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Running head: Identification of Bicyclic Aromatic NAs

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Can GCxGC/MS identification of bicyclic aromatic acids in petroleum fractions help to reveal further details of aromatic hydrocarbon biotransformation pathways?

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RATIONALE: The identification of key acid metabolites ('signature' metabolites) has allowed significant improvements to be made in our understanding of the biodegradation of petroleum hydrocarbons, in reservoir and in contaminated natural systems, such as aquifers and seawater. On this basis, anaerobic oxidation is now more widely accepted as one viable mechanism, for instance. However, identification of metabolites in the complex acid mixtures from petroleum degradation is challenging and would benefit from use of more highly resolving analytical methods.

METHODS: Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC/TOFMS) with both nominal mass and accurate mass measurement was used to study the complex mixtures of aromatic acids (as methyl esters) in petroleum fractions.

RESULTS: Numerous mono- and di- aromatic acid isomers were identified in a commercial naphthenic acids fraction from petroleum and in an acids fraction from a biodegraded petroleum. In many instances compounds were identified by comparison of mass spectral and retention time data with those of authentic compounds.

CONCLUSIONS: The identification of a variety of alkyl naphthalene carboxylic and alkanoic and alkyl tetralin carboxylic and alkanoic acids, plus tentative identifications of a range of alkyl indene and alkyl indane acids, provides further evidence for 'signature' metabolites of biodegradation of aromatic petroleum hydrocarbons. Identifications such as these now offer the prospect of better differentiation of metabolites of bacterial processes (e.g. aerobic, methanogenic, sulphate-reducing) in polar petroleum fractions.

INTRODUCTION

Petroleum acids (also known as naphthenic acids, NA) are constituents of reservoired immature and biodegraded crude oils (e.g. Watson et al., 2002; Meridith et al., 2000), of produced water discharges from oil production platforms, of the processing of petroleum deposits such as oil sands and of contaminated aquifers (e.g. reviewed by Head et al., 2006, 2010; Headley and McMartin., 2004; Headley et al., 2009).

Petroleum acids in produced and process waters and in degraded oil spills probably originate mainly from aerobic processes (e.g. reviewed by Atlas, 1981; Britton, 1984; Leahy and Colwell,1990; Atlas and Bartha,1992). However, the argument that, at least some, acids in reservoired petroleum and contaminated aquifers originate from the anaerobic oxidation of hydrocarbons has now gained widespread acceptance (e.g. reviewed by Agrawal and Geig, 2013; Head et al., 2010). Evidence for this proposal has originated partly from the identification by gas chromatography-mass spectrometry (GC-MS) of some known ‘signature’ acid metabolites of anaerobic oxidation of pure hydrocarbons or of petroleum mixtures. Such metabolites include distinctive fumarate addition products, such as alkylsuccinates (Boll et al., 2002; Widdel and Rabus, 2001. Elshahed et al., 2001; Rios-Hernadez et al., 2003).

Attractive though it is, the use of so-called ‘signature’ acid metabolites of in situ degradation processes is not without disadvantages. Therefore most studies have coupled an enzymatic or genomic approach with that of metabolite identification (reviewed by Agrawal and Geig, 2013). This is necessary partly because the number of compounds made exclusively by any one biodegradation pathway is actually quite

limited: the same compound may originate by different routes (e.g. Figure 1A). Also, identification, by GC-MS, of individual metabolites within the 'supercomplex' mixtures, arising from biotransformation of petroleum hydrocarbons is often difficult.

Advances in analytical methods which would allow further acids to be more easily and better identified might add to the evidence for the prevalent biodegradation mechanisms. Assignment of the absolute stereochemistry of some metabolites by GC-MS of chiral derivatives has allowed pathways to be better elucidated in some cases, for instance (e.g. Jarling et al., 2012) and it has been suggested recently that application of methods such as multidimensional GCxGC/MS might be useful for further 'signature' metabolite analysis (Angarwal and Gieg, 2013). Complex mixtures of unfractionated NA in a commercial sample refined from petroleum have been studied by multidimensional GCxGC/MS and identifications of a number of monocyclic monoaromatic acids made, facilitated by synthesis (Rowland *et al.* 2011), so this recommendation seems sensible.

In the present study we therefore used, first argentation chromatography (Ag^+ solid phase extraction, SPE) to separate and concentrate an aromatic acids fraction from a mixture of commercial NA refined from petroleum (cf. Jones et al., 2012) and then we examined the isolated fraction by GC×GC/MS with both nominal mass and high mass accuracy (cf. West et al., 2013). As a result we now report the identification of numerous series of bicyclic aromatic acids. We then compared these data with those of an acid fraction from a partially biodegraded petroleum and confirmed the presence of many of the same compounds, suggesting that the acids detected may be typical biotransformation products of altered petroleum and indicative of the bacterial processes occurring in reservoir.

EXPERIMENTAL

Authentic naphthalene-1-carboxylic acid, naphthalene-2-carboxylic acid, naphthalene-1-ethanoic acid, naphthalene-2-ethanoic acid, 5,6,7,8-tetrahydro-1-naphthoic acid, 5,6,7,8-tetrahydro-2-naphthoic acid, 1,2,3,4-tetrahydro-1-naphthoic acid, 1,2,3,4-tetrahydro-2-naphthoic acid, 2-methyl-1-naphthoic acid, 4-methyl-1-naphthoic acid, 4,7-dimethylnaphthoic acid, biphenyl-2-carboxylic acid, biphenyl-3-carboxylic acid, biphenyl-4-carboxylic acid, Indane-2-carboxylic acid, indane-2-ethanoic acid and 3-methylindane-2-carboxylic acid were purchased from Sigma (UK).

Alkyltetrahydro acid analogues were obtained by partial hydrogenation (10% Pd/C at 60 bar and 60°C) of the corresponding alkylnaphthoic acids using a ThalesNano H-Cube® continuous-flow reactor (ThalesNano Nanotechnology Inc, Budapest, Hungary), resulting in 1,2,3,4- and 5,6,7,8-tetrahydro acid isomers.

The commercial NA mixture was that obtained for a previous study (Rowland *et al*. 2011a). Such commercial NAs are removed from the middle distillate range of crude oils during refining.

All acids were derivatised by refluxing with BF_3-Methanol and extracted into hexane (Rowland et al. 2011b).

The carboxylic acid fraction of a partially biodegraded crude oil Nigeria: e.g. www.exxxonmobil.com/crude_oil/about_crude_oils.aspx; a medium gravity, low sulphur, naphthenic crude) was obtained by base extraction. Briefly, 15 g of crude oil was extracted (x3; 50 mL total) using a 0.25 M solution of sodium hydroxide (in

30% methanol). Water layers were combined and extracted with hexane (10 mL), acidified to <pH 3 using 37% hydrochloric acid and extracted with a further aliquots of hexane (2 × 15 mL), combined, dried over sodium sulphate and evaporated. The crude oil acid fraction was derivatised by refluxing with BF_3-Methanol and extracted into hexane (Rowland et al. 2011b).

