Video Article

Qualitative Characterization of the Aqueous Fraction from Hydrothermal Liquefaction of Algae Using 2D Gas Chromatography with Time-of-flight Mass Spectrometry

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Abstract

Two-dimensional gas chromatography coupled with time-of-flight mass spectrometry is a powerful tool for identifying and quantifying chemical components in complex mixtures. It is often used to analyze gasoline, jet fuel, diesel, bio-diesel and the organic fraction of bio-crude/bio-oil. In most of those analyses, the first dimension of separation is non-polar, followed by a polar separation. The aqueous fractions of bio-crude and other aqueous samples from biofuels production have been examined with similar column combinations. However, sample preparation techniques such as derivatization, solvent extraction, and solid-phase extraction were necessary prior to analysis. In this study, aqueous fractions obtained from the hydrothermal liquefaction of algae were characterized by two-dimensional gas chromatography coupled with time-of-flight mass spectrometry without prior sample preparation techniques using a polar separation in the first dimension followed by a non-polar separation in the second. Two-dimensional plots from this analysis were compared with those obtained from the more traditional column configuration. Results from qualitative characterization of the aqueous fractions of algal bio-crude are discussed in detail. The advantages of using a polar separation followed by a non-polar separation for characterization of organics in aqueous samples by two-dimensional gas chromatography coupled with time-of-flight mass spectrometry are highlighted.

Video Link

The video component of this article can be found at https://www.jove.com/video/53634/

Introduction

Steady growth in demand for liquid fuels, finite fossil fuel resources, uncertainty of fossil fuel supplies, and concerns over the increasing concentration of greenhouse gases in the atmosphere have increased global awareness for renewable resources[1]. Solar energy (including photovoltaics and solar-thermal), wind energy, hydropower, geothermal, and biomass are the primary renewable sources that could potentially replace fossil-derived energy[2]. Of these, biomass is the only carbon-based alternative energy resource for the production of liquid transportation fuels and high-value chemicals[3]. Biomass includes any organic material such as forest resources, agricultural residue, algae, oilseeds, municipal solid waste, and carbon-rich industrial wastes (*e.g.* from pulp and paper industry or from food processing)[1]. Biomass is classified into two broad categories: lignocellulosic and non-ligneous feedstocks based on compositional characteristics. Lignocellulosic biomass consists of carbohydrates and lignin, while non-ligneous feedstocks have proteins, carbohydrates and lipids/oils[4]. Lignocellulosic feedstocks, derived from terrestrial plants, can only satisfy 30% of the current liquid fuel (gasoline, jet fuel, and diesel) demand if sustainably cultivated and harvested[5,6]. Hence, non-ligneous aquatic microorganisms, such as microalgae and fungi, are considered potential feedstocks for the production of renewable liquid fuels to complement lignocellulosic resources.

Microalgae feedstocks have the potential to satisfy current liquid transportation fuels demand[7,8]. Algae have many advantages: high areal productivity[8], the ability to grow in low-quality, brackish, or sea water[9], and the ability to accumulate energy-dense triglycerides or hydrocarbons[7,8]. Hydrothermal liquefaction (HTL) is a viable and scalable conversion process which utilizes water naturally associated with algal or aquatic feedstocks[10,11]. It is a thermo-chemical process with operating temperatures of 250-400 °C and operating pressures of 10-25 MPa which produces a liquid product, or bio-crude, which can be upgraded into a fuel blend stock. Bio-crude produced from HTL of algae has distinguishable and easily separable organic and aqueous fractions. The organic fraction of bio-crude can be efficiently converted into a refinery ready blend stock via catalytic hydro-treating processes[11]. The aqueous fraction of bio-crude contains ~30% of the total carbon present in the algal feedstock. Although thousands of compounds have been identified in the HTL aqueous stream, the predominant fractions consist of low molecular weight oxygenates (including acids, alcohols, ketones, and aldehydes) formed by the degradation of carbohydrates and lipids, and nitrogen heterocyclics (including pyrroles, pyridines, pyrazines, and imidazoles) derived from protein decomposition[12]. Studies on utilizing the aqueous fraction to improve overall process economics as well as sustainability are ongoing. Synthesis gas can be produced from the aqueous fraction of algae bio-crude via catalytic hydrothermal gasification[10,13,14]. Alternatively, organics in the aqueous fraction can also be catalytically converted to fuel additives and specialty chemicals. Research on optimizing catalytic hydrothermal gasification and catalyst screening studies

for conversion of organics in the aqueous liquid phase is currently underway at the Pacific Northwest National Laboratory (PNNL). For this work, qualitative as well as quantitative characterization of the aqueous fraction of algae bio-crude is required. Since the aqueous fraction of algae bio-crude is considered a waste stream, there are very few studies that have analyzed the aqueous fraction of algae bio-crude[13,15]. Moreover, recent studies concluded that converting this HTL algae water into high-value bio-products would improve the sustainability as well as economics of an HTL-based bio-refinery[11]. Therefore, this study focused on developing a method for qualitative characterization of the aqueous fraction of bio-crude obtained from HTL of algae by two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC × GC–TOF-MS).

GC × GC–TOF-MS is the most promising chromatographic analytical technique to increase resolution (or separation of chemical compounds in a sample), peak capacity (*i.e.* number of resolved peaks), signal-to-noise ratio (for identification of chemical compounds with high confidence), and to avoid co-elution of chemical compounds[16]. In order to maximize resolution, peak capacity, and signal-to noise ratio, two GC columns with different stationary phases are connected in series using a press-fit connector or micro-union[17](see **Figure 1** which is a block diagram of GC × GC–TOF-MS system used in this study). A modulator is located between the press-fit connector and secondary columns to trap, refocus, and re-inject the effluents from the primary column into the secondary column[18]. Modulation occurs on the secondary column in the present study as shown in **Figure 1**. The secondary column is then connected to the TOF-MS via a transfer line assembly.

