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RESEARCH ARTICLE

High-Resolution Mass Spectrometry Study of the Bio-Oil Samples Produced by Thermal Liquefaction of Microalgae in Different Solvents

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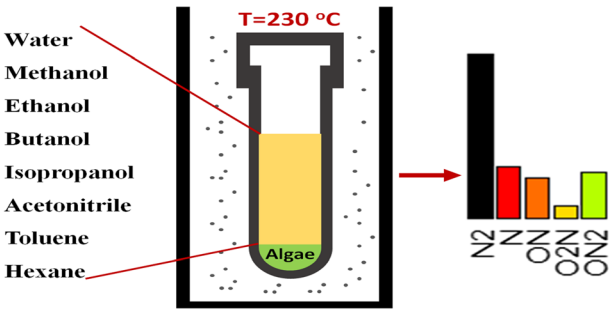

Abstract. We have performed a comparative analysis of the bio-oil produced by thermal liquefaction of microalgae in different solvents using high-resolution Orbitrap mass spectrometry and GC-MS approach. Water, methanol, ethanol, butanol, isopropanol, acetonitrile, toluene, and hexane were used as solvents in which the liquefaction was performed. It was observed that all resulting oils demonstrate a considerable degree of similarity. For all samples, compounds containing 1 and 2 nitrogen atoms dominated in the positive ESI spectra, while a relative contribution of other compounds was small. In negative ESI mode, compounds having 2 to 7 oxygens were observed. Statistical analysis revealed that products can be combined in two groups depending on the solvent used for the liquefaction. To the first group, we can attribute the products obtained by using protic (alcohols) and to the second by using aprotic (acetonitrile, toluene) solvents. Nevertheless, based on our results, we concluded that solvent possesses a minor impact on molecular composition of bio-oil. We suggested that the driving force of the liquefaction reaction is the thermal dehydration of the carbohydrate in algae, resulting in water formation, which could be the trigger of the producing of bio-oil. To prove this hypothesis, we performed the reaction with the dry algae in the absence of the solvent and observed the formation of bio-oil.

Keywords: Bio-oil, Mass spectrometry, Petroleum, Dissolved organic matter, FT ICR, ESI

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Introduction

The conversion of the biomass to the biofuel is the promising approach both for the utilization of a waste and for producing the alternative fuel [1, 2]. Currently, there are two well-developed technologies that are used for the preparation of commercially available fuel: the conversion of the lipid fraction of the biomass to the biodiesel (yield ~97%) [1, 3] and carbohydrates to bioethanol [4, 5]. However, the common disadvantage of these technologies is an only partial conversion of the biomass resulting in the considerable amount of intact residue, which needs to be utilized. As alternative way, the thermal treatment of the biomass was suggested. The production of so called bio-oil is usually performed via a thermochemical conversion such as pyrolysis [6] or gasification [7]. During such processes, a considerable amount of energy is spent on the drying of samples, which makes the overall process less

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profitable. For pyrolysis, the moisture content of the biomass, as a rule, should not exceed 20%. Therefore, a removal of 1 kg of water from the biomass requires about 2.5 MJ of thermal energy, while the calorific value of dry biomass is about 15 MJ/kg [8, 9]. Therefore, the high moisture content of biomass raises inconvenient issues for its pyrolysis. To overcome this problem, a hydrothermal liquefaction (HTL) [8–10] approach can be used. In this process, a wet biomass is subjected to elevated temperature (up to 350 °C) and pressure (up to 20 MPa). In this process, the high moisture content is favorable that makes it energetically effective and potentially interesting for commercial application.

The conventional approach for the production of bio-oil implies HTL in water followed by the extraction of the bio-oil by different organic solvents [11] such as dichloromethane [12], chloroform [13], acetone [14], and hexane [15]. Usage of other solvents and their mixtures [16–21] for the HTL process was also reported. Liu and Zhang studied the liquefaction of pinewood in the presence of various solvents (water, acetone, and ethanol) [20], Yuan et al. performed the liquefaction of microalgae in methanol, ethanol, and 1,4-dioxane [20].

Treatment of biomass by different solvents could change the molecular composition of the bio-oil and, consequently, its properties, e.g., the solubility of the oil. This influences the efficiency of the bio-oil recovery process. However, in the previous studies, only GC-MS was used for the characterization of the molecular composition of the produced oils. The biofuel obtained by the HTL process is a complex mixture containing thousands of individual compounds, and the high-resolution mass-spectrometry (HRMS) [21] is the most informative approach for the study of such samples on the molecular level. HRMS have already proved its indispensability for the study of samples such as petroleum [22], dissolved organic matter [23, 24], essential oils [25], biofuel [26], and wood pyrolysis products [27, 28]. The visual representation of the high-resolution spectra is usually performed using the Kendrick mass defect diagram [29] and the Van Krevelen diagram [30]. Kendrick mass defect diagram allows detection of the compounds which belong to the same homology series formed by -CH_2-, or other repeating structural unit. Van Krevelen diagram allows to relate molecules to different classes (saturated compounds, tannins, sugars, proteins, etc.) based only on elemental composition. Also, several methods based on the performing of various ion-molecular reactions such as ozonation [31], Paternò–Büchi [32] reaction, H/D exchange [33–42], and some others [43–45] were proposed for the obtaining of structural information about the investigated substance. Previously, we have used H/D exchange approach to detect functional groups in molecules of bio-oil and observed absence of the exchange for positively ionized molecules; based on this, we concluded that some molecules may relate to onium compounds [46].

In this paper, we report the study of bio-oils obtained by the hydrothermal liquefaction of the *Spirulina platensis* microalgae (which is promising candidate for production of the bio-oil, because their cultivation is rather simple and cheap [47, 48]) in eight different solvents (water, methanol, ethanol, butanol, isopropanol, acetonitrile, toluene, and hexane) using high-resolution Orbitrap mass-spectrometry and GC-MS approach. Comparing molecular composition of the obtained oils, we are trying to reveal the effect which the solvent plays during HTL process.

