Selective profiling of carboxylic acid in crude oil by halogen-labeling combined with liquid chromatography and high-resolution mass spectrometry

Khoa Huynh[1†], Karen Louise Feilberg[1], Jonas Sundberg[1]
[1] DTU Offshore, Technical University of Denmark, 2800 Lyngby, Denmark
[†] Corresponding author, email address: akhoa@dtu.dk

Abstract

Carboxylic acids are a small but important compound class within petroleum chemistry, contributing significantly to the behavior of crude oil, e.g., in production, processing, and its environmental impact. A more detailed structural information is fundamental to improve our understanding of their influence on petroleum properties. However, due to matrix effects, structural diversity and low abundance, selective characterization of carboxylic acids in crude oil remains challenging. In this work, we present a new methodology for profiling such compounds by liquid-liquid extraction and selective derivatization using 4-bromo-N-methylbenzylamine (4-BNMA) followed by liquid chromatography and electrospray ionization Orbitrap high-resolution mass spectrometry (LC-ESI-Orbitrap HRMS). The fragmentation of 4-BNMA derivatives produces a pair of unique product ions, m/z 169 and 171, which enables the identification of chromatographic sections containing carboxylic acids. The mass spectra of these fractions are extracted, with the acids further computationally isolated based on the unique isotope pattern from the incorporated bromine atom. The method was optimized and validated using seven acid standards and designs of experiments (DOEs), assuring robustness and sensitivity for non-target screening purposes. This method successfully detected up to 380 carboxylic acids in six Danish North Sea crude oil, with up to two carboxyl groups and other heteroatom functionalities. The results indicated that the most populated species are fatty acids (double bond equivalents, DBE, = 1) and naphthenic acids (DBE = 2 – 6). Besides classical carboxylic acids detected, we observed other acids containing multiple functional groups with nitrogen, oxygen, and sulfur atoms.

1 Introduction

Carboxylic acids play an essential role in petroleum chemistry.[1-4] Due to their amphiphilic nature, carboxylic acids in are surface active and contribute to the interfacial behavior and stability of water-oil emulsion systems, which is critical in production systems.[5, 6] The acidic fractions are associated with catalyst poisoning and corrosion of equipment and pipelines, leading to problems in production and refining.[7, 8] However, carboxylic molecules are also valuable markers providing important information about the migration, maturation, and degradation of petroleum. Furthermore, due to their toxicity, aromatic and naphthenic acids are of environmental concern.[9-11]

A detailed characterization of carboxylic acids in crude oil is therefore desirable but remains a significant challenge. It is well-recognized that their structural and compositional diversity and the complexity of crude oil matrices are analytically challenging.[12, 13] Several attempts have been made to separate and enrich carboxylic acids from petroleum samples for further analysis. Several studies employed liquid-liquid extraction (LLE) due to its simplicity and convenience.[4, 14, 15] Other approaches have been explored to improve selectivity and efficiency, including (micro) solid-phase extraction,[16-21] magnetic dispersive extraction,[22] and ionic liquid extraction[23-25]. Analysis without prior sample treatment using high-resolution mass spectrometry (HRMS), including Orbitrap and Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), allows determining a large number of compounds via direct infusion due to the high resolving power and mass accuracy.[26] High levels of heteroatomic compositional detail are acquired accordingly, including the molecular composition, elemental distribution, degree of saturation, and abundance.[27-30] However, these methods do not provide molecular structures or connectivity. Thus, despite the instrumental advantages and improvements of direct infusion HRMS, hyphenated techniques are beneficial to increase structural coverage and information. Therefore, recent method developments have focused on HRMS coupled with liquid and or gas chromatography, including multidimensional separations.[31-35] Ultimately, developing new, more effective analytical strategies within both sample preparation, analytical separations and data processing is essential to increase our understanding of petroleum acids.

Herein, we describe a new methodology for profiling carboxylic acids in crude oil using a halogenated amine tag with subsequent LC separation and precursor ion search by Orbitrap HRMS. The acidic fraction was first isolated by LLE using a basic aqueous-solvent solution. The carboxylic acids in the extractant were then activated and derivatized with 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) and 4-bromo-N-methylbenzylamine (4-BNMA), respectively (Figure 1). The method is based on Marquis et al. who observed that 4-BNMA can (i) facilitate complete derivatization of small mono- and poly-carboxylic acids, (ii) feature a favorable fragmentation characteristic – bromine isotopic pattern, for unknown acid identification, and (iii) improve selectivity in reversed-phase LC.[36] The fragmentation of derivatives to phenyl bromine ion pair (m/z 169 and 171) is an indicator to profile unknown carboxylic acid molecules. Thus, the selectivity of the derivatization is combined with computational isolation of the unique resulting fragmentation pattern. This approach can significantly simplify the complex crude oil matrix and data processing while increasing confidence in the formula assignments. The method performance was comprehensively optimized and evaluated, focusing on extraction and derivatization, assessing method sensitivity, and matrix effects. The applicability was demonstrated by the analysis of six North Sea crude

oils. Up to 380 carboxylic acid constituents were identified, primarily aliphatic and naphthenic acids with a smaller fraction of molecules containing additional heteroatoms (i.e., nitrogen and sulfur).

2 Methodology

2.1 Chemical and reagents

Carboxylic acid standards and derivatizing reagents were purchased from Sigma Aldrich and used as received. Adipic acid (> 99%), benzoic acid (≥ 99.5%), butyric acid (≥ 99%), 2-naphthoic acid (98 %), octanoic acid (≥ 99%), 4-phenylbultyric acid (99%), 1-pyreneacetic acid (97%), EDC (≥ 97%), and 4-BNMA (97%). LC-MS grade solvents and additives, acetonitrile (ACN), dichloromethane (DCM), methanol (MeOH), toluene (Tol), and formic acid (FA,) were purchased from Sigma Aldrich. Hydrochloric acid 37% (analysis grade) and ammonium hydroxide 25% (extra-pure grade) were received from Thermal Fisher Scientific. The Milli-Q water (> 18 MΩ cm) was provided from a Milli-Q Advantage A10 Ultrapure Water Purification System (Merck Millipore). For the method validation, a model oil was prepared by mixing hexadecane (Sigma Aldrich, ReagentPlus®, 99%) and toluene with an 8:2 v/v ratio followed by the addition of model acids.

The individual stock solutions of acid standards were prepared in DCM in a concentration range of 9 – 12 g L^{-1} (35 – 115 mM) and diluted with DCM, MeOH, or ACN for use as working solutions. Each acid standard in the mixture/model oil was 20 µM for optimization experiments and optimization of ionization efficiency via direct infusion analysis. The stock solutions of EDC and 4-BNMA were prepared freshly in the corresponding solvent before being used with a concentration level of 150 – 210 g L^{-1} (750 – 1350 mM). The intermediate solutions of EDC and 4-BNMA at 30 mM were prepared for low-concentration spiking. Solutions of 0.3% hydrochloric acid in solvents (DCM, MeOH or ACN) were prepared to adjust the extractants pH for EDC – 4-BNMA couplings.

For method demonstration, six crude oils from the Danish region of the North were provided by TotalEnergies DK. The crude oils were sourced from the Halfdan, Dan, Kraka, Valdemar, Skjold, and Tyra West fields and sampled at the separator. Oil data and bulk properties, including total acid number (TAN, mg KOH g^{-1}), density, and compound class distributions, are described elsewhere.[37]

2.2 Liquid chromatography – mass spectrometry

The instrumentation setup consisted of a Vanquish UHPLC system coupled to a Q-Exactive HF Ultra-High-Field Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an Ion Max II electrospray ionization (ESI) source. Separation was achieved using a Phenomenex Kinetic Biphenyl (2.1 × 150 mm, 2.6 µm) LC column fitted with a Phenomenex phenyl guard cartridge (4 × 2.0 mm). The sample injection volume was 20 µL (provided by a Vanquish VF split autosampler, held at 4 °C), and samples were eluted using an acetonitrile–water gradient (Table S1). The Ion Max II consists of a heat ESI probe (HESI-II) with sheath, auxiliary and sweep gas pressure set at 50, 13 and 3 arbitrary units, respectively. The spray voltage was 3.5 kV. The auxiliary gas heater and capillary temperature were set to 420 °C and 260 °C, respectively.

