Quantitative and qualitative analysis of a commercial naphthenic acids mixture using urea adduction, argentation solid phase extraction and gas chromatography-mass spectrometry (GC-MS) of methyl esters

Report to the American Petroleum Institute

Original Report Date: 6th September 2012
Revised 27th September 2012 (rev.01)

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Summary

Previously, qualitative analysis of the methyl esters of a commercial naphthenic acids mixture using comprehensive multidimensional gas chromatography-mass spectrometry ('2D-GCMS') revealed the presence (as esters) of about 2000 compounds. Of these, 20 were tentatively identified from the mass spectra and from those of reference compounds, as individual representatives of wider classes of *n*-acids, methyl and polymethyl (including acyclic isoprenoid) and mono- to tricyclic acids. No tetracyclic acids were identified. Several minor monoaromatic acids and some aromatic hydrocarbons were also identified.

In the present study, semi-quantitative assessments of the proportions of a fraction adducted by urea (in which '*n*-acids' were concentrated) and a urea non-adductable fraction (dominated by 'branched and cyclic acids') and 'monoaromatic' acids, of the commercial naphthenic acids were conducted.

Although recoveries of the methyl esters were quite low through urea treatment (47 ± 6%, n=3) the results were reproducible and GC-MS of the methyl esters of urea adducts and urea non-adducts showed that a clear separation of the highly branched and cyclic acids from the urea adductable acids (e.g. *n*-acids) had indeed been achieved. The proportion of '*n*-acids' (urea adduct) was 12 ± 1% (n=3) of the recovered material. The proportion of non-adductable ('highly branched and cyclic') acids was 88 ± 1% (n=3) of the recovered material, based on gravimetric determinations.

Isolation of the 'monoaromatic' acids from the commercial naphthenic acids mixture was achieved using argentation solid phase extraction of the methyl esters on silica with hexane and hexane:diethylether eluants. Although recoveries were quite low through the treatment (64 ± 4%; n=3), again they were reproducible and GC-MS of the methyl esters of the eluate fractions showed that a clear separation of the 'non-aromatic' acids, from the 'monoaromatic' acids had indeed been achieved. The proportion of 'non-aromatic' acids was 84% ± 5% (n=3), of the recovered material and the proportion of 'monoaromatic' acids was 7% ± 1% (n=3) of the recovered material, based on gravimetric determinations.

The losses in recovery of both processes probably included evaporation of some of the more volatile constituents.

The contributions of the *n*-acids and monoaromatic acids to the toxicity (e.g. Microtox endpoint) of the total commercial acids mixture can now probably be estimated using the published Microtox EC_{50} values of individual *n*- and monoaromatic acids as a guide.

1. Introduction

Previously, qualitative analysis of a commercial naphthenic acids mixture using comprehensive multidimensional gas chromatography-mass spectrometry ('2D-GCMS') (West et al., 2012) allowed separation of about 2000 components and identification of individual acids including: *n*-acids, methyl and polymethyl (including acyclic isoprenoid) branched acids and cyclohexyl, perhydroindan, decalin and adamantane acids, similar to those reported previously in a commercial mixture and tentatively identified by comparison of the spectra with those of reference compounds (cf Rowland et al., 2011a).

Previous studies of the toxicological effects of the commercial naphthenic acids mixture had also reportedly been made by the API (Swigert, personal communications) and studies of the mixture composition had been made for the API by the University of Alberta, Edmonton, Canada following GC-MS analysis of silylated derivatives (Swigert, personal communications). The latter method did not allow individual acids to be identified but did provide a semi-quantitative measure of the acid groups, based on the response of certain ions in the electron ionisation mass spectra of the derivatives. Effectively these assignments allowed a table to be constructed of the relative abundances of certain acid groups defined by carbon number (n) and undersaturation with hydrogen (assumed to be due to hydrogen loss due to ring formation) expressed as the term z in the formula $C_nH_{2n+z}O_2$ where z is zero (acyclic) or an even, negative, integer (e.g. -2,-4,-6 for monocyclic, bicyclic, tricyclic acids).

It was agreed that following qualitative analysis using 2D-GCMS (West et al., 2012), UoPEL would conduct a further semi-quantitative analysis of the acids mixture supplied by API. UoPEL were tasked to provide assessments of the proportion of '*n*-acids' (viz: those adducted by urea), 'branched and cyclic acids' (urea non-adductable) and also of the proportion of 'monoaromatic' acids in the commercial naphthenic acids mixture (the latter using argentation solid phase extraction on silica with hexane and hexane:diethylether eluants: Jones et al., 2012).

2. Materials & Methods

2.1 Samples

A commercial preparation of naphthenic acids (Merichem, CAS No. 1338-24-5, ID No. CP006002) was supplied by API. This was reported by the suppliers to contain 0.3% phenols (Swigert, personal communication).

2.2 Methods

Numerous batches of the commercial preparation of naphthenic acids supplied were refluxed with boron trifluoride (12%) in methanol (Acros Organics, Geel, Belgium) and the products were back-extracted into hexane and evaporated to dryness and weighed to provide the 'methyl esters'. On average 93.0 % (standard error = 0.62%, n=10) of the naphthenic acids supplied was esterifiable by reaction with BF_3-methanol and extractable into hexane.

2.2.1 Urea Adduction

'Methyl esters' of the commercial naphthenic acids mixture (ca. 5 mg in triplicate) were dissolved in hexane/acetone (3 mL, 2:1) and a saturated solution of urea in methanol (1 mL) added dropwise. Solutions were stored in at 4°C overnight. The solvent was removed (N_2 stream, 40°C) and the adduction step repeated (twice). The crystals were rinsed (3 times, hexane, 2 mL) and the combined extract filtered (Pasteur pipette, pre-conditioned cotton wool plug) to afford the non-adduct. Urea crystals were dissolved in water to release the adducted compounds which were then extracted into hexane (3 x, 2 mL).

2.2.2 Argentation solid phase extraction

Argentation solid phase extraction (SPE) was conducted in triplicate on the methylated Merichem commercial acids using 6 mL Discovery® Ag-Ion SPE cartridges (750 mg sorbent; Sigma–Aldrich, Dorset, UK). In brief, cartridges were conditioned with hexane (3 × 6 mL); aliquots of the methylated acids (ca. 4 mg in hexane) were then loaded onto the cartridges. Cartridges were subsequently eluted using hexane (4 × 6 mL; fractions 1 – 4), 95% hexane 5% diethyl ether (4 × 6 mL; fractions 5 – 8) and 100% diethyl ether (6 mL; fraction 9). Fractions were collected and reduced to dryness under a steady stream of nitrogen at 40 °C then weighed to an accuracy of 0.01 mg. Fractions were reconstituted in hexane prior to analysis by GC-MS.

2.2.3 GC-MS

Extracts were examined on an Agilent GC-MSD (Agilent Technologies, Wilmington, DE, USA). This comprised a 7890A gas chromatograph fitted with a 7683B Series autosampler and a 5975A quadrupole mass selective detector. The column was a HP-5MS fused silica capillary column (30m x 0.25 mm internal diameter x 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.0 mL min-1. A 1.0 µL sample was injected into a 300°C splitless injector. The oven temperature was programmed from 40 to 300°C at 10°C min-1 and held for 10 min. Data and chromatograms were monitored and recorded using ChemStation (Revision E.01.00.237, Agilent Technologies, Wilmington, DE, USA) software. The quadrupole mass spectrometer used ionization energy of 70 eV and an ion source temperature of 230 °C. Acquisitions were conducted in full scan mode, with a mass range of 50-550 Da monitored.

