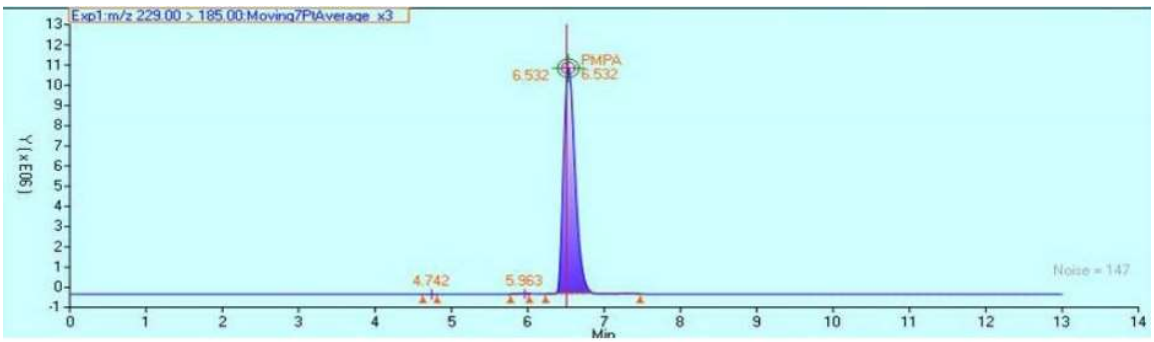
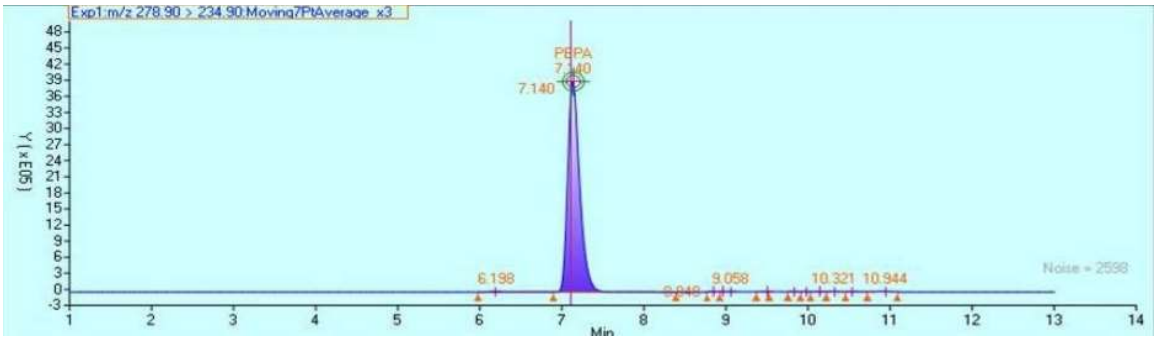
Review of Chemours Report Evaluation of the Analysis of the Branched and Linear Isomers PMPA/PFMOPrA and PEPA/PFMOBA

Overall finding: The LC/MS/MS experiments performed for the evaluation of branched and linear isomers PMPA/PFMOPrA and PEPA/PFMOBA should be repeated with method improvements that better allow for better chromatographic separation and instrument detection of the four individual analytes of interest. Method improvements suggested are for this investigation only. Routine analysis of the isomers would not require implementation of all suggested method changes for the isomer evaluation. However, some of the method changes may help with future routine analysis.

Monitoring of individual analytes: Two solutions containing two analytes were used in this experiment (PMPA/PEPA and PFMOPrA/PRMOBA). One solution for each of the four analytes would provide unambiguous data that any instrument response observed can only be coming from the single analyte in solution.