Methods

The Laboratory Setup for "Solvothermal" Liquefaction

The biomass of *Spirulina platensis* was preliminarily dried in the Binder drying oven at 105 °C. Solvothermal liquefaction of microalgae was carried out as shown in Figure 1. The reactors represent small autoclaves with a volume of 30 cm^3. Three grams of *Spirulina platensis* and 15 cm^3 of the solvent were loaded into the reactor. Then, reactors were sealed and placed into the sand bath heated by the electric heater. The maximum temperature of solvothermal liquefaction experiments was 230 °C. The residence time at maximum temperature was 1 h. The temperature of 230 °C was chosen because it is below

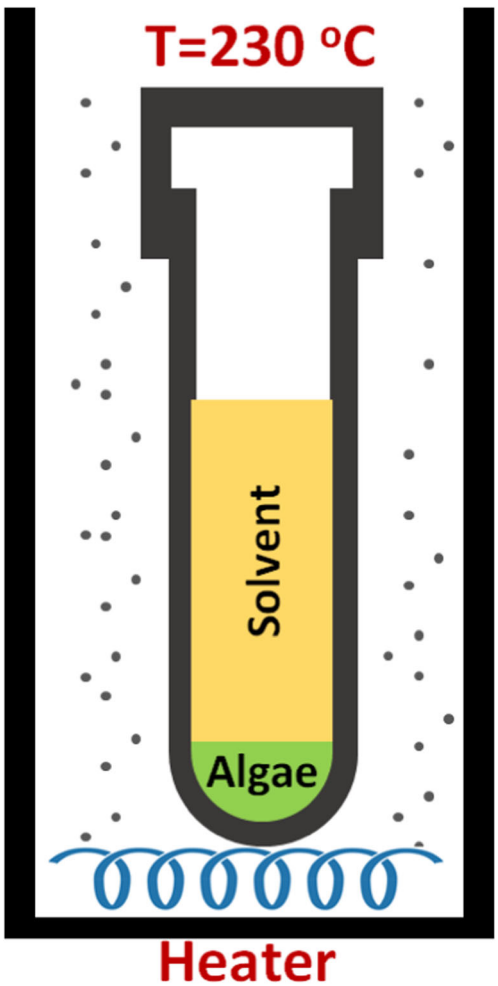

Figure 1. Scheme of laboratory set-up: reactor-autoclave in the sand bath

the temperature of critical point for all solvents (see Table 1). All solvents at this temperature remains liquid (in equilibrium with the gas phase).

At the end of the experiment, the heater was turned off and the reactor was taken out of the oven and cooled down to room temperature. The reactor was then opened and the contents of the reactor, which is a mixture of the solid residue, and the bio-oil dissolved in the solvent, were placed in a plastic tube to settle down the solid residue. After settling, two samples were taken from the tube, the first to determine the yield of the bio-oil, the second for chemical composition analysis. The yield is the ratio of the mass of the residue obtained after the evaporation of the solvent to the mass of the initial biomass loaded into the reactor. The evaporation of the solvent was carried in a Petri dish under the ventilation at room temperature for 12 h, because most of the solvents had a low evaporation temperature. Further, all samples were placed in a drying oven for 4 h at a temperature of 50 °C.

GC-MS Analysis

GC-MS analysis was performed using Agilent 7890, equipped with quadrupole mass spectrometric detector 5977 A with electron impact ionization. For chromatographic separation of components, the following conditions were used: initial temperature of the column 60 °C, isotherm for 3 min, further temperature increases from 60 to 280 °C at a speed of 8°/min, isotherm at 280 °C for 10 min. A capillary column HP-5MS (30 m × 0.32 mm, 0.25 μm) with a stationary phase based on methyl (95%)-phenyl (5%)-phenyl polysiloxane was used. The carrier gas was helium, the flow rate through the column is 1.5 ml/min.

Spectra were registered by full ion current in the range *m*/*z* from 35 to 600 (ionizing electron energy 70 eV), quadrupole temperature 150 °C, ion source temperature 230 °C. The volume of the sample was 1 μl. Identification of compounds was carried out by comparing the obtained mass spectra with the library spectra (NIST 2014), with a probability of coincidence of at least 60%.

ESI MS Analysis

Dried bio-oil samples were dissolved in the MeOH to the concentration 1 g/L. All experiments were performed on an Orbitrap Q-Exactive (Thermo Electron Corp., Bremen, Germany) mass-spectrometer. Ions were generated in positive and negative ESI mode. To prevent samples and line-flow contamination, we used a homemade ESI source, which constitutes of disposable medical syringe (0.3 ml) placed in the syringe pump [49]. High voltage is applied to the syringe needle. Use of the new syringe for each sample excludes any possible contamination. The temperature of the desolvating capillary was set to 200 °C. The infusion rate of the sample was 1 μl/min and the needle voltage was 3000 V. The achieved resolving power was 140,000, each spectrum was the result of the averaging of 100 scans, *m*/*z* range was from 50 to 700. External calibration was performed prior to the analysis using the standard Thermo calibration mixture.

For each peak in the peak list, the following variables were calculated: Kendrick mass $M_{\mathrm{Kendrick}} = M_{\mathrm{IUPAC}} \frac{\left[m_{\mathrm{IUPAC}}^{\mathrm{CH_2}}\right]}{m_{\mathrm{IUPAC}}^{\mathrm{CH_2}}}$ and Kendrick mass defect (KMD) was determined using the formula $KMD = \mathrm{round}(M_{\mathrm{Kendrick}}) - M_{\mathrm{Kendrick}}$. Here $[m]$ means integer part of the mass m. M_{IUPACK} is the IUPAC mass of peak, $m_{\mathrm{IUPAC}}^{\mathrm{H_2}}$ and $m_{IUPAC}^{CH_2}$ are IUPAC masses of the fragment CH_2 and H_2 correspondingly. Molecules that differ only by the number of repeating CH_2 segments have the same KMD. The homology series formed by C_cH_{2c} for molecules with molecular formula $C_cH_{2c+Z}N_nO_oS_s$ are referred to as $ZN_nO_oS_s$. For the accurate assignment of molecular formulas, we used previously described approach based on the weighted Kendrick mass defect histogram [50, 51].

