The titration method was used to study the content of acidic groups on the surface. Briefly, 100 mg of the sample was mixed with 50 ml of 0.1 M NaOH solution (Reahem d.o.o, Novi Sad (F = 0.9799)) and left in a shaker for 24 h. The powder was then separated from the supernatant by centrifugation (5 min at 6000 rpm) and the supernatant was titrated with 0.1 M HCl (Alfapanon, Serbia (F = 0.9934)) solution and methyl orange as an indicator. The equation used to calculate the content of acid groups (X) is:

$$X = \frac{V_{\text{NaOH}}C_{\text{NaOH}} - V_{\text{HCl}}C_{\text{HCl}}}{m} \quad (1)$$

where V_{NaOH} and C_{NaOH} are the volume and the concentration of the NaOH solution, V_{HCl} and C_{HCl} are the volume and the concentration of the HCl solution required to reach the equilibrium point during the titration, and m is the mass of the material.

The performance of the synthesized photocatalysts was investigated using a 10 mg/l Cr(VI) solution, prepared by dissolving $\text{K}_2\text{Cr}_2\text{O}_7$ (Hemo, Belgrade (99.9 %)) in deionized water, with pH adjusted to 3 using concentrated (98 %) H_2SO_4 (Lachema, Czech Republic (98 %)). Briefly, 0.02 g of photocatalyst and 0.133 ml of citric acid solution (20 g/l, Lachner, Czech Republic (99.8 %)), used as a hole scavenger, were added to 40 ml Cr(VI) solution. The above suspension was magnetically stirred in the dark for 30 min before the irradiation to achieve adsorption-desorption equilibrium. To avoid overheating, the reactor used in the experiments had an outer jacket for circulating cooling water and the suspension was constantly stirred during the experiments, which were conducted at a room temperature. A lamp (High pressure Mercury Reprographic, Philips, 125 W) with a filter (GG400 Farbglass SCHOTT) for $\lambda > 400$ nm, *i.e.*, simulated visible (Vis) light, was used as the irradiation source, whose distance and intensity were kept constant during the experiment. The 1 ml suspension was collected after 15 min of irradiation and filtered to remove the photocatalyst. The concentration of Cr(VI) solution was measured using the characteristic absorption peak at 542 nm and 1,5-diphenylcarbazine (Sigma-Aldrich, USA (≥ 99 %)) as an indicator on a UV-Vis spectrophotometer (Shimadzu UV-1800).

RESULTS AND DISCUSSION

According to the results of the EDS analysis, pure CN naturally contains a certain amount of oxygen (Table I), mainly from the oxygen impurities in the precursors.²⁴ After oxygen doping with OA, the results showed that as the OA content increases, more oxygen is present in the samples. Another indication of oxygen doping is the colour of the samples, which is a much deeper brownish red for CN(5) with a higher O content. The oxygen content was increased in all samples after the plasma treatment. It can be seen that the C content was slightly altered, but a greater change occurred in the N content of the modified samples, suggesting that some nitrogen atoms were lost. This leads to the possibility that some of the N in the heptazine units has been replaced by oxygen, which has been reported previously as oxygen atoms could be introduced into CN in the form of lattice oxygen or oxygen-containing functional groups.^{25,26}

TABLE I. Element content (wt. %) of CN and modified CNs

Sample	C	N	O
CN	35.2±0.47	62.8±0.30	2.00±0.17
CN-pt	35.7±0.30	60.8±0.93	3.52±0.62
CN(2)	35.3±1.19	62.0±1.16	2.63±0.03
CN(2)-pt	35.7±0.29	61.2±0.19	3.08±0.48
CN(5)	36.7±2.12	59.6±2.52	3.69±0.49
CN(5)-pt	35.0±0.38	58.5±0.71	6.55±0.50

The XRD patterns of all the CNs (Fig. 1a) showed a characteristic CN phase with two distinct diffraction peaks at $\sim 13.1^\circ$ and $\sim 27.5^\circ$, indicating a typical tri-s-triazine structure.¹² The weak peak at 13.1° corresponds to the (100) plane formed by in-plane repetition of heptazine units with a distance between the heptazine units in the layer (d) of 0.68 nm. Meanwhile, the peak at 27.5° is assigned to the (002) plane, associated with the interplanar stacking of conjugated aromatic rings with a distance between the layers (d) of 0.326 nm, suggesting a layered structure similar to graphite.²⁷ After oxygen doping, the intensity of both peaks decreased with increasing OA content. The weaker intensity of the (100) peak indicates a smaller lateral size of the particles, namely a decrease in the size of the in-plane repeating heptazine unit, while the weaker and broader (002) peak indicates a decrease in the number of layers, implying a reduction in the stacking structures.^{21,23,27} It has also been mentioned that oxygen doping into the CN structure affects the arrangement of the in-plane structural packing units, resulting in more disordered tri-s-triazine motifs, leading to a decrease in the intensity of the (100) peak.^{28,29} Also, with the increase in OA, the position of the (002) peak was slightly shifted to a lower angle gradually, suggesting an increased interlayer distance in O-doped samples.²² Obviously, both the intensity and the position of two significant peaks depend on the amount of OA used in the synthesis.

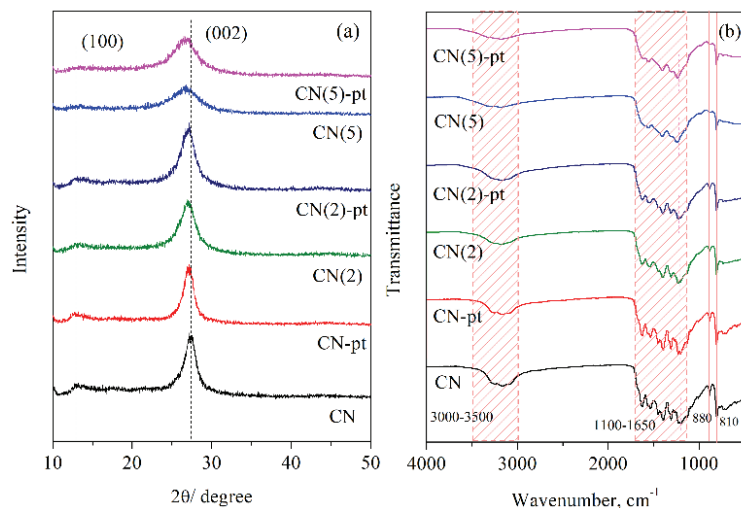


Fig. 1. a) XRD patterns and b) FTIR spectra of the CN photocatalysts.

After the plasma treatment, all samples retained a typical CN structure without any impurity phases, which means that plasma treatment doesn't affect the crystal structure of CN. Compared to pure CN, CN-pt showed no change in the intensity of the diffraction peaks, while the position of the (002) peak was

slightly shifted towards the lower values of 2θ , possibly due to nitrogen vacancies introduced during the treatment.^{7,14,19} However, when the treatment was applied to the previously oxygen-doped samples, it was observed that the intensity of the (002) peak slightly increased compared to the O-doped samples, but was still lower compared to pure CN. Obviously, the plasma treatment had a different effect on the oxygen-doped samples due to the pre-existing changes in their structure.

The surface functional groups and chemical structure of the CN samples were further investigated by FTIR spectroscopy (Fig. 1b). As can be seen, all samples exhibit similar absorption bands and no significant change can be observed compared to the pure CN, which means that the oxygen doping and plasma treatment did not alter the original chemical structure of CN.^{20,27} The broad band in the region $3000\text{--}3500\text{ cm}^{-1}$ belongs to the stretching vibrations of N–H and O–H bonds, present in the free amino groups and adsorbed water on the surface of CN. The bands in the region of $1100\text{--}1650\text{ cm}^{-1}$ are ascribed to the aromatic stretching vibrations of heptazine-derived units, including the C=N stretching mode and C–N out-of-plane bending vibrations.^{12,27} The sharp band at approximately 810 cm^{-1} corresponds to the characteristic breathing mode of tri-s-triazine units in heterocycles and a weak peak at 880 cm^{-1} likely originates from the deformation mode of N–H in amino groups.^{12,22} After the oxygen doping, it can be noticed that the intensity of the bands at $1100\text{--}1650\text{ cm}^{-1}$ is quite reduced, which may be the result of breaking of the triazine ring structure.³⁰ This can further imply that the ring opening probably occurred after introducing defects in the structure. Characteristic peaks of the C–O bond are similar to the C–N, making them difficult to detect by FTIR, but a slight shift in the position of the peak at 1198 cm^{-1} can be observed. This peak is attributed to the C–N stretching vibration and its shift to higher frequencies may indicate a transition from the C–N to the C–O bond, as these C–O vibrations appear at higher frequencies.^{22,23,25} The change in intensity and shape of these bands is more significant after oxygen doping rather than after plasma treatment, so perhaps the conditions applied during the plasma treatment were not strong enough to cause some noticeable changes in the FTIR spectra, as was also shown by the XRD results. It can also be observed that the shape of broad peak at $3000\text{--}3500\text{ cm}^{-1}$ changed; for O-doped samples it became broader with the increase of OA and after the plasma treatment its shape did not change but the intensity was reduced.

The morphological characteristics of CN and modified samples were examined using FESEM (Fig. 2). As expected, the FESEM image of CN showed a porous structure composed of agglomerated, irregular, layered particles. After oxygen doping, it can be observed that the particles became more flaky and the irregular structure became even more porous. When a higher amount of oxalic acid was used, a more porous structure was obtained and the fragmentation of the

layers was more pronounced. Apparently, the calcination of oxalic acid and urea produced gases such as NH_3 and CO_2 , which acted as bubble-forming agents. This resulted in the curling of the nanosheets and the formation of a porous structure in the samples.^{14,26,28,29} Plasma treatment of the CN and O-doped samples did not make a significant difference due to the mild conditions of the DBD system, so a similar morphology was observed.

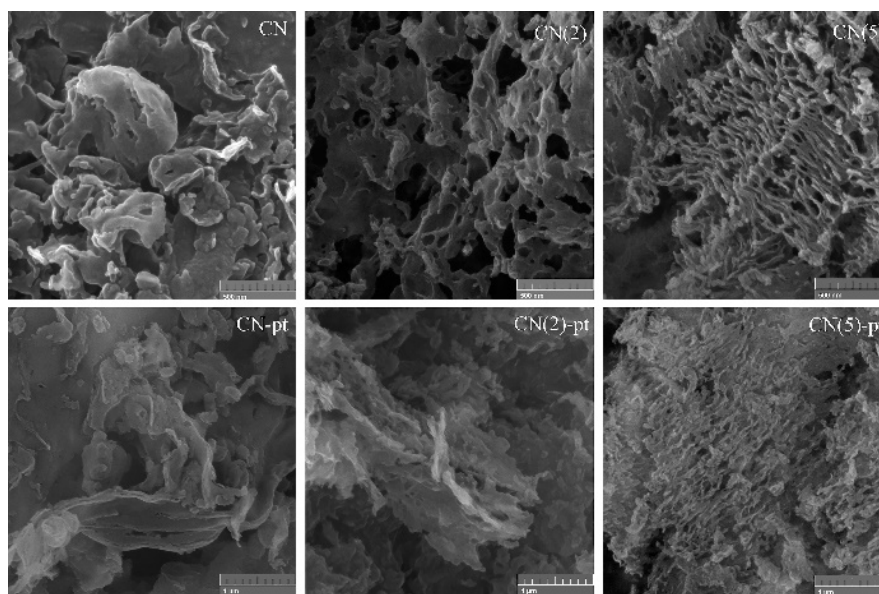


Fig. 2. FESEM photographs of the CN photocatalysts.

UV–Vis diffuse reflectance spectroscopy and photoluminescence (PL) spectroscopy were used to study the optical absorption and electronic properties (Fig. 3). As can be seen in Fig. 3a, the absorption edge of CN (λ_g) was at approximately 416 nm and the calculated band gap energy from the equation $E_g = 1240/\lambda_g$ was 2.98 eV, which is in agreement with previous reports.^{3,28,31} The results showed that a significant increase in visible light absorption was observed after the inclusion of oxalic acid in the CN synthesis, with respect to the OA content. It is known that elemental doping can affect the optical properties of semiconductors and this change confirms the oxygen doping reported previously.^{26,32} From the obtained DRS spectra of the O-doped samples, it can be seen that new absorption bands form between 400 and 600 nm, which has been attributed to the $n \rightarrow \pi^*$ electron transition associated with the lone pairs of the edge nitrogen atoms within the heptazine units.³³ As the two O-doped samples exhibited a significant red shift of the absorption edges, this corresponds to a decrease in band gap energies (Table II) and a higher utilization of visible light.