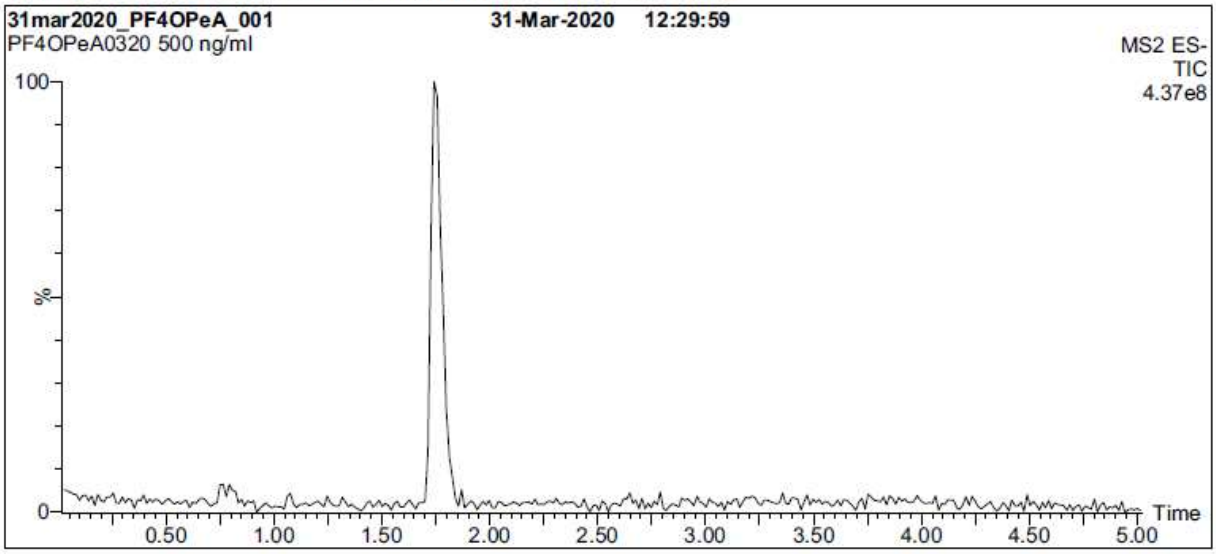
Mass spectrometer signal for PMPA and PEPA: Several of the specific comments and recommendations in this review are related to the amount of instrument response for each isomer. Figures 11 and 12 of the report (below) show that under the instrument conditions used, a signal of 4 x 10^6 to 1 x 10^7 can be achieved for PEPA and PMPA in a Site sample without saturating the instrument detector. This indicates that global mass spectrometer settings and analyte specific parameters are providing good signal for PMPA and PEPA in the field sample. However, similar instrument response was not achieved for the analysis of standard material of PMPA and PEPA. In order to achieve an instrument response of about 1 x 10^6 for the most abundant MRM, a higher concentration stock solution for PMPA and PEPA should be used.





Mass spectrometer signal for PFMOPrA and PFMOBA: The report indicates that there was difficulty achieving a strong MRM signal in the mass spectrometer for PFMOPrA and PFMOBA. Supporting information on the Wellington certificates of analysis for the two linear isomers show a good LC/MS response for the parent ion and a strong signal for the most predominant MRM using LC/MS/MS (see figures below). Sufficient signal is also achieved for monitoring a second MRM transition for each analyte (see figures below). HPLC and mass spectrometer conditions under which the chromatograms in the Wellington C of A were collected are also shown below.