Statistical Analysis

Cluster analysis was performed using build-up R package with functions hclust(…, method="average") and dist(…, method="euclidean"). Principal component analysis (PCA) was performed using distributed function prcomp() from R software.

Elemental Composition

The chemical composition of *Spirulina platensis* and bio-oil samples was analyzed using a Thermo Scientific Flash 2000 HT analyzer. C, H, N, and S contents were determined for liquid samples of bio-oil with oxygen content determined by calculating the difference. The test procedure was repeated five

Table 1. Properties of the Solvents Used

Solvent	Critical temperature, °C	Critical pressure, atm	Boiling point under atmospheric temperature, °C	Density under standard conditions, g/cm^3
Isopropanol	235.6	53	82.4	0.7851
Ethanol	241	62.96	78.4	0.7893
Water	374.15	217	101.4	11.042
Butanol-1	287	48.35	117.25	0.8098
Acetonitrile	272.4	47.70	81.6	0.7875
Toluene	320.8	40	110.6	0.8669
Hexane	234.8	29.61	68	0.6548
Methanol	240	78.63	64.7	0.7918

times for each sample. The content of ash in *Spirulina platensis* was determined by pyrolysis at 800 °C and mass measurements using an analytical balance Sartorius Cubis MSA324S.

Results and Discussion

General Characterization of Microalgae and Bio-Oils

The *Spirulina platensis* microalgae is a well-known culture that is used in the production of many commercial products including healthy food supplements, food for animals, cosmetics, and pharmaceutical products. It is grown usually in an open manner on a large scale. Its elemental and biochemical composition is presented in Table 2. Microalgae under study is mostly proteinaceous with a minor contribution of carbohydrate. After the performing of the liquefaction reaction in different solvents, we have obtained eight viscous samples with strong amine odor and dark-brown color. Product yields and elemental compositions along with solvent polarity are given in Table 3. All products are characterized by high contribution of nitrogen except for the sample obtained by using hexane as a solvent, which possesses only 4% of N by mass. At the same time, bio-oil of this type contains much higher amount of C (68%) compared to other samples, e.g., bio-oil from water consists of 42% of C by mass. Atomic ratios indicate aliphatic character of all bio-oil products, which is consistent with the microalgae bulk elemental composition (Table 2). N/C ratios distinguish the hexane sample. N/C ratio varies from 0.14 for toluene to 0.19 in case of water, which is close to the microalgae. In the case of hexane, N/C was only 0.05, which could be explained by low solubility of N-containing compound in non-polar solvent. Table 3 also provides the higher heating values (HHV) estimated for each bio-oil [52]. All products are characterized by low HHV compared to the bio-crude obtained from proteinaceous source [53]. This could be explained by the low temperature of the liquefaction compared to the conventional > 300 °C [54]. Therefore, comparison of bulk elemental composition showed similarity of the samples. Also, we must notice that traces of solvent used for the hydrothermal liquefaction could disturb the results, so we performed HRMS and GC-MS analysis for deeper comparison of bio-oils.

Mass-Spectrometry

The wide range and narrow range mass spectra in positive ESI mode of the obtained samples are presented in Figure 2. It can be seen, that the general spectrum shape and dominated species are the same for all bio-oils, except the sample obtained in toluene. Spectra in negative ESI mode are shown in Figure 3. It can be seen that in the negative ESI mode only several CHO compounds dominate. For analysis of the obtained mass spectra, we have calculated weighted Kendrick mass defect histogram. Such histogram has an advantage over the regular Kendrick mass defect diagram because it shows relative content of different homology series [50]. Our results are presented in Figure 4. The common feature of all mass-spectra is the major contribution of relatively saturated N-containing compounds, which is typical for crude and bio-oils [55] compounds. N_2 series was the dominant for all samples. These molecules could consist of protein-derived piperazine, pyrimidine, or imidazole structural fragments. Consideration of the minor series revealed differences between samples. In case of bio-oil obtained in methanol, the series 4N was the most pronounced in the spectrum. The core structure, which could be suggested for this series, corresponds to diethylamine ($C_4H_{11}N$). All other samples were contributed mostly by $3N_2$ (ethylpiperazine-like, $C_6H_{14}N_2$), ON and $-1ON_2S$ series. The distinctive feature of the toluene sample was the highest among sample contribution of sulfur-containing compounds as OS, S, and ON_2S series. The relative content of the other compounds belonging to different classes is also shown in Figure 4.

All identified formulae were plotted on DBE vs Molecular mass diagrams [55] presented in Figure 5. Samples obtained from methanol and ethanol are characterized by the richest molecular ensemble (909 and 953 molecular formulas, respectively), which collaborates with the favorable formation of bio-crude in simplest alcohols [56]. DBE vs Mass diagrams revealed differences in bio-oils. Water sample possesses lack of compounds with DBE > 10. This is in agreement with aliphatic character of its elemental composition (Table 3). Other samples except for the toluene are characterized by the similar DBE vs M diagrams with the highest contribution of compounds with DBE < 4. In contrary, toluene sample is composed of unsaturated, likely aromatic compounds with DBE > 8, which collaborates with the bulk elemental analysis.