GC × GC–TOF-MS was used previously for qualitative as well as quantitative analysis of organic samples such as crude oil[16,19], gasoline, jet-fuel, diesel, bio-diesel, and the organic fraction of bio-fuel[20-22] produced from thermo-chemical as well as thermo-catalytic conversion processes[23,24]. For characterization of these organic samples in GC × GC–TOF-MS instruments, a long non-polar column was used as the primary column, while a short polar column was used as the secondary column. This conventional column configuration resolves chemical compounds based on differences in volatility over the first dimension, followed by polarity in the second dimension[18]. Aqueous or water samples from biological processes, food processing, and environmental wastes were also characterized using similar primary/secondary column configurations after the sample had been through preparation steps[17,25-30]. Sample preparation techniques such as derivatization, solid-phase extraction, and organic solvent extraction have all been utilized prior to GC × GC–TOF-MS analysis[17,27-29,31,32]. These techniques were aimed at decreasing the polarity of compounds in the sample for analysis using a conventional column configuration[33]. An alternative strategy was employed in this study based on the nature of the sample (here polar organic compounds in water) utilizing the reverse primary/secondary column configuration for GC × GC–TOF-MS analysis. Since the aqueous fraction of bio-crude produced from HTL has polar compounds[13], a column combination of a primary polar column and a secondary non-polar column was used in the GC × GC–TOF-MS without any upstream sample preparation. This primary/secondary column combination resolves chemical compounds based on differences in polarity over the first dimension, followed by volatility in the second dimension. Limited analytical methods exist in the literature for characterization of aqueous samples using two-dimensional gas chromatography without prior sample processing[15].

The objective of this study was to determine the chemical compounds present in the aqueous fraction of algae bio-crude. To achieve this objective, a GC × GC–TOF-MS data acquisition method was developed with a column combination of polar column (primary) × non-polar (secondary). Klenn *et al*. (2015) suggested that increasing the length of the primary column (especially 60 m GC columns) and lowering the offset temperature of the secondary column with respect to the primary column would maximize peak capacity and resolution[16-18]. Therefore, a 60 m primary column and 5 °C offset temperature of the secondary column with respect to the primary column were used in this study. The optimum modulation period was determined following a protocol described in this study (see section 4). The optimum ramp rate of GC column temperature was determined by a trial and error method and is similar to the value suggested in the literature[16-18]. To discuss the advantages of this column combination for aqueous samples, we have analyzed HTL algae water samples with the conventional column combination of non-polar × polar. Operating parameters suggested in the literature were employed for analyzing the aqueous fraction of algal bio-crude with a non-polar × polar column combination[18].

Protocol

1. Sample Preparation

1. Generate a mixed aqueous/organic product stream via continuous flow HTL of algae according to the reactor design and experimental procedure found in the literature[10,11].
2. Use a gravity separator to separate the product stream into an aqueous phase and organic phase.
3. Filter 10 ml of the HTL aqueous phase using a 0.45 µm syringe filter and store in a refrigerator maintained at 4 °C for GC × GC–TOF-MS analysis.

2. Instrument Components

1. Use a gas chromatograph (GC) equipped with a quad-jet dual stage cooling-based modulator and time-of-flight (TOF) mass spectrometer (MS) for these experiments.
2. Configure the auto-sampler to inject 1 µl of each sample or standard into the GC. Use a randomized block design of sample and standard injections for the auto-sampler sequence as described in the literature[13]. The randomized block design is commonly used in quantitative studies to control for instrument operation. Our laboratory utilizes the design routinely even in comparative studies to verify instrument operation.
3. Connect the primary and secondary column using a press-tight connector before the modulator. Ensure that both edges of both primary and secondary columns are cut straight without sharp edges before connecting to the press-tight connector.
4. Place ferrule on the GC column and then connect primary column to the GC injector so that 5 mm of column is inside the injector.
5. Ensure that glass liner, non-stick liner O-ring and septa for GC injector are new and free of contamination.
6. Use 1/16 x 0.5 mm ID transfer line ferrules to connect the secondary column and transfer line. Place a 0.2 m portion of the secondary column in the transfer line.
7. Ensure that a 0.1 m portion of the secondary column is in the modulator.
8. Use ultrahigh purity helium gas as carrier gas for GC at a flow rate of 1.5 ml min^{-1}.

9. Ensure there is sufficient liquid nitrogen in the Dewar which acts as a coolant in the modulator. The level of the liquid nitrogen in the Dewar can be predicted using a pressure gauge attached to its outlet. A 69 kPa reading of the pressure gauge indicates that the Dewar is full, while 0 kPa indicates that it is empty.

3. Protocols Before Analyzing Samples

1. Ensure there are no major leaks in the instrument. If the vacuum gauge reading of the TOF-MS is higher than 2.7×10^{-5} Pa for 1.5 ml min^{-1} GC column flow rate, this indicates a major leak in the system.
2. Set-up the quality control (QC) method and run in-built 'acquisition system adjustments' protocol to achieve maximum signal response using manufacturer's protocol.
3. Run in-built 'instrument optimization' protocols of QC method, in series - filament focus, ion optic focus and mass calibration tests using manufacturer's protocol. Ensure that mass calibration test passes. This QC method ensures that all the hardware parameters of the instrument are at optimum level.
4. Perform a "leak check" using manufacturer's protocol. Analyze automatically generates leak check report. Ensure that the relative concentration of 28 (N_2), 32 (O_2) and 18 (moisture) ions must be below less than 10%, 3% and 5% of internal standard mass spectra of 69 ion, respectively.
5. Tune the TOF-MS using manufacturer's protocol.
6. Run quality control method as well as TOF-MS tune protocol before and after leak check and also while analyzing samples and standards.

4. Protocol to Determine the Optimum Modulation Period of Modulator

1. Arbitrarily select a long modulation period (*e.g.* 10 sec or 13 sec). Inject a sample as described in 2.2.
2. Identify the retention time in second dimension of the contour plot after which no peaks elutes. Select identified second dimension retention time as optimum modulation period. **Figure 2** clearly elucidate the identification of retention time in second dimension of the contour plot.
3. Increase the modulation period used in step 4.1 and perform the analysis again if "wrap around" is observed[18]. Wrap around phenomena occurs if the peaks in the second dimension elutes below the baseline of first dimension. Example contour plot for 'wraparound' is shown in supplementary information **Figure 3**.
4. Repeat steps 4.2 and 4.3 until optimum value is determined.