Figure 1: **PF4OPeA; LC/MS Data (TIC and Mass Spectrum)**



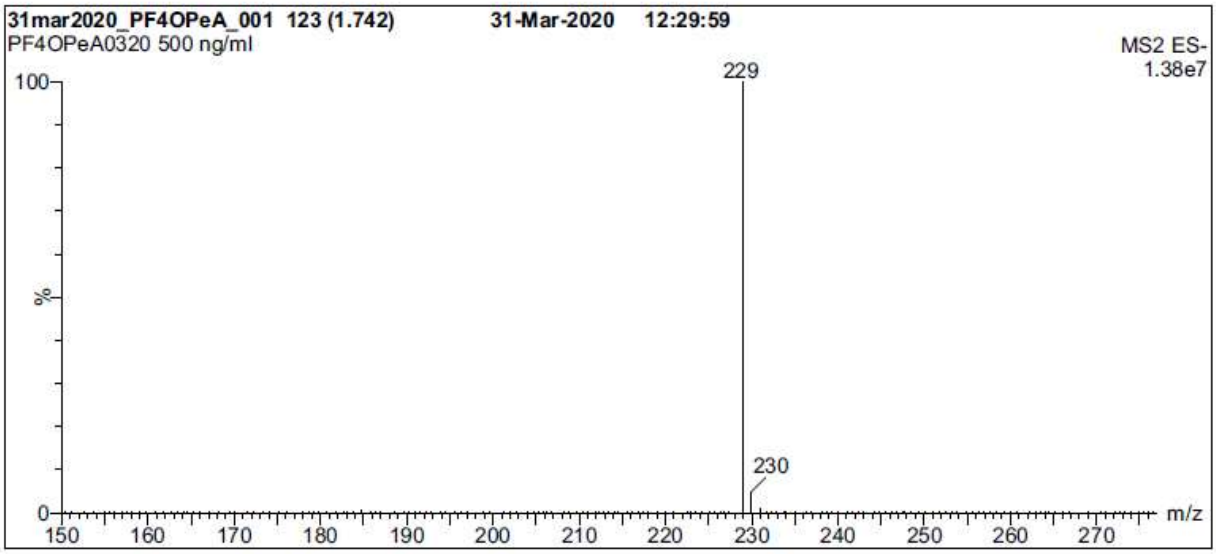


Figure 2: **PF4OPeA; LC/MS/MS