For negative ESI mode, we have observed that compounds having 2 to 7 oxygens dominate in the spectrum, also were observed compounds having one nitrogen atom and from 2 to 7 oxygens. The results of the analyses of data are presented in Figure 6. Because weighted Kendrick mass defect histogram contains too many lines (see Supporting Figure S1), we show Van Krevelen diagram. We can see that independently of the solvent used for HTL, the resulting compounds occupy the same region on the Van Krevelen Diagram, corresponding to unsaturated hydrocarbons and lignin. At the same time, relative

Table 2. Elemental (ash-free) and Biochemical Composition of *Spirulina platensis.* Oxygen Content is Determined by Difference

Sample	C, %	H, %	N, %	S, %	O, %	H/C	N/C	Ash,%
Spirulina platensis	53.04 Proteins 60.7	7.76	12.02 Lipids 12.1	4.24	22.94 Carbohydrates 7.1	1.76	0.19	6.0

Table 3. Bio-Oil Yield, Elemental Composition of the Bio-Oils Obtained by Liquefaction Using Different Solvents

Solvent	Elemental composition								Bio-oil yield, %	Relative solvent polarity
	N, %	C, %	H, %	S, %	O, %	H/C	N/C	HHV, MJ/kg		
Water	9.47	42.40	7.37	1.24	39.52	2.09	0.19	19.38	83	1.000
Methanol	9.31	55.21	7.97	1.20	26.31	1.73	0.14	25.92	84	0.762
Ethanol	9.59	54.52	8.14	1.33	26.42	1.79	0.15	25.88	86	0.654
Butanol-1	9.59	55.27	7.91	1.24	25.99	1.72	0.15	25.91	65	0.586
Isopropanol	9.35	58.26	8.72	1.37	22.30	1.80	0.14	28.30	50	0.546
Acetonitrile	11.89	57.22	8.30	1.29	21.30	1.74	0.18	27.50	18	0.460
Toluene	8.80	54.34	7.50	1.20	28.16	1.66	0.14	24.88	37	0.099
Hexane	4.09	68.50	10.47	1.12	15.82	1.83	0.05	34.67	54	0.009

intensity of the compounds vary with the solvent. We can see that for hexane and toluene, the relative intensity of NO_O compounds is lower compared to other solvents.

For better understanding of the differences and similarities of the obtained bio-oils, we have performed GC-MS analysis and combined those results with HRMS. The observed GC-MS chromatograms are placed in Supporting Information (Figure S2–S9). Figure 7A shows compounds that were detected in all samples by GC-MS, we must emphasize that one of the compounds (*n*-Hexadecanoic acid) was the dominant compound in negative ESI spectra. Also, we performed principle component analysis. In Figure 7, we demonstrate PCA biplot calculated based on all molecular formulas for HRMS and GC-MS data. To obtain Figure 7B_1–B_3, we first determined all molecular formulas present in at least one sample. Then for each sample, we constructed vector values which were equal to the intensity of corresponding *m*/*z* (zero in case if *m*/*z* is not present).

Analyzing data, it is important to remember that in positive ESI mode basic compounds are mainly observed, in negative ESI mode acidic compounds are mainly observed, and GC-MS is optimized for volatile saturated compounds, many of which would not be observed in ESI. Analyzing Figure 7B_1–B_3, we can see that bio-oil obtained from methanol is the most different from the others. For other solvents we did not observe any distinct clustering.

According to our results, despite clear differences in solvent properties, bio-oil molecular compositions vary only by minor components. This is indicative of the common process, which occur during thermal liquefaction. We believe, that dehydration and partial pyrolysis accompany the liquefaction process. Moreover, microalgae possess about 20% of lipids and