When plasma treated, CN-pt showed an absorption edge similar to CN, indicating that the optical band gap was not changed. It was previously reported that the band gap of CN would narrow after introducing nitrogen vacancies, but in this case the band gap remained the same, meaning that the plasma treatment didn't create this type of defect. Nevertheless, the C/N ratio was increased (Table I) and the explanation for this could be the transformation of the terminal amino groups into C–O and C=O groups as a result of treating the CN sample with plasma species.⁷ Interestingly, the band gap energy was affected by plasma treatment of O-doped samples. Plasma treatment and oxygen doping of CN resulted in a synergistic narrowing of the band gap, possibly due to the introduction of oxygen atoms and the pre-existing structural modification during the doping process. A more significant shift can be observed in the CN(5)-pt sample, which was obtained with a larger amount of OA during synthesis and contains more oxygen, meaning that there are already greater changes compared to CN(2). Because of this, carboxyl groups were more easily attached during the plasma treatment, leading to the even higher oxygen content in this sample, giving it the narrowest band gap.³⁴

The position of the valence and conduction bands, *i.e.*, E_{VB} and E_{CB} can be calculated using the empirical formula $E_{VB} = \chi - E_e + 0.5E_g$, where E_{VB} is the valence band potential, χ represents the electronegativity of the semiconductor (4.67 eV for CN), E_e is the energy of free electrons on the hydrogen scale (~4.5 eV), while E_g is the band gap energy of the semiconductor. Furthermore, E_{CB} can be calculated from the equation: $E_{CB} = E_{VB} - E_g$.^{35–37}

TABLE II. Absorption edge, band gap energy, valence and conduction band potentials and surface acidic groups (SAG) of the CN photocatalysts

Sample	λ_g / nm	E_g / eV	E_{VB} / eV	E_{CB} / eV	SAG groups, mmol/g
CN	416	2.98	1.66	–1.32	1.83
CN-pt	414	2.99	1.66	–1.33	6.60
CN(2)	446	2.78	1.56	–1.22	5.70
CN(2)-pt	450	2.76	1.55	–1.21	13.5
CN(5)	484	2.56	1.45	–1.11	8.40
CN(5)-pt	507	2.44	1.39	–1.05	19.5

Although the DRS results suggest that the photocatalytic activity of the obtained samples should be improved under visible irradiation, it doesn't necessarily mean that this will happen. The band gap energy was reduced, but so was the reduction potential of electrons from the conduction band, which could negatively affect the photocatalytic activity of the synthesized samples.

From Fig. 3b it can be seen that CN has a strong PL emission peak at 458 nm, while the spectra of the O-doped samples have one more peak, indicating two recombination centres.³⁸ The position of the new peak (at around 560 nm) is

consistent with the extended absorption range and enhanced visible light absorption, as confirmed by DRS spectra. As expected, CN-pt showed the PL peak at practically the same position as CN. PL spectroscopy was also used to evaluate the transfer and separation efficiency of photogenerated electrons and holes. The intensity of the emission peak is related to the recombination rate of charge carriers, *i.e.*, a lower emission peak means that recombination of charge carriers is suppressed due to better separation. Compared to CN, all samples had lower PL peak intensities, with the greatest suppression for CN(5). The results showed that after plasma treatment of CN, the recombination rate was reduced (sample CN-pt), but when O-doped samples were plasma treated, it led to an increase in peak intensity. It is known that the oxygen atom has more electrons than the nitrogen atom, so assuming that oxygen doping replaces nitrogen atoms, this would result in the generation of an additional electron and a further change in the electronic property of CN. It is possible that the charge density and mobility have been increased following the incorporation of oxygen atoms due to the extended delocalized π -electron system. In addition, the charge redistribution around the dopant caused an electronic polarization effect, creating an internal electric field that favoured carrier separation.^{25,29} This could explain why O-CN samples showed a much lower PL peak intensity and should have a lower electron-hole pair recombination rate. However, the oxygen content was increased in CN-pt (Table I) and it showed a lower recombination rate compared to CN. It has been mentioned that plasma treatment could introduce oxygen-containing functional groups that could alter the charge distribution and attract electrons to the surface. Logically, it was expected that after plasma treatment of O-CN samples, the recombination rate of carriers would be further suppressed due to the synergistic effect of oxygen doping and higher content of oxygen-containing surface functional groups. However, the PL spectra results showed that CN(2)-pt and CN(5)-pt had higher PL emission peak intensities compared to only oxygen-doped samples. Perhaps the distribution of oxygen-containing functional groups was not homogeneous due to the presence of already modified structure, or some other defects were created which acted as recombination centers.

The content of oxygen-containing surface functional groups was also evaluated according to the content of acidic surface groups (Table II). Previous results (Table I) already confirmed that the oxygen content was increased after doping and also after plasma treatment, with the highest oxygen content being present in the CN(5)-pt. Not surprisingly, this sample also had the highest content of acidic groups on the surface. This can be explained by the fact that the previous ring opening during oxygen doping facilitates the attachment of oxygen-containing functional groups to the now more exposed ends. Although CN(5)-pt had the higher content of acidic functional groups on the surface compared to CN(5), the intensity of the PL peak decreased more in the case of CN(5). This is

of course evidence that many factors influence the intensity of the PL peak, and in this case perhaps the lower number of layers in the CN(5) particles, as confirmed by XRD.

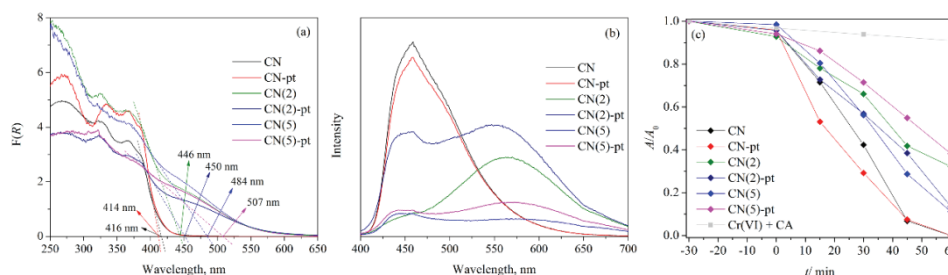


Fig. 3. The a) UV-Vis absorption spectra, b) PL emission spectra and c) photocatalytic reduction of Cr(VI) with the CN photocatalysts under visible irradiation.

In order to verify the impact of oxygen doping and plasma treatment of CN on its photocatalytic performance, the as-synthesized samples were used for a photocatalytic reduction of Cr(VI) under visible irradiation at pH 3, with citric acid (CA) serving as a hole scavenger. The acidic conditions were chosen because the reduction of Cr(VI) to Cr(III) is favoured at lower pH values. To further enhance the photocatalytic efficiency, citric acid was used as a hole scavenger to prevent re-oxidation of Cr(III) and to allow oxidation and reduction reactions to occur simultaneously. It was shown that citric acid has a low reduction potential, as evidenced by the insignificant decrease in Cr(VI) concentration in the experiment without photocatalyst (Cr(VI) + CA in Fig. 3c). Preliminary experiments showed that 30 min is sufficient time to achieve adsorption-desorption equilibrium in the dark for all samples. Fig. 3c shows very small differences in Cr(VI) concentration after 30 min in the dark, indicating that the modifications did not significantly change the adsorption capacity for Cr(VI).

The photocatalytic reduction ability of the synthesized CNs towards Cr(VI) within 60 min was CN-pt > CN > CN(5) > CN(2)-pt > CN(2) > CN(5)-pt. Somewhat expectedly, CN-pt exhibited the best results, slightly better compared to pure CN, since its properties were quite similar to those of CN, except that the PL spectra indicated enhanced separation efficiency of the photogenerated charge carriers. It is likely that during the plasma treatment in air, various oxygen-containing functional groups were introduced (Table II), which resulted in more electrons reaching the photocatalyst surface to participate in the reduction reactions.

In contrast, the remaining samples showed reduced photocatalytic activity compared to the pristine CN. After oxygen doping, it was observed that the band gap energy of the samples decreased, implying an increase in visible light absorption (Fig. 3a). Additionally, the PL spectra suggest that the oxygen-doped samples may exhibit enhanced photocatalytic efficiency due to reduced electron-

hole recombination, but the outcome was contrary to expectations. As mentioned above, the lower intensity of the PL peaks is related to the lower recombination rate of the charge carriers, but another possible explanation for the observed reduction in PL intensity is that the lower number of e^-h^+ pairs was generated after irradiation. This interpretation suggests that the photocatalysts will exhibit reduced photocatalytic activity under visible irradiation due to fewer electrons generated for reduction reactions at the sample surface. In addition, a narrowed band gap results in altered positions of the valence and conduction bands. In this case, this resulted in a decrease in the reduction potential of the electrons, thereby reducing the reduction capacity of the photocatalyst. The lowest photocatalytic efficiency of CN(5)-pt is in agreement with the previous statement as it has the narrowest band gap and the lowest redox capacity.

The explanation of how oxygen doping leads to the extension of the delocalized π -electron system and therefore to a better separation efficiency of the charge carriers could also be questioned here, taking into account the results of photocatalytic experiments.²⁵ The modification strategy that included oxygen doping was the ring structure engineering, *i.e.*, opening of the heptazine ring during the introduction of defects in order to enhance the attachment of oxygen-containing functional groups. With this in mind, it is possible that this led to the breaking of the C–N bonds, as shown by the FTIR spectra, and therefore to the reduction of the π -conjugation system rather than an expansion.

CONCLUSION

In this work, two modification strategies to introduce surface functional groups, oxygen doping and plasma treatment, were successfully applied.

Oxygen doping was evident from the colour change: dark orange of O-doped samples *versus* light yellow of pristine CN. The doping decreased crystallinity, increased porosity and reduced nitrogen content, probably due to heptazine ring opening and defect formation with oxygen atoms replacing nitrogen. This resulted in a narrower band gap and lower reduction and oxidation potentials, which were more pronounced in CN(5) than in CN(2). Mild DBD plasma increase in crystallinity and band gap narrowing, especially for CN(5).

Plasma treatment increased the oxygen content, probably by introducing oxygen-containing functional groups. In previously O-doped CN, existing defects facilitated the incorporation of new functional groups so that CN(2)-pt and especially CN(5)-pt had the highest oxygen content and acidic surface groups. However, except for CN-pt, the photocatalytic activity for Cr(VI) reduction under visible light did not improve. Although the band gap narrowing resulted in improved visible light absorption, it also resulted in a significant decrease in the reduction potential. This was obviously detrimental to the lower activity of the CN photocatalysts for Cr(VI) reduction. CN(5)-pt showed the lowest activity

under these conditions. Notably, the combination of doping and plasma treatment improved the functional group attachment and narrowed the band gap, offering potential for future optimization

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ИЗВОД
МОДИФИКОВАЊЕ КАРАКТЕРИСТИКА ПОВРШИНЕ И ФОТОКАТАЛИТИЧКЕ
АКТИВНОСТИ ЧИСТОГ И g-C₃N₄ ДОПИРАНОГ КИСЕОНИКОМ ПОМОЋУ
ПЛАЗМА ПОСТУПКА

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Графитни угљеник(IV)-нитрид (CN) представља полупроводник изграђен од неметала, који има широку примену у фотокатализи, укључујући и фотокаталитичку редукцију Cr(VI) под дејством видљивог зрачења. Како би се унапредила својства CN синтетисаног термичком полимеризацијом урее, примењене су две методе модификације: а) допирање кисеоником поступком ко-калцинације урее са две различите количине оксалне киселине, и б) плазма третман диелектричним баријерним пражњењем (енгл. *dielectric barrier discharge* (DBD)). Плазма третман је примењен на чист и на CN допиран кисеоником. За карактеризацију фотокатализатора коришћене су XRD, FTIR, FESEM, EDS, PL и DRS методе анализе, као и поступак за одређивање броја површински киселих функционалних група. Резултати су показали да је садржај кисеоника повећан у оба случаја: током допирања кисеоником долази до замене азота кисеоником у решетки, док током плазма третмана кисеоничне функционалне групе се везују на површини. Такође, плазма третманом претходно допираних узорака су функционалне групе које садрже O лакше везане због „отвореног“ хептазинског прстена. Допирање кисеоником је довело до сужавања забрањене зоне, које је било још више изражено након плазма третмана допираних узорака, при чему су смањени и оксидациони и редукциони потенцијал узорака. Иако је побољшана апсорпција видљивог зрачења, ово је допринело смањеној активности за фотокаталитичку редукцију Cr(VI). Најбољи резултати су постигнути са плазма третираним чистим CN, због повећаног садржаја функционалних група које садрже кисеоник што је допринело мањој брзини рекомбинације носилаца наелектрисања.