3. Results & Discussion

The assumption that so-called 'naphthenic acids' comprise only alicyclic acids was commonplace for decades until the highly chromatographically resolving technique known as 2D-GCMS (or by a variety of other synonyms: Adachour et al., 2008) was used to study a commercial mixture of acids treated by heating with boron trifluoride-methanol complex to convert acids to the methyl esters (Rowland et al., 2011a). The

results of the latter and associated examinations showed that although acids in the mixture included so-called straight chain or *n*-acids, methyl and polymethyl branched acids, and monocyclic to tricyclic acids (Rowland et al., 2011a, b), a number of monoaromatic acids (Rowland et al., 2011b), polycyclic aromatic hydrocarbons and phenols were also present (Rowland et al., 2011a; West et al., 2011). The latter may have toxicological significance, in addition to the alicyclic acids (West et al., 2011).

Despite the above reports (Rowland et al., 2011a,b; West et al., 2011) very few, if any, attempts to use 2D-GCMS to characterise commercial naphthenic acids mixtures were made subsequently, even though the method was also shown to be applicable to supercomplex mixtures of acids from other sources, including from oil sands processing (e.g. Rowland et al., 2011c, d, e).

Recently however, in a consultancy work for the API, West et al., (2012) showed that the unprecedented chromatographic resolution reported by Rowland et al., (2011a) could be improved upon still further by use of GCxGC columns with alternative stationary phases, notably with certain ionic liquids and effectively some mixtures could be more or less completely resolved for the first time. For example, the latter conditions were used to examine a mixture of acids supplied by the API and derivatised by heating with boron trifluoride-methanol complex to convert acids to the methyl esters (West et al 2012). From the mass spectra and comparison with spectra from the National Institute of Standards & Testing (NIST) mass spectral library, the major individual resolved components were assigned as due to the methyl esters of *n*-acids. Mass spectra of a selection of further unknowns selected to include members of the z=0, -2, -4, -6 and -8 classes of acids (e.g. acyclic through monoaromatic acids), were also obtained. Also present in the sample were a number of non-acids, identified by comparison of the mass spectra with those of library spectra and of the corresponding best match library spectra. These included polycyclic aromatic hydrocarbons and polycyclic sulphur-containing aromatic hydrocarbons.

Following the study by West et al., (2012), and given the apparent predominance of the individual *n*-acids and occurrence of monoaromatic acids in the mixture, the API requested a semi-quantitative assessment of the proportions of these classes in the mixture.

It was suggested by UoPEL that the well-known technique of urea adduction (Speight, 2007) might be used to obtain a semi-quantitative estimate of the *n*-acids and that a recently published method involving argentation solid phase extraction (Jones et al., 2012) might be used to determine the 'monoaromatic' acids in the naphthenic acids mixture.

3.1 Urea adduction

Treatment of an aliquot (ca. 5 mg) of the methyl esters of the commercial naphthenic acids mixture supplied by the API, with a saturated solution of urea in methanol, allowed subsequent separation of the urea adducted and non-adducted fractions. The experiment was repeated on three separate batches of the methylated acids. Each urea adducted and non-adducted fraction was weighed using a microbalance. The data are shown in Table 1.

Overall, only about 50% of each 5 mg sample was recovered by extraction into hexane. The reasons for this low recovery are unknown but probably include evaporative losses.

However, the reproducibility of the method was good and the proportions of adducted (mainly esters of *n*-acids and simply branched acids) were constant (12 ± 1% of adduct+non-adduct recovered: Table 1). Similarly, the proportions of non-adducted (mainly esters of highly branched and cyclic acids) were constant (88 ± 1% of adduct+non-adduct recovered: Table 1).

GC-MS of the adducted and non-adducted fractions showed that a very clear separation of the esters of *n*- and simply branched acids from the non-adducted esters of highly branched and cyclic acids had been achieved in all three replicates (Figures 1 and 2).

Thus, the adduct fractions comprised mainly *n*-acids ranging from nonanoic to octadecanoic acids, with minor amounts of methyl and ethyl branched acids (Figure 1A-C). These were illustrated by appropriate use of selected ion mass chromatograms (e.g. M^{c}Lafferty ion *m/z* 74; Figure 3A-C). A minor unresolved complex mixture was also present (Figure 1A-C), presumably comprising acids with adductable straight chains (Speight, 2007). The spectra of these did not produce significant M^{c}Lafferty ions, *m/z* 74 (Figure 3A-C). This may indicate that such acids do not contain a γ-H atom (i.e. are trisubstituted at the γ-C), since this is the structural requirement for production of the ion.

In contrast, the non-adduct fractions comprised no detectable *n*-acids (Figure 2A-C) but instead contained significant resolved polymethyl branched acids such as the series of acyclic isoprenoid acids identified previously by 2D-GCMS (West et al., 2012). For instance, the dominant GCMS-resolved acid was assigned from the mass spectrum as the acyclic isoprenoid, 3,7,11-trimethyldodecanoic acid, by comparison with the NIST reference spectrum of the methyl ester. Other acyclic isoprenoid acids were identified previously by the higher resolving 2D-GCMS method (West et al., 2012) but the urea adduction process used herein simplified the mixtures sufficiently for the 3,7,11-trimethyldodecanoic acid to be resolved and identified, even by GCMS (Figure 2A-C).

The distributions of acyclic isoprenoid acids were also illustrated by appropriate use of selected ion mass chromatograms (e.g. M^{c}Lafferty ion *m/z* 74; Figure 4A-C).

A bimodal major unresolved complex mixture was also present in each replicate non-adduct fraction (Figure 2A-C), presumably comprising acids with no adductable straight chains (Speight, 2007). The spectra of some these produced significant M^{c}Lafferty ions, *m/z* 74, resulting in a unimodal unresolved complex mixture in the chromatograms (Figure 4A-C). It appears that the second, more minor mode, of the unresolved complex mixture in the non-adduct (Figure 2A-C), contained acids without a γ-H atom, since this is the structural requirement for production of the *m/z* 74 ion.

In mixtures of acids isolated from oil sands processing (e.g. Rowland et al., 2011e; Jones et al., 2012) this second mode comprised mainly aromatic (or naphtheno-aromatic) acids. Some of the monoaromatic acids identified in a commercial naphthenic acids mixture by 2D-GCMS, lacked γ-H atoms (e.g. alkylphenylpropanoic acids; Rowland et al., 2011b), as did some of those identified in the present mixture by 2DGC-MS previously (West et al., 2012).

3.2 Argentation solid phase extraction

Fractionation of aliquots (ca. 4 mg) of the methyl esters of the commercial naphthenic acids mixture supplied by the API by elution through an argentatious silica solid phase extraction cartridge (cf Jones et al., 2012) yielded an average recovery of 63.5% of the loaded material. This was highly reproducible (standard deviation 4.4%, n=3).

Gravimetry (Figure 5) and GC-MS of the individual fractions (Figures 6A-C) showed that a very clear separation of the esters of 'non-aromatic' acids (fractions 1-3; F1-3) from those of the 'monoaromatic' acids (fraction 5; F5) had been achieved in all three replicates.

