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Fractionation of complex mixtures of naphthenic acids, their characterization and biological activity

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Abstract: Naphthenic acids (NAs) are complex mixtures of cycloaliphatic and alkyl-substituted acyclic carboxylic acids, the overall characteristics of which are determined by the composition of the mixture. A complex mixture of NAs from a commercial fraction of atmospheric oil of the Vojvodina naphthenic crude oil “Velebit” (Serbia) was separated into narrower fractions based on their acidity. Electrospray ionization mass spectrometry analysis of the fractions showed the occurrence of structural differentiation of the acids. By extraction at pH 3–5, about 50 % of the total mass of acids, consisting predominantly of tricyclic and bicyclic structures, was separated. Acids of lower acidity, (about 22 %), separated at pH 9 and 10, and their dominant constituents were acids with three-, four- and five-membered rings. A correlation was found between the dominant structure and the biological activity of the NAs of the fractions. The fraction extracted at pH 8, also with dominant bicyclic and tricyclic structures, showed the highest auxin and gibberellin activities.

Keywords: naphthenic acids; fractionation; structural analysis; biological activity.

INTRODUCTION

Naphthenic acids (NAs) are carboxylic acids that are natural constituents of naphthenic oils.^{1–3} In the process of commercial oil fractionation, the NAs are distributed into fractions and, in some commercial processes, they have to be extracted since they lower the quality of the crude oil product. The amount of NAs commercially extracted in the USA in 1992 was estimated to be 5500–6000 t.^{4,5} About 80 % of the isolated NAs are transformed into their salts, primarily to cop-

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per naphthenates, which is used for wood protection. Besides this, naphthenates are used for the production of a variety of esters, amides, imidazolines and other derivatives that are widely used in the chemical industry. Due to this, but primarily because of the occurrence of NAs in oil sands process waters in Canada, there is a growing interest in the characterization, purification and application of NAs. This can be seen from the number of relevant papers with the key word “naphthenic acids”, especially since the year 2000, when the annual number of papers increased by about eight fold compared to the annual number before 2000.⁶

At high concentrations (above 50 mg L⁻¹), NAs are corrosive and toxic substances,^{4,7} and for these reasons they represent serious contaminants of refinery wastewaters, xenobiotics, and act as environmental pollutants.^{8–10} Low concentrations (up to 0.5 mg L⁻¹) of NAs and their salts have been studied for a long time as substances exhibiting biological activity, such as plant growth hormones,^{11–13} rooting agents^{14,15} or bactericides.¹⁶

Naphthenic acids represent complex mixtures of alkyl-substituted aliphatic and cyclic monocarboxylic acids of the general formula C_nH_{2n-z}O₂, where *n* is the number of carbon atoms and *z* the hydrogen deficiency due to ring formation. NAs may contain many individual structures. Despite of the advances in analytical techniques used to study NAs, a complete picture of the composition of these complex mixtures has yet to be obtained. In view of their complexity, it seems that this is an unattainable goal, although several recent advances have been made, including the identification of individual NA structures in both petroleum and oil sands by multidimensional comprehensive gas chromatography–mass spectrometry.^{17–19} Literature data indicate that NAs from different sources have different structural compositions and ranges of molecular masses, as well as different mass ratios of the acids involved, which determine their chemical and biochemical properties, such as, for example, toxicity.²⁰ One of the approaches to dealing with this problem is to separate a complex NA mixture into narrower fractions according to their particular characteristics, determine the composition of these narrower fractions and their properties, and then correlate these properties to their chemical structures. Frank *et al.*²¹ separated an NA into narrower fractions by fractional distillation of the methyl esters and showed that they differed slightly in respect to their toxicity. Based on these results, the authors established a quantitative structure–activity relationship that attempted to predict the toxicity of NAs.²² Using five NA samples differing in total acid number, Ding-Rong *et al.*²³ attempted to establish a correlation between corrosivity and chemical structure and found that NAs with lower molecular weight and fewer ring structures usually were more corrosive. Apart from fractional distillation, NAs can be also separated in some other ways. Niemi *et al.*²⁴ developed a method of NAs fractionation by supercritical fluid extraction and tested the effects of different fractions on wood decay. Extraction and fractionation of petroleum NAs with solid media