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Enhanced adsorption properties of Algerian bentonite modified by starch and glycerol for methylene blue and methyl red retention

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Abstract: This paper describes the preparation of novel bentonite-starch composites and assesses their effectiveness as adsorbents for removing methylene blue (MB) and methyl red (MR) dyes from aqueous solutions. The adsorbents were characterized using X-ray diffraction and FTIR spectroscopy. This study is aimed to optimize the removal process by investigating the effect of pH, adsorbent dose, contact time and initial concentration. The sorption kinetics of MB and MR dyes were analysed using the pseudo-first order and pseudo-second order models. The experimental results indicate that the pseudo-second order kinetic model provides the best fit. The composite adsorbents exhibited a sorption capacity for MB, ranging from 146.21 to 157.58 mg g⁻¹ for bentonite–starch (Bt@star) and bentonite–starch–glycerol (Bt@star@gly), respectively. The sorption capacity for MR dye was 426.38 mg g⁻¹ for Bt@star and 309.82 mg g⁻¹ for Bt@star@gly. Furthermore, the correlation coefficient values indicate that the adsorption of MB and MR by Bt@star@gly is best described by the Langmuir model, which unequivocally implies that the adsorbent surface is homogeneous, resulting in monolayer adsorption. The Langmuir model also accurately describes the adsorption of MB onto Bt@Star. However, the Freundlich isotherm model is the best fit for the adsorption of MR, indicating the existence of multilayer adsorption. Finally, this study demonstrates that the composite adsorbents prepared herewith exhibit excellent adsorption performance and can be a cost-effective alternative for treating coloured wastewater.

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INTRODUCTION

Organic dyes are synthetic compounds extensively used in industries of textiles, paper and plastics.¹ However, their stability and resistance to degradation make them persistent environmental pollutants.² When discharged into water bodies, these dyes can cause severe water pollution, affecting aquatic life and potentially entering the human food chain.³ The effective removal of organic dyes from wastewater is crucial to prevent environmental contamination and safeguard the public health.⁴

Recently, various methods and technologies have been proposed and utilized for treating wastewater contaminated with dyes. These methods include membrane filtration,⁵ biological treatment,⁶ oxidation, photocatalytic degradation,⁷ adsorption⁸ and coagulation-flocculation.⁹ Researchers widely use the adsorption method as an alternative for dye wastewater treatment.¹⁰ However, the efficiency of this process can be limited by the use of expensive adsorbents.¹¹

One of the most effective techniques for improving the adsorption process is to use various mineral or cement additives. These additives can be of natural origin, such as natural pozzolan, or artificial, such as lime or cement. They can also be mineral waste, such as silica fume, fly ash and so on. These materials exhibit distinct physicochemical and mineralogical characteristics.¹² Furthermore, low-cost materials, such as natural adsorbents, agricultural waste and by-products, have been proven effective in removing dyes.¹³ Clay minerals are considered as alternative materials to achieve this goal. According to many studies, bentonite clay can improve their properties in several fields.¹⁴ Bentonites are clay minerals, formed mainly by smectitic minerals in the form of lamellar silicates. In these minerals, their small particle size, less than 4 μm ,¹⁵ creates a large specific surface area and the presence of charge on the surfaces gives them unique physicochemical properties that allow them to attract substances from aqueous solutions. They are therefore widely used, among other important applications, to adsorb toxic compounds from aqueous media.¹⁶

However, it is a simple fact that clays have a low affinity for negatively charged anionic dyes. It is important to note that the adsorption capacities of clays can be significantly enhanced through a simple modification process using cationic polymers or surfactants. This can be achieved through straightforward ion-exchange reactions that create interactions between the cationic species and the adsorbate.¹⁷ Previous research has shown that using modified montmorillonite instead of raw montmorillonite greatly increases the effectiveness of acid dye removal.¹⁸ It is important to note that the synthesis of modified montmorillonites has already been reported and successfully used for the adsorption of Congo red dye.¹⁹ The study showed that the adsorption efficiency was significantly affected

by the length of alkyl chains in a series of alkyl ammonium bromides. Several authors have successfully modified montmorillonite using unconventional agents,²⁰ including gemini surfactants, to improve the adsorption of organic contaminants.²¹

This paper outlines a new approach for the modification of bentonite with starch and glycerol. The prepared composites were used as adsorbents to remove cationic (methylene blue) and anionic (methyl red) dyes. Various experimental parameters were examined to optimize the adsorption conditions. To assess the adsorption process, some kinetic studies were carried out.

EXPERIMENTAL

Materials

The material used in this study is the natural clay of Maghnia (Algeria), which is supplied by the ENOF company of bentonites.

One portion of the clay was used in the experiments, while the other was used for composite preparation. The procedure given for the preparation of the Bt@star@gly composite consists of mixing 5 g (5 %) bentonite in 45 mL of distilled water and then adding 0.75 g (30 %) glycerol and 0.125 g (5 %) starch to the suspension. On the other hand, the second composite (Bt@star) was prepared by the mixing of equal amounts by weight of bentonite and starch. The mixtures were then refluxed at a temperature of 80 °C for 4 h. After evaporating the water under reduced pressure, the resulting composites were manually ground and purified by stirring for 2 hours in distilled water to remove unreacted starting materials. Finally, the purified bio-composites were dried in an electric oven at 60 °C for 24 h.

The synthesized composites were evaluated for their mineralogical and structural properties using X-ray diffraction (XRD) with CuK α radiation ($\lambda = 1.54 \text{ \AA}$) at 40 kV and 30 mA, on a Rigaku Mini Flex 600. Fourier-transform infrared spectroscopy (FTIR) studies were conducted using the Agilent Cary 600 Series FTIR. The transmission spectra were obtained in the range of 4000–400 cm⁻¹.

Adsorption experiments

The batch equilibrium experiments were conducted to evaluate the adsorption capacities of the prepared composites for methylene blue (MB) and methyl red (MR). In each experiment, 25 mg of the composites were added to 25 mL of dye solutions with initial dye concentrations of 100 mg L⁻¹. The experiments were carried out in beakers with constant stirring at 400 rpm. The pH of the solution was adjusted within the range of 3 to 9 by the addition of drops of 1 M HCl or NaOH solutions. The contact time ranged from 5 to 120 min, while the initial dye concentration ranged from 100 to 500 mg L⁻¹. After adsorption, the adsorbent was separated from the liquid phase by centrifugation at 3000 rpm for 10 min. The supernatant was analysed using an Optizen 1412 UV/Vis spectrophotometer.

The adsorption capacity and removal efficiency were calculated from the initial and final concentrations of the dyes in the solution using the following equations:

$$q_e = (C_o - C_e)V/m \quad (1)$$

$$RE (\%) = 100(C_o - C_e)/C_o \quad (2)$$

where C_o and C_e denote the initial and equilibrium concentrations (mg L⁻¹) of the dye aqueous solution, V represents the volume of the dye solution (L) and m represents the mass (g) of the adsorbent used in each experiment.

RESULTS AND DISCUSSION

Adsorbents characterizations

Raw bentonite, Bt, Bt@star and Bt@star@gly adsorbent materials were characterized using XRD and FTIR techniques. The XRD diffractogram of raw bentonite, shown in Fig. 1a, indicates the presence of different diffraction peaks at 2θ 5.84, 15.7, 19.96, 21.0, 26.8, 35.1, 42.6, 46.0, 50.3, 54.8, 62.2, 68.3 and 73.3° , corresponding to different phases such as montmorillonite, quartz, calcite and magnesite.

These peaks were also observed in the biocomposites Bt@star and Bt@star@gly, with a single characteristic peak at 2θ $5\text{--}6^\circ$ indicating the basal spacing (d -spacing) of the silicate layers. The d -spacing for Bt was measured at 2θ 5.62° , with a value of 15.71 Å. The d -spacing for Bt@star was slightly shifted to a higher angle, 2θ 5.97° , with a value of 14.77 Å. This shift in the d -spacing suggests that the presence of starch polymer constricted the interlayer galleries of bentonite. It should also be noted that the chains of the polymer are mainly located on the outer surface of the bentonite clay. Angkawijaya *et al.* reported similar behaviour when modifying bentonite with chitosan.²²

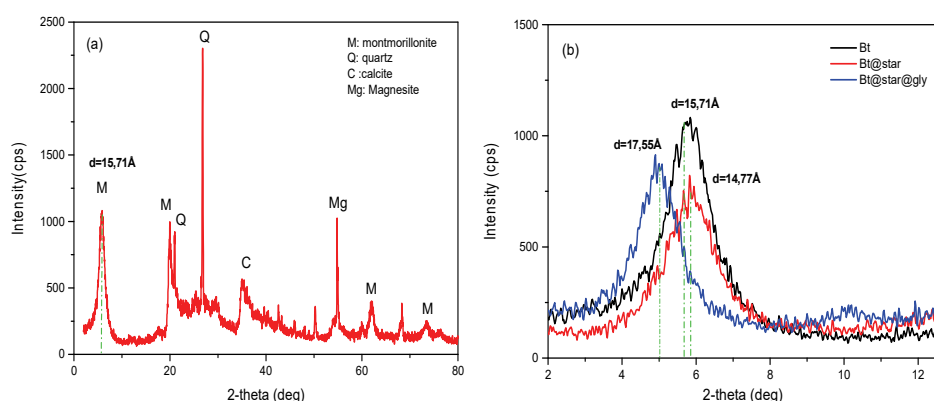


Fig. 1. XRD patterns of raw Bt (a); comparative d -basal spacing of Bt, Bt@star and Bt@star@gly (b).

Furthermore, as shown in Fig. 1b, the d -spacing for Bt@star@gly shifted to a lower angle of 2θ 5.03° with a value of 17.55 Å. This increase in the d -spacing value confirms the success of the modification and the presence of glycerol in the basal space. These findings are consistent with results obtained using other molecules and macromolecules to modify bentonite, such as hexadecyltrimethylammonium bromide, cationic surfactant cetyltrimethyl ammonium bromide, graphene oxide, gelatine and poly(*N*-vinylpyrrolidone).^{18,23–26}

The XRD results are also in agreement with FTIR analysis. Fig. 2 displays the characteristic bands of corn starch, raw bentonite Bt and the biocomposites Bt@Star and Bt@Star@glyc.

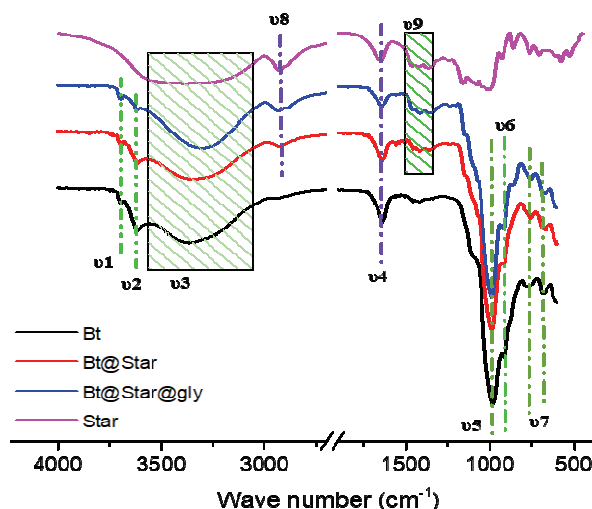


Fig. 2. FTIR spectra of the adsorbent materials.

The bands (v1 and v2) of raw bentonite at 3625 and 3701 cm^{-1} are caused by the vibrations of the OH groups of the water molecules adsorbed on the surface of the sample Bt. A broad absorption envelope (v3) was observed in the range from 3537 to 2987 cm^{-1} , which is attributed to the OH^- vibration of physically adsorbed water. Additionally, the bending mode of water was observed at 1635 cm^{-1} (v4).²⁷ The IR spectrum of Bt indicates the presence of the intense band (v5) centered at 981.51 cm^{-1} , which is related to the stretching vibration of the Si–O bonds. The 922 cm^{-1} (v6) band reveals the presence of amorphous SiO_2 .²⁸ Moreover, the bands at 688 and 775 cm^{-1} (v7) are attributed to the Al–O–Si–O bond and silanol group, respectively.

The FTIR spectra of Bt@Star and Bt@Star@glyc unquestionably exhibited a profile similar to that of raw bentonite. In addition, the spectra confirm the successful modification with the emergence of new bands at 2930 (v8) and around 1400 cm^{-1} (v9). These bands are attributed to the stretching vibration of the C–H bonds and the symmetric deformation of CH_2 groups and asymmetric stretching of C–H bonds.²⁹ The Bt@Star spectrum and the Bt@Star@glyc spectrum are clearly distinguishable. The two bands in question are more prominent in the Bt@Star@glyc composite. This is due to the presence of similar absorption bands in the glycerol spectra.³⁰ This observation unequivocally confirms the fixation of glycerol in the bentonite lattice, as previously demonstrated by XRD.