5. Experimental Parameters of Instrument Set-up

1. Install a polar (60 m x 0.25 mm x 0.5 µm film thickness) capillary column as the primary column and a non-polar (2.3 m x 0.25 mm x 0.5 µm film thickness) capillary column as the secondary column. Bake both the primary and secondary column for at least 2 hr to remove trace amounts of moisture, air and contaminants associated with new GC columns.
2. Use ultrahigh purity helium gas as carrier gas for GC at a flow rate of 1.5 ml min^{-1}.
3. Set the GC injector to a temperature of 260 °C and a split ratio of 1:250.
4. Employ the following temperature program for the primary column: a constant temperature of 40 °C for 0.2 min followed by a temperature ramp to 260 °C at 5 °C min^{-1}, followed by a constant temperature of 260 °C for 5 min.
5. Maintain the modulator temperature 5 °C higher than that of the secondary column and the secondary column temperature at 5 °C higher than that of the primary column.
6. Use an optimum modulation period of 4 sec with 0.8 sec of hot pulse and 1.2 sec of cold pulse. This value is determined based on the protocol described in section 4.
7. Set transfer line temperature to 270 °C.
8. Set the acquisition delay or solvent delay to 0 sec.
9. Set the lower and higher range of m/z as 35 and 800, respectively.
10. Set the MS detector acquisition rate at 400 spectra/sec.
11. Maintain the MS detector voltage at 150 V higher than the optimized value.
12. Maintain the MS ion source temperature at 225 °C.

6. Data Analysis

1. Perform data processing using the software supplied by the instrument manufacturer.
2. Select the following tasks in the data analysis method - Compute baseline, find peaks above the baseline, library search and calculate are/ height.
3. Track the baseline through the data file. Enter baseline offset as 0.5.
   Enter expected peak width of 15 sec in the first dimension and 0.15 sec in the second dimension.
4. Set signal-to-noise ratio as 5,000 and similarity values of >850 for identification of compounds.
5. Select a commercially available mass spectral library to identify chemical compounds present in samples and set the library search mode to forward.
6. Process the data files using this data analysis method using manufacturer's protocol. It requires at least 1 hr to process a data file.

Representative Results

A total ion chromatogram (TIC) obtained for the aqueous fraction of algae bio-crude analyzed with a column combination of polar × non-polar is shown in **Figure 4**. Retention times and similarity or match factor values of compounds identified by searching against a National Institute of Standards and Technology (NIST) library are tabulated in **Table 1**. Oxygenates (such as cyclopenatanone, furanic compounds and dianhydromannitol) and organic acids (including acetic acid, propanoic acid and butanoic acid) were observed in HTL algae water[34]. These chemicals could be formed from the degradation of the algae carbohydrate fraction during HTL[13]. In addition to oxygenates, the aqueous phase has nitrogen containing compounds (N-compounds) such as pyridine, pyrazine, acetamides, succinimide and their alkyl-derivatives. Presumably, these compounds are the degradation products of proteins in algal biomass[4,35].

The high-intensity peaks identified in the contour plot for the aqueous fraction of algae bio-crude were validated by analyzing standards. Standards containing organic acids and N-compounds were prepared and analyzed in GC × GC–TOF-MS. Total ion chromatogram of the organic acids standard and N-compound standards are shown in **Figure 5**. Retention time and similarity values of the standards are tabulated in **Table 2** and correspond to the identified chemical compounds in HTL algae water. Column bleed was observed for both standards and samples at high temperatures (>250 °C). This column bleed has been previously reported in the literature for polar GC columns[18]. Carbon dioxide (CO_2) was observed in HTL algae water whereas it was not seen in the standards (see **Figures 4** and **5**). This indicates that the aqueous fraction of algae bio-crude has dissolved CO_2, which may be produced during the HTL of algal feedstocks[11].

The aqueous fraction of algae bio-crude was also analyzed with the conventional column combination of non-polar × polar which was widely used in the literature[17]. The total ion chromatogram of HTL algae water from a GC × GC–TOF-MS analysis with a non-polar primary separation followed by a polar secondary separation is shown in **Figure 6**. As shown in **Figure 6**, organic acids and N-compounds present in the aqueous fraction of algae bio-crude elute with more than one peak. Acetic acid and other organic acids elute throughout the duration of the analysis, especially in the first dimension. Retention times and similarity/confidence values of the compounds identified by searching against a NIST library are tabulated in **Table 3**. Peak capacity of the conventional column configuration (24, see **Table 3**) is lower than that of polar × non-polar (50, see **Table 1**) while using same data analysis method. It can be concluded that peak capacity, peak shapes, and resolution of the HTL algae water were poor for the analysis where the non-polar is the primary and the polar is the secondary separation. Therefore, this column configuration of non-polar × polar is not suitable for qualitative as well as quantitative characterization of aqueous algae bio-crude without prior sample preparation.

A long modulation period (see the secondary axis of **Figure 6**) was necessary to characterize the aqueous fraction of algae bio-crude for the non-polar × polar configuration. As previously shown in **Figure 4**, a short modulation time of 4 sec was sufficient for the characterization of HTL algae water using a column combination of polar × non-polar. Since a short modulation time is recommended for GC × GC analysis[16-18] to retain the separation obtained in the first dimension, this is another advantage of using polar × non-polar for characterization of HTL algae water.

GC × GC–TOF-MS analysis of aqueous algae bio-crude with a polar × non-polar column configuration produces symmetrical peak shape, improves peak capacity and high resolution when compared to a conventional column configuration of non-polar ×polar. Hence, GC × GC–TOF-MS analysis described using polar × non-polar can be employed for quantification of chemical compounds present in aqueous fraction of algae bio-crude without any sample preparation techniques.

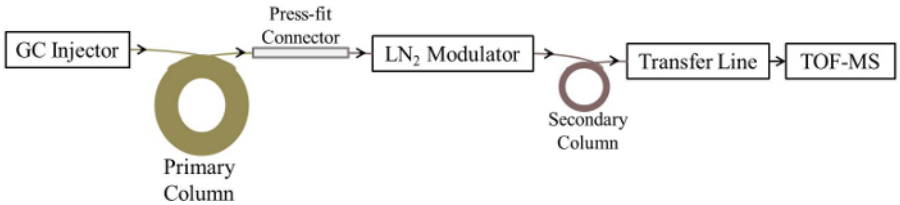

Figure 1: Block flow diagram of GC × GC-TOF-MS used in this study. Please click here to view a larger version of this figure.