The precursor ion and fragmentation of acid standard derivatives were studied by direct infusion of solutions containing 20 µM of individual derivatives using a syringe pump with a flow rate of 40 µL min^{-1}

combined with a 400 µL min^{-1} auxiliary flow of ACN:H_2O (50:50, v/v) with 1% FA to mimic the detection during the chromatographic process. The experiments were measured in full scan mode (200 – 1000 m/z) and all-ion fragmentation (AIF) mode to identify the carboxylic derivative with the ion-pair fragment of 169 and 171 m/z. The normalized collision energy (NCE) was optimized at 35 arbitrary units. For optimization experiments, the responses of acid derivatives were recorded with selected ion monitoring mode (Targeted-SIM), using a precursor ion inclusion list provided from direct infusion study (Table 2). For profiling of unknown carboxylic acids, we applied the full MS/AIF scanning mode with a sequence of full MS scan (without high-energy collision dissociation cell – HCD fragmentation) followed by AIF with NCE applied. Full scan spectra (200 – 1000 m/z, resolution 120 000) and AIF spectra (144 – 194 m/z, resolution 120 000 and NCE = 35) were acquired. Chromatograms and mass spectra data were processed using the XCalibur software (Thermo Scientific) and exported as comma separated files for further processing as described below.

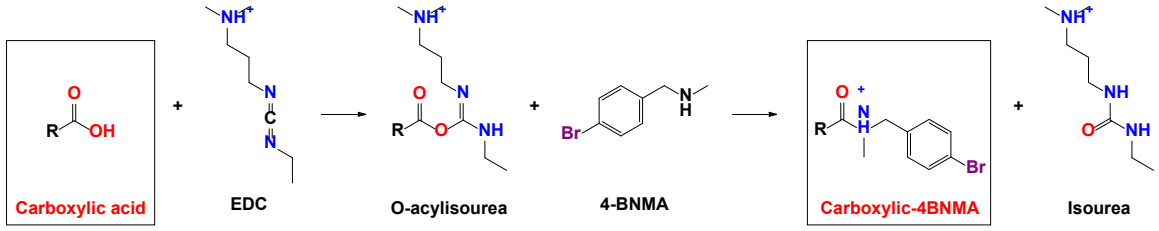


Figure 1. Simplified schematic of EDC activation followed by 4-BNMA derivatization of a generic carboxylic acid.

2.3 Carboxylic acid derivatization optimization

Figure 1 shows the two-step derivatization of carboxylic acid, i.e., carbamide activation with EDC and, subsequently, substitution with 4-BNMA. The carboxyl (-COOH) activation with EDC prevents acid-base reaction with 4-BNMA, making the acid susceptible to nucleophilic substitution.[38] The derivatization was optimized with respect to EDC and 4-BNMA concentrations (corresponding to reagent: -COOH molar ratios in the range of 1 to 100), EDC and 4-BNMA coupling times (t_1 and t_2), and solvent (DCM, ACN, EtOH, MeOH). Seven acid standards were used with molecular structures and reactions scheme shown in Figures 2 and S1.

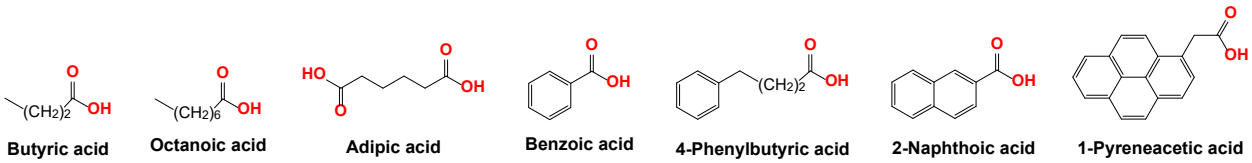


Figure 2. Molecular structure of the model carboxylic acids

Briefly, a 980 µL mixture of acid standards (each at a concentration of 20 µM) was placed in a 2 mL vial. To this solution, 5 – 15 µL of EDC stock solution (in DCM, ACN, EtOH or MeOH) was added and the mixture was kept at 60 °C for t_1 hours. The reaction was continued by adding 5 – 15 µL of 4-BNMA stock solution held at 60 °C for t_2 hours. Finally, the solution was cooled to room temperature, dried under a low nitrogen

gas flow for 10 minutes, and reconstituted in 1 mL ACN-H_2O (1:1, v/v). Before analysis, the sample was filtered using a syringe filter (0.2 µm, polytetrafluoroethylene).

A design of experiment (DOE) software – MODDE® (Umetrics, Sweden), was used for setup experiments and data analysis. DOE_1 with generalized subsets design (GSD) examined the influence of factors on reactions (Table 1), including factors 1 and 2 (EDC and 4-BNMA concentrations = 160 – 16000 µM), factors 3 and 4 (t_1, t_2 = 0.5 – 24 hours), and factor 5 (solvents). The responses were integrated peak areas of seven carboxylic-4BNMA derivatives measured by LC-Orbitrap HRMS.

2.4 Optimization of carboxylic acid extractions

DOE_2 with a two-level full factorial design (FFD) was used to define the optimal extraction condition of acidic fractions with LLE. The model oil consisted of seven carboxylic acid standards was used. Three factors were examined, including ammonium hydroxide concentration (corresponding to pH 6.8 – 10.5 at 25 °C), water content, and solvent types (Table 1). For extractions, 200 µL model oil was extracted two times with 600 µL designed solutions by shaking at 1000 rpm for 2 hours. The extractants were isolated by centrifugation at 4400 rpm for 15 mins and collected into 2 mL amber vials. In the case of using ammonium hydroxide for LLE, 0.3% hydrochloric acid solution was used to adjust the extractant's pH to 6.5 before EDC – 4-BNMA labeling. The optimal labeling condition obtained from DOE_1 were applied for DOE_2: 320 µM EDC (molar ratio EDC: -COOH 2:1), 1600 µM 4-BNMA (molar ratio 4-BNMA: -COOH 10:1), t_1 = 0.5 hours, t_2 = 0.5 hours, and T = 60 °C. For experiments with ACN, t1 was increased to 12.5 hours. Samples were cooled to room temperature, dried with nitrogen gas, and reconstituted in 1 mL ACN-H2O (1:1, v/v), followed by filtering with 0.2 µm syringe membrane before analysis.

Table 1. Examined factors in DOE_1 and DOE_2.

DOE_1			DOE_2		
Factors	**Low (-)**	**High (+)**	**Factors**	**Low (-)**	**High (+)**
EDC (mM)	0.16	16	NH_4OH %	0 (pH = 6.8)	0.01 (pH = 10.5)
4-BNMA (mM)	0.16	16	H_2O %	0	10
t_1 (hours)	0.5	24	Solvents	ACN, MeOH	
t_2 (hours)	0.5	24			
Solvents	ACN, DCM, MeOH, EtOH				

2.5 Optimized sample preparation conditions

The following optimal conditions were applied for the analysis of crude oils: 0.5 mL crude oil was extracted two times with 1.5 mL of 10% v/v aqueous acetonitrile solution with 0.01% ammonium hydroxide. A total of 3 mL extractant was collected into a 4 mL amber vial by centrifugation at 4400 rpm for 15 mins. To the extractant, 10 µL EDC stock solution was added and held at 60 °C for 0.5 hours before adding 10 µL 4-BNMA stock solution and keeping it at the same temperature for 1 hour. For LC-Orbitrap HRMS analysis, 3 mL extractant was dried under the nitrogen stream and reconstituted in 1 mL ACN-H_2O (1:1, v/v). Notably, the optimal concentrations of reagents were held at EDC: -COOH and 4-BNMA: -COOH molar

ratios of 2:1 and 10:1, respectively. The carboxylic acid concentration in six crude oil samples was estimated based on their reported TAN, assuming that only monocarboxylic acids engender acidity.[37]