Methylene blue and methyl red dyes removal

Effect of pH. Numerous studies have shown that pH is a critical parameter in the adsorption process, as it affects both the functional groups of the adsorbent and the adsorbate. Therefore, this study investigated the removal of MB and MR dyes by native starch (Star), raw bentonite (Bt) and the composites Bt@Star and Bt@Star@glyc at three different pH values: 3, 6 and 9, using HCl (1 mol L⁻¹) and NaOH (1 mol L⁻¹). The results are presented in Fig. 3.

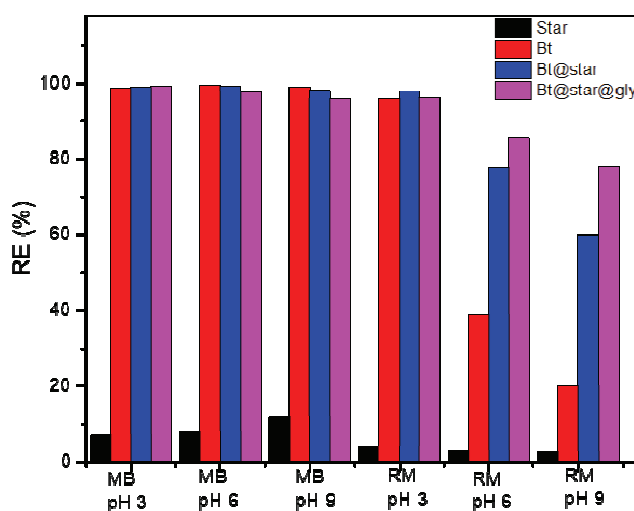


Fig. 3. MB and MR adsorption efficiency RE at different pH for Star, Bt, Bt@star and Bt@star@gly. [MB] = 100 mg L⁻¹, [MR] = 100 mg L⁻¹, V_{MB} and V_{MR} = 25 mL, m_{ads} = 25 mg, t = 60 min, T = 20 °C, 400 rpm.

Upon comparing the four adsorbents, the experimental data clearly show that the native corn starch has the lowest retention capacity, making it unsuitable for MB and MR retention.

The results for MB dye clearly show that Bt, Bt@star and Bt@Star@gly have a significant and similar retention capacity (*RE*) at both pH 3 and 6 (Fig. 3). At pH 3, the *RE* is 98.53, 98.99 and 99.19 % for Bt, Bt@star and Bt@Star@gly, respectively. At pH 6, the *RE* is 99.46, 99.23 and 97.53 % for Bt, Bt@star and Bt@Star@gly, respectively.

The retention efficiency is also very significant for the MR dye at pH 6. The value increased when starch and glycerol modifiers were added to the raw bentonite. The values were 95.95 % for Bt, 97.73 % for Bt@star and 96.19 % for Bt@Star@gly. The results clearly show that the addition of starch and glycerol has no negative effect on the adsorption capacity of the bentonite portion for the dyes.

The result for MR at pH 6 is undoubtedly the most important for the part in question. Fig. 3 clearly shows a significant increase in *RE* from 38.74 % for Bt to 77.71 % for Bt@Star and 85.58 % for Bt@Star@gly. This result definitively confirms the crucial role of modifying Bt with starch and glycerol, which creates a structure that interacts more effectively with cationic (MB) and anionic (MR) dyes.

In contrast, it is clear that the retention efficiency of MR dye consistently decreases for all adsorbent materials as the pH varies from 3 to 6 and then to 9. This can be mainly explained by the partial deprotonation of the adsorbent surface, which gives rise to a negative charge that prevents the interaction with MR molecules that already carry a negative charge specific to their carboxylate groups.³¹

Effect of contact time

Fig. 4a and b show the effect of contact time on the adsorption capacity of MB and MR dyes. In these experiments, 25 mg of each adsorbent was added to 25 ml of the dye's solutions with a concentration of 100 mg L⁻¹.

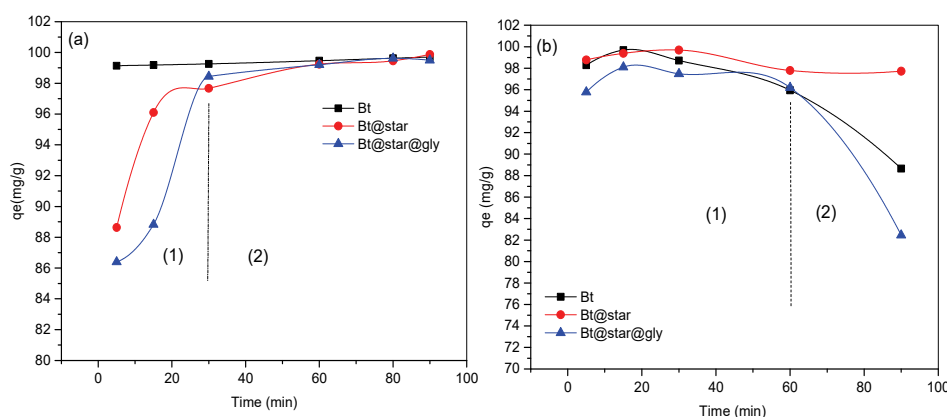


Fig. 4. Time-dependent q_e (mg g⁻¹) of MB (a) and MR (b) removed by Bt, Bt@star and Bt@star@gly. [MB] = 100 mg L⁻¹, [MR] = 100 mg L⁻¹, V_{MB} and V_{MR} = 25 mL, m_{ads} = 25 mg, pH 6, T = 20 °C, 400 rpm.

As shown in Fig. 4a, the adsorption of MB on the adsorbents Bt, Bt@star and Bt@star@gly is a fast process which can be divided into two distinct phases for the two composites. The first phase lasts for 30 min. During this time, the majority of the MB molecules are fixed on the surface of the adsorbents, then diffuse into the pores and the basal space of the biocomposites at a significant adsorption rate. In the second phase, the saturation of the active sites of the adsorbents resulted in a significant reduction in adsorption rate, with only a few molecules being adsorbed.

After 80 min of contact between the adsorbent and the adsorbate (second phase), the adsorption of MB reached a plateau, indicating that equilibrium had been reached. The presence of the plateau proves that the adsorption of MB has reached equilibrium under the considered experimental conditions and also proves that the adsorption rate is balanced by the desorption rate.

Fig. 4b clearly shows that the adsorption of MR on the used adsorbent materials is rapid. Fig. 4 also demonstrates that the retention of MR is highly efficient, with a q_e value of more than 95 mg g^{-1} in only 5 min of contact time. Fig. 4b also shows a contrasting reverse behaviour to that of Fig. 4a, with minimal fluctuation in retention capacity from 5 to 60 min (Part 1), then a gradual desorption of the adsorbed MR molecules on Bt@star and Bt@star@Gly (Part 2).

The experimental data were fitted with pseudo-first-order and pseudo-second-order models using Eqs. (3) and (4):

$$\text{Pseudo-first-order equation: } \log(q_e - q_t) = \log q_e - \frac{K_1}{2.303} t \quad (3)$$

$$\text{Pseudo-second-order equation: } \frac{t}{q_t} = \frac{1}{K_2 q_e^2} + \frac{1}{q_e} t \quad (4)$$

where $K_1 \text{ (min}^{-1}\text{)}$ is the pseudo-first order adsorption rate constant, $K_2 \text{ (g mg}^{-1} \text{ min}^{-1}\text{)}$ is the pseudo-second order adsorption rate constant and $q_e \text{ (mg g}^{-1}\text{)}$ is the adsorption capacity at equilibrium. These constants were determined from the slopes and intercepts of the lines obtained by plotting t/q_t versus t .

The graphical representations of this model are represented in Fig. 5 and the kinetic parameters obtained from the fitting are given in Table I. The comparison between calculated adsorption capacities $q_{e,\text{cal}}$ and experimental $q_{e,\text{exp}}$ values for each dye clearly suggests that the pseudo second order model describes the adsorption of MB and MR better. Similar results were reported by Mohammedi *et al.*^{32–34}

In addition, the correlation coefficients (R^2) confirm that the adsorption of MB and MR on each adsorbent studied is described by the pseudo-second order model, indicating a multistage adsorption process. The adsorption rate is therefore dependent on the number of active sites in the adsorbent material. These results are consistent with the previous studies, which also found that the adsorption of methylene blue and methyl red on various materials follows the pseudo-second order model.^{35–37}

Effect of MB and MR dose on the adsorption performance and isotherm study. The effect of initial dye concentration was investigated in the range of 100 to 500 mg L^{-1} . Fig. 6a–c show that the amount of dye retained by each adsorbent increases with increasing initial dye concentration. For an initial concentration of MB equal to 500 mg L^{-1} , the adsorption capacity achieved was 203.78 mg g^{-1}

($RE = 40.75\%$), 146.21 mg g^{-1} ($RE = 29.24\%$) and 157.58 mg g^{-1} ($RE = 31.51\%$) for Bt, Bt@star and Bt@star@gly, respectively. The MR dye q_e values are substantial, proving that the three materials are excellent adsorbents for removing both anionic and cationic dyes. The obtained adsorption capacities are equal to 309.81 mg g^{-1} ($RE = 61.96\%$), 426.38 mg g^{-1} ($RE = 85.27\%$) and 309.82 mg g^{-1} ($RE = 61.96\%$) for Bt, Bt@star and Bt@star@gly, respectively. To compare, the Table II clearly shows that the composites used in this study have better adsorption capacities ($q_{\max}$) than many other materials reported so far in the literature.^{29,38–45}

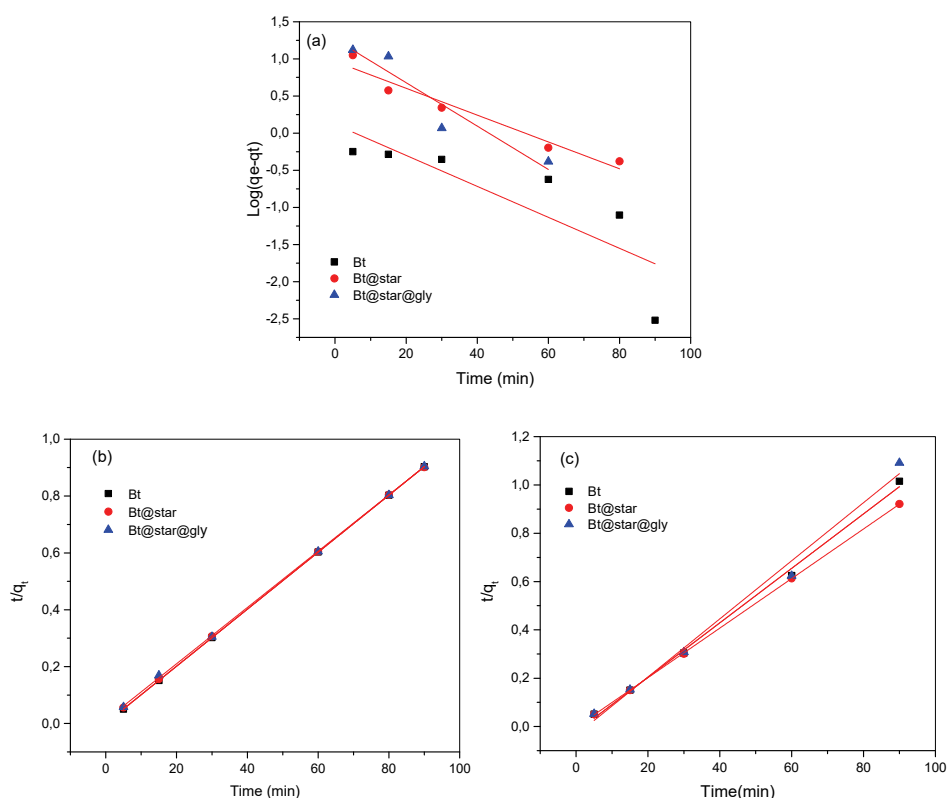


Fig. 5. Linear fit by using the pseudo-first order model of the retention of MB (a) and the linear fit by using pseudo-second order model for the retention of MB (b) and MR (c).