was also performed on silica deposited with KOH, by which NA fractions of different acidity could be recovered from crude oils.²⁵ Separation of the acid components of crude oil on anionic ion-exchange resins resulted in carboxylic acids with a molecular mass distribution that differed from that of the normal alkanes in the crude oil.²⁶ Ashumov *et al.*²⁷ reported fractionation of NA mixtures by stepwise degradation of their soaps. The naphthenic acids were saponified with sodium hydroxide and then the soaps were decomposed gradually with HCl into different fractions.

Naphthenic acids are mainly weak acids with pK_a values in the range from 5 to 6.²⁸ They dissolve well in organic solvents, whereas their solubility in water depends on the pH. In a very acidic medium, when the NAs are present in the protonated form, they are practically insoluble, whereas in a very basic medium, they dissolve in the water as their salts. Headley *et al.*²⁸ showed that the mass profiles of water-soluble NAs differed significantly depending on the pH of the medium. This fact indicates that different classes of these acids are dissolved at different pH values. Based on this study,²⁸ and knowing the properties of NAs, such as their solubility in aqueous media and their pK_a values, it can be supposed that it is possible to fractionate a mixture of NAs by dissolving them as salts at a high pH and then, by addition of a strong mineral acid, achieve a successive and selective separation of the water-insoluble acids, which can then be readily extracted with an organic solvent.

The aim of the presented work was to investigate the possibility of fractionation of NAs based on their solubility in aqueous media of given pH values and then characterize the obtained fractions and study their biological activity of the type of the plant hormones auxin and gibberellin. These tests were realized in an attempt to explain the effect of these fractions on plant rooting, observed in a previous study.²⁸

EXPERIMENTAL

General methods

All employed chemicals were purchased from Fluka.

Naphthenic acids were isolated from an atmospheric oil fraction (distillation interval 168–290 °C) of Vojvodina crude oil “Velebit“ by the optimized procedure of alkaline extraction.²⁹ Further purification was performed by triple repeated alkaline extraction and drying with anhydrous Na_2SO_4 . The average molecular mass of the naphthenic acids was determined²⁹ (ASTM D3238) to be 262, and this value was used in the further experiments.

Fractionation of NAs

The mixture of NAs (1.00 g, 3.82 mmol) was mixed with distilled water (300 mL) at room temperature and then 5 % NaOH solution (≈ 10 mL) was added under constant stirring until pH 11 was attained. After complete dissolution of the NAs, 5 % H_2SO_4 was added in small portions (1 mL) under cooling and constant stirring. When the pH had decreased by one unit, the insoluble NAs, separated on the top of the solution, were extracted three times with diethyl ether (30.0, 30.0 and 20.0 mL). Successive addition of acid and lowering of the pH by

one unit was continued until pH 2 was achieved. The extracts were dried over anhydrous Na₂SO₄ and the diethyl ether was removed by evaporation under reduced pressure.

Characterization of NAs

Group-structural analysis was performed by mass-spectrometric fragmentation to quasi-molecular ions by a soft ionization technique in either the positive or negative ion mode. Low-resolution high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC–ESI–MS) spectra were recorded on a Finnigan LCQ advantage MAX spectrometer (Waltham, USA). The spectra encompassed a molecular series of protonated and sodiated molecular ions of the acids [M+H]⁺ and [M+2H]⁺ or [M+23]⁺ recorded in the positive ion mode in 0.1 % trifluoroacetic acid in acetonitrile or acetonitrile–water mixture 1:1 and 0.1 % formic acid, as well as a series of [M–H][–] recorded in the negative ion mode in a solution of 0.1 % triethylamine in acetonitrile–water (70:30) with the addition of 2 % NH₄OH. The injected sample volume was 50 μL, and the reference was the molecular ion of 5-cyclohexylpentanoic acid. The search range was 50–600 amu. Spectra were analyzed by the Xcalibur program (Thermo Fisher Scientific).