3 Results and discussions

3.1 Precursor and product ions of acid derivatives

Direct infusion of solutions containing individual derivatives was conducted to verify labeling reactions. The measured precursor masses of carboxylic-EDC (O-acylisourea) and carboxylic-BNMA conformed to the corresponding theoretical masses and molecular structures (Table 2 and Figure S1). Both labeling products are highly sensitive in positive ESI mode because the tertiary amine structure gives a permanent positive charge. The carboxylic-EDC intermediates ($m/z_{carboxylic\text{-}EDC}$) were detected without the 4-BNMA labeling step, confirming the successful EDC activation. By adding 4-BNMA, the intermediates were transformed completely to carboxylic-BNMA, and no carboxylic-EDC was detected. In AIF mode, the carboxylic-BNMA precursor ions produced the product ion pair of m/z 168.965 and 170.963 in high abundance, which agrees with the fragmentation of biological carboxylic acids obtained by Bryce et al.[36] Based on the 4-BNMA molecular structure, m/z 168.965 and 170.963 were assigned to phenyl bromine ions $^{79}BrC_7H_6^+$ and $^{81}BrC_7H_6^+$, respectively. The chromatographic retention time of those 4-BNMA derivatives was also determined by analysis of the acid standards mixture using LC-Orbitrap MS (Table 2, Figure 3).

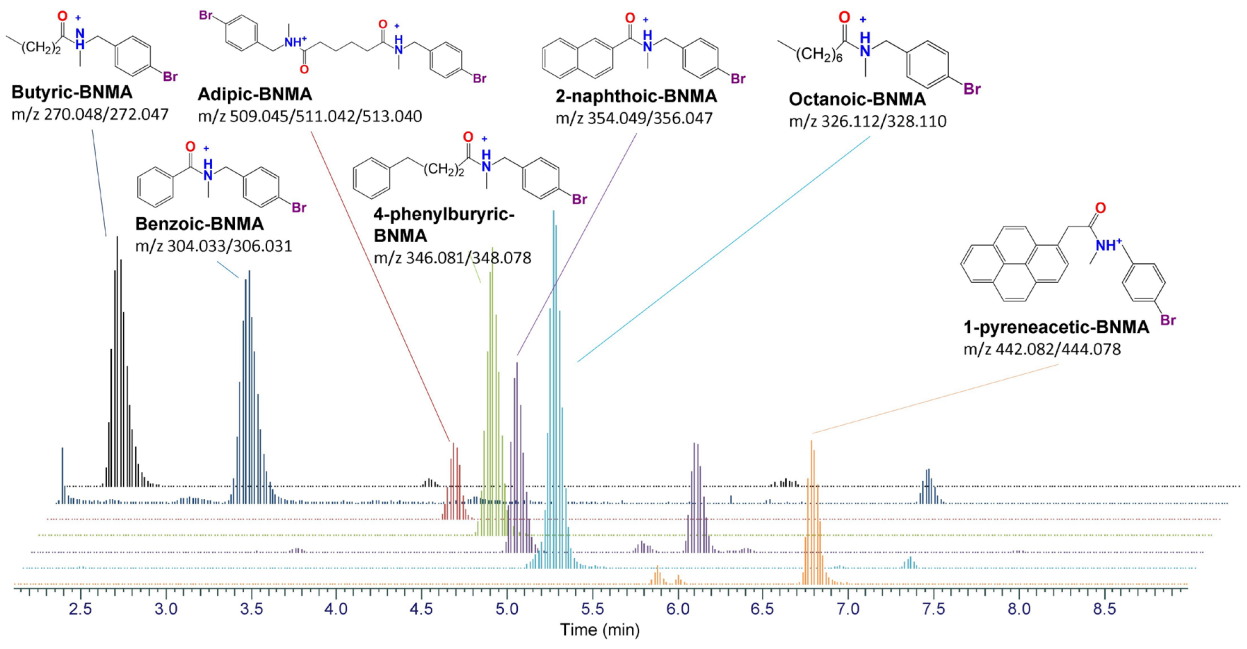


Figure 3. Typical LC-ESI-Orbitrap MS chromatogram obtained for 7 carboxylic acids derivatized with 4-BNMA using Phenomenex Kinetic Biphenyl 3.0 x 150 mm, 5 µm LC column.

Table 2. Precursor ions of EDC intermediates ($m/z_{carboxylic\text{-}EDC}$) and 4-BNMA derivatives ($m/z_{carboxylic\text{-}4BNMA}$), and product ions of carboxylic-BNMA (m/z_{AIF}) obtained by direct infusion into (+) ESI-Orbitrap MS. The chromatographic retention time (RT) of carboxylic-BNMA obtained by LC-Orbitrap MS analysis.

Compound	RT (min)	MW (g mol^{-1})	$m/z_{carboxylic\text{-}EDC}$	$m/z_{carboxylic\text{-}4BNMA}$	m/z_{AIF}
Butyric acid	2.41	88.11	244.202	270.048/272.047	168.965/170.963
Benzoic acid	3.21	122.12	278.190	304.033/306.031	
Adipic acid	4.45	146.14	457.361	509.045/**511.042**/513.040	
4-Phenylbutyric acid	4.74	164.2	320.230	346.081/348.078	
2-Naphthoic acid	4.93	172.18	328.210	354.049/356.047	
Octanoic acid	5.19	144.21	300.262	326.112/328.110	
1-Pyreneacetic acid	6.77	260.29	418.250	442.082/444.078	

3.2 Optimization of extraction and derivatization conditions

Previous EDC/N-methylated amine coupling methods require mixing the sample with EDC and amine simultaneously at concentrations above 2.4×10^6 and 4.7×10^4 folds of total carboxyl concentration (-COOH) for incubation at 60 °C, 1 hour.[36, 39] There reported methods have had several drawbacks, including (i) greatly excessed reagents without removal, (ii) the coupling times were not thoroughly investigated, and (iii) high water content caused EDC hydrolysis. Herein, we optimized the EDC/4-BNMA derivatization and LLE steps using DOE techniques. The supporting information describes the full DOE setup and analysis details, validation of DOE and plot information. Initially, individual FFD sets with four quantitative factors (1 – 4) were carried out to find optimal reaction conditions in a specific solvent – ACN or EtOH, as they are favourable for LLE of acids in crude oil.[4, 14, 15] Because of the potential of labeling approach in different solvents involving other extraction techniques, DOE_1 was setup with inclusion of factor 5. The GSD allows (i) combining individual DOEs to add a complete design of all possible factor compositions – sequential experimentation, and (ii) reducing experiments for a mix of quantitative and multilevel qualitative factors.

As the results, factors 1 to 5, and the interactions 1*2, 1*3, 1*5, 2*3, 3*4, and 4*5 showed notable effects for seven acid derivatives responses (Figure 4). The coefficient plot displayed significant changes (with a confidence interval away from 0) in responses when a factor varies from low to high value. Individually, factors 1 to 5 showed positive and negative influences on the response, meaning they are not necessarily the highest values of factors that maximize reaction efficiency. There were adverse effects when increasing individual factors 1 and 3 but minor effects with factors 2 and 4 (Figure 4). EDC concentration and t_1 should be considered cautiously due to O-acylisourea intermediate stability and its reaction with 4-BNMA, which influences carboxylic-BNMA production (Figure 1). 4-BNMA concentration and t_2 have low impacts on most responses, except for the high impact of 4-BNMA concentration on benzoic-BNMA. Figure 4 showed substantial positive effects with ACN and DCM, while MeOH and EtOH reduced the derivatization efficiency. The result indicates that the substitution with 4-BNMA (S_N2) favoured aprotic solvents, which lack hydrogen bonding to nucleophiles and thus promote the reaction rate. Also, solvents with low dielectric constants (ε_{DCM} = 9.1) can eliminate the rearrangement of the O-acylisourea to the

stable N-acylurea.[40] Because of crude oil miscibility in DCM, ACN was selected for the derivatization as suitable for the LLE stage, although DCM has a more substantial impact. Additionally, positive correlations between most interaction factors and responses indicated that the consequential effects of one factor depend on the level of the other factor (Figures 4, S5, S6).