TABLE I. Characteristic parameters obtained by pseudo-first order; pseudo-second order

Dye	Material	$q_{e,\text{exp}}$ mg g^{-1}	1 st order			2 nd order		
			K_1 / min^{-1}	$q_{e,\text{cal}} / \text{mg g}^{-1}$	R^2	$K_2 / \text{mg g}^{-1} \text{min}^{-1}$	$q_{e,\text{cal}} / \text{mg g}^{-1}$	R^2
MB	Bt	99.69	0.0479	1.31	0.703	0.1137	99.70	1
	Bt@star	99.69	0.0415	9.22	0.950	0.0134	100.50	0.999
	Bt@star@gly	99.87	0.067	18.34	0.904	$8.06 \cdot 10^{-3}$	101.01	0.9998

TABLE I. Continued

Dye	Material	$q_{e,exp}$ mg g ⁻¹	1 st order			2 nd order		
			K_1 / min ⁻¹	$q_{e,cal}$ / mg g ⁻¹	R^2	K_2 / mg g ⁻¹ min ⁻¹	$q_{e,cal}$ / mg g ⁻¹	R^2
MR	Bt	99.69	Not applicable			0.0113	88.65	0,9961
	Bt@star	99.61	Not applicable			0.0103	97.46	0,9999
	Bt@star@gly	98.09	Not applicable			0.0119	83.33	0,9901

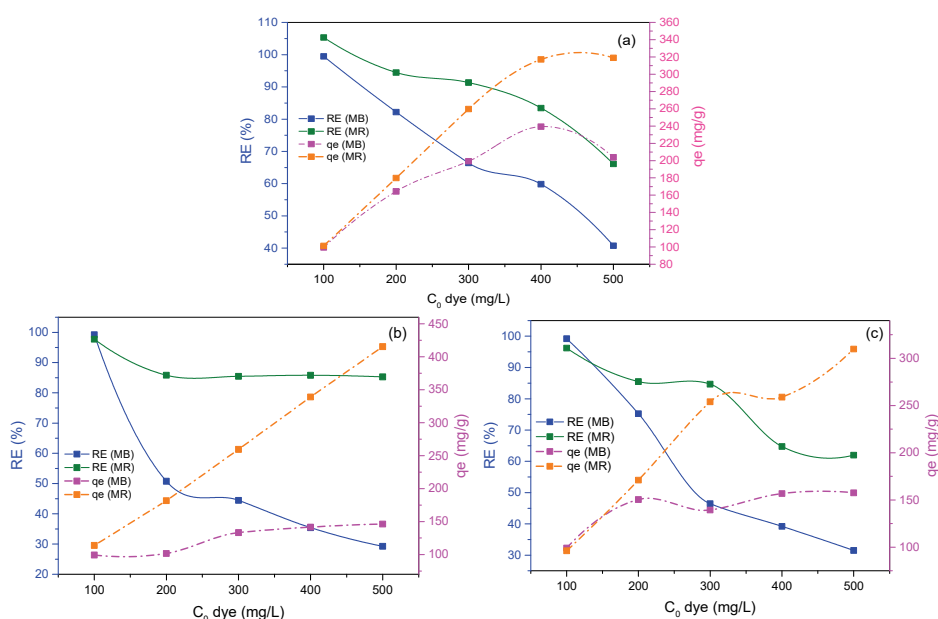


Fig. 6. MB and MR removal capacity (q_e) and removal efficiency (RE) as a function of the initial dye concentration by using: a) Bt, b) Bt@star and c) Bt@star@gly. V_{MB} and V_{MR} = 25 mL, m_{ads} = 25 mg, pH 6, T = 20 °C, 400 rpm, t = 60 min.

In order to obtain information about the adsorption isotherms, the experimental data were fitted to the Langmuir and Freundlich adsorption models using Eqs. (5) and (6), respectively. The curves obtained are shown in Figs. 7a–d:

$$\text{Langmuir equation: } \frac{C_e}{q_e} = \frac{1}{K_1 Q_{\max}} + \frac{C_e}{Q_{\max}} \quad (5)$$

$$\text{Freundlich equation: } \log q_e = \log K_F + \frac{1}{n} \log C_e \quad (6)$$

where K_1 is the Langmuir constant (L mg⁻¹), $Q_{\max}$ is the maximum adsorption capacity (mg g⁻¹), C_e the concentration at equilibrium (mg L⁻¹), K_F the Freundlich constant (mg g⁻¹) and n is the adsorption intensity.

Table III shows the values obtained for the constant parameters of the Langmuir and Freundlich adsorption isotherms. The correlation coefficient values clearly

TABLE II. Maximal adsorption capacity $q_{\max}$ for MB and MR removal by different materials

Dye	Material	$q_{\max} / \text{mg g}^{-1}$	Reference
MB	Hydroxyapatite@starch composite	45.51	29
	Calcined hydroxyapatite	38.93	37
	Biogas plant waste	147	38
	Starch biocryogel	34.84	39
	Diatomite	66.7	40
	Montmorillonite modified tea waste biochar	27.89	41
	Bt	203.78	This work
	Bt@star	146.21	
	Bt@star@gly	157.58	
MR	Biogas plant waste	115	38
	Bark of Hopbush	36.64	42
	Orange peel	111.11	43
	Carbon clay/alginate membrane	248.14	44
	Anionic surfactant	53.59	45
	Bt	309.81	This work
	Bt@star	426.38	
	Bt@star@gly	309.82	

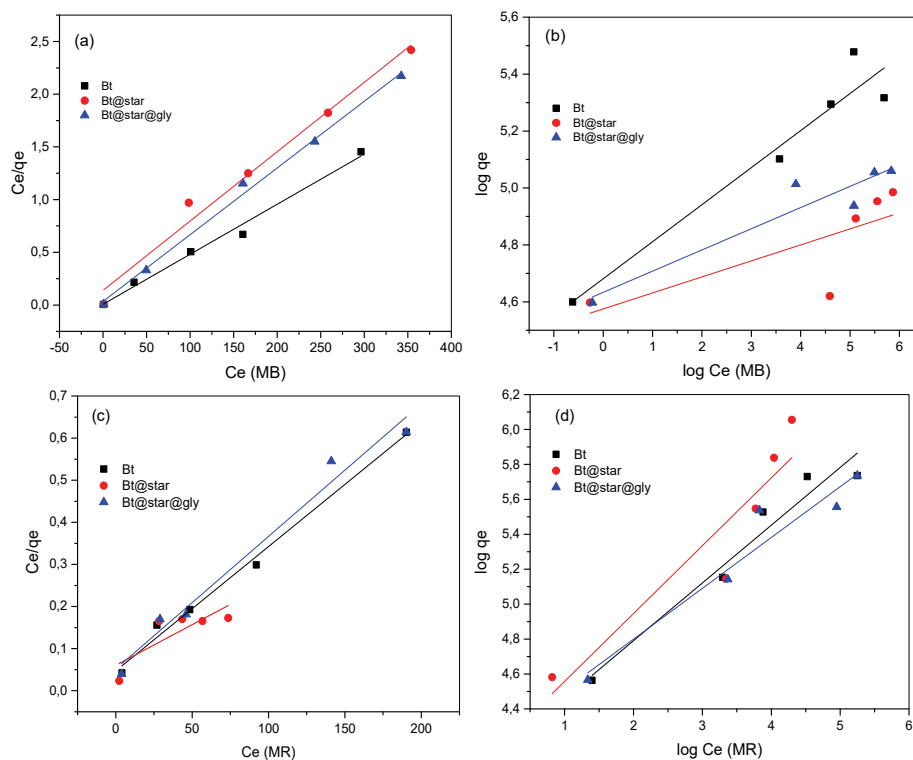


Fig. 7. Linear fits of the experimental data of MB (a, b) and MR (c, d) by using Langmuir and Freundlich isotherm models, respectively.

indicate that the adsorption of MB and MR dyes onto Bt and Bt@star@gly is better described by the Langmuir model. The applicability of the Langmuir model suggests that the adsorbent surface is homogeneous, resulting in the monolayer adsorption. This unequivocally demonstrates that all the binding sites present in Bt and Bt@star@gly are energetically equivalent.

TABLE III. Isotherm parameters for MB and MR adsorption by Bt, Bt@star and Bt@star@gly samples according to Langmuir and Freundlich models

Material	Dye	Freundlich			Langmuir		
		n	K_F	R^2	$q_{\max} / \text{mg g}^{-1}$	K_L	R^2
Bt	MB	7.6863	107.8994	0.909	210.97	0.661	0.986
	MR	3.0257	62.1592	0.938	338.98	0.063	0.989
Bt@star	MB	17.7935	97.0474	0.441	151.98	0.051	0.978
	MR	2.5753	64.6831	0.827	518.13	0.032	0.543
Bt@star@gly	MB	13.4408	102.8734	0.874	157.98	0.184	0.994
	MR	3.4376	67.8771	0.913	317.46	0.061	0.973

The Langmuir model also describes the adsorption of MB by the composite material Bt@Star. Nevertheless, the Freundlich isotherm model is the best fit for the adsorption of MR, indicating the existence of multilayer adsorption. This explains the important adsorption capacity of 426.38 mg g^{-1} at an initial MR concentration of 500 mg L^{-1} .

CONCLUSION

The present investigation showed the significant results regarding the adsorption of methylene blue and methyl red using bentonite and its composites. The described modification significantly improves the adsorptive capacity of bentonite, making these composites highly effective adsorbents for anionic dyes. Furthermore, the study proves that using readily available natural materials is the most effective way to minimize both starting material costs and experimental expenses. The application of adsorption on modified bentonite is undoubtedly a promising approach for the removal of pollutants from water.

ИЗВОД

ПОБОЉШАНА СВОЈСТВА АДОРПЦИЈЕ АЛЖИРСКОГ БЕНТОНИТА МОДИФИКОВАНОГ СКРОБОМ И ГЛИЦЕРОЛОМ ЗА ЗАДРЖАВАЊЕ МЕТИЛЕНСКОГ ПЛАВОГ И МЕТИЛ-ЦРВЕНОГ

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У раду је описана припрема нових композита бентонит–скроб и оцењена је њихова ефикасност као адсорбенса за уклањање боја метиленског плавог (МВ) и метил-црвеног

(MR) из водених раствора. Адсорбенси су окарактерисани коришћењем рендгенске дифракције и FTIR спектроскопије. Студија је имала за циљ да оптимизује процес уклањања боја испитивањем утицаја рН, дозе адсорбенса, времена контакта и почетне концентрације. Кинетика сорпције МВ и MR боје је анализирана коришћењем модела псеудо-првог и псеудо-другог реда. Експериментални резултати показују да кинетички модел псеудо-другог реда најбоље одговара. Композитни адсорбенси су показали сорпциони капацитет за МВ у распону од 146,21 до 157,58 mg g⁻¹ за бентонит–скроб (Bt@star) и бентонит–скроб–глицерол (Bt@star@gly), редом. Капацитет сорпције за MR боју био је 426,38 mg g⁻¹ за Bt@star и 309,82 mg g⁻¹ за Bt@star@gly. Штавише, вредности коефицијента корелације показују да се адсорпција МВ и MR од стране Bt@star@gly најбоље описује Ленгмировим моделом. Ово недвосмислено имплицира да је површина адсорбенса хомогена, што резултира једнослојном адсорпцијом. Ленгмир модел такође тачно описује адсорпцију МВ на Bt@star. Међутим, Фројндлихов модел изотерме је најбољи за адсорпцију MR, што указује на постојање вишеслојне адсорпције. Коначно, ова студија показује да композитни адсорбенти који су овде припремљени показују одличне резултате адсорпције и могу бити исплатива алтернатива за третман обојене отпадне воде.

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Biosorptive removal of cobalt (II) ion from wastewater using pomegranate peel activated carbon as biosorbent

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Abstract: Cobalt is used to link the components during integrated circuits (ICs) fabrication. Cobalt ion is common in chemical mechanical planarization (CMP) spent slurry. The role of cobalt is indispensable in the semiconductor industry and its presence in the wastewater is inevitable. Cobalt metal ions are toxic heavy metals that can cause serious health issues such as heart disease, nausea, vision sterility, thyroid damage, bone defects and diarrhea. This work investigates the potential application of *Punica granatum* (pomegranate) peel-activated carbon (PPAC) as an adsorbent for cobalt metal ion removal by adsorption. The work also examines the influence of initial cobalt ion concentration, pH, contact duration and adsorbent dose on the removal efficiency (*RE*) and adsorption capacity (*AC*) of Co^{2+} and the results of the findings are discussed. The Co(II) adsorption kinetic study revealed that a pseudo-2nd-order is best fitted with a rate constant of $\sim 0.00358 \text{ g mg}^{-1} \text{ min}^{-1}$. The adsorbent utilized in this work was assessed using SEM, EDAX, FTIR, XRD and TGA. EDX composition of post-adsorbent usage showed the presence of cobalt. Cobalt ion adsorption onto the PPAC was best fitted with the Freundlich isotherm model. Co(II) adsorption was found to be endothermic based on the thermodynamic characteristics evaluated for the carbon. At ambient temperature ($\sim 303 \text{ K}$) and neutral pH, PPAC was found to have a maximum *AC* of $\sim 85 \text{ mg g}^{-1}$ with a *RE* of $\sim 90 \%$.