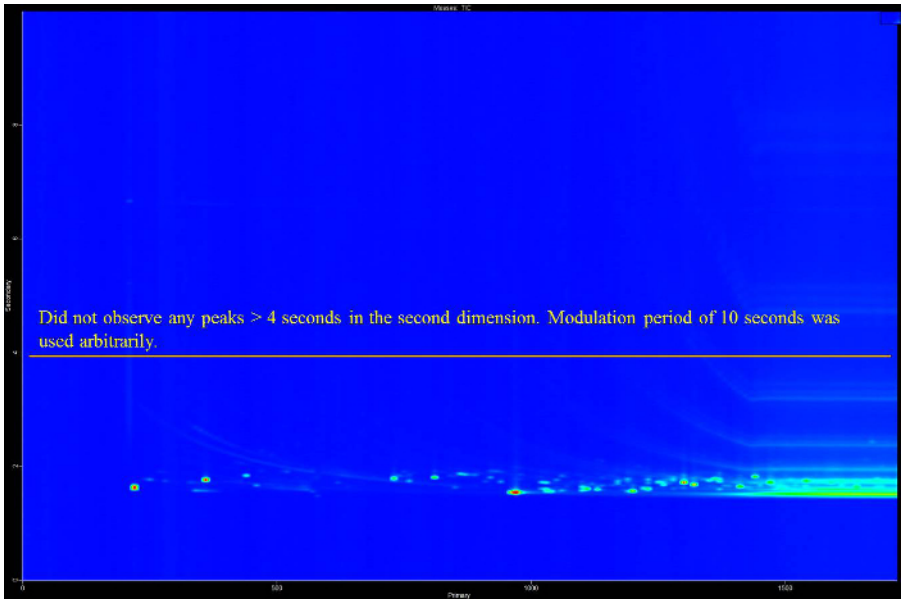

Figure 2: Contour plot of HTL algae aqueous fraction obtained using column combination of polar × non-polar for determining optimum modulation time. 10 seconds was randomly selected. No peaks were observed >4 sec in second dimension. Therefore, 4 sec was identified as optimum modulation time. Please click here to view a larger version of this figure.

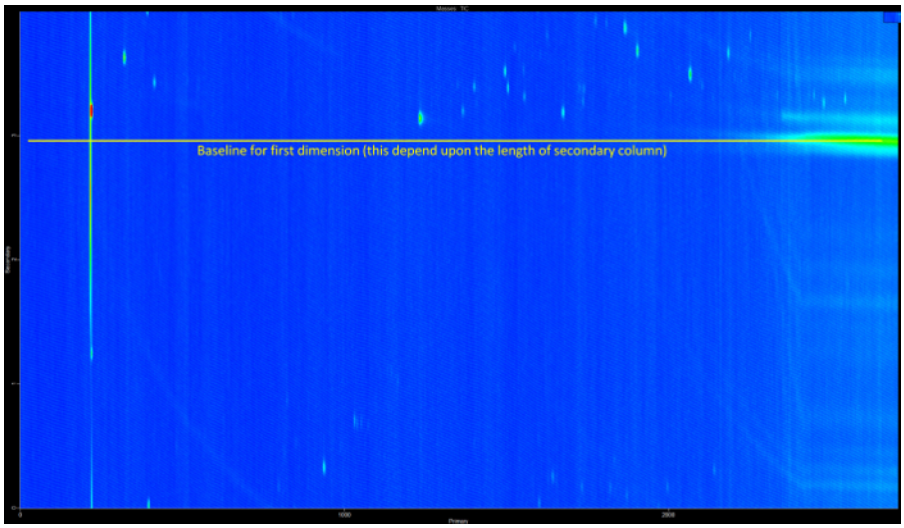

Figure 3: Contour plot of HTL algal aqueous fraction that shows 'wrap around' phenomena. Wrap around phenomena occurs if the peaks in the second dimension elutes below the baseline of first dimension. 3.5 m secondary column length was used to obtain this contour plot. This plot was collected to clearly explain wrap around phenomena. Please click here to view a larger version of this figure.

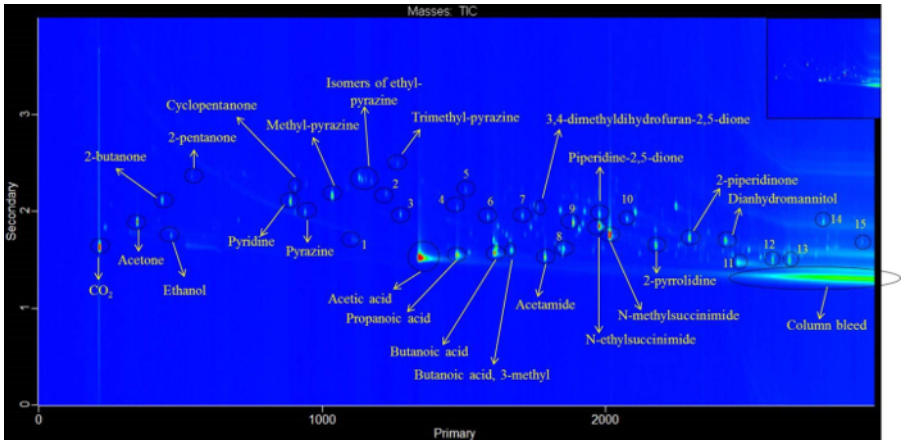

Figure 4: Contour plot of HTL algae aqueous fraction obtained using column combination of polar × non-polar. Chemical compounds were identified using NIST 2008 library. The units of primary and secondary axis are seconds. The similarity values of identified chemical compounds are tabulated in **Table 1**. 1 → 1-hydroxy-2-propanone; 2 → 2-cyclopenten-1-one, 2-methyl; 3 → *N,N*-dimethyl acetamide; 4 → 2-cyclopente-1-one, 3-methyl; 5 → 2-cyclopenten-1-one, 2,3-dimethyl; 6 → 3-pentenoic acid, 4-methyl; 7 → 2-pyrrolidinone, 1-methyl; 8 → propanamide; 9 →1H-Imidazole, 1-methyl-4-nitro-; 10 → *N*-propyl succinimide; 11 → glycerin; 12 → 3-pyridinol; 13 → 2,5-pyrrolidinedione; 14 → acetamide, *N*-(2-phenylethyl); 15 → *N*-(2-hydroxyethyl) succinimide. Please click here to view a larger version of this figure.