The desirability plots (Figures 5, S8-10) visualize the possibility of maximizing carboxylic-BNMA responses (scoring 0 to 1) when varying parameters. As shown in Figure 4, low concentrations of EDC and 4-BNMA with short coupling times at 60 °C should ideally maximize the reaction efficiency in ACN. The EDC activation time – t_1, should be considered carefully; mixing EDC simultaneously with 4-BNMA resulted in a low yield of acid derivatives. The result is in accord with Quincy et al. study indicating that the derivatization of carboxylic acids by EDC and amine could be efficient at low molecular ratios between reagents and total -COOH.[41] However, these findings are contrary to that of Bryce et al., who used excessive amounts of EDC and 4-BNMA without considering coupling times.[36] Therefore, we suggest the optimal condition for EDC/4-BNMA coupling of carboxylic acid using ACN solvent, with molar ratios of EDC: -COOH 2:1 and 4-BNMA: -COOH 10:1, t_1 = 0.5 hours, t_2 = 1 hour at 60 °C.

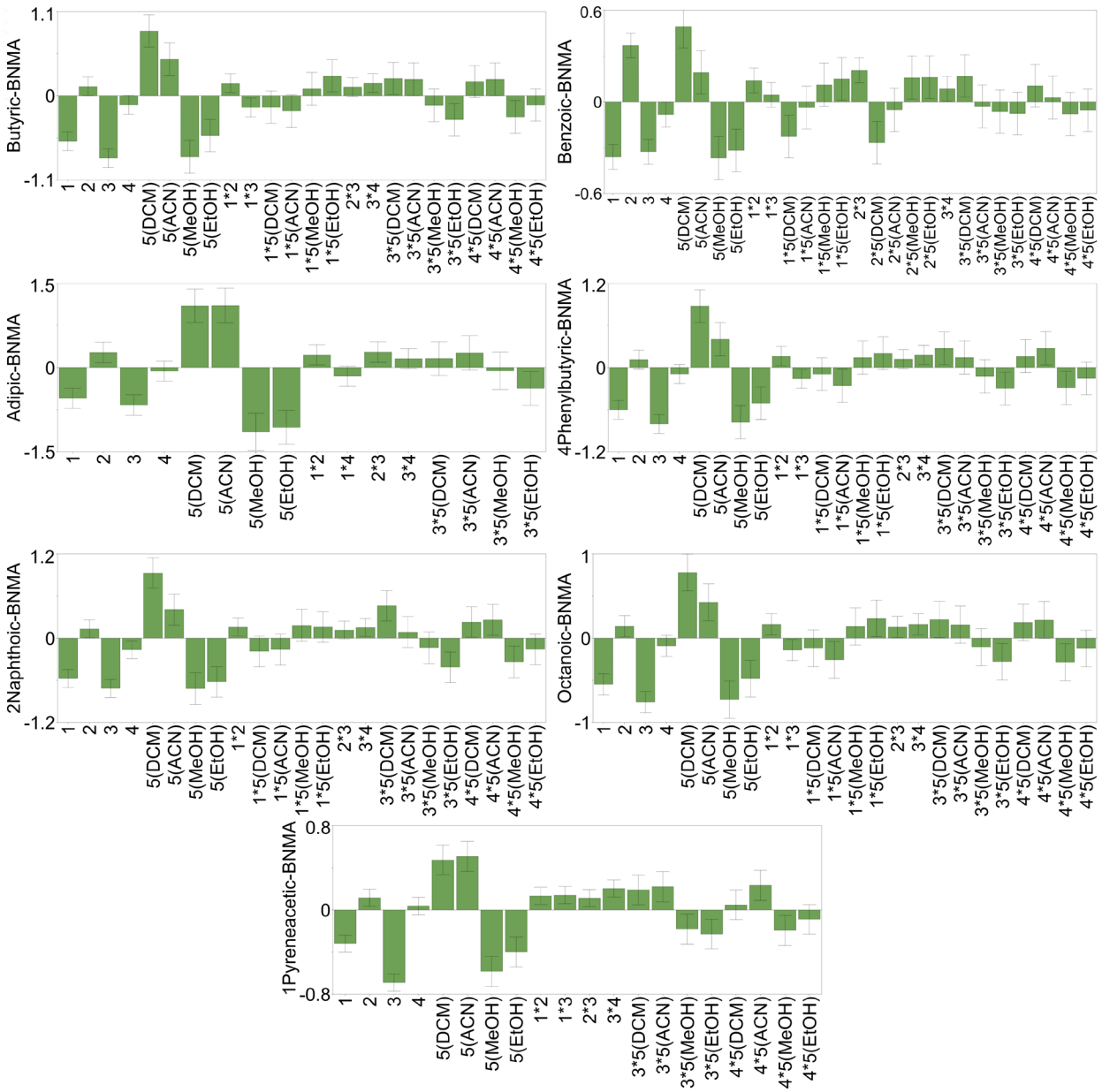
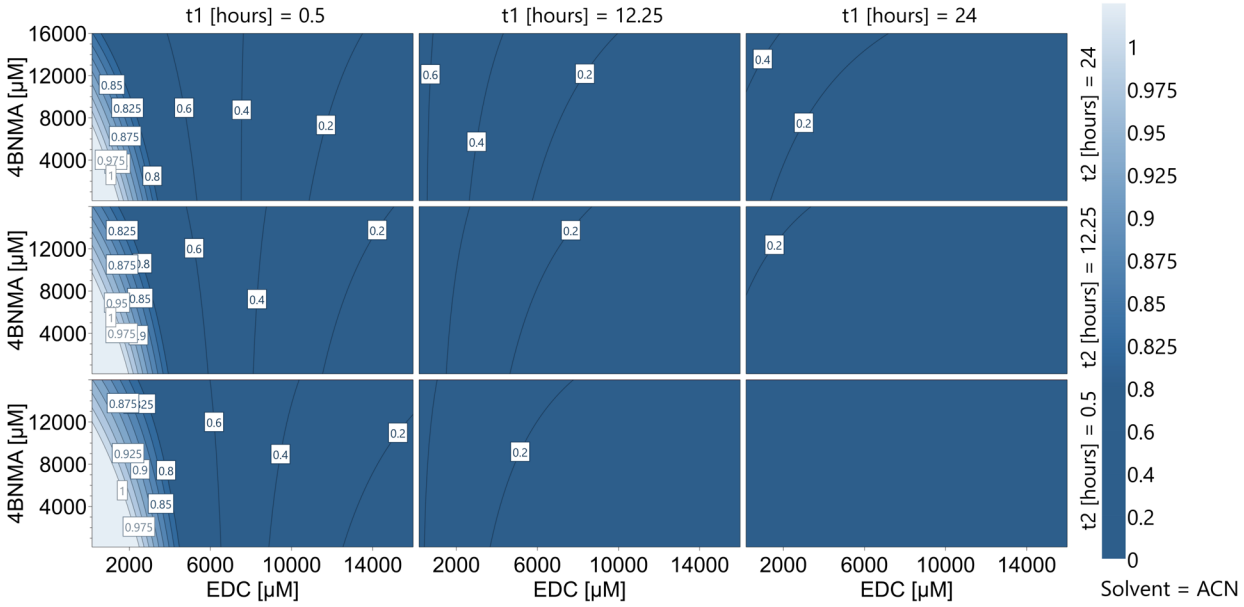


Figure 4. Scaled and centered coefficients plots of all individual and interaction factors in DOE_1 for seven acid derivatives. The responses – carboxylic-BNMA derivatives peak areas, were transformed to improve the DOE during model analysis (supporting information).