Keywords: semiconductor; adsorption capacity; Freundlich; pseudo-second order; Langmuir.

INTRODUCTION

Cobalt, copper, nickel, zinc and other heavy metal ions are frequently detected in waste effluent from mineral extraction, tanneries, as well as from the electronic, electroplating and petrochemical sectors. Since heavy metals cannot be broken down by nature, they often accumulate in living organisms and can

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cause several illnesses.¹ Heavy metal ion concentration in wastewater is significantly greater than those advised by environmental regulations. Several techniques are being adapted to extract these metal ions from industrial effluents before disposal. The semiconductor industry uses cobalt as interconnect material in the manufacturing of integrated circuits (IC). However, during the chemical mechanical planarization of cobalt, the spent slurry might contain the cobalt ions, which are discarded as waste.^{2,3} The allowable cobalt concentrations in drinking water, inland surface water and irrigation water are 0.01, 0.05 and 1 mg L⁻¹ respectively.^{4,5} A deficit in cobalt inhibits the production of new red blood cells, but excessive intake into the body can be extremely dangerous.⁶ Cobalt poisoning can result in blindness, deafness and critical illness like heart failure.⁶ Environmentalists are deeply concerned with the efficient removal of heavy metals before their release into the environment due to toxicity even at low dosages.⁷

Various treatment methods have been employed, including ion exchange through the membrane, filtration, adsorption, chemical precipitation and coagulation–flocculation to remove the cobalt metal ion. However, the adsorption process has been proven to be simpler, more advantageous and more efficient than other methods.⁸ As agricultural wastes are inexpensive and do not pollute the environment, agro-based adsorbents are preferred to remove heavy metals from aqueous solutions.

Several adsorbents such as hazelnut,¹ lemon peel,⁹ orange peel,⁶ black tea waste,¹⁰ rice husk¹¹ and eucalyptus-based¹² activated carbon have been reported for the exclusion of cobalt ions from wastewater. However, pomegranate peel (PP, *Punica granatum*) powder-based adsorbent has not been reported for the adsorption of cobalt yet. Pomegranate fruit is popular in both raw and processed forms and is consumed as juice, preserves and wine. The pomegranate skin can make as much as 30 % of the fruit overall, yet it is frequently thrown out as a leftover waste. Gallic acid, ellagic acid, flavone derivatives and hydroxycinnamic acids are present in PP.¹³ PP contains lignocellulosic waste and as a result, there has been an increase in research on valorization of this waste in recent years.¹⁴ Recently, adsorbents such as polypyrrole-modified carbon nanotubes¹⁵ and Ca(OH)₂ modified quartz rock particles¹⁶ were utilized to remove the cobalt metal ion from the wastewater. Those reported adsorbents were relatively expensive when compared with the proposed adsorbent. Nowadays, fruit peels are transformed into biosorbents to remove harmful pollutants, which would increase the economic worth of these inexpensive biowastes. The current study explores the possibility of using PP-activated carbon as an adsorbent to eliminate the cobalt metal ions from the simulated semiconductor wastewater.

EXPERIMENTAL

Chemicals

Analytical-grade chemicals were used in this work. Cobalt (II) sulphate heptahydrate, hydrochloric acid and potassium hydroxide were procured from Loba Chemie Pvt. Ltd in India. Cobalt sulphate solution pH was maintained using HCl or KOH, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ was used for the preparation of metal ion solution.

PP-activated carbon preparation

PP was gathered from the fruit market in Raipur, Chhattisgarh. Before the treatment, PPs were cleaned using DIW and dehydrated peel in a hot air oven at 80 °C for 48 h. The dehydrated peels were finely powdered with the help of a mixer grinder and sieved through 355-micron mesh. PP dried powder was thermally activated at 300 °C for 30 min. Eventually, the powder was washed with 0.1 M HCl solution several times until the pH reached 7. Finally, the thermally triggered carbon powder was dehydrated in a hot air oven for 30 min at 70 °C.

Characterization

The surface morphology of PP, PP-activated carbon (PPAC, pre-adsorption) and PPAC post-adsorption of Co (PPACC) were analyzed with Zeiss Evo-18 model scanning electron microscopy (SEM). Oxford, INCA 250 model EDS was deployed for the elemental analysis. Bruker alpha model FTIR was employed to reveal the surface functional group of PP, PPAC and PPACC and the surface area was examined using a Smart Sorb 92/93 model Brunauer–Emmett–Teller (BET) analyzer. Hitachi Nexta HTA 300 TGA was employed to understand the thermal performance of the adsorbent. The crystalline nature of the sample was determined using XRD of PAN Analytical 3kW X'pert, which emits $\text{CuK}\alpha$ radiation source ($\lambda = 0.015418 \text{ \AA nm}^{-1}$) with 2θ in the range between 20 and 90° (3.5° min⁻¹ scan rate). The point of zero charge (PZC) experimental procedure reported in the literature was adapted in this work.⁶ Thermally activated PP powder was used in this study.

Batch adsorption

Unless mentioned otherwise, all the adsorption batch experiments were executed at 25 °C, with 0.5 g adsorbent dosage in 100 ml metal ion solution of 500 ppm at pH 7 for 90 min contact duration. The effect of initial concentration (10–1000 ppm), pH (2–11), adsorbent dose (0.1–1 g) and contact time (15–150 min) on the *AC* and *RE* were studied. Metal ion solution with adsorbent was kept in a shaker for 90 min at a rate of 300 rpm. After filtration of the sample, the amount of the cobalt ion in the filtrate was measured using atomic absorption spectroscopy (AAS) of ECIL AAS4141 at 241 nm wavelength. All the trials were performed in triplicate and the average *AC* was stated with the standard deviation. The equation used for the calculation of *AC* and *RE* is presented in Eqs. S-1 and S-2 (Supplementary material to this paper).

RESULTS AND DISCUSSION

Characterization

TGA/DSC/DTA analysis. The thermal response of PP powder was explored employing differential thermogravimetry (DTG), differential scanning calorimetry (DSC) and thermogravimetry (TG) analyses in an N₂ environment from atmospheric temperature to 1000 °C with a warming rate of 10 °C min⁻¹ and the findings are presented in Fig. 1a. TG outcome reveals that the powder underwent

significant weight loss in the first 500 °C and remains almost constant thereafter. The initial stage of weight loss of ~8.6 % (*i.e.*, up to 100 °C) is ascribed to the exclusion of moisture content, while the later phase of weight loss of ~60.4 % is due to the breakdown of hemicellulose and lignin.¹⁷ The DSC peak shows that the thermal decomposition of hemicellulose and lignin in PP powder is an exothermic process.

FTIR analysis. Fig. 1b illustrates the FTIR spectra of PP, PPAC and PPACC used in this study. Cobalt ions adhere to the adsorbent surface through the active sites. Chemical functional groups *viz.*, amine, hydroxyl, carbonyl and amide group are liable for the adsorption. FTIR spectra of PP show a robust peak at 677, 944, 1523, 2326 and 3733 cm^{-1} that represents the O–H stretching of the phenolic group,¹⁸ symmetrical stretching of N–C=O group,¹⁹ symmetric stretch of –COO group,²⁰ O=C=O stretching of the carbon dioxide group²¹ and OH group,²² respectively. However, those peaks disappear in the PPAC sample due to the thermal treatment of PP. But there are two peaks, *viz.*, 1706 and 2928 cm^{-1} corresponding to the ketonic group²³ and –C–H of CH₂ (asymmetrical stretching) group,²⁴ respectively, in the PPAC sample which is responsible for the cobalt metal ion adsorption. Those peaks get suppressed in the PPACC sample due to the adsorption of the cobalt metal ion. A new peak at 2353 cm^{-1} ascribed to C–O of carboxylic acids, alcohols, esters and ethers is formed for the PPACC sample.²⁵

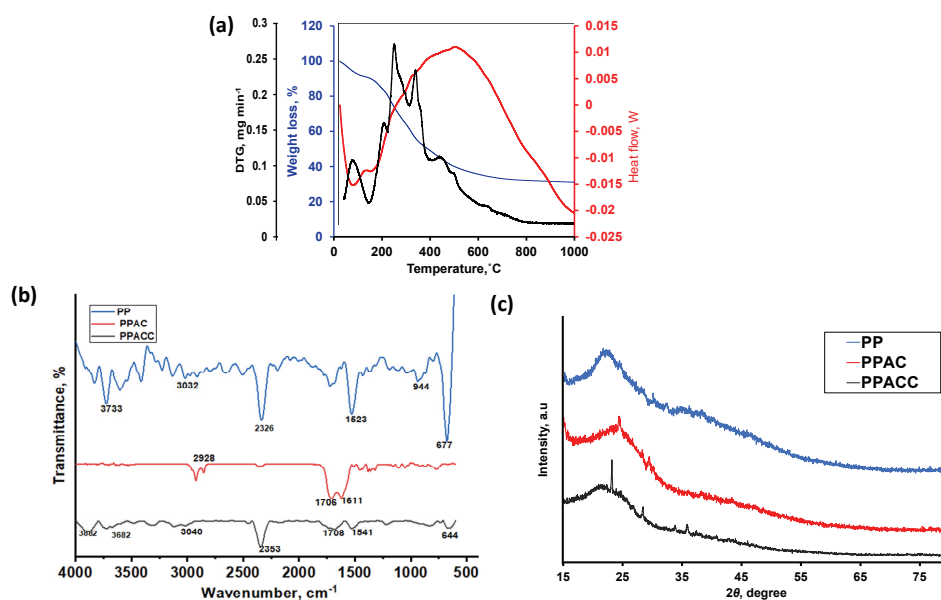


Fig. 1. a) TGA, DSC and DTG curve of PP; b) FTIR spectra of PP, PPAC and PPACC; c) X-ray diffraction spectra of PP, PPAC and PPACC.

XRD analysis. Fig. 1c shows the XRD spectra of PP, PPAC and PPACC. PP showed a broad peak at near 25° with lower intensity. However, the same peak becomes relatively less broad (*i.e.*, sharper) and slightly shifted toward the lower diffraction angle for the PPACC sample. All the XRD spectra revealed the presence of non-cellulosic *viz.*, hemicellulose and lignin in the PP and the absence of sharp diffraction peaks of crystalline cellulosic material.¹⁷ PPACC samples showed peaks of cobalt sulfate, which matches well with the reported literature.²⁶

BET surface area. The AC was found to escalate with a rise in the surface area, which plays a crucial role. PP, PPAC and PPACC samples were used to evaluate the surface area. PP sample claimed the least surface area of $0.298 \text{ m}^2 \text{ g}^{-1}$, while PPAC exhibited $4.056 \text{ m}^2 \text{ g}^{-1}$. The thermal activation method has increased almost 15 times the surface area when compared with the adsorbent before activation.^{27,28}

SEM and EDX. The surface morphology and the elemental composition of PP, PPAC and PPACC samples were probed using SEM and EDX respectively and the images and composition are presented in Fig. 2a–c. Literature suggests

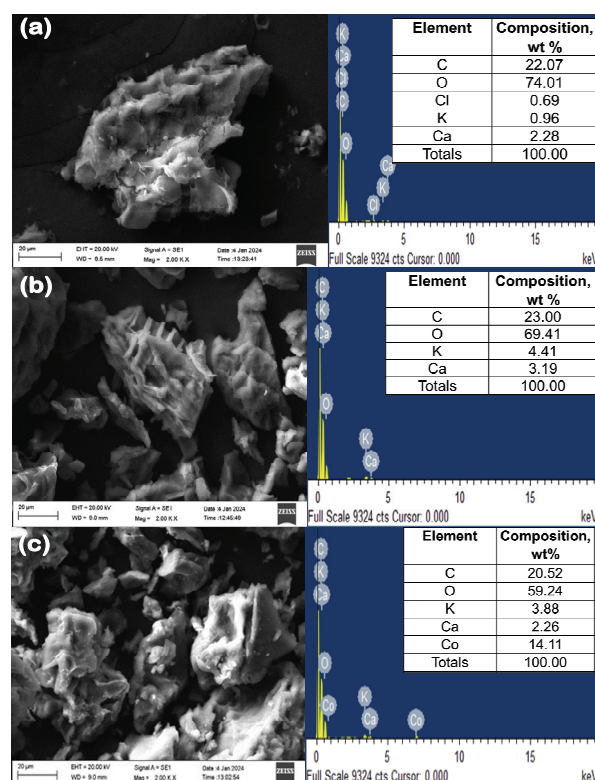


Fig. 2. SEM micrographs with EDAX analysis of (a) PP (b) PPAC and (c) PPACC.

that the PP sample contains lignin, crude fiber and other fluid substances and the fibers are stuck together.²⁹ PPACC samples exhibited the presence of cobalt, which confirmed the adsorption of cobalt on the adsorbent.