The methylated commercial NA extracts were sub-fractionated by argentation solid phase extraction (SPE) using 6 mL Discovery® Ag-Ion SPE cartridges (750mg sorbent; Sigma-Aldrich, Dorset, UK). In brief, cartridges were conditioned with hexane (3 × 5 mL). Methylated commercial NA samples (5 mg in hexane) were then loaded onto the cartridges which were subsequently eluted using hexane (4 × 5 mL), 95% hexane: 5% diethyl ether (4 × 5 mL), 100% diethyl ether (1 × 5 mL), 100% methanol (1 × 5 mL). Fractions were collected and reduced to dryness under a steady stream of nitrogen at 40 °C.

Two-dimensional comprehensive gas chromatography/time-of-flight mass spectrometry (GCxGC/TOFMS) analyses were conducted as described previously (West et al. 2013). Briefly, analyses were conducted using a model 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a ZX2 GCxGC cryogenic modulator (Zoex, Houston, TX, USA) interfaced with a BenchTOFdx™ time-of-flight mass spectrometer (Almsco International, Lantrisant, UK) operated in positive electron ionisation mode and calibrated with perfluorotributylamine. The scan speed was 50 Hz. The first-dimension column was a 95% dimethyl polysiloxane fused-silica capillary HP-5ms (30m × 0.25 mm × 0.25 μm; Agilent Technologies J & W, Wilmington, DE, USA) and the second-dimension column was a 50% phenyl polysilphenylene-siloxane BPX50 (3m × 0.1 mm × 0.1 μm;

SGE, Melbourne, Australia). Samples (1 μL) were injected at 280 °C splitless. The oven was programmed from 40 °C (held for 1 min), heated to 300 °C at 5 °C min^{-1} and then at 10 °C min^{-1} to 320 °C (held for 10 min). The modulation period was 5 sec. The mass spectrometer transfer line temperature 280 °C and the ion source temperature 300 °C. Data processing was conducted using GC Image™ version 2.3 (Zoex, Houston, TX, USA).

High resolution GC×GC/TOFMS analyses were undertaken using an Agilent 7890A gas chromatograph fitted with a Zoex ZX1 thermal modulator interfaced with a AccuTOF GCv TOF mass spectrometer (Jeol Inc., Peabody, MA, USA) operated in positive ion mode. The scan speed was 25 Hz. The first-dimension column was a 100% dimethyl polysiloxane DB-1 (30m × 0.25 mm × 0.25 μm; Agilent Technologies J & W) and the second-dimension column was a 50% phenyl methylpolysiloxane DB-17 (3m × 0.1 mm × 0.1μm; Agilent Technologies J & W). Helium was used as the carrier gas at a constant flow of 0.7 mL min^{-1}. Samples (1 μL) were injected at 250 °C splitless. The oven was programmed from 40 °C (held 2 min), heated to 310 °C at 2.5 C min^{-1} (held 10 min). The modulation period was 5 sec. The mass spectrometer transfer line temperature 300 °C and the ion source temperature 280 °C. The scan range was *m/z* 40–550.

RESULTS

Examination of an aromatic NA fraction (as methyl esters) isolated from petroleum by Ag^+ SPE followed by GC×GC/MS in both nominal and accurate mass modes (cf. Rowland et al., 2011; West et al., 2103) resulted in highly resolved chromatograms allowing electron ionisation (EI) mass spectra containing molecular and fragment

ions of many individual aromatic acids to be obtained. The aromatic fraction was dominated by a range of C_8-C_{16} alkylbenzoic acids (as methyl esters), reported previously (Rowland et al, 2011), but a range of bicyclic mono- and diaromatic acids was also present. We now provide details of these identifications.

Bicyclic diaromatic acids

We identified the methyl esters of naphthalene-1-carboxylic acid and naphthalene-2-carboxylic acid by the much improved resolution by GCxGC/MS compared with GC-MS (e.g. Figure 1B,C) and interpretation of the resultant library-searchable EI mass spectra (Figure 2(A) and 2(C)). We were able to firmly identify individual isomers by comparing the data with the mass spectra and GC×GC retention times of the esters of purchased reference acids (Fig. 2(B) and 2(D)). For instance, the suspected molecular ion ($M^{+\cdot}$, *m/z* 186) was observed in the spectra of unknowns I and II (Fig. 2(A) and 2(C)) and the accurate mass of this ion was measured by GC×GC/HRMS as *m/z* 186.068> This is within <0.5 mDa of that required for $C_{12}H_{10}O_2$ (theoretical mass 186.0675) and is consistent with the molecular ion of a methyl ester of a $C_{11}H_8O_2$ bicyclic diaromatic acid. Major fragment ions were assigned to loss of a methoxy group (*m/z* 155.049, B^+; $C_{11}H_7O$) and loss of the methylated carboxy group (*m/z* 127.054; $C_{10}H_6$) from the molecular ion, as commonly observed in the mass spectra of methyl esters of carboxylic acids. Many of the same ions were present in the spectra of the reference acids (methyl esters; Fig. 2(B) and 2(D)). The GC×GC retention times of unknown I were the same as those of naphthalene-1-carboxylic acid reference acid (methyl ester), whilst those of unknown II matched those of naphthalene-2-carboxylic acid (methyl ester). Thus, we firmly assign acids I and II

as naphthalene-1-carboxylic acid (methyl ester) and naphthalene-2-carboxylic acid (methyl ester) respectively.

Naphthalene-2-carboxylic acid has been identified as a product of numerous bacterial processes (both aerobic and anaerobic: Figure 1A).However, in addition, we were able to identify numerous methyl (C_1) and dimethyl (C_2) naphthalene carboxylic acid isomers by interpretation of the spectra from first principles. The spectra of the methyl esters of the methyl naphthalene carboxylic acids (e.g. Fig. 2(E)) were characterised by abundant molecular ions (e.g. $M^{+\cdot}$, *m/z* 200.084; $C_{13}H_{12}O_2$), within <0.8 mDa of that required for $C_{13}H_{12}O_2$ (theoretical mass 200.0832) and were dominated by a base peak ion (*m/z* 169.064; $C_{12}H_9O$) due to benzylic cleavage and resulting in loss of a methoxy group. The spectra also contained ions consistent with losses of a methyl group (*m/z* 185.104; $C_{13}H_{13}O$) and of a methyl carboxy substituent (*m/z* 141.069; $C_{11}H_9$). The mass spectra of all isomers of the unknowns were virtually identical to those of 2-methyl- and 4-methyl naphthalene-1-carboxylic acids reference standards (methyl esters: Fig 2(F)) but the GC retention times in the first GC dimension were slightly less than those of the unknowns. We therefore suggest that the unknowns are other isomers of methyl naphthalene carboxylic acids, (a total of fourteen isomers are possible), most probably with the (now methylated) carboxy group in the 2-position. The question now arises: which of the known bacterial transformation processes (cf Fig. 1(A)), produces these metabolites?

Spectra consistent with dimethyl naphthalene acid homologues (as methyl esters) were also obtained (e.g. Fig. 2(G). The spectra were tentatively assigned from the strong molecular ion ($M^{+\cdot}$, *m/z* 214.099), within <0.2 mDa of that required for

$C_{14}H_{14}O_2$ (theoretical mass 214.0988) bicyclic diaromatic acid methyl ester, and abundant ion at *m/z* 155.085 (<0.5 mDa for $C_{12}H_{11}$) due to loss of the methylated carboxy substituents (50%); the base peak ion was at *m/z* 183.082 (<1.6 mDa for $C_{13}H_{11}O$) consistent with loss of a methoxy group. We obtained a reference sample of 4,7-dimethylnaphthalene-1-carboxylic acid and examined this by GC×GC/MS (Fig 2(H)). The mass spectrum and both first and second GC retention time of the reference sample were extremely similar to the unknowns; however, the GC retention times were not identical to those of any of the unknowns.