Data (Selected MRM Transitions)**

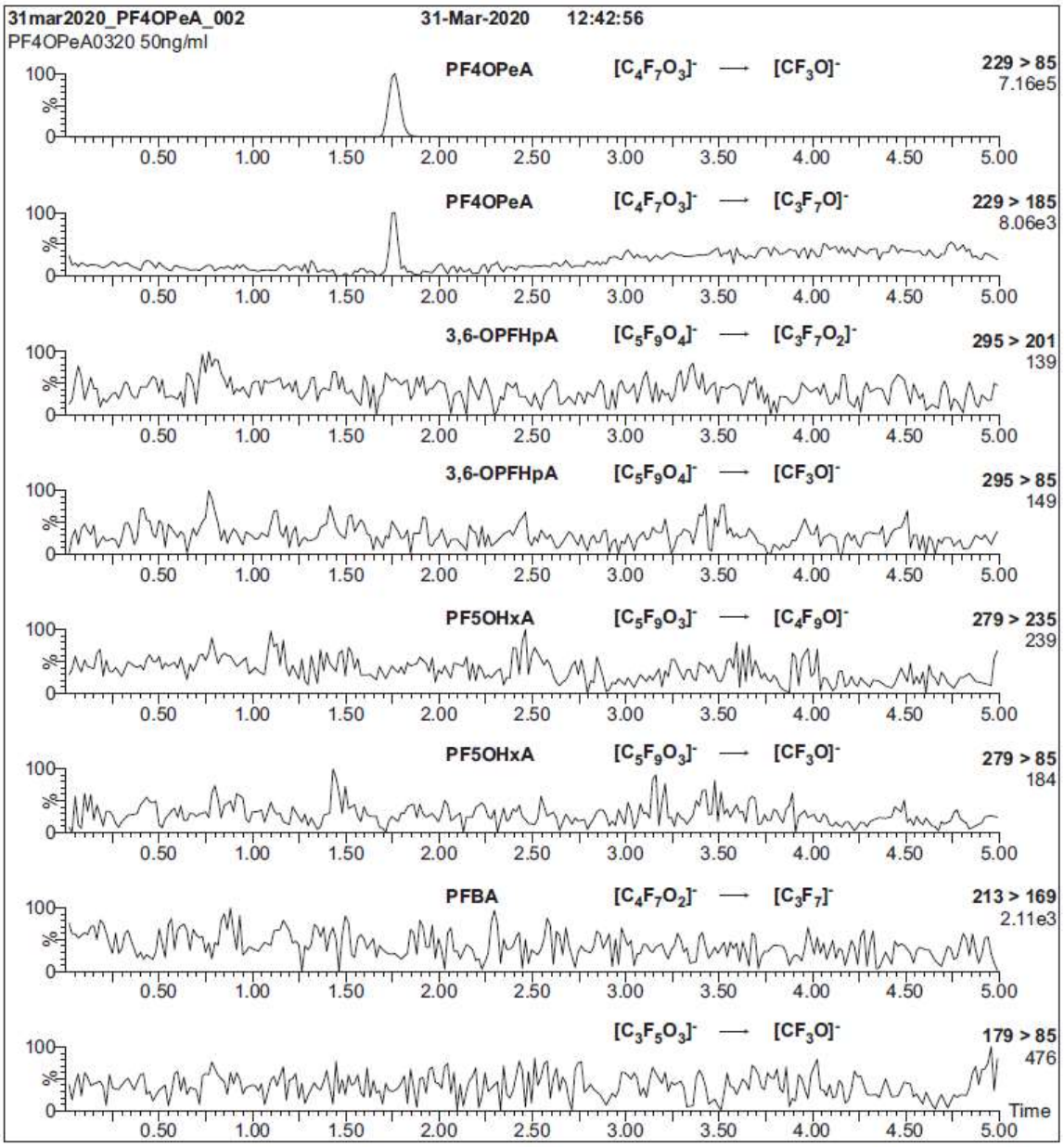
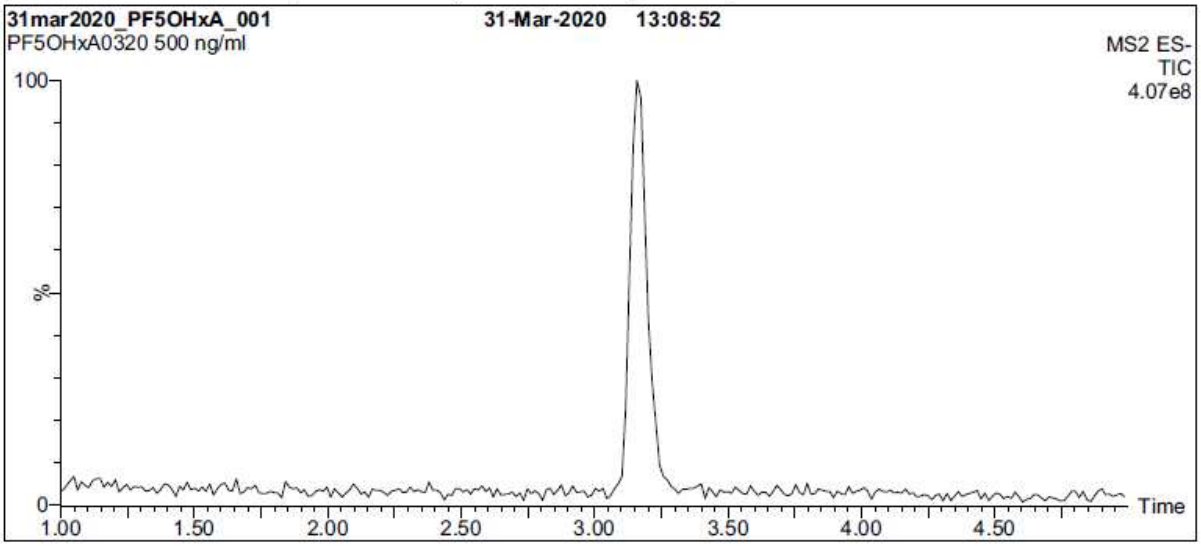