As confirmation, mass spectra of some of the more resolved acids (methyl esters) in the putative 'monoaromatic' fraction F5 were obtained by GCMS, and these compared favourably with those assigned previously to methyl esters of monoaromatic acids by 2D-GCMS (Figure 7; West et al., 2012; Rowland et al., 2011b).

As with the simplification of the mixtures produced by urea adduction (Section 3.1, above) the argentation solid phase extraction improved the resolution of this fraction F5 such that even GCMS spectra of some components were assignable to monoaromatic acids (Figure 7). For example, the spectrum in Figure 7A was tentatively assigned to the methyl ester of a methylphenylbutanoic acid and the spectrum in Figure 7C was tentatively assigned to the methyl ester of a trimethylbenzoic acid. Many more compounds were assignable previously from 2D-GCMS spectra (West et al., 2012).

Calculated as a percentage of the material recovered, the 'non-aromatic' acids comprised 84 ± 5 % % of the total and the 'monoaromatic' acids 7 ± 1%; again reproducibility was high (Figure 5).

4. Conclusions

Recoveries of methyl esters were quite low through urea treatment (47 ± 6%, n=3) and through SPE treatment (64 ± 4%; n=3). Reasons for the reproducible but low recoveries through urea and argentation treatments are unknown but probably included evaporation of some of the more volatile constituents once converted to methyl esters.

However, ***of those recovered*** through fractionation processes, a commercial mixture of naphthenic acids contained about 84% 'non-aromatic' acyclic and alicyclic monoacids. About 12% of the total acids were urea adductable *n*- or simply branched acids and about 88% were non-adductable highly branched, cyclic acids and aromatic acids. About 7% comprised 'monoaromatic' acids. Thus about 72% was non-

adductable highly branched and cyclic acids. Additional analyses made for API by Fedorak reported 12% highly branched acids (Swigert, personal communication). If the methods used by Fedorak and herein are comparable, this would suggest the proportion of cyclic acids was 60%. The remaining proportion (9%) comprised a variety of non-acids and uncharacterised material (Figure 8) of which 0.3% was phenols according to suppliers details and an unknown proportion of polycyclic aromatic hydrocarbons (PAH) and heteroaromatics (S-PAH; West et al., 2012).

Thus, combining all the sources of information, of the 93.0 % (standard error = 0.62%, n=10) of the naphthenic acids supplied which was esterifiable by reaction with BF_3-methanol and extractable into hexane :

Cyclic acids 60%
Highly branched acids 12%
n- and simply branched acids 12%
'Monoaromatic' acids 7%
Other 9% (of which, 0.3% phenols, unknown % PAH and S-PAH)

Total 100% of the naphthenic acids esterifiable and extractable into hexane or 93% of naphthenic acids supplied by API.

The contributions of the *n*-acids and monoaromatic acids to the toxicity (e.g. Microtox endpoint) of the total commercial acids mixture can now probably be reasonably estimated using the published Microtox EC_{50} values of individual *n*- and monoaromatic acids as a guide, should this be desirable. For example, Jones et al., (2011) published Microtox EC_{50} values of individual *n*- and monoaromatic acids as well as values for acyclic isoprenoid and simply branched acids and a variety of cyclic acids, examples of which were identified in the present mixture. Johnson et al., (2011) and Rowland et al., (2011f) also published Microtox EC_{50} values for a number of other alicyclic and monaromatic acids. Tollefsen et al. (2012) published values for the EC_{50} of some of the same acids to Rainbow Trout hepatocytes.

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Figure Legends

Figure 1. A-C. Total ion current chromatograms urea adductable methyl esters of API supplied naphthenic acids (replicate experiments).

Figure 2 A-C. Total ion current chromatograms urea non-adductable methyl esters of API supplied naphthenic acids (replicate experiments).

Figure 3A-C. Extracted ion mass chromatograms (*m/z* 74) resulting from GCMS of urea adductable methyl esters of API supplied naphthenic acids (replicate experiments).

Figure 4A-C. Extracted ion mass chromatograms (*m/z* 74) resulting from GCMS of urea adductable methyl esters of API supplied naphthenic acids (replicate experiments).

Figure 5. Relative contributions of fractions produced by Ag+ SPE chromatography. Error bars represent 1 relative standard deviation of the mean (n = 3).

Figure 6. GCMS of Ag+ SPE fractions: A. All fractions. B. Fraction 2 ‘non-aromatic’ monacids. C. Fraction 5 ‘monoaromatic’ monoacids.

Figure 7. Mass spectra of methyl esters of components resolved by GCMS extracted ion mass chromatography in Ag+ SPE putative ‘monoaromatic ‘ fraction 5 (F5).

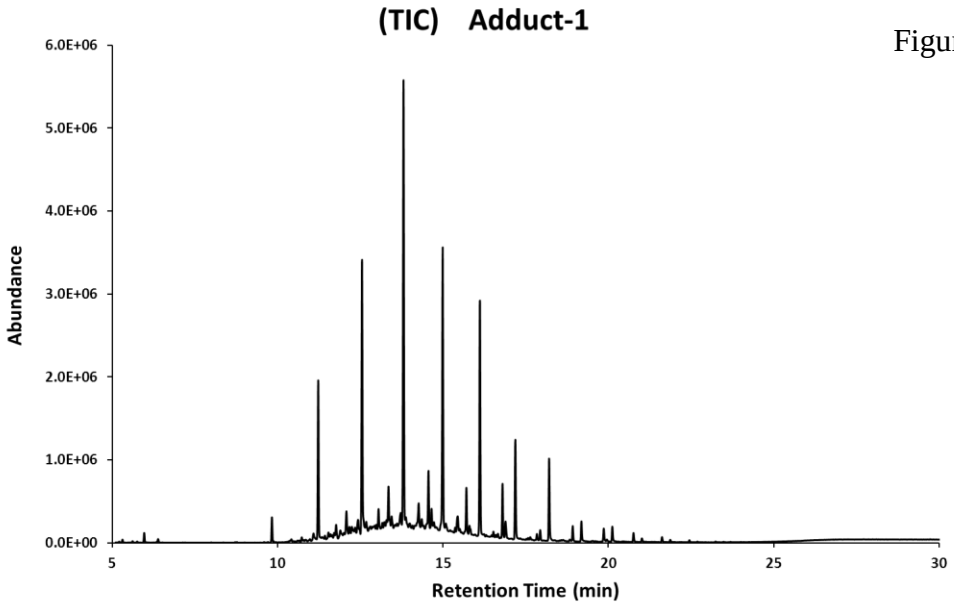
Figure 8. Pie chart showing approximate proportions of classes of acids and unknowns (data normalised assuming 100% recoveries: i.e. that losses in recovery due to evaporation etc were proportional for each class).