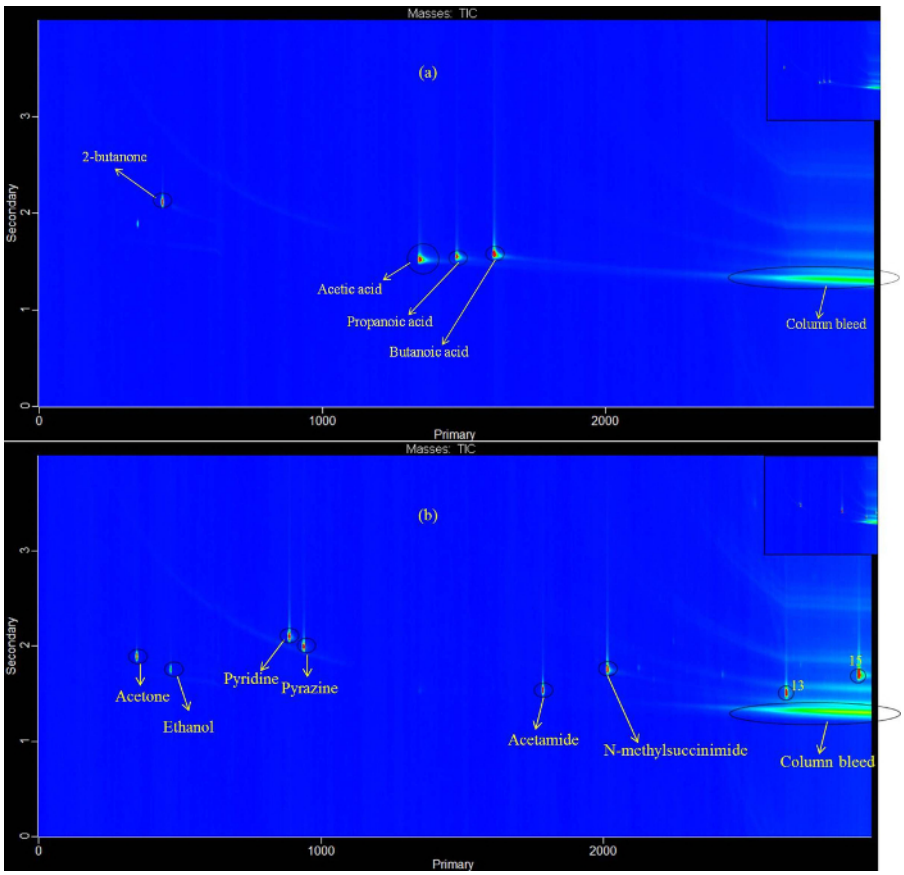

Figure 5: (**a**) Contour plot of standard containing acetic acid, propanoic acid, butanoic acid, and 2-butanone using column combination of polar × non-polar. (**b**) contour plot of standard containing acetone, ethanol, pyridine, pyrazine acetamide, *N*-methylsuccinimide, succinimide, and *N*-(2-hydroxyethyl)succinimide using column combination of polar × non-polar. The similarity values of standards are tabulated in **Table 2**. Please click here to view a larger version of this figure.

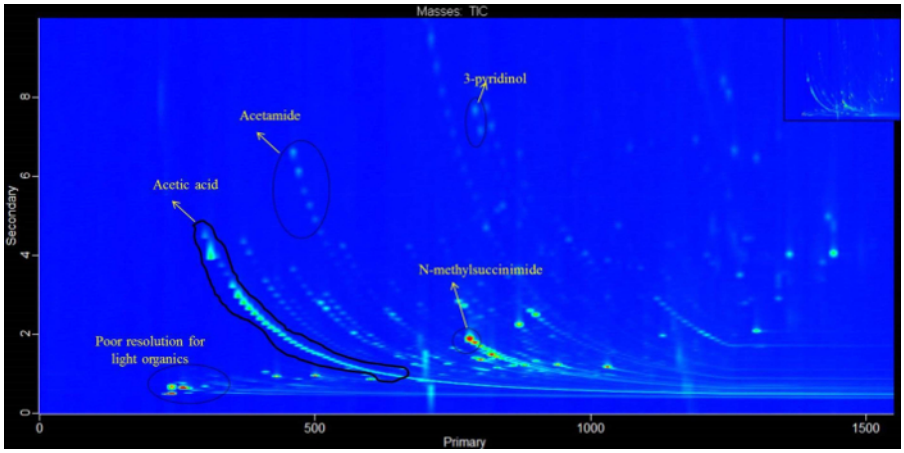

Figure 6: Contour plot of HTL algae aqueous fraction obtained using column combination of non-polar × polar. This figure shows poor resolution of light organics, organic acids and N-compounds. The similarity values of identified chemical compounds are tabulated in **Table 3**.
Please click here to view a larger version of this figure.

Name	R.T. (sec)	Similarity
Carbon dioxide	215, 1.64	999
Acetone	347, 1.89	967
2-Butanone	435, 2.12	965
Ethanol	467, 1.75	949
2-Pentanone	539, 2.36	942
3-Pentanone	539, 2.41	940
Pyridine	887, 2.11	967
Cyclopentanone	903, 2.25	962
Pyrazine	939, 1.99	945
Pyridine, 2-methyl-	943, 2.28	950
Pyrazine, methyl-	1035, 2.16	964
Pyridine, 3-methyl-	1087, 2.25	947
2-Propanone, 1-hydroxy-	1107, 1.71	950
Pyrazine, 2,5-dimethyl-	1131, 2.35	950
Pyrazine, 2,6-dimethyl-	1139, 2.33	953
Pyrazine, ethyl-	1151, 2.34	954
Pyrazine, 2,3-dimethyl-	1171, 2.32	963
2-Cyclopenten-1-one, 2-methyl-	1223, 2.19	960
Pyrazine, 2-ethyl-6-methyl-	1235, 2.54	926
Pyrazine, trimethyl-	1263, 2.49	944
Acetamide, *N,N*-dimethyl-	1275, 1.97	957
Acetic acid	1339, 1.53	963
Pyrrole	1443, 1.65	970
Propanoic acid	1475, 1.55	953
2-Cyclopenten-1-one, 3-methyl-	1475, 2.04	956
2-Cyclopenten-1-one, 2,3-dimethyl-	1503, 2.22	884
Propanoic acid, 2-methyl-	1515, 1.58	929
3-Pentenoic acid, 4-methyl-	1583, 1.95	897
Acetamide, *N*-ethyl-	1603, 1.71	950
Butanoic acid	1607, 1.58	941