Determination of the auxin and gibberellin activities of the NA fractions

The auxin activity of the total NAs and its fractions was determined by the test of inhibition of germination of brown mustard (*Brassica juncea* L.), based on counting the germinated seeds after treatment with potassium naphthenates at a concentration of 1.0 mg dm^{–3} and the corresponding concentrations of 3-indoleacetic acid (IAA).³⁰ The gibberellin activity of the total NAs and its fractions (potassium salts, 1.0 mg dm^{–3}) was measured by the barley-endosperm test, using the corresponding concentrations of gibberellin GA₃ for comparison.³¹ The experiments were repeated three times. Statistical significance was tested by one-way Anova followed by comparisons of means by the Duncan multiple range test (*p* < 0.05).

RESULTS AND DISCUSSION

Separation of NAs into narrower fractions

By the described fractionation of total NA preparation, the major part of the NAs was separated in the acidic range (pH 2–5, 62.5 %), then in the basic range (pH 9–10, 22.3 %), and the smallest in the neutral range (pH 6–8, 13.2 %) (Fig. 1). Bearing in mind that the procedure allows the gradual protonation of the acids and their separation into fractions, the obtained results suggest the existence of two groups of acids – weaker ones that were separated in the basic range and stronger ones that were separated in the acidic range.

Comparative structural analysis of the isolated NA fractions and the total NAs

Acids separated at pH 4. At pH 4, 23.9 % of the total NA mass was isolated, which means that the majority of acids had a higher relative acidity. The mass ratios of the NA classes as correlated with the homologous *z*-series in the mixture of acids extracted from the aqueous solution of the total NAs at pH 4 are presented in Table I. There were no pentacyclic structures among the isolated acids and the dominant acids being were structures having 18 to 21 carbon atoms in their molecule (36.5 %); additionally, the ratio of fatty acids was very small (9.7 %).

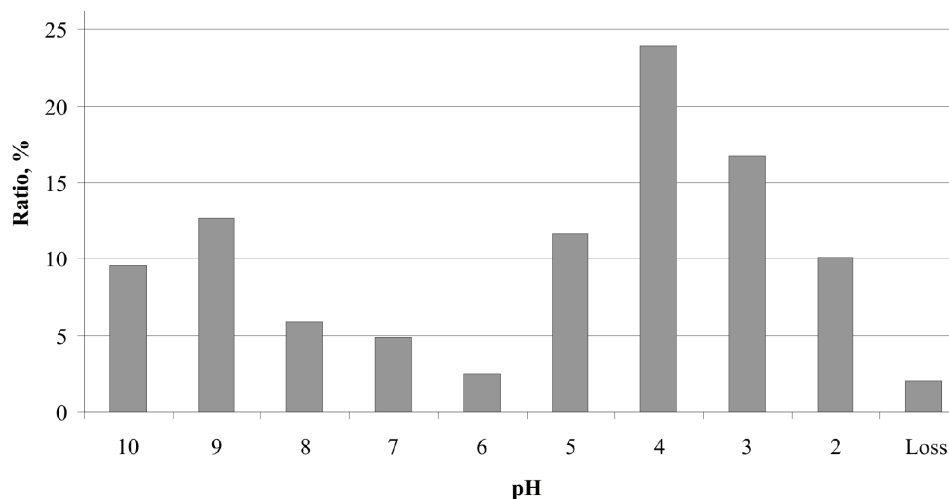


Fig. 1. Mass proportions (%) of particular NAs separated at different pH values.

TABLE I. Distribution of NA classes isolated at pH 4, based on the m/z values in the ESI-MS spectrum

Carbon number	Percentage of naphthenic acids by z number					Percentage by carbon number
	0	2	4	6	8	
14	0.00	0.00	0.56	0.00	0.00	0.56
15	0.00	0.87	0.44	0.00	0.00	1.31
16	0.00	1.06	1.37	1.06	0.00	3.49
17	0.62	0.00	2.62	3.56	0.00	6.80
18	0.94	1.87	3.37	5.05	0.00	11.23
19	1.12	1.81	3.43	5.18	1.18	12.72
20	1.18	1.87	1.68	6.24	4.43	15.40
21	1.25	2.31	3.24	6.11	4.56	17.47
22	1.25	1.87	2.06	3.56	3.56	12.30
23	1.44	1.12	1.50	2.06	2.00	8.12
24	1.18	0.00	1.25	1.56	0.75	4.74
25	0.75	0.00	1.12	1.06	0.62	3.55
26	0.00	0.00	0.75	0.62	0.00	1.37
27	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.50	0.44	0.00	0.94
Total	9.73	12.78	23.89	36.50	17.10	100.00