Table Legends

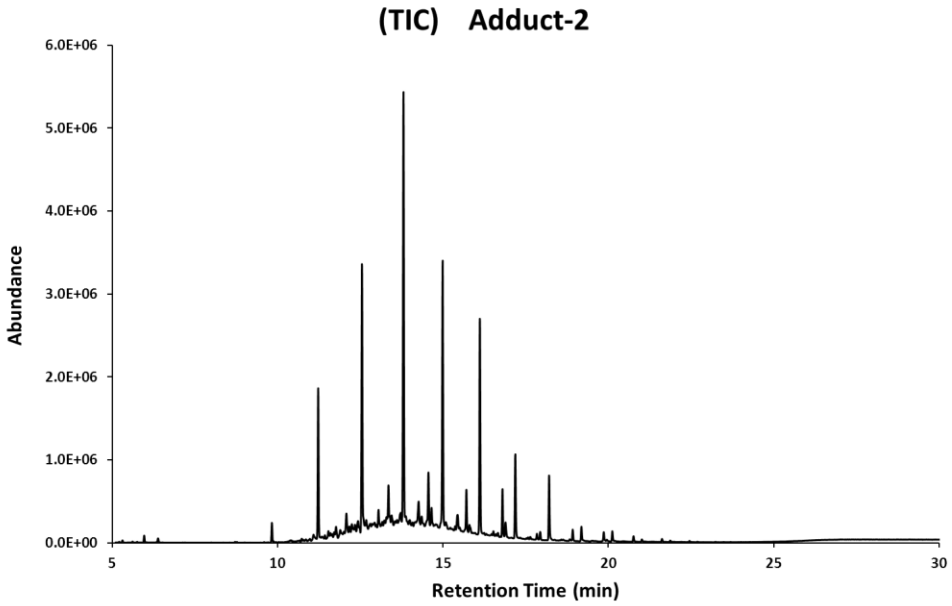
Table 1. Gravimetry of urea adduction/non-adduction fractions

Figure 1

(A)


(TIC) Adduct-1
Abundance
6.0E+06
5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
5
10
15
20
25
30
Retention Time (min)

(B)


(TIC) Adduct-2
Abundance
6.0E+06
5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
5
10
15
20
25
30
Retention Time (min)

(C)

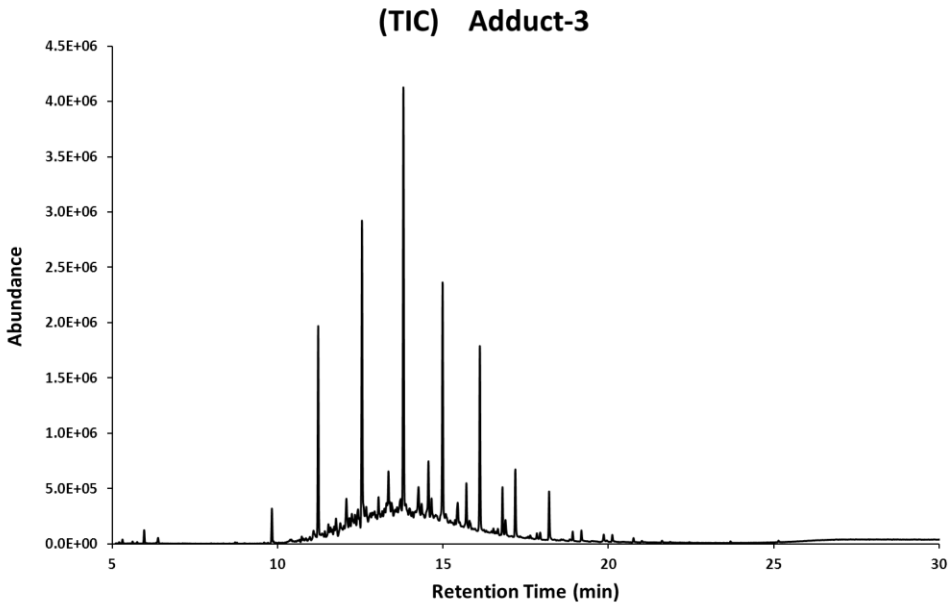
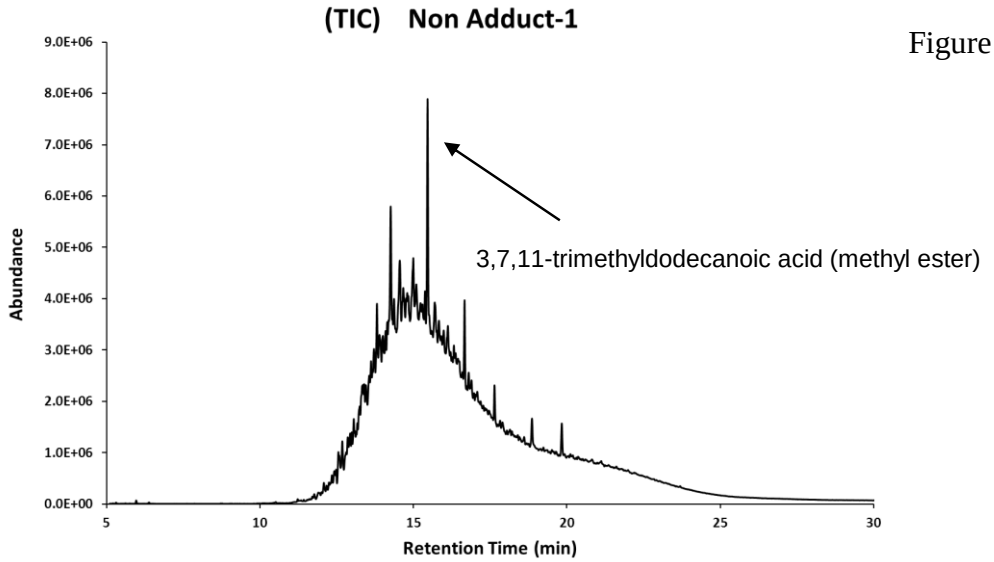
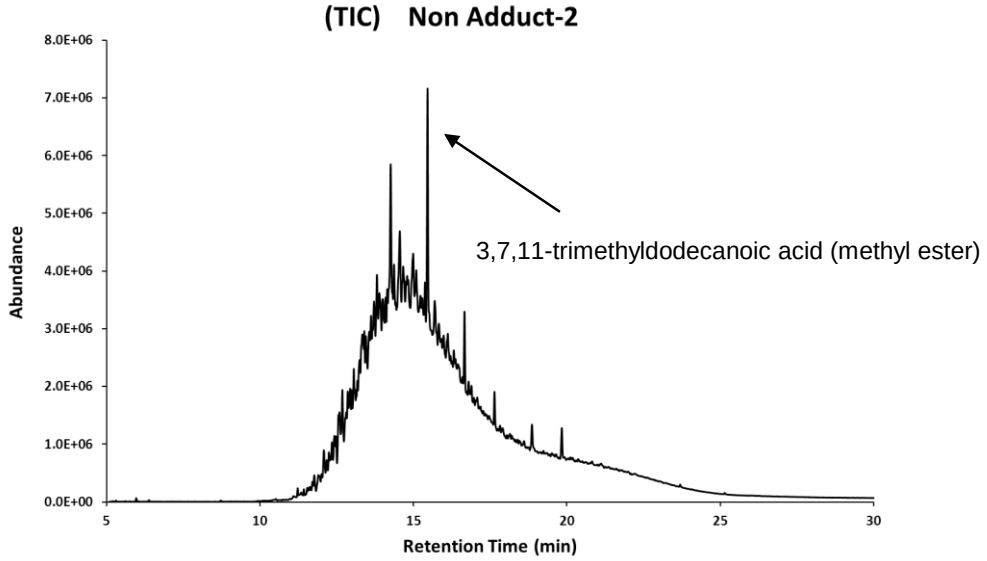

(TIC) Adduct-3
Abundance
4.5E+06
4.0E+06
3.5E+06
3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
5
10
15
20
25
30
Retention Time (min)

Figure 2

(A)


(TIC) Non Adduct-1
Abundance
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

(B)


(TIC) Non Adduct-2
Abundance
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

(C)