Also identified by comparison of the mass spectra and GC×GC retention times with authentic reference compounds, were naphthalene-1-ethanoic and naphthalene-2-ethanoic acids and biphenyl-3-carboxylic acid and biphenyl-4-carboxylic acid.

Bicyclic monoaromatic acids

Two isomers of tetrahydro-2-naphthoic acid (methyl ester) and one isomer of tetrahydro-1-naphthoic acid (methyl ester) were identified, first by interpretation of the mass spectra (Fig. 3(A), 3(C) and 3(E)), all of which were characterised by molecular ions of *m/z* 190 (consistent with the methyl ester of a C_{12} bicyclic monoaromatic acid) and secondly by comparison with the spectra and GCxGC retention times of reference samples (methyl esters) of each of the four possible isomers, 1,2,3,4-tetrahydro-2-naphthoic acid (Fig. 3(B)), 5,6,7,8-tetrahydro-2-naphthoic acid (Fig. 3(D)), 5,6,7,8-tetrahydro-1-naphthoic acid (Fig. 3(F)) and 1,2,3,4-tetrahydro-1-naphthoic acid (Fig. 3(G)), respectively.

Spectra consistent with various isomers of C_1 (MW=204) and C_2 (MW=218) substituted tetrahydro naphthoic acids were also observed, as were the

corresponding $C_{0\text{-}2}$ isomers of isomers with an ethanoic acid side chain (Fig. 4 and Supplementary Figures S1 and S2).

Also present were isomers of indanoic acids. Indane-2-carboxylic acid was identified by interpretation of the mass spectrum (Fig. 5(A)), and comparison with the mass spectra and GC×GC retention times of the ester of a purchased reference compound (Fig. 5(B)). The accurate mass of the suspected molecular ion was measured by GC×GC/HRMS as *m/z* 176.099, within <0.8 mDa of that required for $C_{11}H_{12}O_2$ (theoretical mass 176.0832), also present was a base peak ion (*m/z* 116.065; C_9H_8) due to loss of the methylated carboxy group followed by H-transfer. Many of the same ions were present in the spectrum of the reference compound (Fig. 5(B)). The GC×GC retention times of the unknown were the same as those of a purchased indane-2-carboxylic acid (methyl ester).

Indane-2-ethanoic acid was identified in the same manner (Fig. 5(C) and 5(D)). The accurate mass of the suspected molecular ion (Fig. 5(C)) was measured by GC×GC/HRMS as *m/z* 190.098, within <0.8 mDa of that required for $C_{12}H_{14}O_2$ (theoretical mass 190.0988). Major fragment ions were assigned to loss of a methoxy group (*m/z* 159) and loss of the methylated carboxy group and H-transfer (*m/z* 116) from the molecular ion, as sometimes observed in the mass spectra of methyl esters of carboxylic acids. The same ions were present in the spectrum of the reference compound (Fig. 5(D) and GC×GC retention times of the unknown were the same as those of indane-2-ethanoic acid reference acid (methyl ester).

Additionally, we tentatively identified an isomer of methyl indanoic acid (Fig. 5(E)) by spectral interpretation and comparison with the reference compound of an isomer (Fig. 5(F)). The spectrum of the unknown methyl indanoic acid (Fig. 5(E)) was

characterised by a molecular ion ($M^{+\cdot}$ *m/z* 190.099) within <0.2 mDa of that required for $C_{14}H_{12}O_2$ (theoretical mass 190.0988) and was dominated by a base peak ion (*m/z* 130.079; $C_{10}H_{10}$) due to loss of the methylated carboxy group. The spectrum also contained ions consistent with losses of a methyl group (*m/z* 175) and of a methyl carboxy substituent (*m/z* 159). The mass spectra of the unknown was very similar to that of the 3-methylindane-2-carboxylic acid reference (methyl ester: Fig 5(F)) and the 1st and 2nd dimension GC retention times, whilst not identical, were very close to those of the unknown. We therefore suggest that the unknown is another isomer of methylindane carboxylic acid.

Having identified a range of bicyclic di- and mono- aromatic acids in the commercial NA aromatic fraction we compared the GC×GC retention times and mass spectra with those of an acid fraction isolated by simple base extraction from a partially biodegraded Nigerian crude oil. Many of the same acids were identified by GCxGC/MS of the methyl esters, even in this total acid extract (Supplementary Information - Table S1), suggesting that many of these acids are probably present in biodegraded oils more generally and that the products we identified in the commercial NA mixture are perhaps typical of those of biodegraded petroleums.

DISCUSSION

The biodegradation of petroleum has a significant impact on the quality and hence on the economics of exploration for, and exploitation of, this primary energy source (reviewed by Head et al., 2010). Furthermore, biodegradation processes serve to alter the composition of petroleum residues spilled into the environment or introduced by process waters (e.g. Atlas and Hazen, 2011). It is therefore important that the biodegradation processes leading to the formation of metabolites, such as

carboxylic acids, are better elucidated. Firm identification of as many acids as possible can only improve an understanding of the pathways.

Whilst petroleum acids in aqueous fractions, such as produced or aquifer waters, sometimes occur as relatively simple mixtures with some components amenable to identification even by GC-MS (e.g. Barman Skaare et al., 2007; Lang, 2011), identification of the key metabolites in more complex mixtures in petroleum or resulting from processing of oil sands, has proved more challenging. The high chromatographic resolving power of GCxGC, especially when coupled with the high mass spectral resolution of HRMS, has allowed new information to be obtained about the occurrence of individual acids in such 'supercomplex' mixtures (West et al., 2013a, b).

By analogy, we were able herein, to compare the mass spectra and GCxGC retention times of numerous bicyclic mono- and diaromatic acids (as methyl esters) in a petroleum and in a petroleum–derived commercial naphthenic acids mixture, with those of authentic compounds. This was achieved by combining the fractionation of the esters of alicyclic from aromatic acids by silver ion chromatography with GCxGC/MS analyses of the sub-fractions in both nominal and high mass accuracy modes. This extended and improved on previous reports of more tentative identifications in somewhat simpler mixtures by GC-MS and also bodes well for a more thorough description of petroleum acids in future, following this initial study.

Numerous workers have used identification of particular acids as supportive evidence for the assignment of different biodegradation pathways (e.g. Figure 1 (A)). For instance, aerobic degradation of hydrocarbons to acids has long been known

(e.g. Wang and Shao, 2013) and some metabolic pathways, including biodegradation of polycyclic aromatic hydrocarbons and alkyl aromatics, are well described (e.g. Cerniglia, 1992; Miyachi et al., 1993; Rowland et al., 1986). Contributions to the long debate on whether the in–reservoir biodegradation of petroleum is predominately an aerobic or an anaerobic process have also been made possible, partly by the identification in biodegraded crude oils, of metabolites of petroleum hydrocarbons characteristic of anaerobic pathway(s) (e.g. Aitken et al., 2004). Anaerobic hydrocarbon degradation has been demonstrated via sulfate, nitrate and iron reduction, as well as methanogenesis (e.g. Rueter et al., 1994; Rabusand Widdel, 1995; Lovley et al.,1989; Zengler et al., 1999; Anderson and Lovley, 2000).