Acids separated at pH 8. Only 5.9 % of total acids were extracted at pH 8, and an analysis of the HPLC–ESI-MS spectra showed that dominant series of acids were bicyclic ($z = 4$, 32.5 %) and tricyclic ($z = 6$, 32.5 %) structures (Table II). The ratio of fatty acids was only 7.2 %; tricyclic acids were present at approximately the same level as at pH 4, but there are significantly more bicyclic structures with 20 to 25 C atoms in their molecule. No pentacyclic structures were found in this mixture.

TABLE II. Distribution of NA classes isolated at pH 8, based on the m/z values in the ESI-MS spectrum

Carbon number	Percentage of naphthenic acids by z number					Percentage by carbon number
	0	2	4	6	8	
15	0.00	0.00	0.78	0.00	0.00	0.78
16	0.00	0.00	0.00	0.00	0.00	0.00
17	0.00	0.00	0.88	0.00	0.00	0.88
18	0.00	0.00	0.78	0.97	0.00	1.75
19	0.97	0.00	1.07	1.07	0.00	3.11
20	0.97	1.07	2.72	2.92	1.85	9.53
21	0.88	0.78	3.41	3.99	0.00	9.06
22	0.97	2.24	5.26	3.02	0.00	11.49
23	1.70	1.75	4.87	5.26	0.00	13.58
24	0.78	1.07	3.31	7.01	4.77	16.94
25	0.00	2.82	3.60	2.72	0.00	9.14
26	0.00	5.26	2.72	2.34	2.14	12.46
27	0.88	0.00	1.65	2.14	1.75	6.42
28	0.00	1.17	1.46	1.07	1.17	4.87
Total	7.15	16.16	32.51	32.51	11.68	100.00

Acids separated at pH 10. Structures with the lowest acidity are the NAs that were insoluble in an aqueous medium of pH 10, and their ratio in the total mixture was 9.6 %. While the acid series with $z = 4$ and $z = 6$ were dominant in the fractions isolated at pH 4 and 8, this was not true for the fraction of weakest acids, isolated at pH 10 (Table III). In this fraction, the ratio of acyclic acids was 25.3 %, while pentacyclic acids ($z = 10$) appeared for the first time (12.0 %). Table III shows that structural differentiation occurred since the molecular ions of pentacyclic acids (12.0 %) of the series $[M+H]^+$ were evidenced. In the spectrum, acyclic z -class of acids were dominant (25.3 %), with the prevailing structures having 19, 20 and 21 C atoms (16.3 %). The ratio of tetracyclic acids was 17.7 %, with the dominant molecules having 22, 23 and 26 C atoms; the dominant tricyclic acids also had 22 to 26 C atoms in their molecules.

There was a sharp differentiation of acids not only into classes (z -series), but also within the same class with respect to the number of C atoms. Fatty acids, mono-, bi-, tri- and tetracyclic acids were differently distributed in all three fractions, as can be seen in Fig. 2. Pentacyclic acids, the content of which in the total mixture was the lowest, are the evidently weakest acids and hence the least soluble, so that they were concentrated in the fraction isolated at pH 10. At this pH, most of the fatty acids were isolated, whereas decreasing the pH resulted in less and less tricyclic structures in the mixture, which indicates their increasing average acidity. In this way, proof was obtained for the possible fractionation of NAs by their extraction with diethyl ether from aqueous media of different pH values. It is to be expected that naphthenic acids from different sources could be frac-

tionated in a similar way and the obtained results would depend on the composition of the mixture of naphthenic acids.