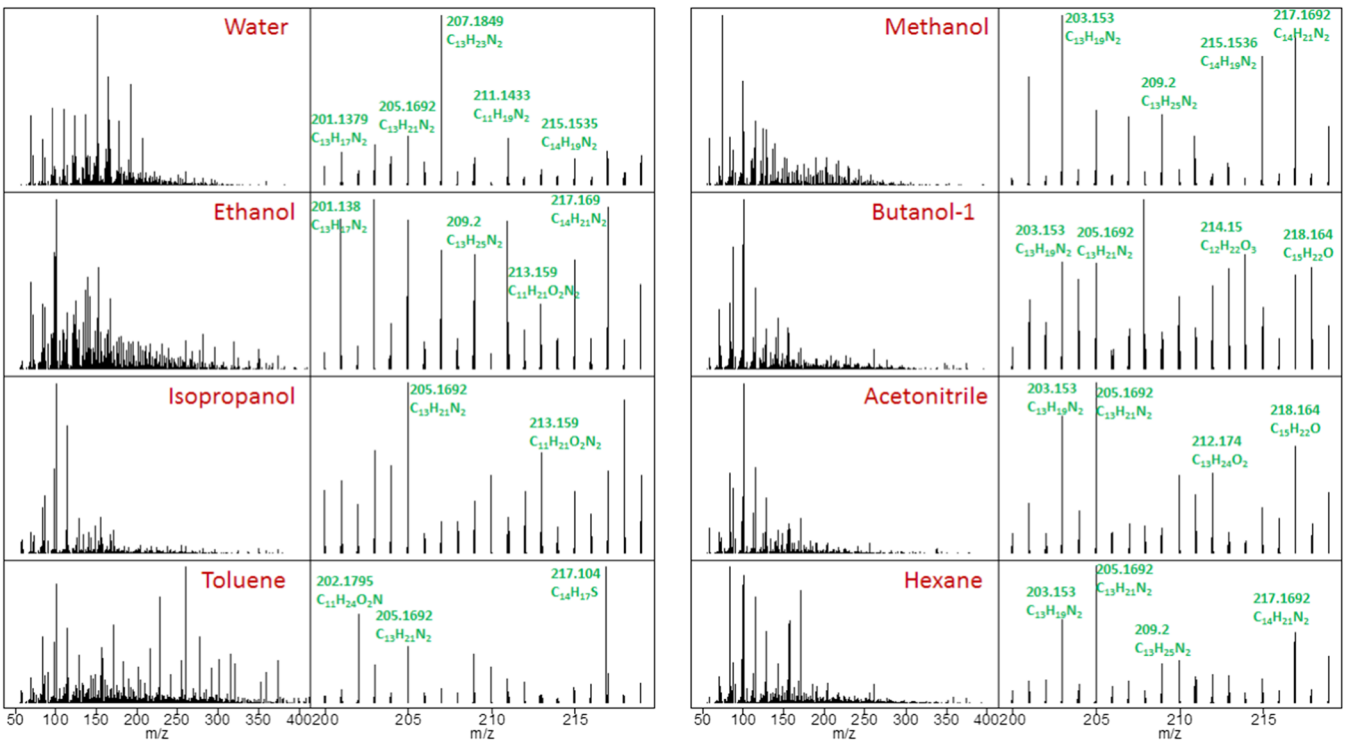

Figure 2. The wide- and narrow-range mass spectra of the obtained bio-oils in positive ESI mode

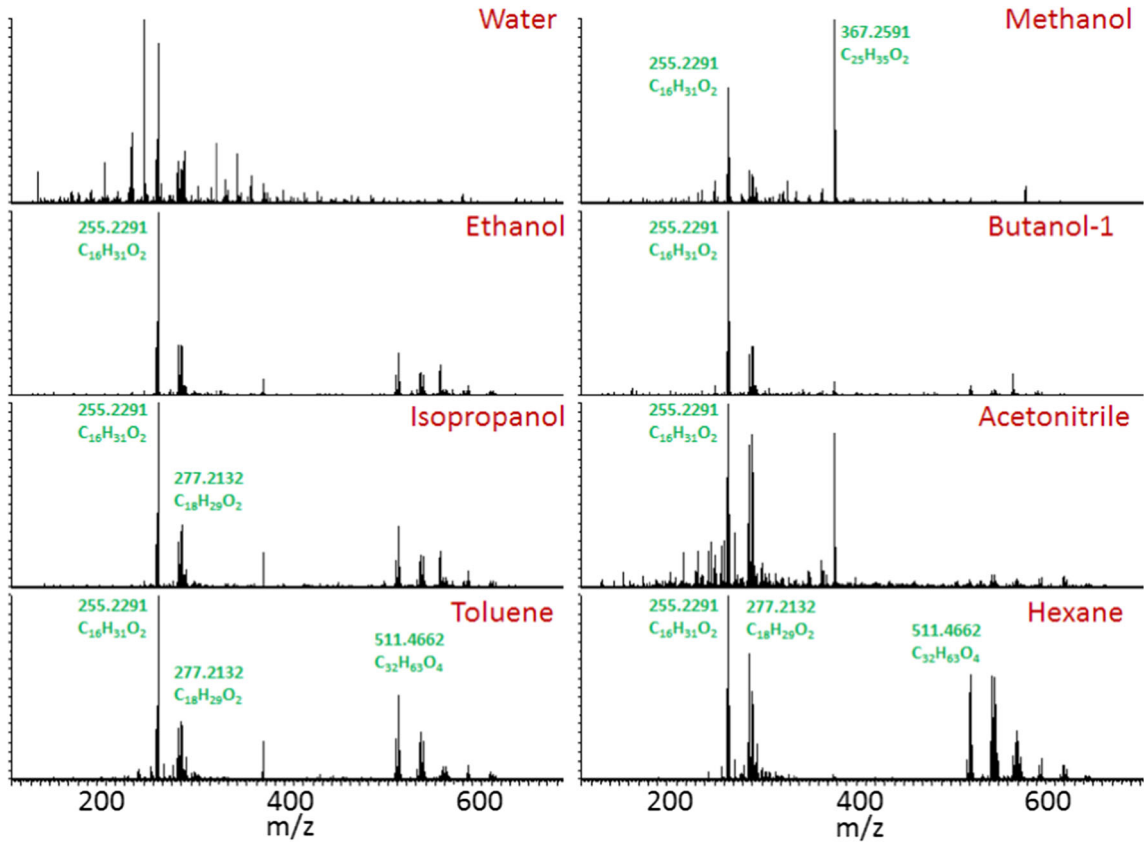

Figure 3. The wide range mass spectra of the obtained bio-oils in negative ESI mode