205

206 **Figure 5.** The average desirability distribution plot of carboxylic derivatives in DOE_1 with **ACN solvent**.
207 The possibility of achieving the desired target is increased from 0 to 1 score, with a score of one being
208 the highest ability for maximizing all the responses.

209 In previous studies, 70% alcohol aqueous solution and sodium hydroxide have been applied to extract
210 carboxylic acids from crude oil.[4, 32, 42, 43] Barrow et al. dissolved crude oil in acetonitrile at 100 μg mL^{-1} with
211 1% ammonium hydroxide addition for direct infusion to nano-ESI FT-ICR MS.[27] In this study, acetonitrile
212 and methanol were selected as solvents for LLE of crude oil acids, with the relative polarity of 0.460 and
213 0.762, respectively.[44] Ammonium hydroxide was selected to assist the deprotonation of carboxylic acids
214 and promote their solubility in the aqueous-solvent solution, improving extraction efficiency. Because of
215 the significant influences of aprotic and protic solvents on derivatization, the labeling conditions were
216 adjusted based on DOE_1 to achieve equivalent responses in DOE_2. For experiments with ACN, the
217 adjustment of t_1 was to normalize the difference between ACN and MeOH based on the desirability plots
218 at a score of 0.4 – maximum score for MeOH (Figures 4 and S10).

219 The coefficient plot showed that high ammonium hydroxide concentration and ACN positively enhanced
220 the derivative responses (Figure 6). In contrast, the responses were adversely affected by high water
221 content, MeOH, and interactive factors (NH_4OH*H_2O, $NH_4OH*MeOH$). The desirability plot (Figure 7)
222 showed that increasing water content when the ammonium hydroxide concentration is below 0.006%
223 could descend the average derivative responses. Adding water increases the solubility of organic acids in
224 the extractant and enhances the extraction efficiency.[45] However, excessive water molecules may
225 hydrolyze EDC into 1-(3-(dimethylamino)propyl)-3-ethylurea, which dramatically lowers the derivatization
226 efficiency at 60 °C in the next stage.[46, 47] A previous study found that 10% of water in the reaction solution
227 should be an optimal condition to dissolve analytes and derivatives, which benefits the reactions.[39] Also,
228 the extractant pH should be considered carefully by adjusting the ammonium hydroxide concentration
229 due to the influence on the partitioning of acidic species. Hemmingsen et al. observed exclusively low-
230 molecular weight acid species ($m/z < 400$) in pH 7 EtOH:H_2O fraction and intermediate- to high-molecular-

weight species (400 < m/z < 600 and m/z > 600) in pH 10 fraction.[32] Thus, the optimal LLE condition for carboxylic acids from crude oil is using a 10% aqueous-acetonitrile solution with 0.01% ammonium hydroxide (Figure 7). The optimal extraction and labeling conditions were defined based on the DOE prediction for model compounds and applied to presented carboxylic acids in crude oil.

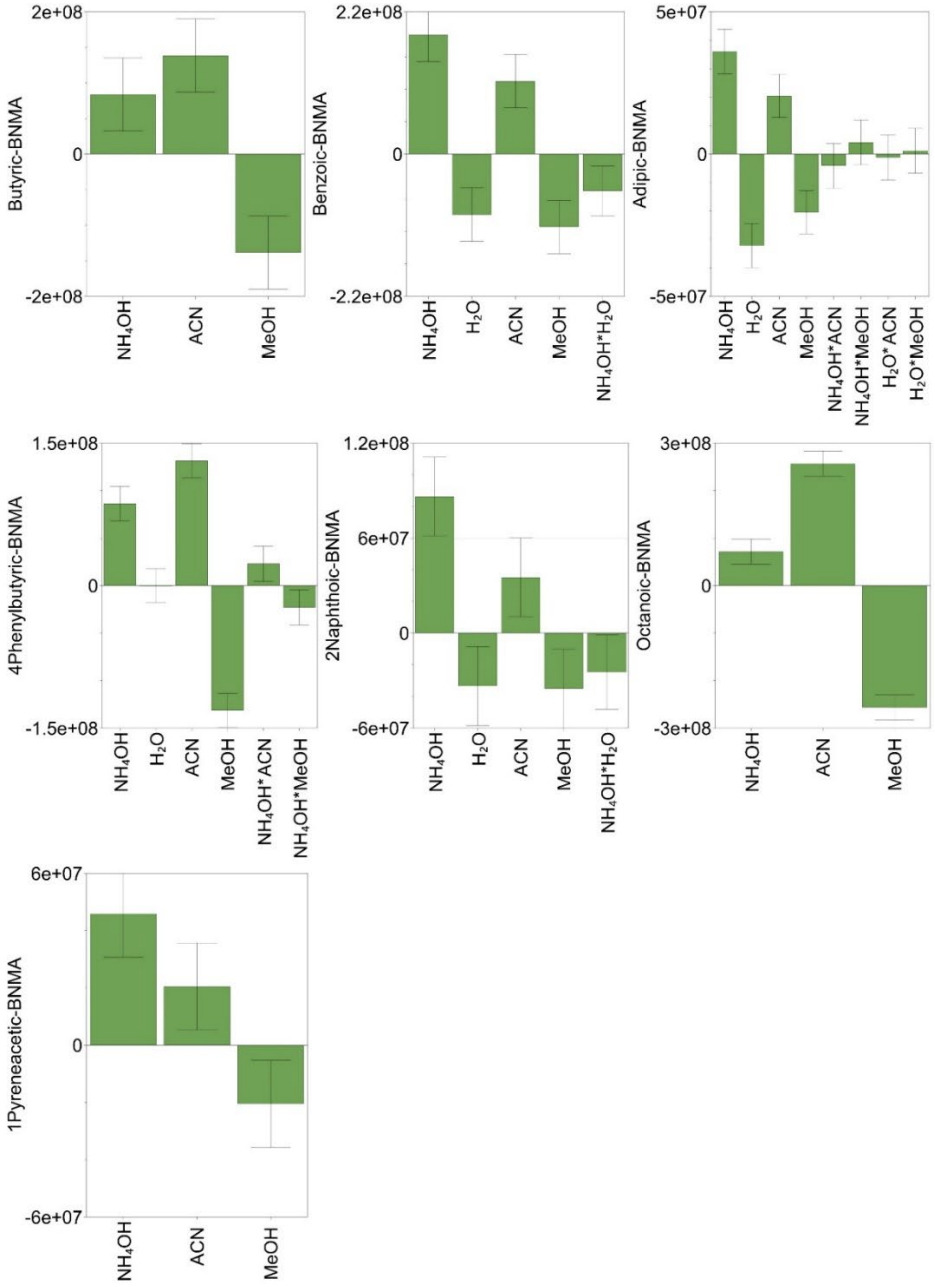


Figure 6. Coefficients plots of all individual and interaction factors in DOE_2 for seven acid derivatives.

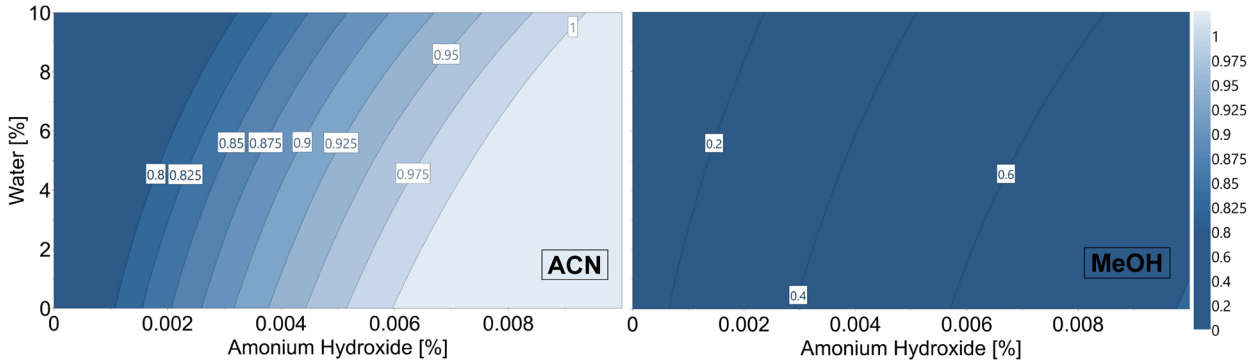


Figure 7. The average desirability distribution plot for carboxylic derivatives in DOE_2