Acetamide, *N*-methyl-	1615, 1.63	963
Propanamide, *N*-methyl-	1663, 1.70	956
Butanoic acid, 3-methyl-	1667, 1.60	928
2-Pyrrolidinone, 1-methyl-	1703, 1.96	936
3,4-Dimethyldihydrofuran-2,5-dione	1759, 2.05	719
Acetamide	1783, 1.53	976
1,2-Cyclopentanedione	1819, 1.67	888
Propanamide	1847, 1.57	870
1H-Imidazole, 1-methyl-4-nitro-	1883, 1.88	671
2,5-Pyrrolidinedione, 1-ethyl-	1975, 1.85	936
Piperidine-2,5-dione	1975, 1.98	798
2,5-Pyrrolidinedione, 1-methyl-	2011, 1.76	960
2,5-Pyrrolidinedione, 1-propyl-	2075, 1.92	861
2-Pyrrolidinone	2175, 1.65	976
2-Piperidinone	2295, 1.73	959
Dianhydromannitol	2419, 1.70	944
Glycerin	2463, 1.47	888
3-Pyridinol	2586, 1.50	921
2,5-Pyrrolidinedione	2646, 1.50	923
N-[2-Hydroxyethyl]succinimide	2902, 1.69	941

Table 1: Similarity values and retention time of chemical compounds present in HTL algae water using column combination of polar × non-polar. Compounds were identified using the NIST 2008 Library. The scale of similarity values is 0-999. Higher similarity values correspond to a closer match of the spectra obtained for that sample to that for the compound in the NIST database. R.T. represents retention time of chemical compounds (primary, secondary).

Name	**R.T. (sec)**	**Similarity**
Acetone	347 , 1.89	952
2-Butanone	435, 2.12	934
Ethanol	467 , 1.76	952
Pyridine	887, 2.10	947
Pyrazine	939, 1.99	928
Acetic acid	1339, 1.53	981
Propanoic acid	1471, 1.56	948
Butanoic acid	1603, 1.59	935
Acetamide	1783, 1.54	961
2,5-Pyrrolidinedione, 1-methyl-	2011, 1.76	957
2,5-Pyrrolidinedione	2642, 1.52	940
N-[2-Hydroxyethyl]succinimide	2902, 1.71	935

Table 2: Retention time and similarity values of standards analyzed using polar × non-polar. Compounds were identified using the NIST 2008 library. The scale of similarity values is 0-999. Higher similarity values correspond to a closer match of the spectra obtained for the standard to that for the compound in the NIST database. R.T. represents retention time of chemical compounds (primary, secondary).

Name	R.T. (s)	Similarity
Carbamic acid, monoammonium salt	234 , 0.521	999
Carbamic acid, monoammonium salt	234 , 0.653	981
Trimethylamine	243 , 0.540	922
Acetone	243 , 0.648	927
Dimethyl ether	243 , 0.720	932
Dimethylamine	252 , 0.578	925
2-Butanone	261 , 0.684	933
Acetic acid	261 , 3.139	963
Methanethiol	306 , 0.550	924
Pyrazine	333 , 1.157	949
Pyridine	342 , 1.063	950
Cyclopentanone	378 , 1.032	944
Pyrazine, methyl-	405 , 1.217	954
Acetamide, *N*-methyl-	414 , 4.850	887
2-Cyclopenten-1-one, 2-methyl-	504 , 1.409	951
Pyrazine, 2,5-dimethyl-	513 , 1.207	919
Pyrazine, 2,3-dimethyl-	522 , 1.265	905
2,5-Pyrrolidinedione, 1-methyl-	801 , 4.178	955
Quinuclidine-3-ol	828 , 2.750	680
2,5-Pyrrolidinedione, 1-ethyl-	873 , 3.058	889
2-Piperidinone	954 , 5.474	954
Caprolactam	963 , 2.458	746
N-[2-Hydroxyethyl]succinimide	1089 , 2.429	857
N-[2-Hydroxyethyl]succinimide	1260 , 2.278	814
1-Phenethyl-pyrrolidin-2,4-dione	1791 , 3.742	788
5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine	2016 , 4.608	787

Table 3: Similarity values and retention time of chemical compounds identified in HTL algae water using column combination of non-polar × polar. Compounds were identified using the NIST 2008 library. The scale of similarity values is 0-999. Higher similarity values correspond to a closer match of the spectra obtained for the sample to that for the compound in the NIST database. R.T. represents retention time of chemical compounds (primary, secondary).

Discussion

Results clearly illustrate the ability of the column combination of polar × non-polar to resolve polar compounds and light volatiles present in the aqueous fraction of algae bio-crude without prior sample preparation techniques. Drastic peak tailing was observed for organic acids and N-compounds while using the non-polar× polar column combination. This peak tailing was not observed for the early eluting light organics. This behavior has been reproducible when verifying the instrument is free of leaks (the vacuum in TOF-MS was below 2.7×10^{-5} Pa for GC carrier gas flow rate of 1.5 ml min^{-1}). It would be expected that if there was an issue with dead volume in the press tight connector or if the cold jet flow rate would be excessive that the behavior would be observed across the chromatogram. However, even late eluting compounds (not identified on the figure) do not tail. Therefore, we conclude that this is a result of the aqueous sample injection/column configuration combination.

The split ratio is the volume of sample entering the column versus the amount lost to the split flow. The higher the split ratio the smaller the amount of sample introduced onto the column. Generally this produces more efficient peaks which would improve peak capacity. Determining the proper split ratio for samples can prevent problems from column overloading (split ratio too low) or issues with compound detection (split ratio too high). Therefore, a split ratio of 1:250 was used in the GC × GC–TOF-MS data acquisition methods for both column combinations to prevent column loading and also to improve peak capacity.

Similarity values for chemical compounds identified are in the range of 850-999. This indicates that chemical compounds are identified with more than 85% confidence. This was achieved by using an MS acquisition rate of 400 spectra/second in GC × GC–TOF-MS data acquisition methods. A 400 spectra/second acquisition rate improves the signal-to-noise ratio of peaks which increases the similarity values of identified chemical compounds[17]. Higher similarity values enable us to identify chemical compounds with high confidence. However, this high MS acquisition

rate results in a long data analysis time. Therefore, it is recommended to use a 200 spectra/sec MS acquisition rate for quantification of these samples which decreases the data analysis time.