TABLE III. Distribution of NA classes isolated at pH 10, based on the m/z values in the ESI-MS spectrum

Carbon number	Percentage of naphthenic acids by z number						Percentage by carbon number
	0	2	4	6	8	10	
13	0.00	0.00	1.33	0.00	0.00	0.00	1.33
14	0.98	1.00	0.78	0.00	0.00	0.00	2.76
15	1.02	0.00	0.00	0.00	0.00	0.00	1.02
16	0.78	1.33	0.44	0.78	0.00	0.00	3.33
17	1.56	1.11	0.00	1.06	0.00	0.00	3.73
18	2.39	1.11	1.67	1.56	0.00	0.00	6.73
19	3.89	2.05	1.67	0.00	0.00	0.00	7.61
20	4.11	1.44	1.44	0.00	1.44	0.00	8.43
21	4.83	1.11	1.11	2.00	1.33	0.00	10.38
22	3.50	1.89	1.78	2.22	3.66	0.00	13.05
23	1.33	0.00	1.22	1.67	3.05	2.50	9.77
24	0.89	1.00	1.00	1.50	2.33	2.22	8.94
25	0.00	0.89	0.89	1.56	0.00	2.44	5.78
26	0.00	1.00	1.00	1.89	3.11	1.56	8.56
27	0.00	0.00	1.00	0.00	1.89	1.67	4.56
28	0.00	0.00	0.00	0.00	0.00	0.83	0.83
29	0.00	0.00	0.00	1.56	0.89	0.78	3.23
Total	25.28	13.93	15.33	15.80	17.70	12.00	100.00

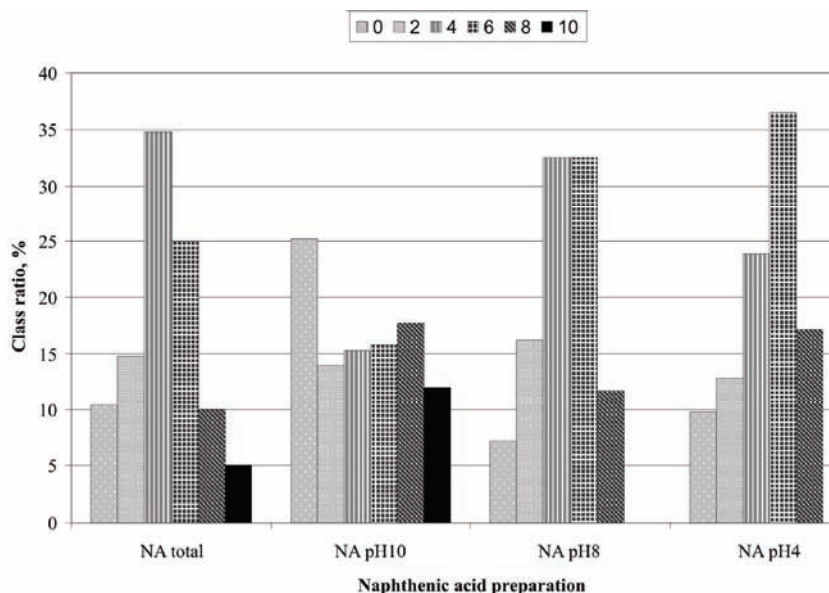


Fig. 2. Comparative distribution of the total NAs and NA classes isolated from aqueous solutions at pH 10, 8 and 4.

Auxin and gibberellin activities of the total NAs and its fractions

In respect of auxin activity, the total NA preparation and the fraction separated at pH 10 exhibit the same activity which corresponds to an IAA concentration of 0.05 mg L^{-1} , whereas the fraction separated at pH 4 showed a somewhat lower activity (16.4 % lower than that of the total preparation). The highest auxin activity (19.6 % higher than that of the total preparation) was exhibited by the fraction separated at pH 8, which corresponded to an IAA concentration of 0.5 mg L^{-1} (Fig. 3).