3.3 Method validation

Both DOEs showed high R^2, Q^2, model validity, and reproducibility values (Figures S3, S11), implying good valid models. The observed vs. predicted plots (Figures S7, S13) were on the diagonal line, indicating good model predictions. The optimal conditions were experimentally examined with three replicates to evaluate the DOEs prediction, which responses were identical to predicted values. A series of calibration standards mixtures (1 – 50 µM/acid) in 10% aqueous ACN solutions were derivatized and analyzed using the optimized protocol. Typical LC chromatograms of 7 carboxylic derivatives are shown in Figure 3 for the optimized method. Calibration curves (Figure S14) for the standard derivatives were established to determine the limit of detection (LODs in Table 2), utilizing the method for the characterization of unknown carboxylic acids. The LODs were calculated using the ratio of 3.3 times the intercept's standard deviation and the slope of calibration functions.[48] For selected acids, the sensitivities were different, ranging from 0.9×10^6 to 5.5×10^6 arbitrary peak area units per µM. All compounds observed linearity in signal response up to 50 µM. The LODs for carboxylic acids ranged from 1.8 to 33 ng g^{-1} or 8.6 to 101 nM (Table 2). A spiked crude oil at 50 µM each acid and a standard mixture were analyzed to estimate the matrix effect. The matrix effect (ME %) was evaluated by comparison of carboxylic-BNMA response in spiked oil and a mixture of standards in aqueous ACN (Table 2). As observed, the ME (%) values ranging between 9% and 36% for seven carboxylic acids suggested low-moderate matrix effects of crude oil on this method. Overall, the results suggest that the optimized method is applicable for characterizing unknown carboxylic acids in crude oil. We also evaluated the proposed identification workflow (Figure 8) by analyzing the spiked crude oil consisting of carboxylic acid standards. All standards were identified with carboxylic-BNMA mass annotation and tag removal approach.

Table 2. LODs, analytical reproducibility (relative standard deviation, RSD of 5 replicates of 1.5 µM carboxylic-BNMA standard solutions calculated by peak areas) and matrix effect (ME %) achieved for each carboxylic acid. The ME was calculated by the following equation: $ME\ \% = \frac{Peak\ area_{Standard+matrix}}{Peak\ area_{Standard}} \times 100.$

Carboxylic acids	LOD (ng. g^{-1})	RSD (%)	ME %
Butyric acid	3.3	1.5	9
Benzoic acid	6	3.0	17

Adipic acid	5.2	3.3	14
4-Phenylbutyric acid	1.8	2.4	22
2-Naphthoic acid	11	2.1	33
Octanoic acid	6.5	1.8	14
1-Pyreneacetic acid	33	3.7	36

3.4 Sample analysis and data processing

An identification workflow was developed to identify unknown carboxylic acids based on the phenyl bromine ion pair of derivatives fragments (Figure 8). The product ions from AIF scans were matched against the isotopic pattern of m/z 169 and 171, with an abundance ratio of approximately 1:1. Accordingly, the carboxylic-BNMA derivative peaks were integrated with the full-MS chromatogram corresponding to the integrated AIF LC peaks. Based on the merged full-MS chromatographic peaks, a derivatives m/z list was summarized and filtered with a signal-to-noise ratio above 3. The derivatives were annotated using Formularity.[49] The original carboxylic acid formulas were computationally generated by removing the labeling group using an in-house developed Python script. The PyC2MS software was used for the treatment and visualization of carboxylic acid data.[50] Detailed information regarding the Formularity and computational removal of 4-BNMA label are provided in the supporting information.

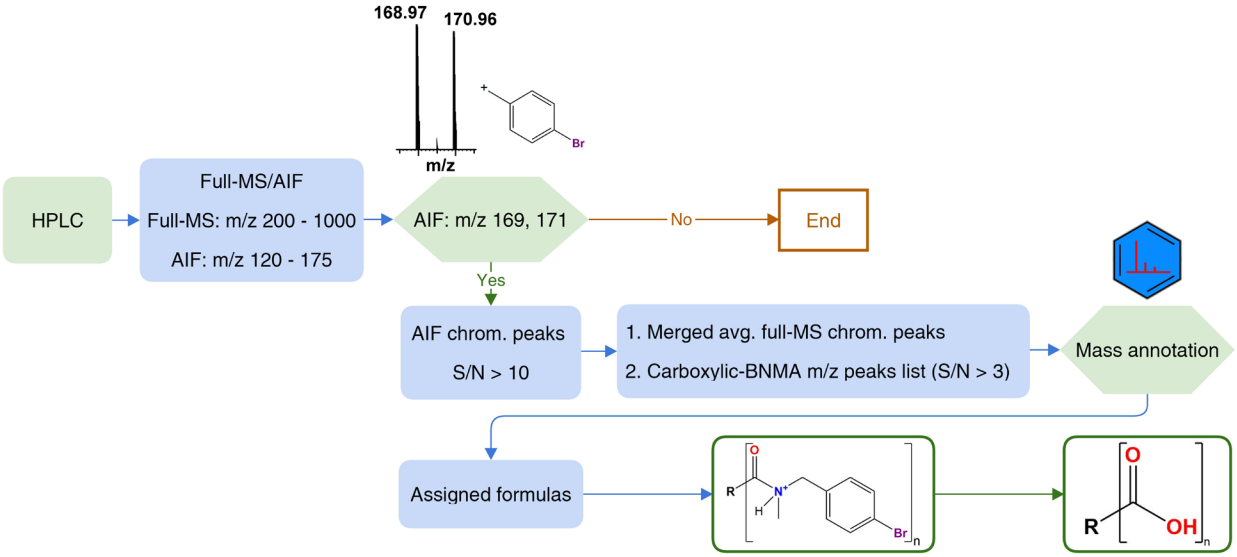


Figure 8. Schematic for carboxylic acids identification workflow using phenyl bromine isotopic ion pair.

Kujawinski et al. developed a Compound Identification Algorithm (CIA) function to assign formulas consisting of C, H, O, N, S, and P to the HRMS m/z list.[49, 51] The CIA assigns accurate mass using an assisting database (CIA DB) and formula expansion features based on building blocks (CH_2, H_2, O_2, or homologs series). The universal CIA DB comprises over 29 million pure hypothetical formulas limited to CHNSO and validated based on the seven golden rules, having a monoisotopic mass below 1500 Da.[52] Featuring CIA, the Isotopic Pattern Algorithm (IPA) function was developed for annotating compounds containing multi-isotopic elements.[53] In this study, we compiled an in-house IPA DB containing 2.9 million formulas based on a subset of the CIA DB, extracting formulas with 2 – 6 oxygen atoms (corresponding to mono-, di- and

tri-carboxylic acids). The list of formulas was derivatized in-silico, and the isotope peak pattern of the resulting formula was calculated using Ecipex software.[54] Full details of the CIA DB transformation and IPA DB compilation are given in the supporting information.

By analyzing three averaged fractions from the Halfdan sample, we observed separations of compounds from low to high DBE and C number distribution by increasing retention time (Figure 9). The number of compounds in each fraction increased from 22 compounds (Fraction 1) to 30 (Fraction 2) and 133 (Fraction 3). The molecular size and degree of unsaturation of detected acids were also increased in the latter fractions, i.e., the early fractions contained carboxylic-BNMA derivatives with small aliphatic, monocyclic, and monoaromatic carboxylic acid structures. In contrast, the later fractions contained acid derivatives with bigger aliphatic, multiple cyclic, and aromatic structures. The AIF LC chromatogram presented an excellent separation of m/z 169/171 peaks produced by corresponding carboxylic-BNMA derivatives. Compared to Figure 3, the elution order of model acids followed the same C and DBE distribution trends corresponding to the three oil fractions. Carboxylic acid retention increased by increasing the number of aromatic rings and carbon sidechain (benzoic < 4-phenyl butyric < 1-pyreneacetic acid). A labeled bromine phenyl group also improved the separation of aliphatic carboxylic acids, such as butyric and octanoic acids, which otherwise can be challenging to adequately retain in reverse phase LC. Overall, the biphenyl LC column features selectivity to aromatic and moderately polar analytes by π-π interactions. It thus benefits the carboxylic-BNMA derivatives containing the bromine phenyl group(s). The LC separation has met our expectations to simplify the sample matrix and improve selectivity for derivatives.