Some acids, particularly succinic or methyl succinic diacids, resulting from fumarate addition to both aromatic and non-aromatic hydrocarbons, do indeed appear to be distinctive ‘signature’ metabolites for pathways such as sulfate reduction (e.g. Annweiler et al., 2000; Gieg and Suflita, 2002; Safinowski et al., 2006; Jarling et al., 2012 and references therein). However, such metabolites were not detected in experiments where crude oil was degraded under methanogenic conditions in the laboratory (Aitken et al., 2013). The products of this process are therefore unknown, although laboratory incubations of individual or simple mixtures of bicyclic alkyl aromatic hydrocarbons, such as polymethylnaphthalenes, show that oxidation to the corresponding acids is possible during methanogenesis (Berdugo-Clavijo et al., 2012).

Thus, some acids are common to more than one pathway (Figure 1(A)); in such instances, conclusions about the degradation pathways have usually required

supporting evidence from simultaneous enzymatic or genomic studies (e.g. Berdugo-Clavijo et al., 2012; Gieg et al., 2010; Jones et al., 2008; Sherry et al., 2013).

The results of the present study allowed, not only identification of naphthoic acids substituted in both 1- and 2- positions, but also of some of the corresponding methyl, and dimethyl-substituted isomers (Figure 2), by comparison of spectra with those of authentic compounds. This supports previous, more tentative, identifications by GC-MS in diesel-contaminated ground water (Gieg and Suflita, 2002). Such compounds are known products of methanogenic (Berdugo-Clavijo et al., 2012) and aerobic (e.g. Rowland et al., 1986; Miyachi et al., 1993 and references therein) oxidation of naphthalene and alkylnaphthalenes. However, when detected in the presence of alkylsuccinates and/or tetrahydronaphthoic acids, which are known intermediates in the anaerobic sulfate reduction pathway for naphthalene degradation (Annweiler et al., 2002; Aitken et al., 2004), they have usually been assigned to an origin from anaerobic processes. No alkylsuccinates were detected in the present samples, but GCxGC/MS allowed firm identification of numerous tetrahydronaphthoic acids (Figures 3; S1, S2), again mainly by comparison with data for authentic compounds. Furthermore, several tetrahydronaphthyl acids with ethanoic acid side chains were firmly identified (Figure 4). In this instance we refrain for the moment, from concluding that these products are necessarily solely the result of anaerobic processes however; it seems at least possible that aerobic degradation of alkyltetralins might also produce these acids by alpha- and beta oxidation of the alkyl chains. A similar explanation might suffice for the occurrence of the indanoic acids (Figure 5). Although 5-indanoic acid has been identified in anoxic groundwater associated with tar oil contamination and suggested to form through anaerobic

processes (Annweiler et al., 2001; Lang, 2011), the co-occurrence herein of the methyl substituted isomers and ethanoate derivatives at least leaves open the possibility of production of these acids by alpha- and beta oxidation of the alkyl chains of alkylindanes. The latter hydrocarbons are widespread in petroleum, judging from GCxGC/MS studies of petroleum-contaminated shellfish (Booth et al., 2008). Studies of the products of aerobic and anaerobic oxidation (e.g. sulfate reduction, methanogenesis) of petroleum hydrocarbons in controlled experiments (cf. Berdugo-Clavijo et al., 2012; Aitken et al., 2013) by the highly resolving chromatographic and mass spectrometric GCxGC/MS methods might allow such hypotheses to be tested.

CONCLUSIONS

Use of GCxGC/MS with nominal and accurate mass resolution allowed the biodegradation products of numerous aromatic petroleum hydrocarbons to be identified firmly, even in the complex mixtures of acids in and from petroleum. The method has considerable potential for unravelling the complex distributions of acids in petroleum, generally.

The studies herein only allowed identification of mono-acid primary metabolites, which could be the products of aerobic and/or anaerobic biodegradation; 'signature' products of anaerobic degradation, alkylsuccinate diacids, were not found- but these may be transient intermediates, so no conclusions can yet be drawn about the originating mechanisms for the acids. Other di-acids have been identified by the same methods in heavily degraded oil sands process water samples recently (cf. Lengger et al. 2013). These may represent more oxidised end members of aerobic and/or anaerobic biodegradation (cf. Holowenko et al., 2001; Gieg et al., 2010; An et al., 2013) and will be the subject of future studies of petroleum fractions.

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FIGURE LEGENDS

Figure 1. (A) Summary of some aromatic hydrocarbon biotransformation pathways known to produce naphthalene-2-carboxylic acid. (B) Contour plot of a summed extracted ion chromatogram (*m/z* 186 + 200 + 214) of an aromatic acidic fraction (methyl esters) of commercial naphthenic acids refined from petroleum. Colours indicate the intensities of the responses (green>yellow>pink). (C) Extracted ion chromatogram (*m/z* 186 + 200 + 214) showing individual resolved peaks due to the methyl esters of naphthalene-1-, naphthalene-2-carboxylic acids and methyl (C_1) and dimethyl/ethyl (C_2) analogues.