Point of zero charge (PZC). The pH dependence on the adsorption process can also be elucidated with the aid of the PZC result, which is presented in Fig. S-1 of the Supplementary material. PZC for PPAC was ~ 6 , which indicates that the surface charge is negative for $\text{pH} < 6$ and is positive for $\text{pH} > 6$. In the acidic pH, the surface gets protonated by OH, COOH and NH_2 groups, while in the basic pH, those functional groups get deprotonated form and render a negative charge to the surface.²⁷ The negative outer charge of the adsorbent enhances the electrostatic attraction of the positive cobalt ion.

Effect of concentration

The impact of cobalt ion concentration on *AC* and *RE* is depicted in Fig. 3a. It was observed that the *AC* escalated steadily with a rise in the metal ion concentration and reached $\sim 150 \text{ mg g}^{-1}$ and the highest *RE* of $\sim 98 \%$ was recorded at 10 mg L^{-1} . However, the *RE* was found to decline continuously with a rise in the metal ion strength. An analogous trend was described in the literature for the

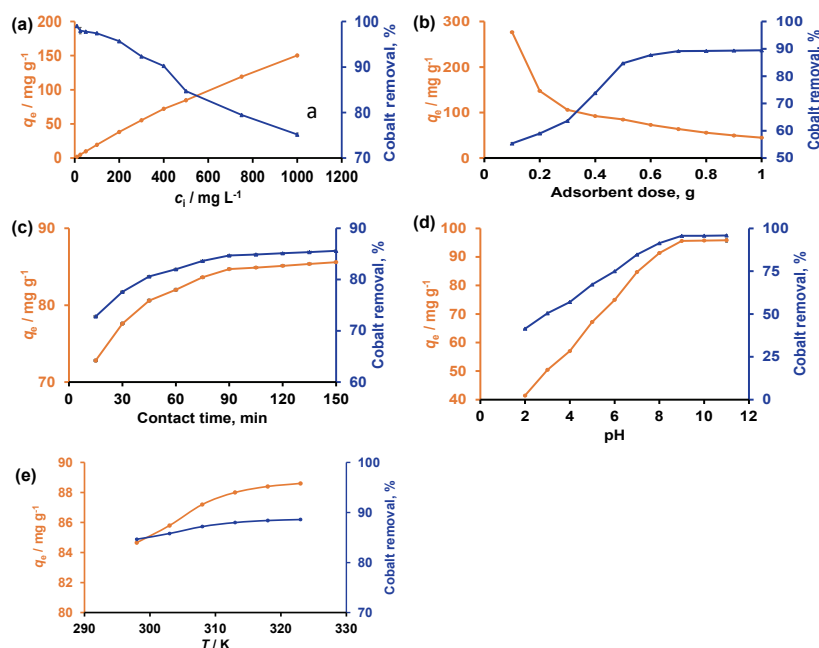


Fig. 3. Effect of: a) initial concentration, b) adsorbent dosage, c) contact duration, d) pH and e) temperature on the adsorption capacity (q_e) and cobalt ion removal efficiency using PPAC, C_i : 10 to 1000 mg L^{-1} , pH: 2 to 11, time: 15 to 150 min, T: 298 to 328 K, dosage: 0.1 to 1.0 g.

removal of chromium ions using PP as an adsorbent.³⁰ This could be accredited to the saturation of the active sites available on the adsorbent surface with cobalt ions, which limits the removal and hence reduces the overall removal efficiency.^{30,31} As the molar ratio between the cobalt metal ion and the adsorbent active sites is moderate at a lower initial concentration, the adsorption process happens quickly. If the molar ratio increases, the adsorption rate becomes lower, which ultimately reduces the *RE*, *i.e.*, leading to the exhaustion of the adsorbent active sites. Šoštarić *et al.* proposed the electrostatic repulsive force between the adsorbent and the adsorbate might also cause a decrease in the *RE*.³²

Effect of adsorbent dose

Fig. 3b shows the impact of PPAC dose on *AC* and *RE*. Cobalt *RE* increases from ~55 to ~90 % with a rise in the adsorbent dosage and then gets saturated. However, the *AC* decreases continuously with an escalation in the adsorbent dose. As the metal ions to the adsorbent active site ratio decreases with an increase in the dosage, the *AC* gets suppressed. However, the sites increase substantially with the adsorbent dose. As the active site number increases, the probability of the metal ion getting adsorbed on the surface increases, hence, the *RE* increases and finally gets saturated due to the limitation of the ions in the solution.³⁰ A similar trend was also reported by Anita *et al.* and explained the trend based on the accomplishment of the equilibrium.³³

Effect of contact time

Fig. 3c presents the influence of contact duration between the adsorbent and the cobalt metal ion on *AC* and *RE*. Both showed an increasing trend up to 90 min of contact time and then stabilized out. The *AC* enhanced from ~75 to ~85 mg g⁻¹ with an increase in the contact duration from 15 to 150 min. The *RE* increased from ~75 to ~85 % with the contact duration from 15 to 150 min. The adsorption of the cobalt metal ion on PPAC was found to be much faster in the initial stage and then gets saturated after the equilibrium time of 90 min is reached. The reason is that the Co(II) ions get adhered on the adsorbent surface immediately, as the active sites are more in number in the initial stages and get exhausted at the later stage.^{30,34,35}

Effect of pH

Fig. 3d elucidates the influence of pH on *q_e* and *R* using PPAC as an adsorbent. *AC* and *RE* are found to be higher on the alkaline side than on the acidic side. There is a steep rise in the value of both parameters from pH 5 to 9. In the lower pH, the H⁺ compete with the adsorbate for the active sites present on the adsorbent surface. However, the cobalt ion has relatively fewer chances to occupy the active sites when compared to the H⁺.¹³ *AC* and *RE* tend to increase with an increase in the pH¹³ and were found to be higher near the neutral pH.³⁶

Beyond pH 8, the cobalt solution tends to precipitate out due to the formation of cobalt hydroxide.¹ This was established by the color change in the solution from pink to blue.

Effect of temperature

The influence of temperature on the *AC* and *RE* of cobalt was found to be similar in magnitude and the results are shown in Fig. 3e. *AC* increases continuously with the temperature from ~85 to ~89 mg g⁻¹. This dismisses the possibility of the physisorption of metal ions. Lower *AC* at low temperatures might be due to challenges for the diffusion of metal ions into the pores. However, at the elevated temperature due to rapid collision, the ions are forced to enter the pores of the adsorbent.^{37,38}

Adsorption isotherms

Adsorption equilibrium isotherms provide insight relationship between q_e and equilibrium concentration (C_e). The aqueous phase adsorption process may be ascertained using a range of isotherms. Langmuir, Freundlich and Temkin isotherm models are very commonly used for adsorption studies⁶ and Eqs. S-3 to S-8 (Supplementary material) represents the linear and non-linear forms of isotherm. Langmuir adsorption is valid for homogeneous monolayer adsorption, while Freundlich isotherm is applicable for heterogeneous multilayer adsorption.³⁹ Temkin isotherm considers the heat of adsorption of all the molecules drops steadily with adsorption. Langmuir, Freundlich and Temkin adsorption isotherms are presented in Fig. 4 i-a–c, respectively, for the linear forms and in Fig. 4 ii-a–c for the non-linear forms. Fitting parameters of various isotherms for both linear and non-linear equations are tabulated in Table I. The best fit was obtained for the Freundlich isotherm, as the correlation coefficient value was found to be greater than the other two isotherms for both the linear and non-linear cases. This confirms that the adherence of metal ions on the adsorbent surface is heterogeneous with multilayer adsorption. Adsorption of the cobalt ion on the surface of the adsorbent is viable and favourable, as Langmuir separation factor (R_L) < 1 and $1/n$ < 1 in Freundlich isotherm.

Kinetic study

Kinetics studies were performed to evaluate the rate of adsorption, rate constant, order and rate controlling step. The impact of contact duration on *AC* and cobalt *RE* data was utilized for this study. Pseudo-first order (PFO), pseudo-second-order (PSO) and intra-particle kinetic diffusion equations are presented in Eqs. S-9 to Eq. S-11 (Supplementary material). All the models were mapped with the data and are shown in Fig. 5a–c. PSO model was found to be best fitted, as the correlation coefficient was found to be a higher value of 0.9998.³⁰ Kinetic fitting parameters were calculated using the slope and intercept from Fig. 5a–c

and are presented in Table II. The experimental and predicted value of AC is also reported in Table II. Experimental AC ($q_{e,exp}$ or q_e) is found to be comparable with the $q_{e,pred}$ for the PSO system. However, there exists a significant deviation in the adsorption values for the pseudo-first-order system. Chemisorption is the rate-limiting step, as the PSO system fits the data.⁴⁰

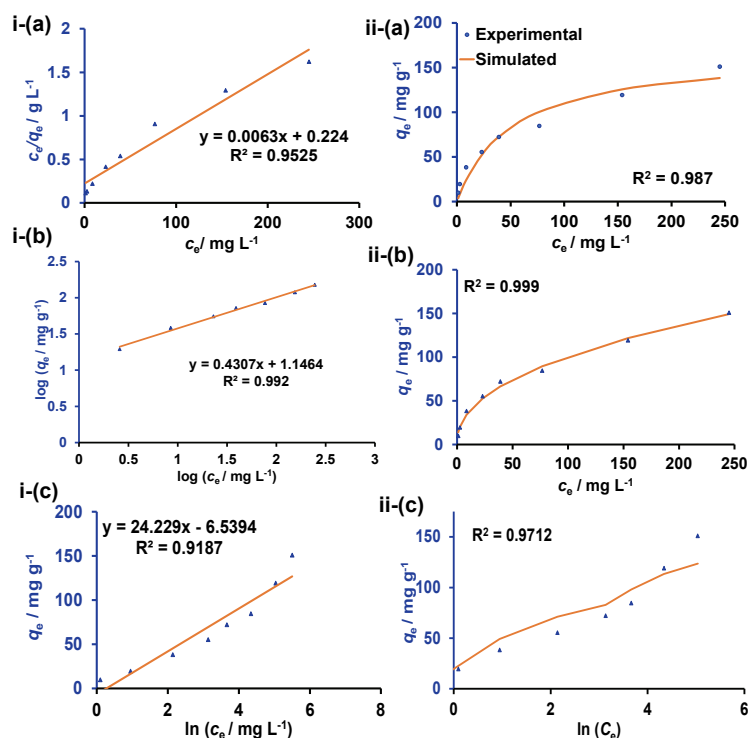


Fig. 4. i) Linearized plot and ii) non-linear plots of: a) Langmuir, b) Freundlich and c) Temkin adsorption isotherm models.

TABLE I. Parameters of various isotherms of Co(II) ion adsorption on PPAC

Isotherm	Parameter	Linear	Non-linear
Langmuir	$q_m / \text{mg g}^{-1}$	158.7	100.6
	K_L / mg^{-1}	0.0285	0.0040
	R^2	0.9525	0.9871
Freundlich	K_f / g^{-1}	14.8007	8.6837
	N	2.3218	0.5011
	R^2	0.992	0.999
Temkin	$A / \text{L g}^{-1}$	86.95	0.04
	$b / \text{J g mol}^{-1} \text{mg}^{-1}$	0.0035	116.23
	R^2	0.998	0.971

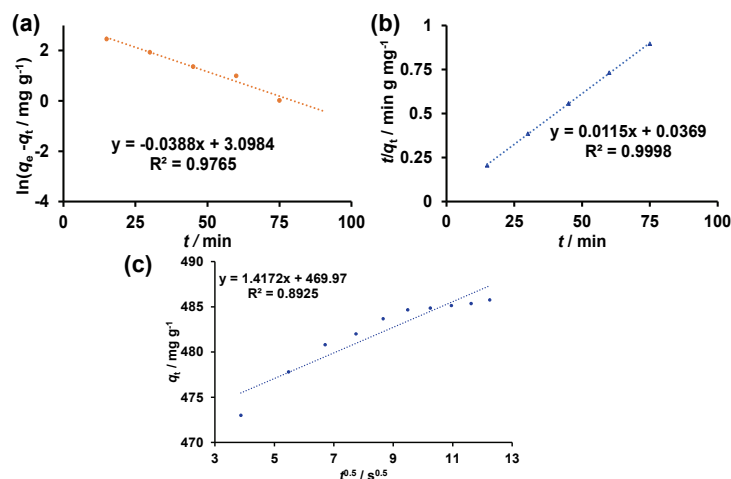


Fig. 5. Kinetics models: a) PFO, b) PSO and c) intra-particle diffusion model for cobalt adsorption on PPAC.