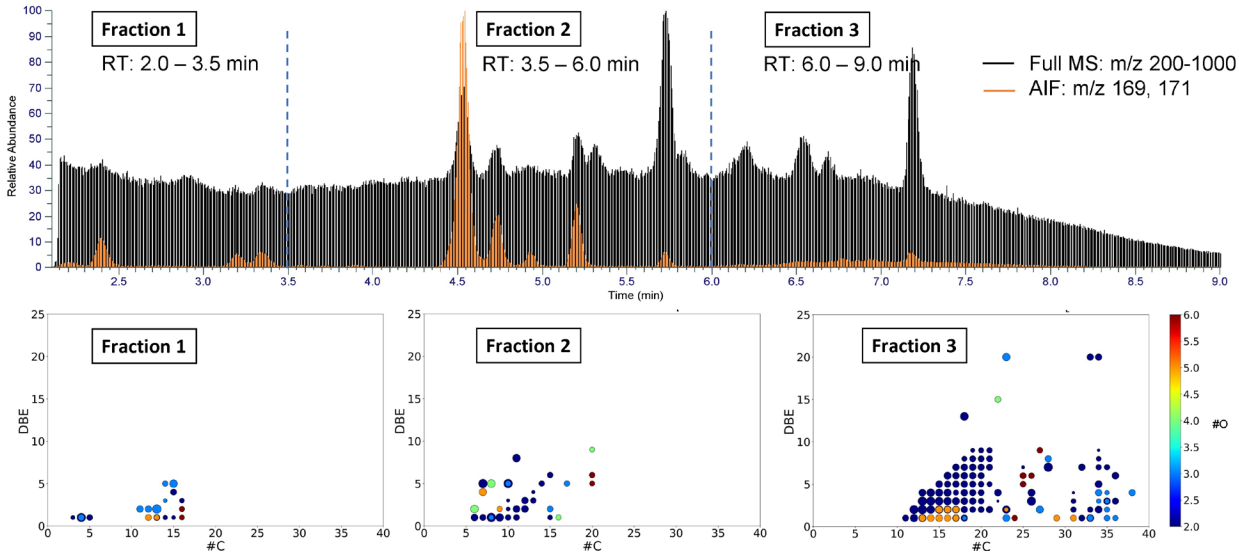


Figure 9. Full-MS (black) and AIF (orange) LC chromatograms of Halfdan crude oil with the corresponding DBE vs. C plots for three averaged fractions.

3.5 Profiling of carboxylic acids in North Sea crude oils

The methodology was employed to characterize unknown carboxylic acids in six Danish North Sea crude oils. Seven carboxylic acid standards were spiked to crude oils for quality control purposes to monitor the identification process. The number of full MS chromatographic fraction/peaks was averagely 8 – 10

fractions, depending on detected AIF chromatographic peaks (S/N > 10) at m/z 169 and 171. All identified acids were merged to evaluate presented carboxylic acids in crude oil samples. Due to irrelevances when merging averaged full-MS LC peaks, the m/z intensity of carboxylic-BNMA was not used for data analysis, but the number of assigned formulas was. Generally, 125 to 380 compounds are identified containing carboxyl functional group(s) with up to 50 C and 6 O in samples. Those are primarily monocarboxylic acids with or without additional NSO functionalities (Figure 10C) and very few diacids (< 1.8%) in the O_4 and O_6 classes. CHO and CHNO, including O_2, O_3, O_4, O_5, O_6, N_1O_2, N_1O_3, N_1O_4, and N_2O_2 compounds, are the most populated classes, followed by CHSO and CHNSO classes (O_2S_1 and $N_1O_2S_1$) as shown in Figures 10A and 10B. CHO class with O_2 (for Halfdan, Valdemar, and Tyra West samples) and O_4 (Dan, Kraka, and Skjold samples) subclasses are the most dominant species, accounting for 42 – 60% of the assigned compounds. Multiple oxygen-containing monoacids (O > 2) are suspected to be carboxylic compounds with additional oxygen functional group(s). The nitrogen-containing carboxylic acids are the second populated species (26 – 41%), of which N_1O_2 and N_1O_3 compounds are the most prominent. The presence of nitrogen and sulfur atoms in detected carboxylic acids implied multi-functional group molecules occurring in those crude oil samples other than classical naphthenic acids.[30, 55]

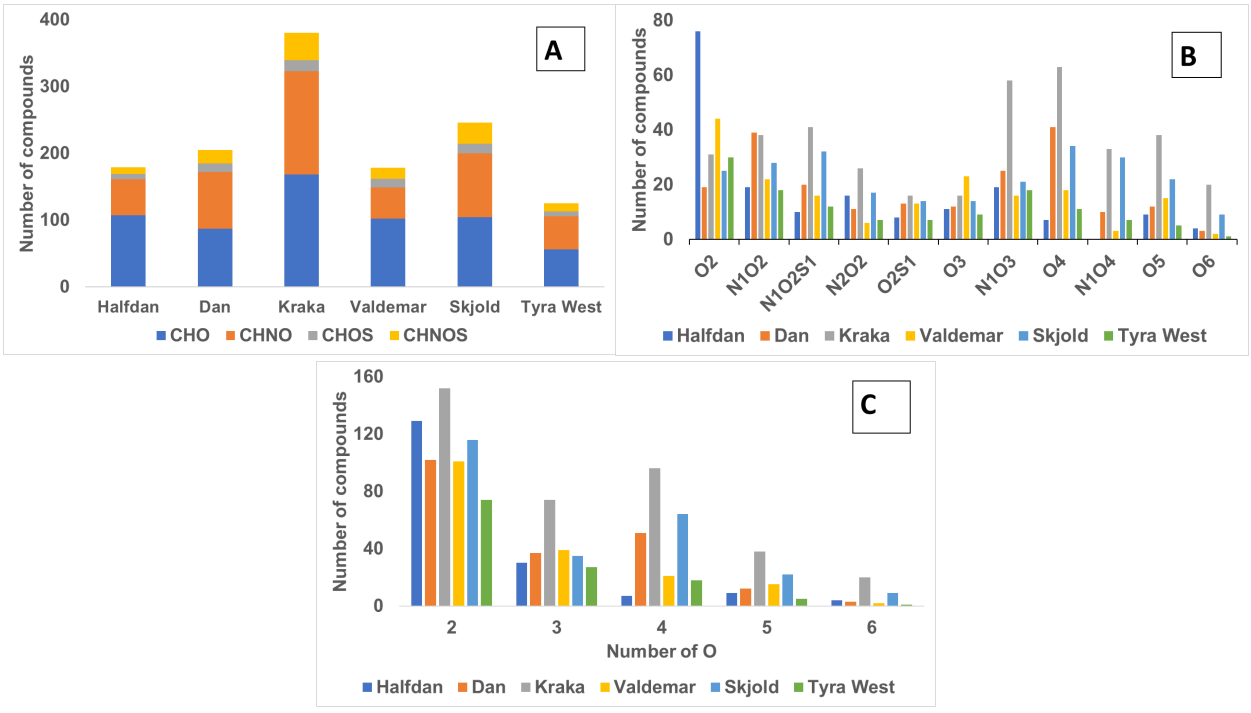


Figure 10. Overview of heteroatom classes population (A and B), and oxygen distribution (C) of detected carboxylic acids in North Sea crude oil samples.