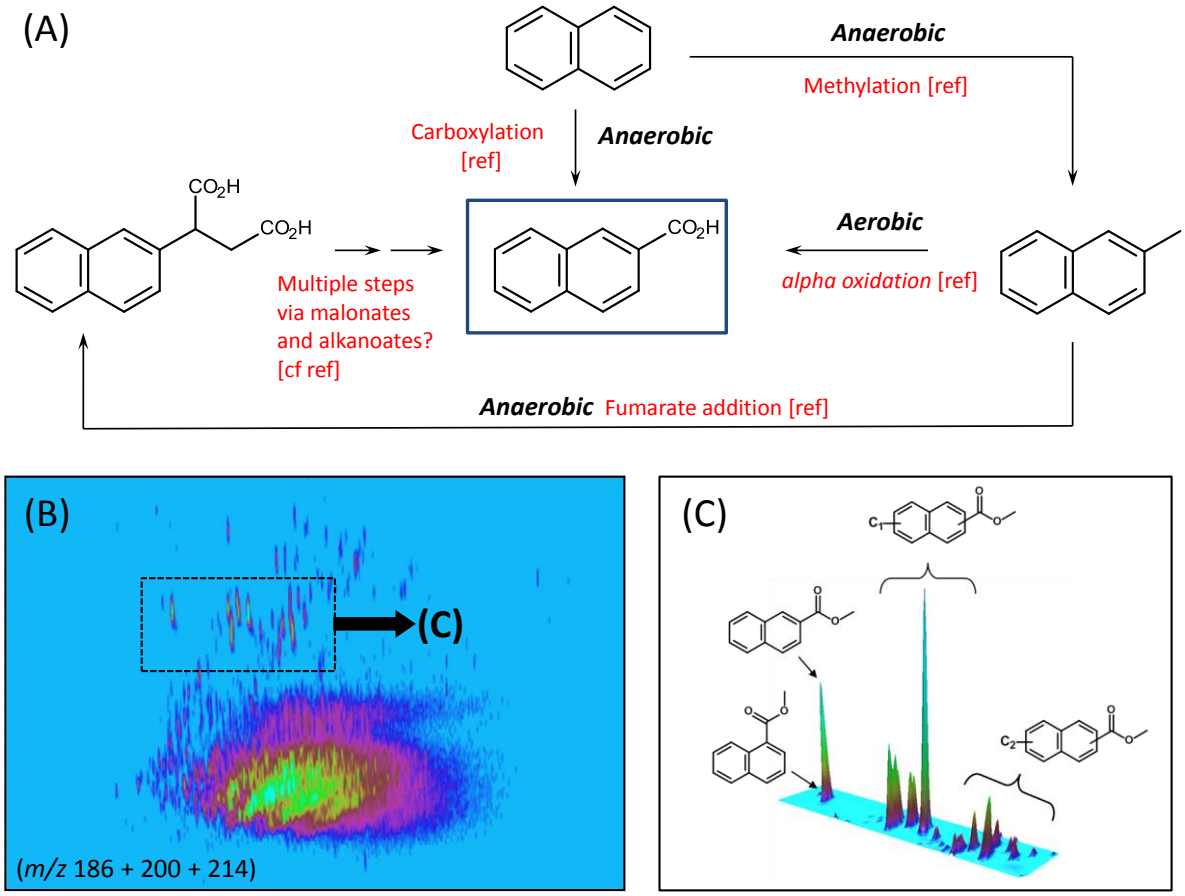
Figure 2. Electron ionisation mass spectra of methyl esters of examples of bicyclic diaromatic acids: (A) unknown I; (B) authentic naphthalene-1-carboxylic acid; (C) unknown II; (D) authentic naphthalene-2-carboxylic acid; (E) unknown III; (F) authentic 4-methylnaphthalene-1-carboxylic acid; (G) unknown IV; (H) authentic 4,7-dimethylnaphthalene-1-carboxylic acid from GC×GC/TOFMS analysis of methyl esters of commercial NA.

Figure 3. Electron ionisation mass spectra of methyl esters of tetrahydro naphthoic acids: (A) unknown I; (B) authentic 1,2,3,4-tetrahydro-2-naphthoic acid; (C) unknown II; (D) authentic 5,6,7,8-tetrahydro-2-napthoic acid; (E) unknown III; (F) authentic 5,6,7,8-tetrahydro-1-naphthoic acid; (G) authentic 1,2,3,4-tetrahydro-1-naphthoic acid from GC×GC/TOFMS analysis of methyl esters of commercial NA.

Figure 4. Electron ionisation mass spectra of methyl esters of tetrahydro ethanoic acids: : (A) unknown I; (B) authentic 1,2,3,4-tetrahydro-2-ethanoic acid; (C) unknown II; (D) authentic 5,6,7,8-tetrahydro-2-ethanoic acid; (E) unknown III; (F) authentic 5,6,7,8-tetrahydro-1-ethanoic acid; (H) Unknown IV; (G) authentic 1,2,3,4-tetrahydro-1-ethanoic acid from GC×GC/TOFMS analysis of methyl esters of commercial NA.

Figure 5. Electron ionisation mass spectra of methyl esters of indane acids: : (A) unknown I; (B) authentic indane-2-carboxylic acid; (C) unknown II; (D) authentic Indane-2-ethanoic acid; (E) unknown III; (F) authentic 3-methylindane-2-carboxylic acid from GC×GC/TOFMS analysis of methyl esters of commercial NA.

Figure 1.



NB: Add [ref] numbers once all references have been added to the text.

Figure 2.

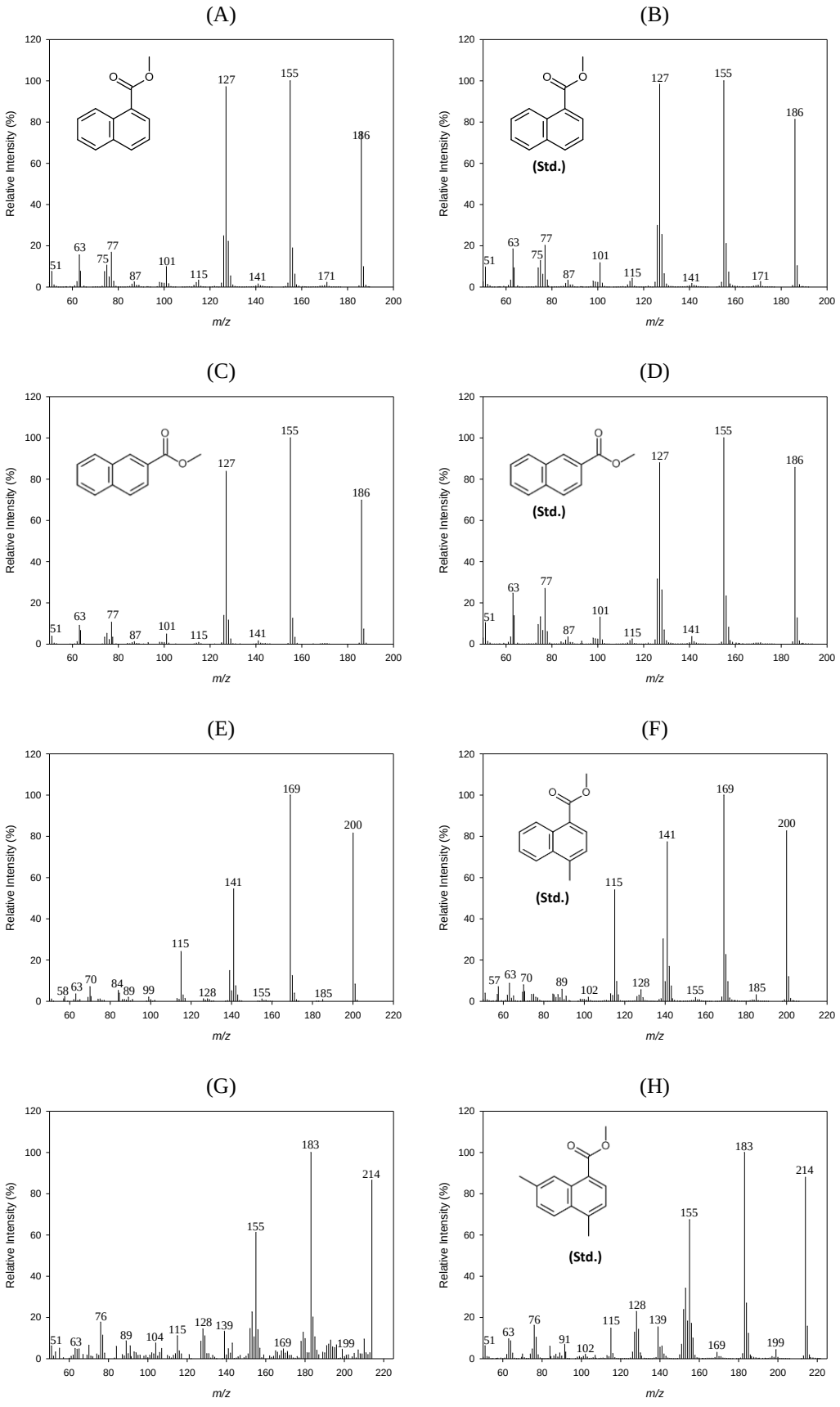


Figure 3.