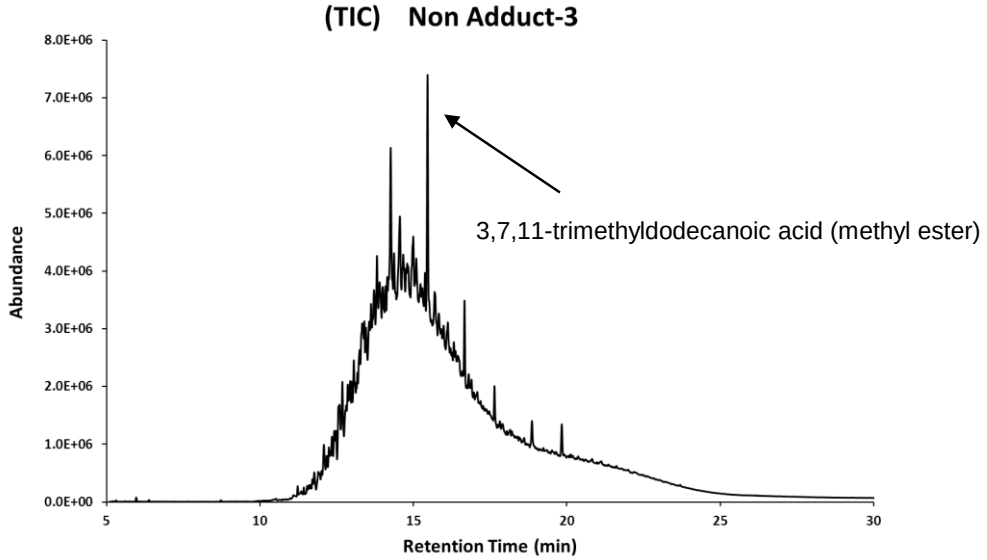
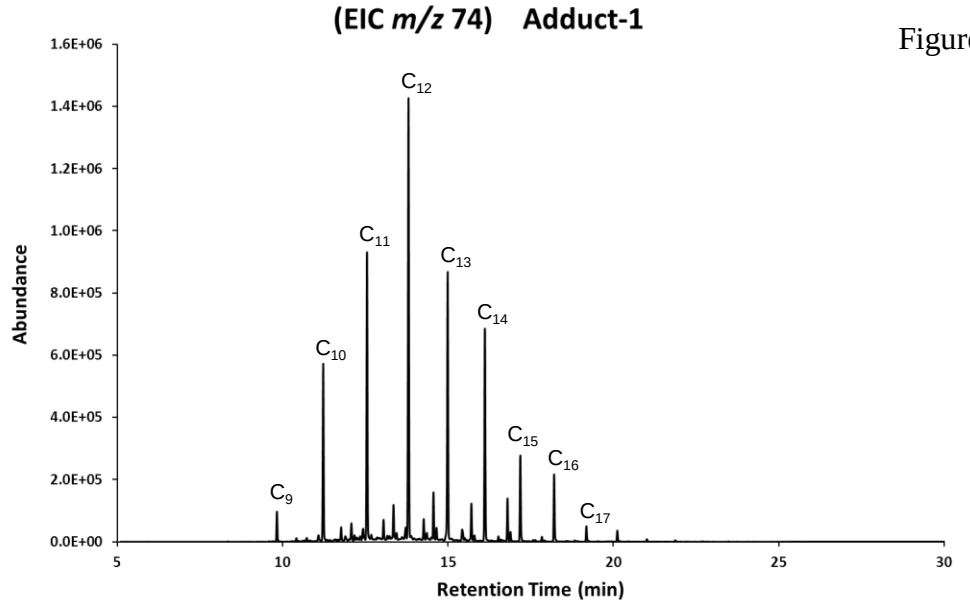
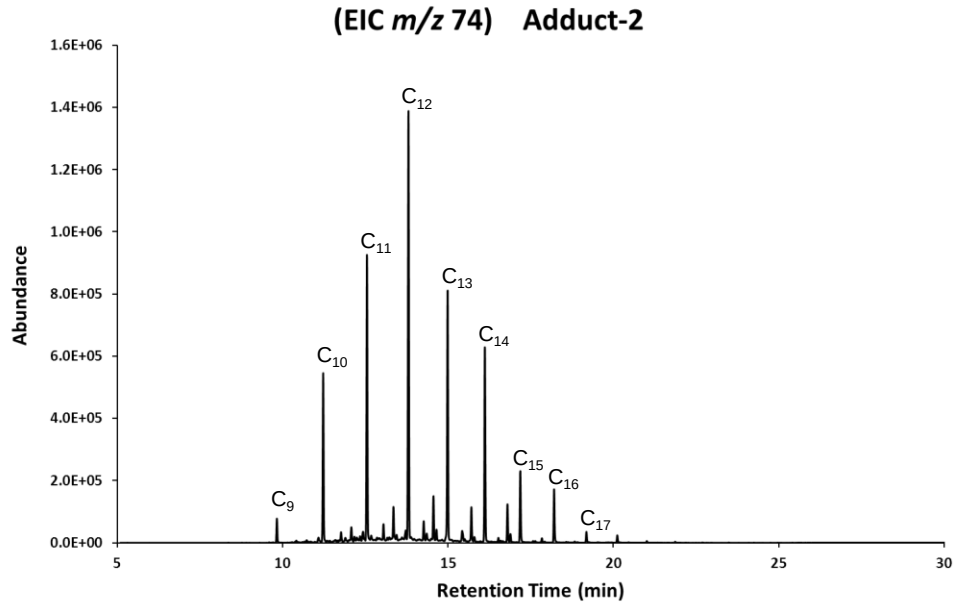

(TIC) Non Adduct-3
Abundance
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

Figure 3

(A)


(EIC m/z 74) Adduct-1
Abundance
Retention Time (min)
C9
C10
C11
C12
C13
C14
C15
C16
C17

(B)


(EIC m/z 74) Adduct-2
Abundance
Retention Time (min)
C9
C10
C11
C12
C13
C14
C15
C16
C17

(C)