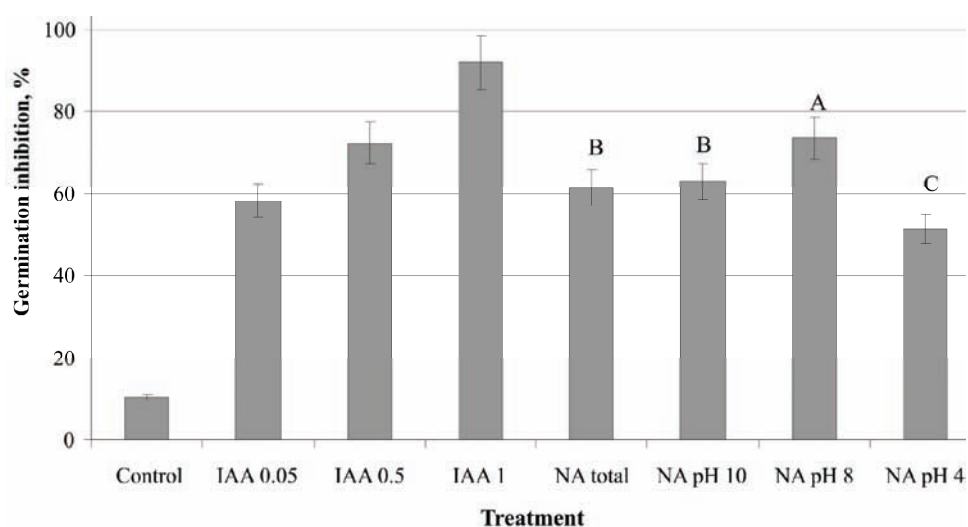


Fig. 3. Auxin activity of aqueous solutions of potassium naphthenates and the three NA fractions. Control (treatment with water, without IAA or K-naphthenates); IAA 0.05 – IAA 1 (treatments with IAA, concentrations: 0.05, 0.50 and 1.0 mg L^{-1}); NA total (treatment with total K-naphthenates, concentration: 1.0 mg L^{-1}); NA pH 10, NA pH 8, NA pH 4 (treatments with NA fractions obtained at the corresponding pH values, concentration: 1.0 mg L^{-1}). The bars represent the standard deviation. Columns labeled with different letters are significantly different ($P < 0.05$, the Duncan multiple range test).

The separated NA fractions showed much greater mutual differences with respect to the gibberellin activity (Fig. 4). Again, the fraction separated at pH 8 showed the highest activity (92.3 % higher than that of the total preparation), which corresponds to a GA_3 concentration between 1×10^{-3} and $1 \times 10^{-2} \text{ mg L}^{-1}$. The fraction separated at pH 4 showed an insignificantly higher activity compared to that of the total preparation, whereas the fraction separated at pH 10 exhibited a significantly lower activity (46.1 % lower than that of the total preparation). The NA fraction separated at pH 8 was characterized by the dominant presence of NAs with bicyclic and tricyclic structures, which were present in equal ratios.