TABLE II. Kinetic parameters of various models for Co(II) ion adsorption on PPAC

Kinetic study	Parameter	Linear
PFO model	$q_{e, \text{exp}} / \text{mg g}^{-1}$	84.7000
	$q_{e, \text{pred}} / \text{mg g}^{-1}$	22.1600
	K_1 / min^{-1}	0.0388
	R^2	0.9765
PSO model	$q_{e, \text{exp}} / \text{mg g}^{-1}$	84.7000
	$q_{e, \text{pred}} / \text{mg g}^{-1}$	86.9500
	$K_2 / \text{g mg}^{-1} \text{min}^{-1}$	0.0035
	R^2	0.9998
Intra-particle diffusion model	$K_3 / \text{g mg}^{-1} \text{min}^{-1}$	1.4172
	R^2	0.8925

Thermodynamic parameters

Enthalpy (ΔH°) and entropy (ΔS°) changes were estimated from the Van't Hoff plot between $\ln k_c$ and $1/T$ and the plot is presented in Fig. 6. Equations used for determining the thermodynamic properties are provided in Eqs. S-12 to Eq. S-14 (Supplementary material). The positive value of ΔH° and ΔS° denotes the endothermic nature and a raise in the randomness respectively. Gibbs energy change (ΔG°) was estimated using the enthalpy and entropy values. The thermodynamic property provides a deep insight into the adsorption mechanism and the determined values are presented in Table III. A negative ΔG° value represents the spontaneity of the adsorption process.^{34,41,42}

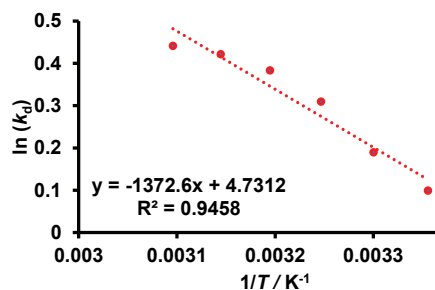


Fig. 6. Thermodynamic plot for the adsorption of Co(II) onto PPAC.

TABLE III. Thermodynamics parameter for cobalt ion adsorption on PPAC at different temperatures

C_i mg L ⁻¹	ΔH° kJ mol ⁻¹	ΔS° J mol ⁻¹ K ⁻¹	ΔG° / kJ mol ⁻¹					
			298 K	303 K	308 K	313 K	298 K	323 K
500	11.42	39	-0.31	500	11.42	39	-0.31	500

Comparative study

The *AC* of PPAC towards the cobalt ion is assessed with the various adsorbents stated in the literature and the summary is presented in Table IV. PPAC showed a higher *AC* of 85 mg g⁻¹ in comparison with most of the adsorbents reported in the literature. The proposed adsorbent follows pseudo-2nd order kinetics, Freundlich isotherm and the adsorption process is endothermic in nature. Kinetic study results reported in the present work match well with the other adsorbents utilized in the literature.^{1,6,7,9,10,11,12,38} While most of the literature reported the Langmuir adsorption isotherm, however few literatures have reported Freundlich and Dubinin–Radushkevich (D–R) model. Unlike the chemical activation or thermochemical activation process, the thermal activation process is less expensive. The yield obtained in this study was ~55 %, which may be limitation of this study, as the continuous column study requires more adsorbent.

TABLE IV. Assessment of adsorption capacity of various adsorbents specified in the literature and the pomegranate peel

Adsorbent	q_e mg g ⁻¹	Activation meth. ^a	Kinetic study ^b	Process ^c	Isotherm ^d	Ref.
Activated carbon hazelnut	13.88	TC	2 nd	Endo	L	1
Orange peel	5.128	C		Exo	L	6
Activated carbon	51	TC	1 st	Endo	L	7
Lemon peel	22	T		Exo	L	9
Black tea waste	13.77	TC	2 nd	–	L	10
Rice husk	35	TC		–	L	11
Eucalyptus	55.5	C		–	L	12
Rice husk	123	TC		Endo	D–R	42
Mesoporous carbon	1.59	TC		Exo	F	44
Macroalgae <i>Padina pavonica</i>	17.98	C		Exo	L	45

TABLE IV. Continued

Adsorbent	q_e mg g ⁻¹	Activation meth. ^a	Kinetic study ^b	Process ^c	Isotherm ^d	Ref.
Ca(OH) ₂ modified quartz rock particles	47.112	C	2 nd	Exo	L	16
Sodium borohydride	26.29	C		Exo	L	15
Pomegranate peel	85	T		Endo	F	This study

^aTC – thermochemical; C – chemical; T – thermal; ^b 1st – pseudo 1st order; 2nd – pseudo 2nd order; ^c Exo – exothermic; Endo – endothermic; ^d L – Langmuir; D-R – D-R model, F – Freundlich

CONCLUSION

Co(II) metal ions from simulated semiconductor industry wastewater were removed by adsorption on PPAC. Several factors *viz.*, inceptive metal ion concentration, temperature, dosage, pH and contact duration were varied to determine their effect on the AC. Adsorption of Co(II) ions on PPAC shadowed PSO kinetics. Freundlich isotherm fitted well for the adsorption data with linear/non-linear correlation coefficient of 0.992/0.99, respectively. Maximum cobalt ion adsorption of ~85 mg g⁻¹ was obtained at neutral pH without the formation of cobalt hydroxides. The evaluation of thermodynamic parameters for PPAC demonstrated that the adsorption of Co(II) ions was endothermic with a positive value of ΔH° , whereas the rise in degree of spontaneity is confirmed by a negative value of ΔG° and increase in disorder of the adsorption process is indicated by the positive value of ΔS° . PPAC, a low-cost adsorbent from agricultural waste, is proven for the exclusion of Co(II) ions from wastewater. The preparation of proposed adsorbent is simpler, cheaper and environmentally benign, as the pomegranate peel, thrown as waste material, was utilized. The desorption study of the spent PPACC, *i.e.*, regeneration of adsorbent can be considered as future research.

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/12938>, or from the corresponding author on request.

ИЗВОД

БИОСОРПТИВНО УКЛАЊАЊЕ ЈОНА КОБАЛТА(II) ИЗ ОТПАДНИХ ВОДА
КОРИШЋЕЊЕМ АКТИВНОГ УГЉА ОД КОРЕ НАРА КАО БИОСОРБЕНТА

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Кобалт се користи за повезивање компоненти током производње интегрисаних кола (IC). Јони кобалта се уобичајено налазе у истрошеној суспензији хемијско механичке планаризације (СМР). Улога кобалта је незаменљива у индустрији полупроводника и његово присуство у отпадним водама је неизбежно. Јони кобалта су токсични и могу изазвати озбиљне здравствене проблеме као што су болести срца, мучнина, оштећење

вида, оштећење штитне жлезде, дефекте костију и дијареју. Овај рад истражује потенцијалну примену активног угља добијеног од *Punica granatum* (нар, РРАС) као адсорбента за уклањање јона кобалта. У раду се испитује утицај почетне концентрације јона кобалта, рН, трајање контакта и доза адсорбента на ефикасност уклањања (*RE*) и адсорпциони капацитет (*AC*) Co^{2+} , и разматрају нађени резултати. Кинетичка студија адсорпције Co(II) показала је да се псеудо-други ред најбоље уклапа са константом брзине од $\sim 0,00358 \text{ g mg}^{-1} \text{ min}^{-1}$. Адсорбент који је коришћен у раду је процењен помоћу SEM, EDAX, FTIR, XRD и TGA. EDX састав узорка након адсорпције показао је присуство кобалта. Адсорпција јона кобалта на РРАС најбоље одговара моделу Freundlich изотерме. Утврђено је да је адсорпција Co(II) ендотермна, на основу термодинамичких карактеристика процењених за угљеник. На температури спољашње средине ($\sim 303 \text{ K}$) и неутралном рН, утврђено је да РРАС има максималну *AC* од $\sim 85 \text{ mg g}^{-1}$, са *RE* од $\sim 90 \%$.

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SUPPLEMENTARY MATERIAL TO
**Biosorptive removal of cobalt (II) ion from wastewater using
pomegranate peel activated carbon as biosorbent**

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Adsorption capacity was calculated using the Eq. S-1

$$q_e = \frac{C_i - C_e}{W} V \quad (\text{S-1})$$

q_e denotes the amount of Co(II) ions adsorbed at equilibrium per unit mass of the adsorbent (mg g⁻¹)

C_i represents the initial concentration of cobalt (II) ion (mg L⁻¹)

C_e represents the final concentration of cobalt (II) ions (mg L⁻¹)

V represents the volume of the solution (ml)

W represents the mass of PPAC (adsorbent) (g)

Cobalt metal ion removal efficiency (R) was calculated using Eq. S-2

$$R (\%) = \frac{C_i - C_e}{C_i} \times 100 \quad (\text{S-2})$$

ISOTHERM AND KINETIC MODEL

Langmuir Isotherm Model (linear form)

$$\frac{C_e}{q_e} = \frac{1}{q_m K_L} + \frac{C_e}{q_m} \quad (\text{S-3})$$

q_m denotes the maximum adsorption capacity (mg g⁻¹)

K_L represents the empirical constant corresponding to the affinity of the binding sites and the adsorption energy (L mg⁻¹)

Langmuir Isotherm Model (Non linear form)

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (\text{S-4a})$$

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Separation Factor

$$R_L = \frac{1}{1 + K_L C_1} \quad (S-4b)$$

R_L = Separation factor, which gives the information whether the adsorption is favourable or not. R_L ranging between 0 and 1 represents the feasibility of adsorption process.

Freundlich Isotherm Model (Linear form)

$$\log q_e = \log K_f + \frac{1}{n} \log C_e \quad (S-5)$$

K_f = Freundlich constant related to adsorption capacity (mg g⁻¹)

n = adsorption density parameter relating the degree of surface heterogeneity

Freundlich Isotherm Model (Nonlinear form)

$$q_e = K_f C_e^{\frac{1}{n}} \quad (S-6)$$

Temkin Isotherm Model (Linear form)

$$q_e = B \ln A + B \ln C_e \quad (S-7)$$

A = Temkin constant (L g⁻¹)

Temkin Isotherm Model (Non-linear form)

$$q_e = B \ln A C_e \quad (S-8a)$$

B refers to the heat of adsorption, which is defined below

$$B = \frac{RT}{b} \quad (S-8b)$$

b is the Temkin constant (J mol⁻¹).

KINETIC STUDY

Pseudo first order model

$$\ln(q_e - q_t) = \ln(q_e) - K_1 t \quad (S-9)$$

q_t = capacity of adsorption at a particular time ' t ' (mg g⁻¹)

K_1 = pseudo first order rate constant (min⁻¹)

Pseudo second order model

$$\frac{t}{q_t} = \frac{1}{K_2 q_e^2} + \frac{t}{q_e} \quad (S-10)$$

K_2 = pseudo second-order rate constant (g mg⁻¹ min⁻¹)

Intra particle diffusion model

$$q_t = K_{int} t^{0.5} \quad (S-11)$$

K_{int} = intra particle diffusion rate constant (mg g⁻¹ min^{-1/2})

Thermodynamic parameters were obtained from the following equation

$$\ln K_d = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (S-12)$$

$$K_d = \frac{q_e}{c_e} \quad (S-13)$$

$$\Delta G = \Delta H - T\Delta S \quad (S-14)$$

K_d = distribution coefficient (L g⁻¹)

ΔG = Gibbs free energy (kJ mol⁻¹)

ΔH = change in enthalpy (kJ mol⁻¹)

ΔS = change in entropy (kJ mol⁻¹ K⁻¹)

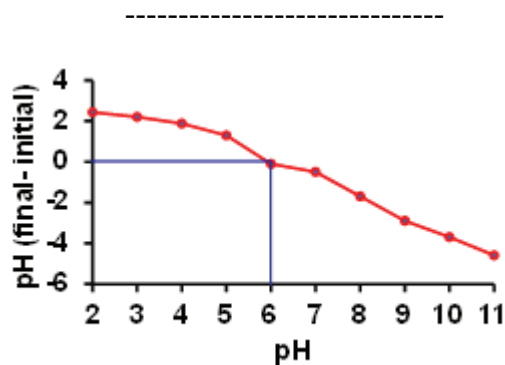


Fig. S1. Point of zero charge on PPAC using NaCl of 0.01 mol/L.