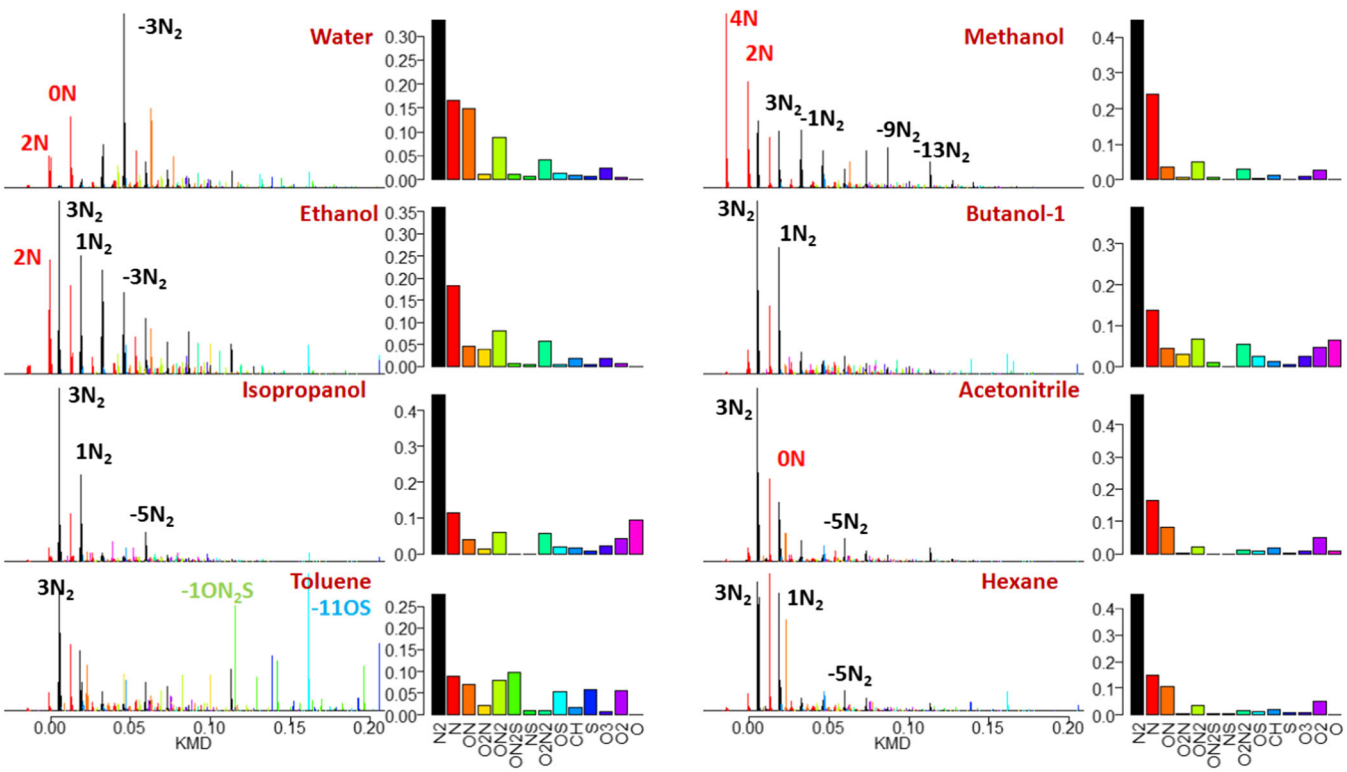

Figure 4. The weighted Kendrick mass defect histogram and relative content of compounds belonging to different classes. Positive ESI mode

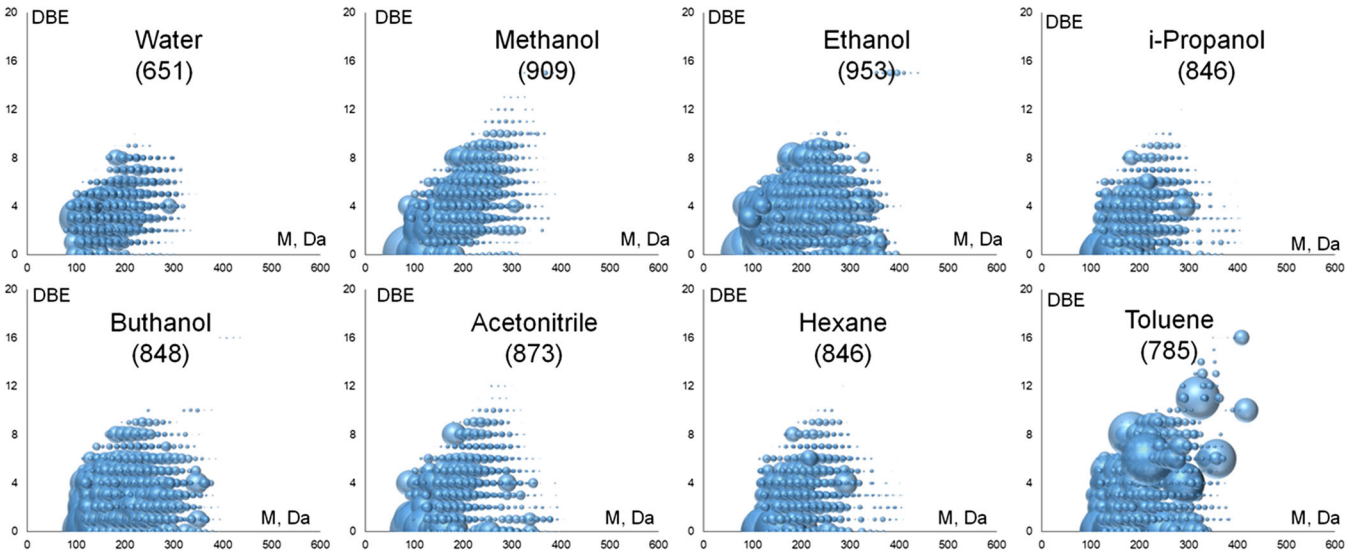

Figure 5. DBE vs molecular mass diagrams for all samples under study. Number of identified molecular compositions are in brackets. Positive ESI mode.

carbohydrates (Table 2), which possess a number of secondary alcohol. Thermal dehydration leads to water formation and, consequently, change liquefaction pathway in all cases to water-like process.

To support this hypothesis, we performed the thermal liquefaction of the dry microalgae biomass in the absence of solvent. In this case, only the water produced during thermal decomposition of the carbohydrates and glycerides should be considered as a reactive species. The experiment was performed under two temperatures: 230 and 350 °C. Under the 230 °C such process is known as torrefaction during which the hydroxyl groups are removed thus producing hydrophobic material [57, 58]. The process under 350 °C could be referred to the low-temperature pyrolysis, taking into account that in classical pyrolysis, the volatile compounds are removed from the reactor. In both processes we have obtained bio-oil, though under 230 °C, the major part of bio-mass remained solid. The results of mass spectrometric analysis are presented in Figure 8.