Double bond equivalents (DBE) and carbon number distribution for observed carboxylic acids in all samples are displayed in Figures 11 and 12. The m/z abundance was log-transformed, and the number of O is color-coded, as shown. The most populated species in all crude oil are fatty acids (DBE = 1), mainly having 2 O and up to 5 O. The following dominant species are DBE 2 and 3 compounds, likely naphthenic acids containing one and two rings. The remaining population with DBE ≥ 4 is likely multiple ring naphthenic acids, as seen below the aromatic planar in Figure 11. Very few aromatic carboxylic acids (DBE

> 5) were observed using this method, consisting of -COOH and other O- and N- functional groups. These results are in agreement with Hemmingsen et al. observations using FTICR-MS, which showed that O_2 species are dominant carboxylic acids in North Sea crude oil, with DBE = 3 being the most abundant.[32] Naphthenic acids with two rings appear to be the most common carboxylic acids after aliphatic acids. Also, the results accord with previous observations that acid constituents in similar crude oils range from aliphatic carboxylic acids to acids with aromatic rings and multiple functional groups.[37] The CHNO class with dominant N_xO_y compounds has been commonly suspected as nitrogen-containing carboxylic acids bearing pyrrole and pyridine cores.[56, 57] The N_1O_2 subclass was commonly observed with DBE above ten could be monocarboxylic carbazoles and benzocarbazoles, caused by biodegradation processes.[58, 59] However, most of the observed nitrogen-containing acids (CHNO) by the current method had low DBE values up to 7, suggesting the presence of pyrrole, indole, and indoline carboxylic acids. Possibilities for O_2S_1 compounds with DBE < 4 could be sulfide or thiophene carboxylic acids.

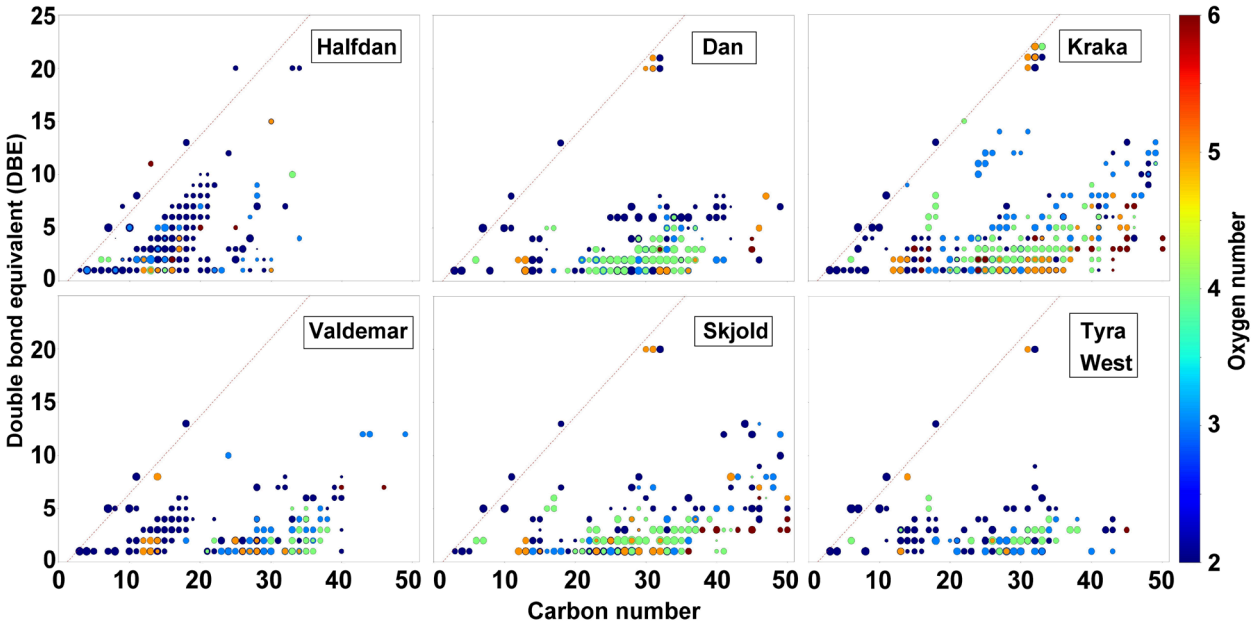


Figure 11. Number of oxygen color-contoured plots of double bond equivalent (DBE) versus carbon number for identified carboxylic acids in six Danish North Sea crude oils. The red-dotted line indicates planar aromatic limit.

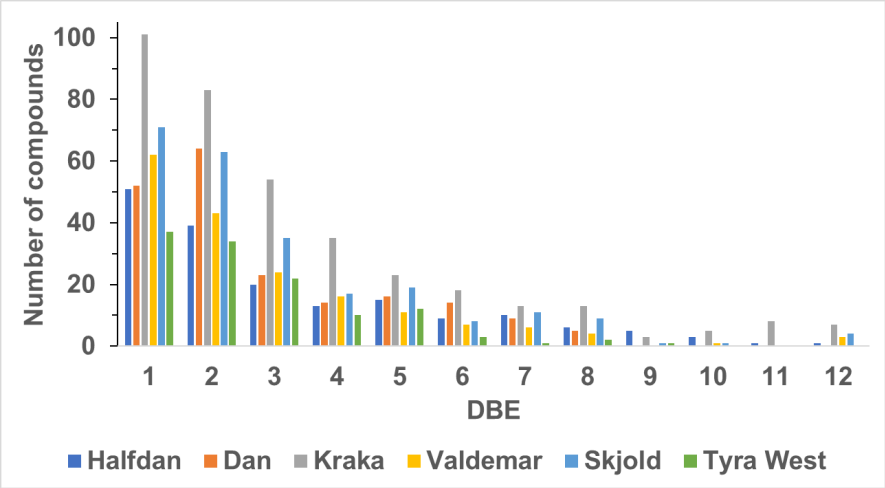


Figure 12. Distribution of DBE up to 12 in six crude oil samples.

4 Conclusions

Overall, we successfully developed a highly selective method for the non-target characterization of carboxylic acids in crude oil. The unique fragmentation pathway of carboxylic-BNMA derivatives and coupled LC separation offer a practical approach to simplifying the complexity of HRMS data by targeting a product ion pair, i.e., m/z 169/171. The potential of this approach for different desirable extraction techniques and LC-HRMS systems has been proved through comprehensive optimization and validation in this work. Importantly, the findings contribute to a comprehensive understanding of carboxylic acids in North Sea crude oils, which are essential to petroleum chemistry and environmental studies.

The crude oil matrix was still complex, as seen in a full-scan chromatogram with significant background, requiring more selective separation instead of LLE. A significant drawback of the method is the required manual intervention during data processing to extract regions of interest. It thus requires automated data analysis, which is beneficial, especially when analyzing larger sample batches. Further workflow development focusing on data processing using commercially available or built-in software is suggested.

Method capabilities are not limited to crude oil matrix but apply to other complex organic matter and water samples with suitable extraction methods. Marquis et al. successfully featured 4-BNMA derivatization for quantifying and confirming the number of carboxylic groups in cells and tissues.[36] As products of crude oil and gas exploration, naphthenic acids in produced water have raised concerns due to toxicity to aquatic environments, requiring detailed characterizations.[60-62] Therefore, we aim toward employing the developed method to characterize water-soluble acids in produced water, giving more detailed molecular information about carboxylic acids and supporting petroleum chemistry studies.

Author contributions

Khoa Huynh conceptualized the study, developed and validated the methodology, designed experiments, analyzed and visualized data. Jonas Sundberg supervised the study, revised the identification workflow, and developed the data processing scripts. Khoa Huynh wrote the manuscript. Khoa Huynh and Jonas

Sundberg performed scientific and grammatical revisions to the final version of the manuscript. Karen Louise Feilberg supervised the project and acquired funding.

Declaration of competing interest

The authors declare that there is no competing interest that could influence this paper.

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