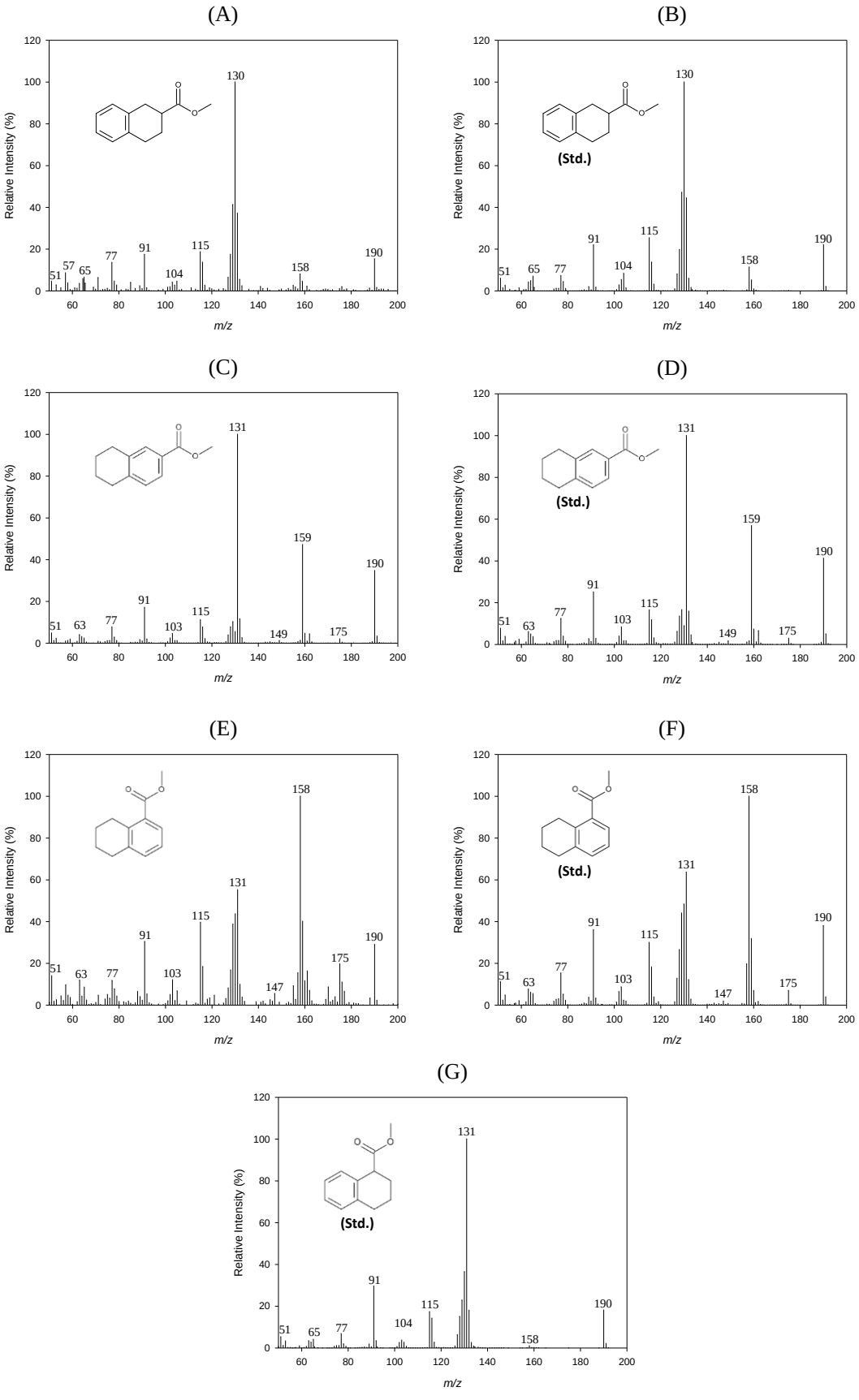


Figure 4.