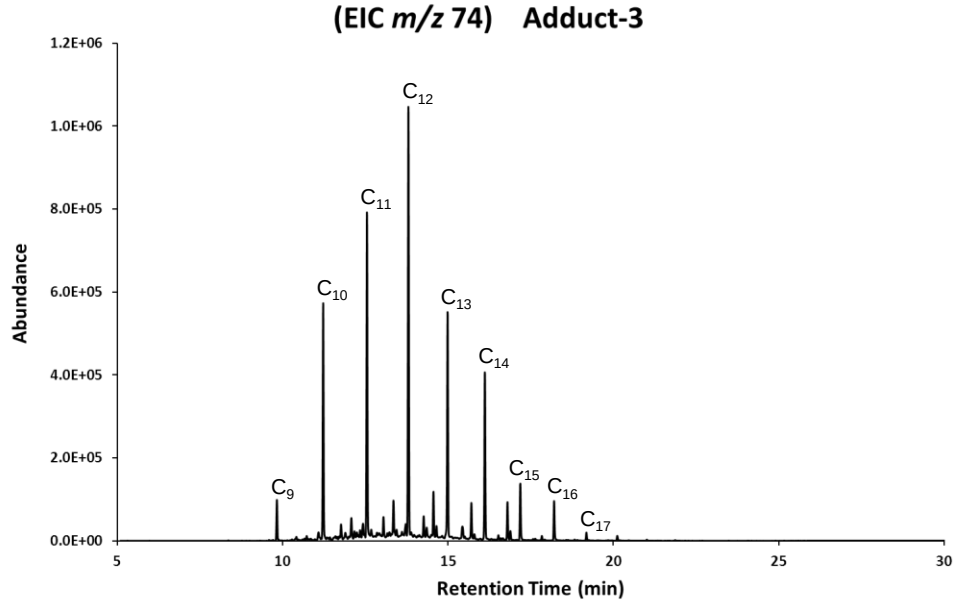
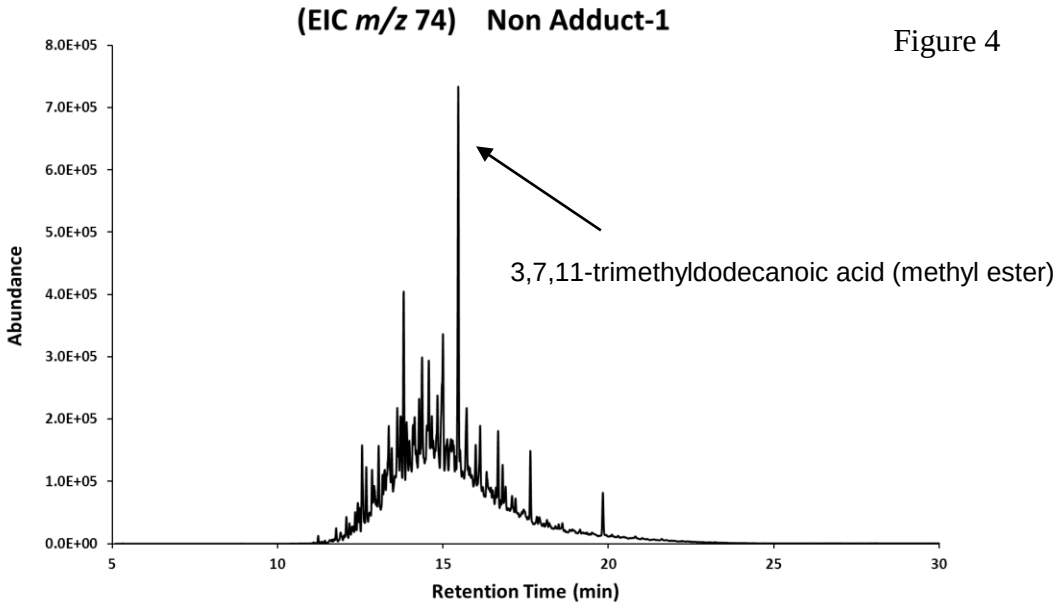
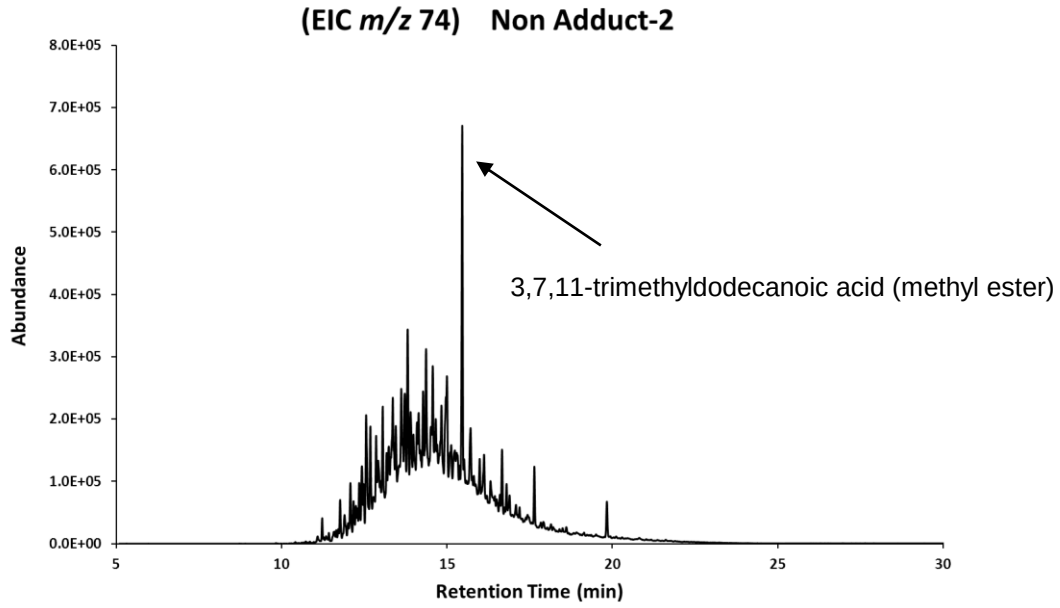

(EIC m/z 74) Adduct-3
Abundance
Retention Time (min)
C9
C10
C11
C12
C13
C14
C15
C16
C17

Figure 4

(A)


(EIC m/z 74) Non Adduct-1
Abundance
8.0E+05
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
5
10
15
20
25
30
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

(B)


(EIC m/z 74) Non Adduct-2
Abundance
8.0E+05
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
5
10
15
20
25
30
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

(C)

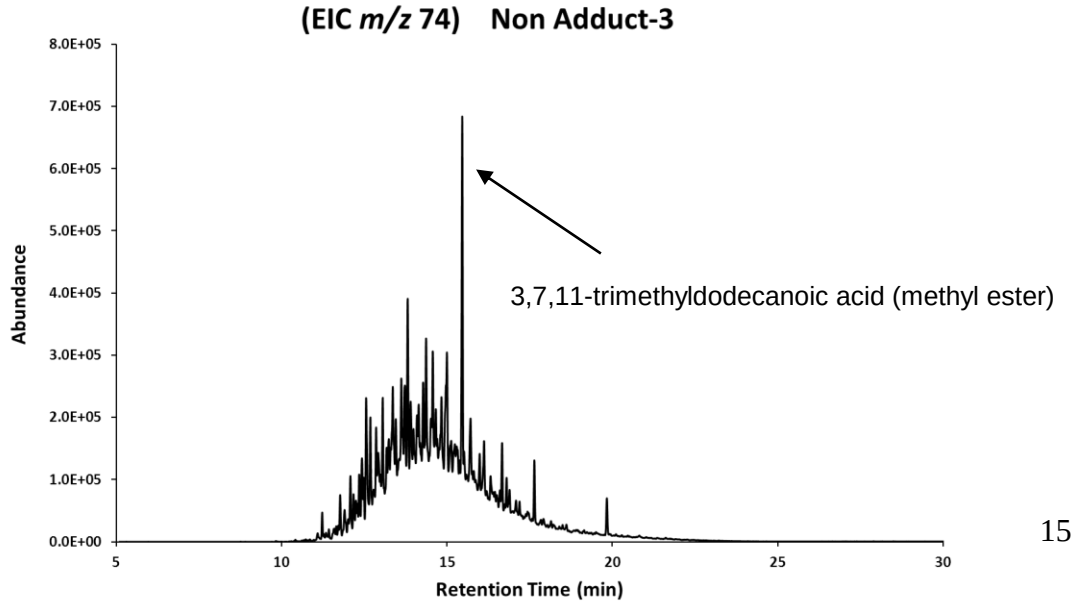

(EIC m/z 74) Non Adduct-3
Abundance
8.0E+05
7.0E+05
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
5
10
15
20
25
30
Retention Time (min)
3,7,11-trimethyldodecanoic acid (methyl ester)