The GC × GC–TOF-MS data acquisition method developed for characterizing aqueous algae bio-crude with polar × non-polar could be further improved by increasing the length of the secondary column. By increasing the length of the secondary column, resolution can be improved in the second dimension which enables the separation of isomers present in the sample[16,17]. Peak capacity could also be further improved with increase in the length of the secondary column. HTL algae waters characterized in this paper are dilute[11] (contain approximately 3 total wt% of carbon) and may not require a longer secondary column. However, this recommendation could be beneficial during characterization of complex and concentrated aqueous samples.

Since the maximum programmable temperature of the polar column is 260 °C, this method cannot elute high boiling point chemical compounds such as long chain fatty acids, mono-glycerides, di-glycerides, triglycerides and oligomers of amino acids as well as sugars[16]. Samples containing these compounds, when analyzed, may contaminate the GC injector and columns. Contamination of GC injector and columns leads to peak tailing, change in the retention time of chemical compounds, and high noise or low signal-to-noise ratio of the MS detector which are undesirable for qualitative as well as quantitative characterization. Hence, when utilizing this column combination for analyzing aqueous samples containing high boiling point chemical compounds analysts should employ appropriate quality control methods.

The chemical compounds identified in the aqueous fraction of algae bio-crude have a wide variety of applications. Pyridine, pyrazine and their alkyl derivatives are intermediate chemicals for production of agrochemicals, drugs[36,37], and are widely used as solvents in homogenous catalysis[38,39]. Similarly, derivatives of succinimide also have a wide variety of applications including polymer intermediates, detergents[40], clinical drugs[41,42], fuel additives and lubricating oil additives[40]. The organic acids present in HTL algae water can be used as a feedstock in catalytic processes to produce ketones or esters for easy separation from the aqueous phase[43].

The GC × GC–TOF-MS method developed for the column combination of polar × non-polar in this paper can also be employed to analyze water sample from biological process, food processing, and environmental wastes. Researchers used this column combination for characterization of organic samples[44-47]. It is reported that this column combination is best for effective separation of different classes of hydrocarbons - aliphatic, aromatics, alkyl benzene and binuclear aromatics[44-46]. Therefore, utilizing a polar separation for the first dimension of separation and non-polar for the second dimension of separation would be suitable column configurations for characterization of both aqueous as well as organic fraction of bio-crude produced from hydrothermal liquefaction of biomass.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Huber, G. W., Iborra, S., & Corma, A. Synthesis of Transportation Fuels from Biomass:Chemistry, Catalysts, and Engineering. *Chem. Rev.* **106**, 4044-4098 (2006).
2. Mata, T. M., Martins, A. A., & Caetano, N. S. Microalgae for biodiesel production and other applications: A review. *Renew. Sustain. Energy Rev.* **14**, 217-232 (2010).
3. Vispute, T. P., Zhang, H., Sanna, A., Xiao, R., & Huber, G. W. Renewable Chemical Commodity Feedstocks from Integrated Catalytic Processing of Pyrolysis Oils. *Science.* **330**, 1222-1227 (2010).
4. Maddi, B., Viamajala, S., & Varanasi, S. Comparative study of pyrolysis of algal biomass from natural lake blooms with lignocellulosic biomass. *Bioresour. Technol.* **102**, 11018-11026 (2011).
5. Kim, S., & Dale, B. E. Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioenergy*. **26**, 361-375 (2004).
6. von Blottnitz, H., & Curran, M. A. A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. *J. Clean. Prod.* **15**, 607-619 (2007).
7. Hu, Q. *et al.* Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* **54**, 621-639 (2008).
8. Georgianna, D. R., & Mayfield, S. P. Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature.* **488**, 329-335 (2012).
9. Amaro, H. M., Guedes, A. C., & Malcata, F. X. Advances and perspectives in using microalgae to produce biodiesel. *Appl. Energy.* **88**, 3402-3410 (2011).
10. Elliott, D. C., Biller, P., Ross, A. B., Schmidt, A. J., & Jones, S. B. Hydrothermal liquefaction of biomass: Developments from batch to continuous process. *Bioresour. Technol.* **178**, 147-156 (2015).
11. Elliott, D. C. *et al.* Process development for hydrothermal liquefaction of algae feedstocks in a continuous-flow reactor. *Algal Res.* **2**, 445-454 (2013).
12. Sudasinghe, N. *et al.* High resolution FT-ICR mass spectral analysis of bio-oil and residual water soluble organics produced by hydrothermal liquefaction of the marine microalga Nannochloropsis salina. *Fuel* **119**, 47-56 (2014).
13. Panisko, E., Wietsma, T., Lemmon, T., Albrecht, K., & Howe, D. Characterization of the aqueous fractions from hydrotreatment and hydrothermal liquefaction of lignocellulosic feedstocks. *Biomass Bioenergy.* **74**, 162-171 (2015).