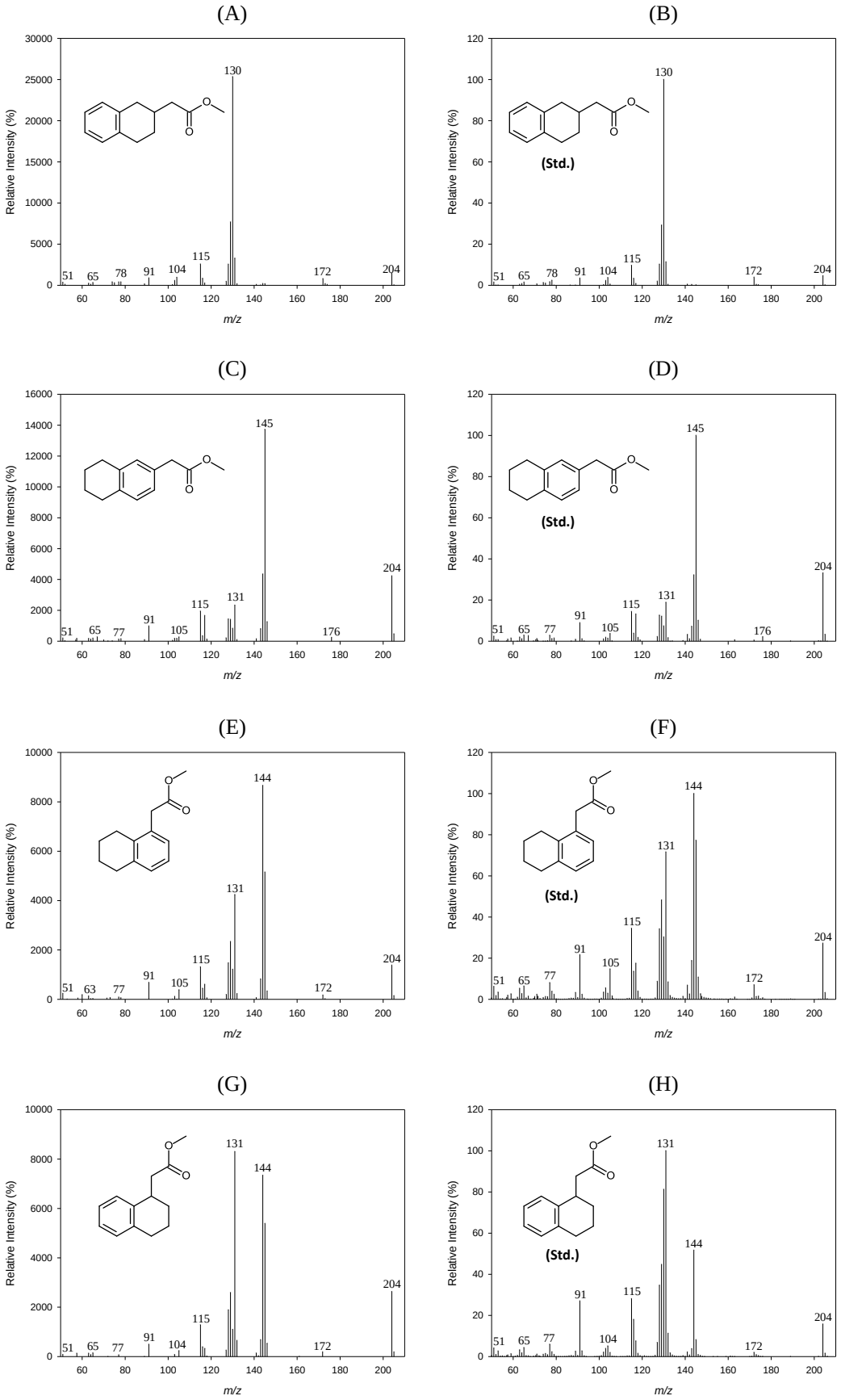
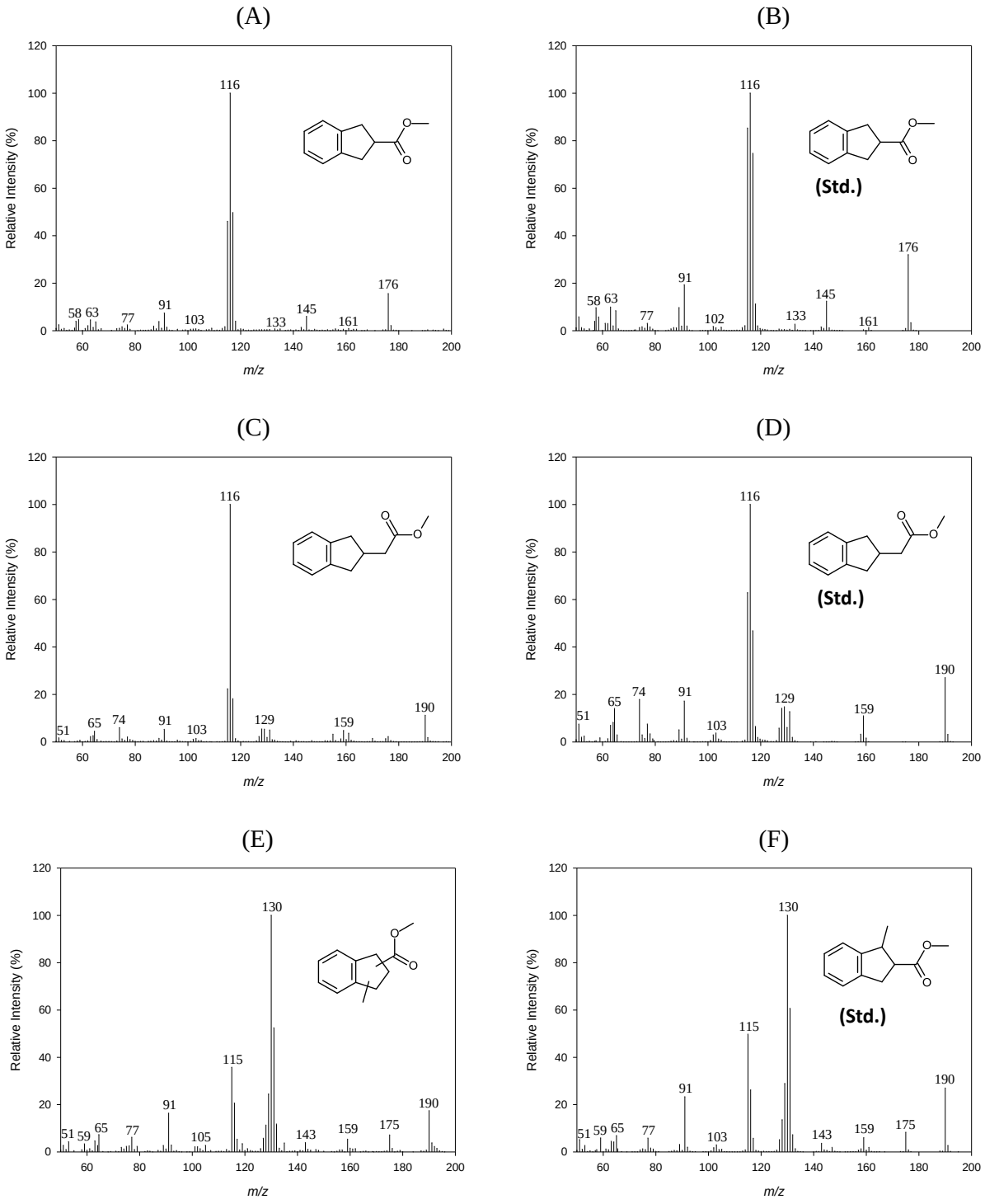


Figure 5.


(A)
(B)
(C)
(D)
(E)
(F)
Relative Intensity (%)
m/z
(Std.)
116
176
91
58 63
77
103
133
145
161
102
74
65
51
129
159
190
130
115
59
105
143
175

ONLINE SUPPLEMENTARY INFORMATION

Can GCxGC/MS identification of bicyclic aromatic acids in petroleum fractions help to reveal further details of aromatic hydrocarbon biotransformation pathways?

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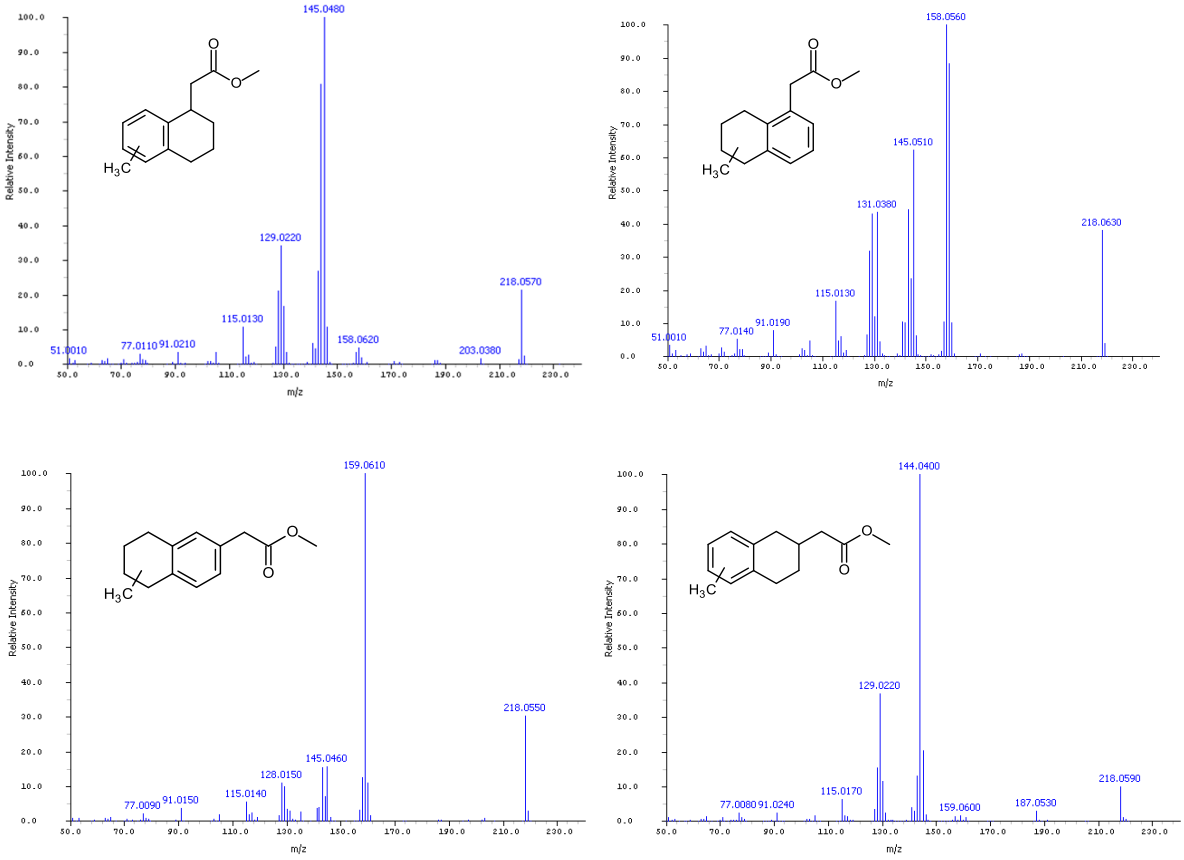
Figure - S1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129.0220
218.0570
115.0130
158.0620
203.0380
77.0110
91.0210
51.0010
158.0560
145.0510
131.0380
218.0630
115.0130
77.0140
91.0190
51.0010
159.0610
218.0550
145.0460
128.0150
115.0140
77.0090
91.0150
144.0400
129.0220
218.0590
115.0170
77.0080
91.0240
159.0600
187.0530
Relative Intensity
m/z