Figure 5

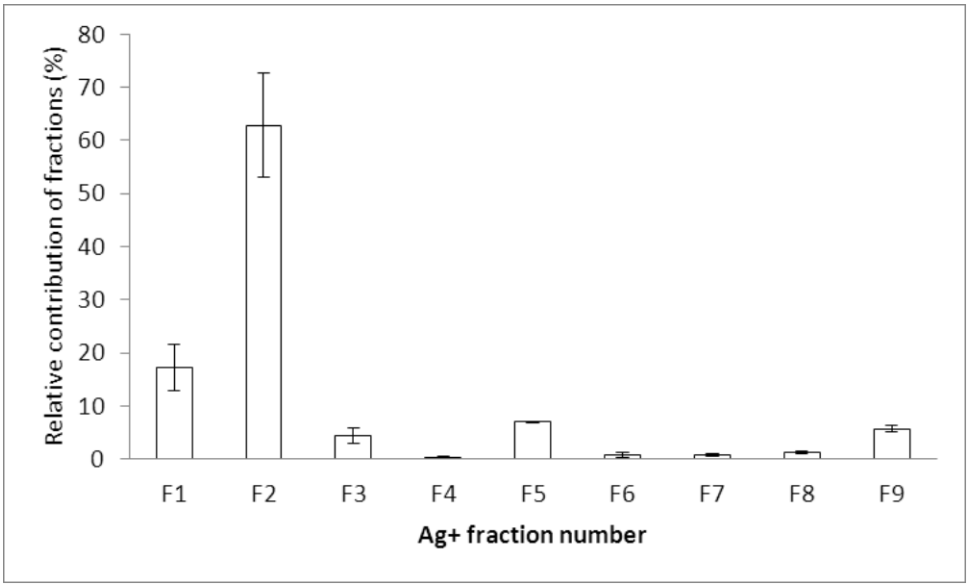
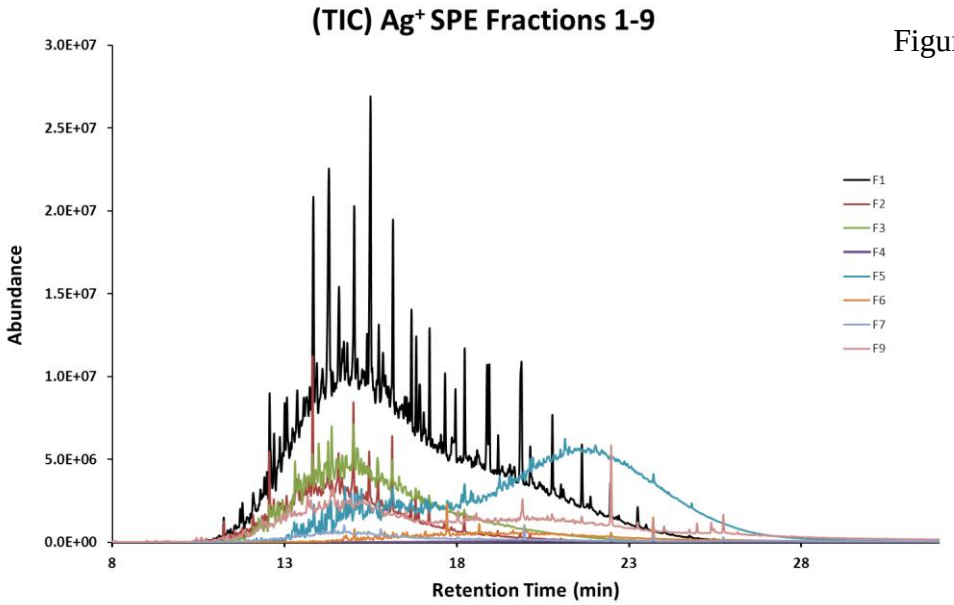
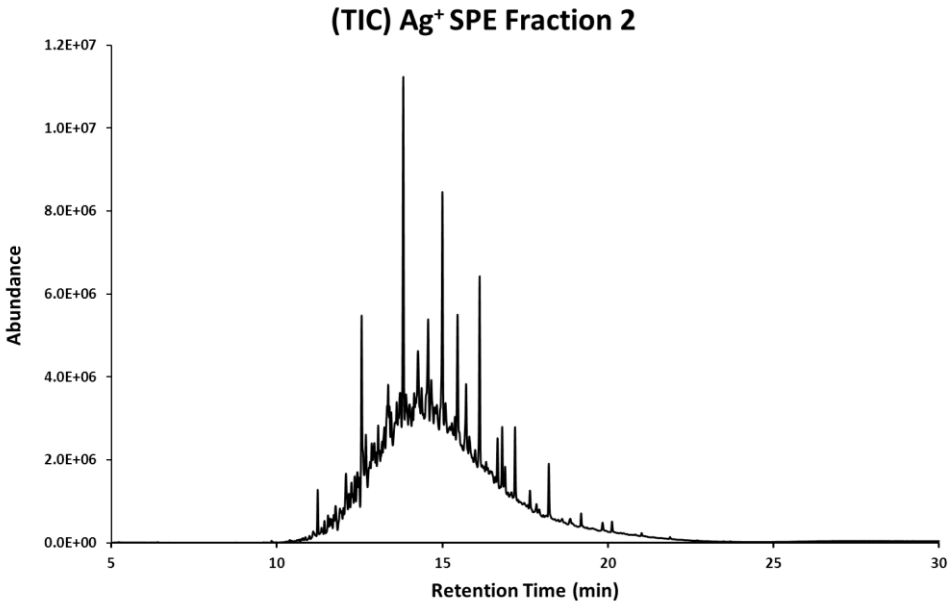

Relative contribution of fractions (%)
80
70
60
50
40
30
20
10
0
F1
F2
F3
F4
F5
F6
F7
F8
F9
Ag+ fraction number

Figure 6

(A)


(TIC) Ag+ SPE Fractions 1-9
Abundance
Retention Time (min)
F1
F2
F3
F4
F5
F6
F7
F9

(B)


(TIC) Ag+ SPE Fraction 2
Abundance
Retention Time (min)

(C)

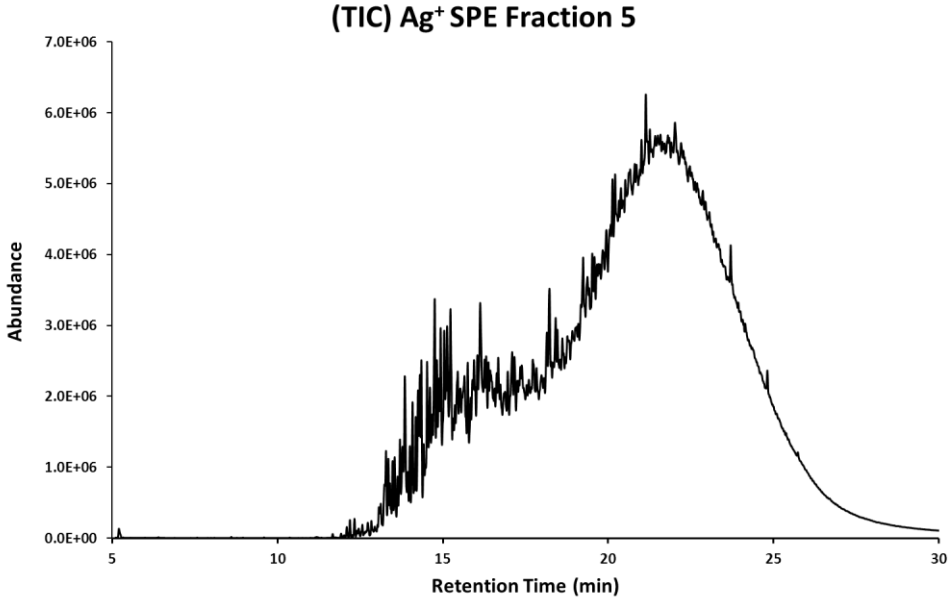

(TIC) Ag+ SPE Fraction 5
Abundance
Retention Time (min)