14. Onwudili, J. A., Lea-Langton, A. R., Ross, A. B., & Williams, P. T. Catalytic hydrothermal gasification of algae for hydrogen production: Composition of reaction products and potential for nutrient recycling. *Bioresour. Technol.* **127**, 72-80 (2013).
15. Villadsen, S. R. *et al.* Development and Application of Chemical Analysis Methods for Investigation of Bio-Oils and Aqueous Phase from Hydrothermal Liquefaction of Biomass. *Energy Fuels* **26**, 6988-6998 (2012).
16. Klee, M. S., Cochran, J., Merrick, M., & Blumberg, L. M. Evaluation of conditions of comprehensive two-dimensional gas chromatography that yield a near-theoretical maximum in peak capacity gain. *J. Chromatogr. A.* **1383**, 151-159 (2015).
17. Seeley, J. V., & Seeley, S. K. Multidimensional Gas Chromatography: Fundamental Advances and New Applications. *Anal. Chem.* **85**, 557-578 (2013).
18. Mostafa, A., Edwards, M., & Gòrecki, T. Optimization aspects of comprehensive two-dimensional gas chromatography. *J. Chromatogr. A*. **1255**, 38-55 (2012).
19. Zhu, S. *et al.* A simple model for separation prediction of comprehensive two-dimensional gas chromatography and its applications in petroleum analysis. *Anal. Methods* **6**, 2608-2620 (2014).
20. Almeida, T. M. *et al.* Preliminary Studies of Bio-oil from Fast Pyrolysis of Coconut Fibers. *J. Agric. Food Chem.* **61**, 6812-6821 (2013).
21. Rathsack, P. *et al.* Analysis of pyrolysis liquids from scrap tires using comprehensive gas chromatography-mass spectrometry and unsupervised learning. *J. Anal. Appl. Pyrolysis* **109**, 234-243 (2014).
22. Tessarolo, N. S. *et al.* Assessing the chemical composition of bio-oils using FT-ICR mass spectrometry and comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry. *Microchem. J.* **117**, 68-76 (2014).
23. Djokic, M. R., Dijkmans, T., Yildiz, G., Prins, W., & Van Geem, K. M. Quantitative analysis of crude and stabilized bio-oils by comprehensive two-dimensional gas-chromatography. *J. Chromatogr. A.* **1257**, 131-140 (2012).
24. Vendeuvre, C., Ruiz-Guerrero, R., Bertoncini, F., Duval, L., & Thiebaut, D. Comprehensive two-dimensional gas chromatography for detailed characterisation of petroleum products. *Oil Gas Sci. Technol.* **62**, 43-55 (2007).
25. Guo, Q. *et al.* Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry for the screening of potent swampy/septic odor-causing compounds in two drinking water sources in China. *Anal. Methods* **7**, 2458-2468 (2015).
26. Ma, H. *et al.* Analysis of human breath samples of lung cancer patients and healthy controls with solid-phase microextraction (SPME) and flow-modulated comprehensive two-dimensional gas chromatography (GC [times] GC). *Anal. Methods* **6**, 6841-6849 (2014).
27. Lamani, X., Horst, S., Zimmermann, T., & Schmidt, T. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS). *Anal. and Bioanal. Chem.* **407**, 241-252 (2015).
28. Skoczynska, E., Leonards, P., & de Boer, J. Identification and quantification of methylated PAHs in sediment by two-dimensional gas chromatography/mass spectrometry. *Anal. Methods*. **5**, 213-218 (2013).
29. Tobiszewski, M., Bigus, P., & Namiesnik, J. Determination of parent and methylated polycyclic aromatic hydrocarbons in water samples by dispersive liquid-liquid microextraction-two dimensional gas chromatography-time-of-flight mass spectrometry. *Anal. Methods.* **6**, 6678-6687 (2014).
30. Freitas, L. S. *et al.* Analysis of organic compounds of water-in-crude oil emulsions separated by microwave heating using comprehensive two-dimensional gas chromatography and time-of-flight mass spectrometry. *J. Chromatogr. A* **1216**, 2860-2865 (2009).
31. Gunatilake, S. R., Clark, T. L., Rodriguez, J. M., & Mlsna, T. E. Determination of five estrogens in wastewater using a comprehensive two-dimensional gas chromatograph. *Anal. Methods*. **6**, 5652-5658 (2014).
32. Ljungkvist, G., Larstad, M., & Mathiasson, L. Determination of low concentrations of benzene in urine using multi-dimensional gas chromatography. *Analyst.* **126**, 41-45 (2001).
33. Schummer, C., Delhomme, O., Appenzeller, B. M. R., Wennig, R., & Millet, M. Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. *Talanta.* **77**, 1473-1482 (2009).
34. Yang, H. P., Yan, R., Chen, H. P., Lee, D. H., & Zheng, C. G. Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel.* **86**, 1781-1788 (2007).
35. Du, Z. *et al.* Microwave-assisted pyrolysis of microalgae for biofuel production. *Bioresour. Technol.* **102**, 4890-4896 (2011).
36. Scriven, E. F. V., & Murugan, R. in *Kirk-Othmer Encyclopedia of Chemical Technology.* John Wiley & Sons, Inc., (2000).
37. Higashio, Y., & Shoji, T. Heterocyclic compounds such as pyrrole, pyridines, pyrrolidine, piperidine, indole, imidazol and pyrazines. *Appl. Catal. A: Gen.* **260**, 251-259 (2004).
38. Ndaji, F. E., & Thomas, K. M. The kinetics of coal solvent swelling using pyridine as solvent. *Fuel*. **72**, 1525-1530 (1993).
39. Fillon, H., Gosmini, C., Nédélec, J.-Y., & Périchon, J. Electrosynthesis of functionalized organodizinc compounds from aromatic dihalides via a cobalt catalysis in acetonitrile/pyridine as solvent. *Tetrahedron Lett.* **42**, 3843-3846 (2001).
40. Silin, M. A., Ivanova, L. V., Burov, E. A., Koshelev, V. N., & Bordubanova, E. G. Synthesis and testing of polyalkenyl succinimides as components of detergent additives for motor fuels. *Pet. Chem.* **52**, 272-277 (2012).
41. Bialer, M. Chemical properties of antiepileptic drugs (AEDs). *Adv. Drug Deliv. Rev.* **64**, 887-895 (2012).
42. Bellina, F., & Rossi, R. Synthesis and biological activity of pyrrole, pyrroline and pyrrolidine derivatives with two aryl groups on adjacent positions. *Tetrahedron*. **62**, 7213-7256 (2006).
43. Snell, R. W., & Shanks, B. H. CeMOx-Promoted Ketonization of Biomass-Derived Carboxylic Acids in the Condensed Phase. *ACS Catal.* **4**, 512-518 (2014).
44. Manzano, C., Hoh, E., & Simonich, S. L. M. Improved Separation of Complex Polycyclic Aromatic Hydrocarbon Mixtures Using Novel Column Combinations in GC × GC/ToF-MS. *Environ. Sci. Technol.* **46**, 7677-7684 (2012).
45. van der Westhuizen, R. *et al.* Comprehensive two-dimensional gas chromatography for the analysis of synthetic and crude-derived jet fuels. *J. Chromatogr. A* **1218**, 4478-4486 (2011).
46. Omais, B. *et al.* Investigating comprehensive two-dimensional gas chromatography conditions to optimize the separation of oxygenated compounds in a direct coal liquefaction middle distillate. *J. Chromatogr. A* **1218**, 3233-3240 (2011).
47. Wildschut, J., Mahfud, F. H., Venderbosch, R. H., & Heeres, H. J. Hydrotreatment of Fast Pyrolysis Oil Using Heterogeneous Noble-Metal Catalysts. *Ind. Eng. Chem. Res.* **48**, 10324-10334 (2009).