Figure S2

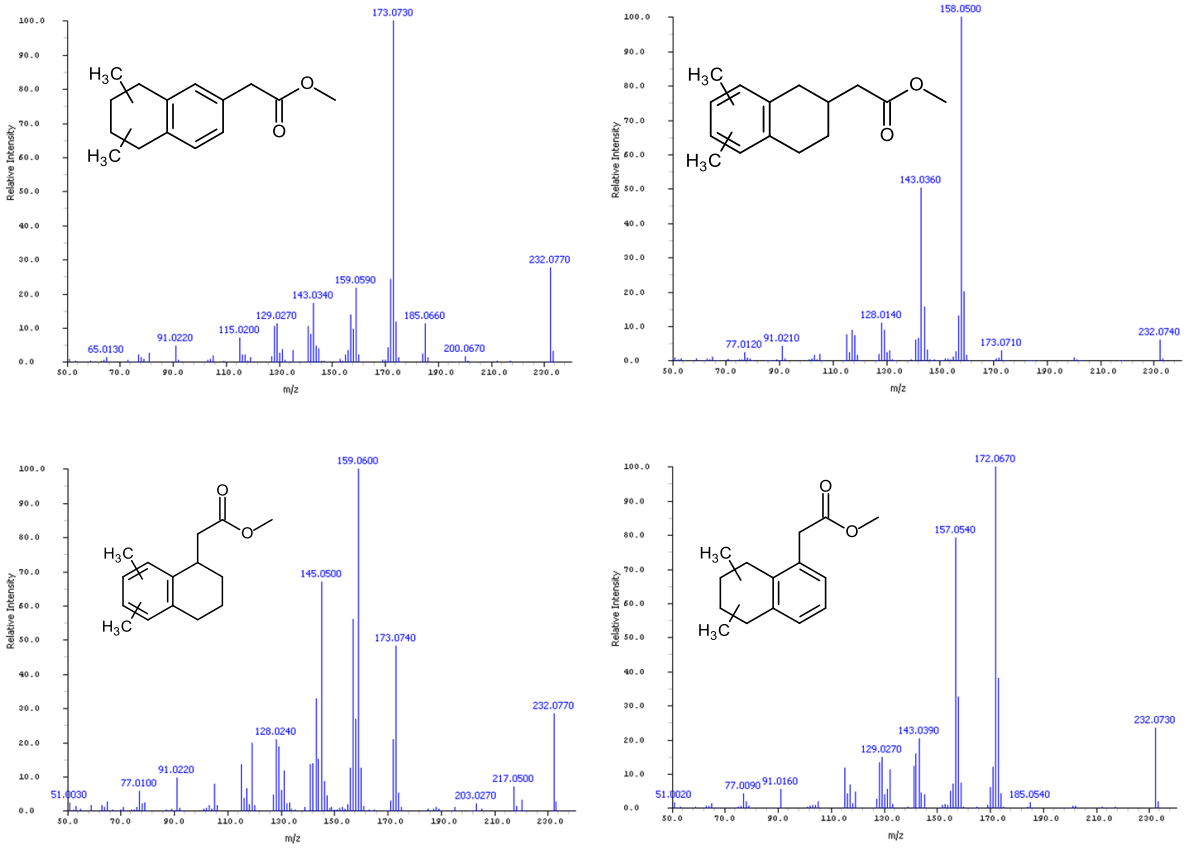
Relative Intensity
m/z
173.0730
232.0770
159.0590
143.0340
129.0270
185.0660
115.0200
91.0220
65.0130
200.0670
158.0500
143.0360
128.0140
91.0210
77.0120
173.0710
232.0740
159.0600
145.0500
173.0740
232.0770
128.0240
91.0220
77.0100
51.0030
217.0500
203.0270
172.0670
157.0540
143.0390
232.0730
129.0270
77.0090
91.0160
51.0020
185.0540

Table – S1

Compound	Commercial NA Aromatic Fraction	Biodegraded Crude NA Fraction	MW
Bicyclic Diaromatic Acids			
Naphthalene-1-carboxylic acid	●	●	186
Naphthalene-2-carboxylic acid	●	●	186
C1 naphthalene carboxylic acid *isomers*	●	●	200
Naphthalene-1-ethanoic acid	●	○	200
Naphthalene-2-ethanoic acid	●	○	200
Biphenyl-2-carboxylic acid	○	○	212
Biphenyl-3-carboxylic acid	●	○	212
Biphenyl-4-carboxylic acid	●	○	212
C2 naphthalene carboxylic acid *isomers*	●	●	214
Bicyclic Monoaromatic Acids			
Indane-2-carboxylic acid	●	●	176
Indane-2-ethanoic acid	●	●	190
3-methylindane-2-carboxylic acid	●	●	190
1,2,3,4-tetrahydro-1-naphthoic acid	○	○	190
5,6,7,8-tetrahydro-1-naphthoic acid	●	○	190
1,2,3,4-tetrahydro-2-naphthoic acid	●	○	190
5,6,7,8-tetrahydro-2-naphthoic acid	●	●	190
C1 1,2,3,4-tetrahydro-1-naphthoic acid	●	○	204
C1 5,6,7,8-tetrahydro-1-naphthoic acid	○	○	204
1,2,3,4-tetrahydro-1-ethanoic acid	●	○	204
5,6,7,8-tetrahydro-1-ethanoic acid	●	○	204
1,2,3,4-tetrahydro-2-ethanoic acid	●	○	204
5,6,7,8-tetrahydro-2-ethanoic acid	●	○	204
C1 tetrahydro ethanoic acid *isomers*	●	●	218
C2 tetrahydro ethanoic acid *isomers*	●	○	232

● detected

○ not detected