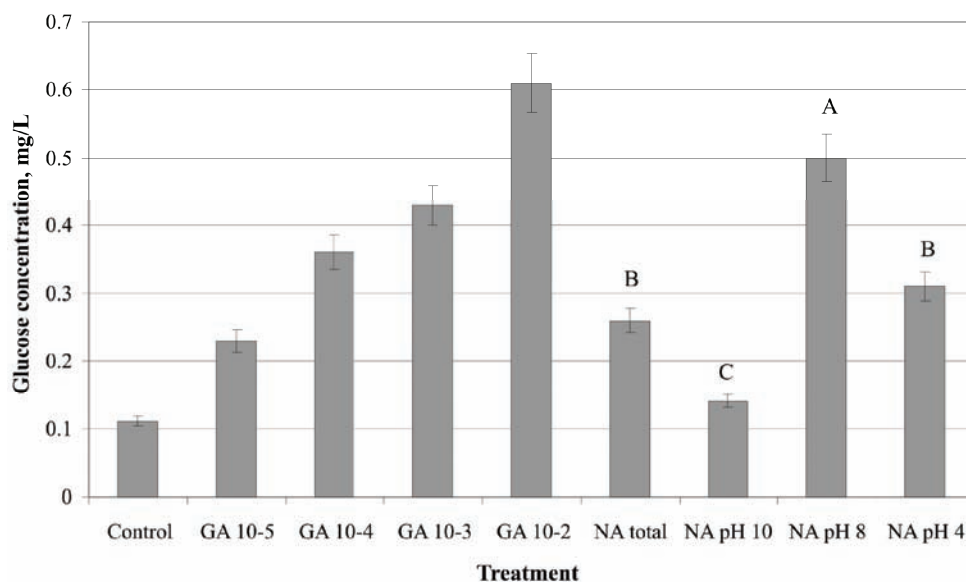


Fig. 4. Gibberellin activity of aqueous solutions of the NA fractions. Control (treatment with water, without GA or K-naphthenates); GA 10-5; GA 10-4; GA 10-3; GA 10-2 (treatments with GA₃, concentrations: 1×10^{-5} , 1×10^{-4} , 1×10^{-3} and 1×10^{-2} mg L⁻¹, respectively); NA total (treatment with total K-naphthenates, concentration 1.0 mg L⁻¹); NA pH 10, NA pH 8, NA pH 4 (treatment with NA fractions obtained at the corresponding pH values, concentration 1.0 mg L⁻¹). The bars represent the standard deviation. Columns labeled with different letters are significantly different ($P < 0.05$, the Duncan multiple range test).

As is evident from Fig. 3, the treatment of brown mustard seeds with potassium naphthenate caused inhibition of their germination, which may be a result of an activity similar to that of plant hormones of the auxin-type, although phytotoxic activity cannot be excluded. Namely, it is known that naphthenic acids can exhibit phytotoxic effects which depend on the concentration and plant species.³²⁻³⁴ That the activity of the total NAs and its fractions is similar to that of the plant hormones auxin is supported by the recently emerging hypothesis that the synthetic auxin 1-naphthaleneacetic acid (NAA) has a similar structure to those of the NAs in the $z = 4$ family. Although NAA has an aromatic ring structure whereas the NAs are aliphatic, there is a possibility that the NAs operate through the same receptors as the auxin NAA.³⁵ In addition, it was recently established that one or more of the compounds present in the naphthenic acid mixtures bind to the androgen receptor in a manner similar to that of flutamide, a powerful metabolite for binding to androgen receptors.³⁶

CONCLUSIONS

Complex NA mixtures can be separated into narrower fractions based on the differences in their acidity. The obtained fractions differed in respect to their

group-structural composition. Pentacyclic acids, the content of which in the total NA mixture was otherwise the lowest, concentrate in the fraction isolated at pH 10. The differences in the compositions of the obtained fractions led to differences in their biological activity, and the fraction separated at pH 8 showed the highest auxin- and gibberellin-type activities. Based on the composition of this fraction, it can be concluded that such activities are due to the dominant presence of NAs with bicyclic and tricyclic structures in an equal ratio.

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ИЗВОД

РАЗДВАЈАЊЕ КОМПЛЕКСНЕ СМЕСЕ НАФТЕНСКИХ КИСЕЛИНА,
ЊИХОВА КАРАКТЕРИЗАЦИЈА И БИОЛОШКА АКТИВНОСТ

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Нафтенске киселине (НК) су комплексна смеша циклоалифатичних и алкил-супституисаних ацикличних карбоксилних киселина чије особине у многоме зависе од састава смеше. Комплексна смеша нафтенских киселина из комерцијалне фракције атмосферског уља војвођанске нафтенске нафте “Велебит” раздвојена је на уже фракције на основу различите киселости. Структурном ESI-MS анализом ужих фракција утврђено је да том приликом долази до структурне диференцијације киселина. Екстракцијом на pH 3–5 издвојено је око 50 % од масе укупних киселина са најдоминантним трицикличним и бицикличним структурама. Киселина мање киселости у смеси има око 22 %, издвојене су на pH 9 и pH 10, а доминантне структуре киселина су са три, четири и пет кондензованих прстенова у молекулу. Утврђено је да постоји корелација доминантних структура и биолошке активности нафтенских киселина ужих фракција. Фракција екстрахована на pH 8 има доминантне би- и три-цикличне структуре киселина и показује највећу ауксинску и гиберелинску активност.

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