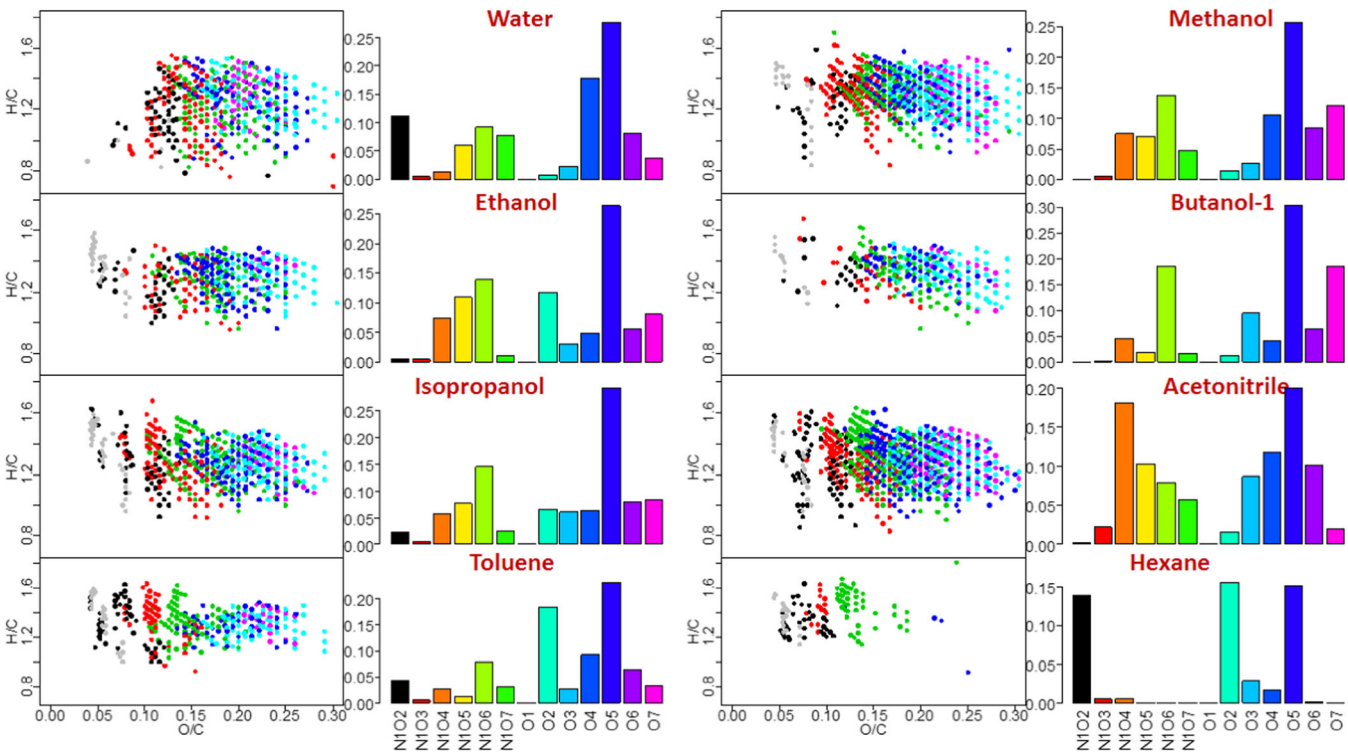

Figure 6. The Van Krevelen diagram and relative content of compounds belonging to different classes. Negative ESI mode