Figure 7

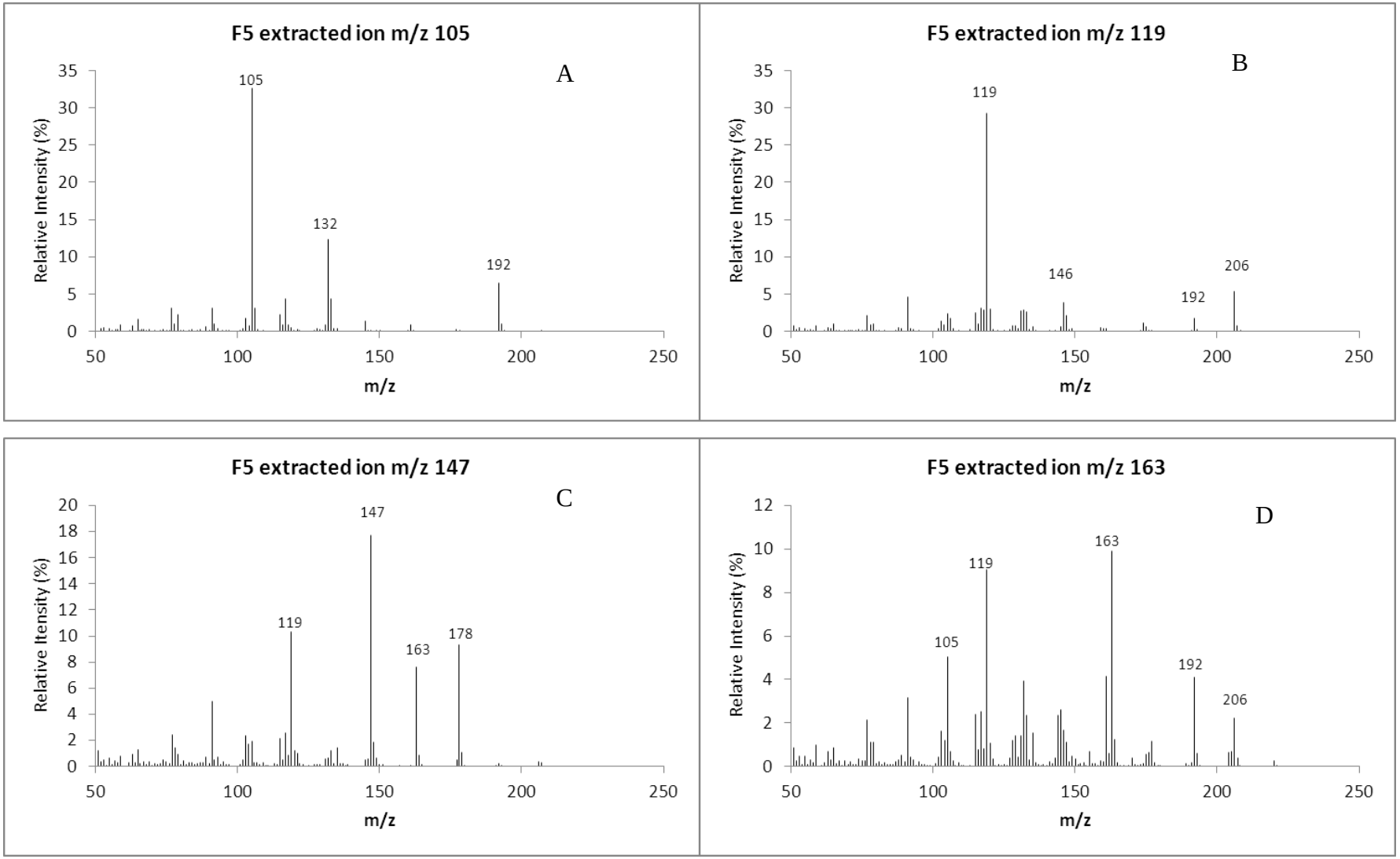

F5 extracted ion m/z 105
A
105
132
192
Relative Intensity (%)
m/z
F5 extracted ion m/z 119
B
119
146
192
206
Relative Intensity (%)
m/z
F5 extracted ion m/z 147
C
147
119
163
178
Relative Itensity (%)
m/z
F5 extracted ion m/z 163
D
163
119
105
192
206
Relative Intensity (%)
m/z

Figure 8

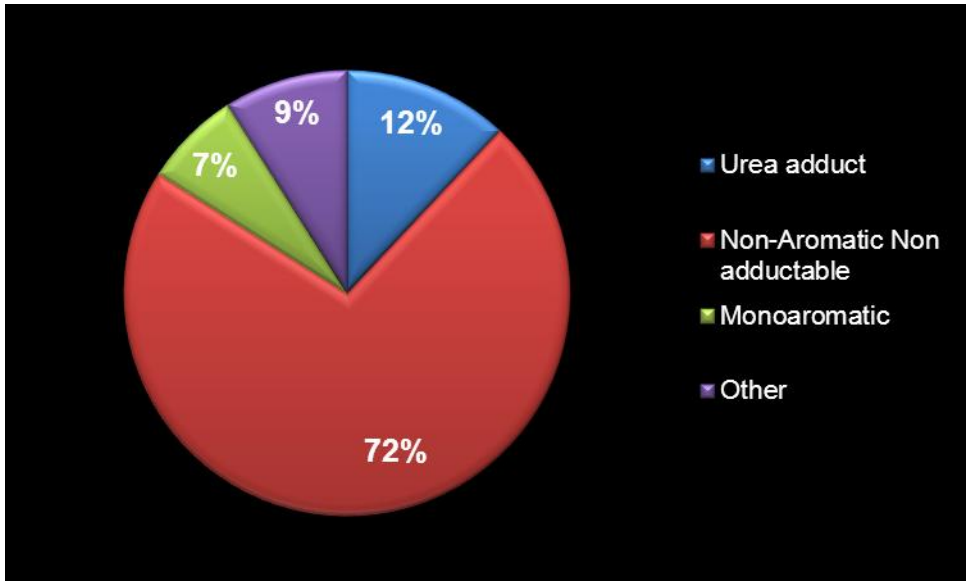


Table 1

Sample	API Napthenic Acids ME (mg)	Non-Adduct Fraction (mg)	Adduct Fraction (mg)	Non-Adduct + Adduct (mg)	% Recovered
Rep 1	4.90	2.44	0.111	2.55	52
Rep 2	5.00	1.90	0.090	1.99	40
Rep 3	4.70	2.18	0.100	2.28	49
Average	*4.87*	*2.17*	*0.10*	*2.27*	*47*
SD	*0.15*	*0.27*	*0.01*	*0.28*	
% RSD	*3.1*	*12.4*	*10.5*	*12.3*	