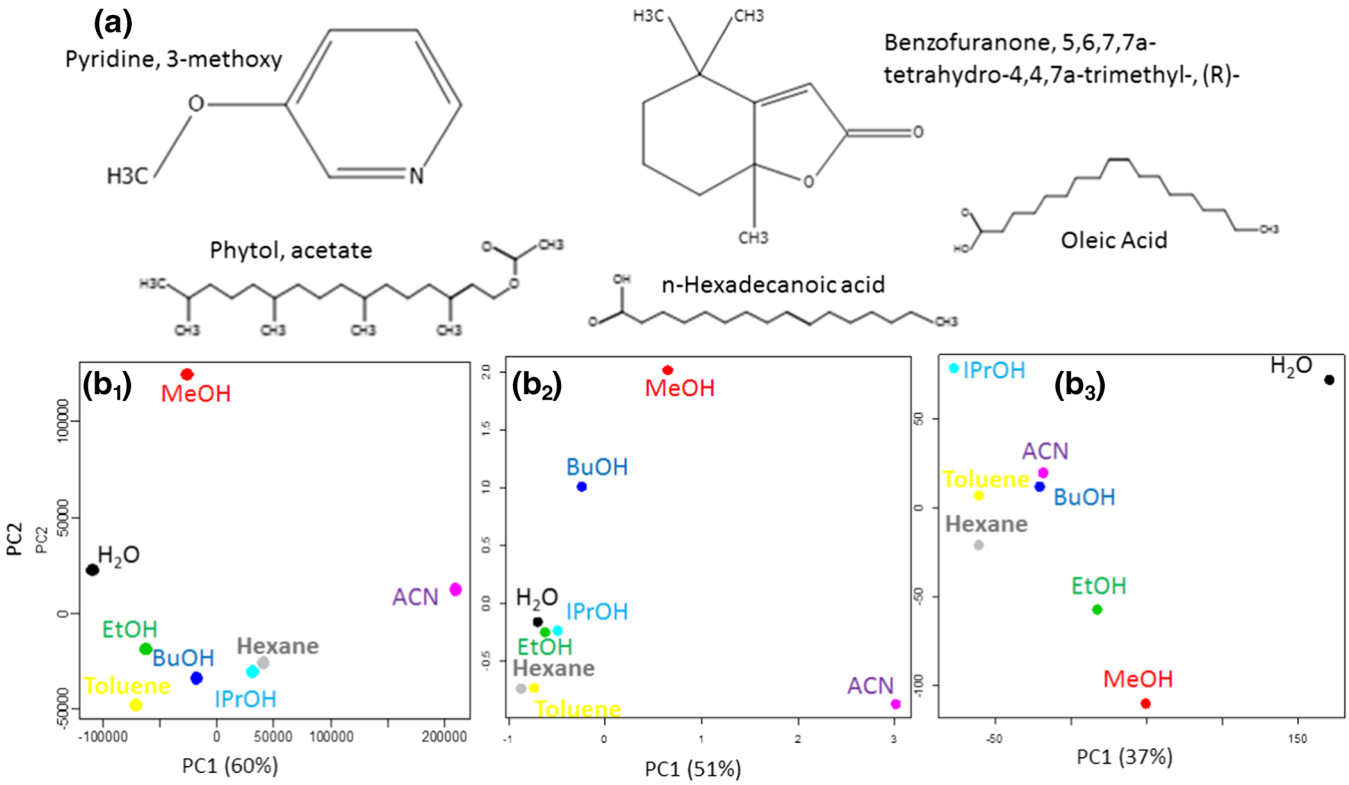

Figure 7. (a) Compounds observed using GC-MS in all samples. (b_1), (b_2) (b_3): (PCA1, PCA2) plot calculated based on the all molecular formulas and their intensity for pos-ESI-MS, neg-ESI-MS, and GC-MS data correspondingly

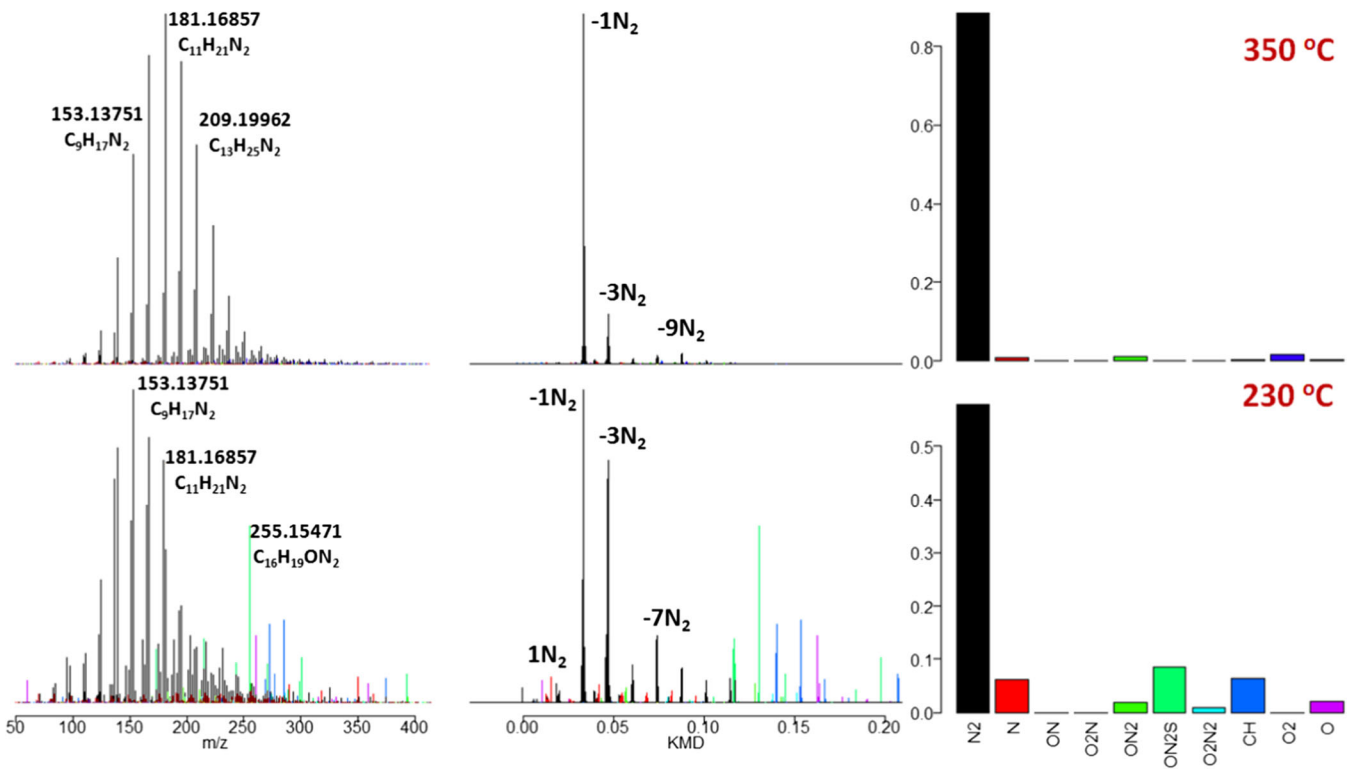

Figure 8. Mass spectrum, weighted Kendrick diagram, and classes of detected compounds for the bio-oil produced without solvent

We could see that in both cases, N_2 class dominate in the spectrum and that bio-oil obtained under low temperature is more diverse. This supports our suggestion.

Conclusion

We have analyzed bio-oils obtained by thermal liquefaction in different solvents. Despite significant differences in physical-chemical properties of the solvents used, we observed similar molecular compositions with a minor variation in all bio-oils, which was shown by using high-resolution mass spectrometry and GC-MS approach. For all samples, we observed that N_2, N, and ON classes dominate in the positive ESI spectrum. In negative ESI mode, we observed compounds having 2 to 7 oxygens; *n*-Hexadecanoic acid was dominant compound which was also detected by GC-MS. Statistical analysis performed using all detected *m*/*z* allowed to distinguish between bio-oils produced using protic and aprotic solvents. Based on overall similarity of the obtained bio-oils, we suggest that yields of the liquefaction depend on the solubility of the product in the particular solvent rather than on the differences in the reaction mechanism. We believe that water elimination from secondary alcohol presenting in carbohydrates and lipids strongly affects the reaction pathway, which results in formation of the similar products. We performed the torrefaction of the dry biomass in the sealed reactor. The obtained bio-oil was similar to those obtained using solvents, which supports our hypothesis.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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