Figure 1: **PF5OHxA; LC/MS Data (TIC and Mass Spectrum)**



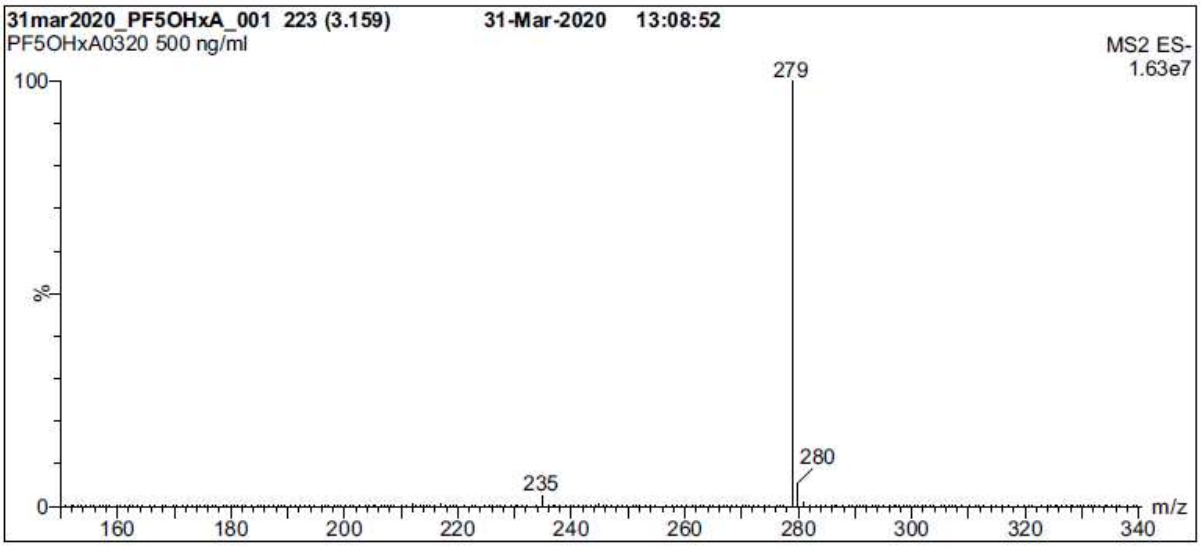
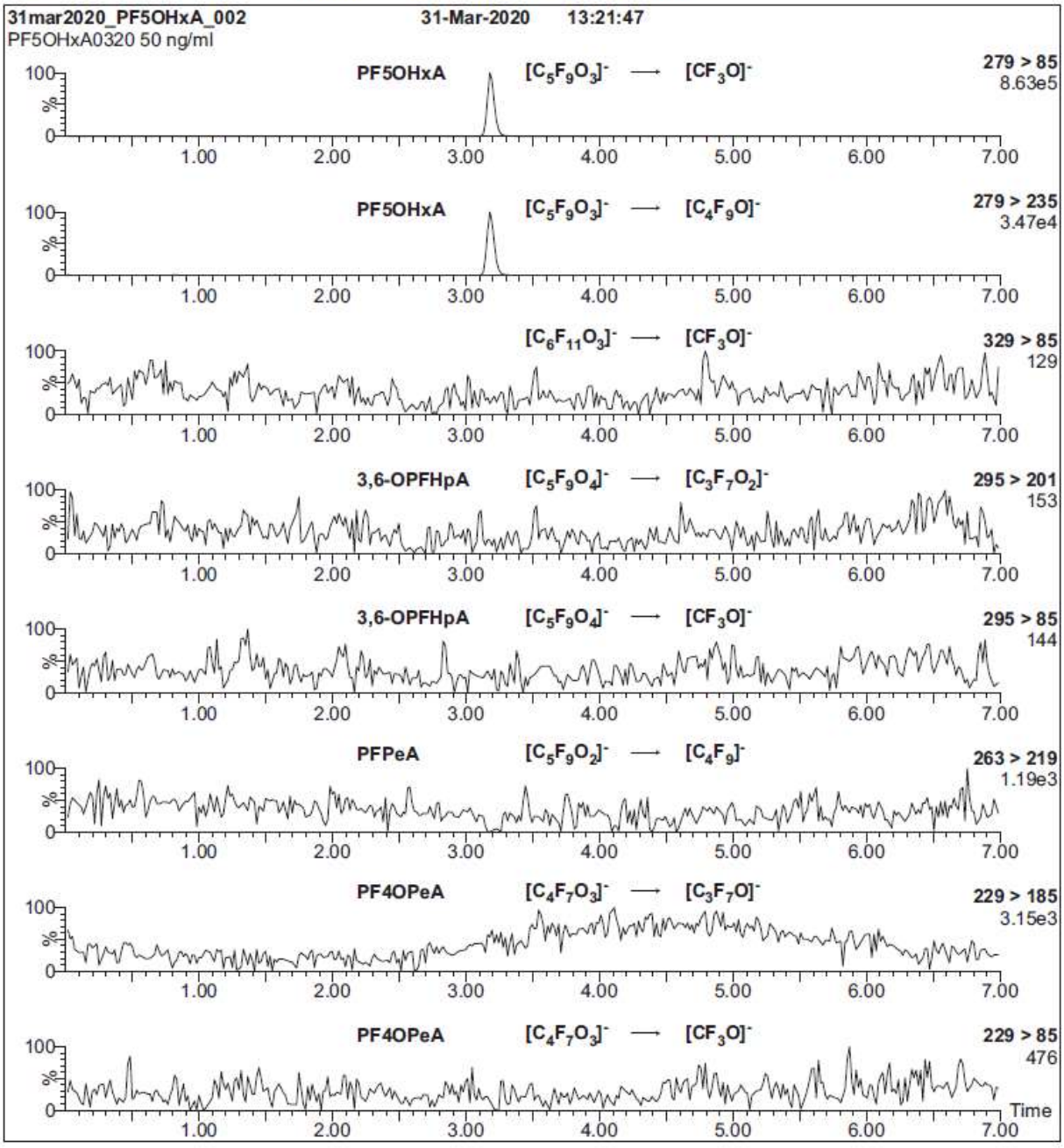


Figure 2: PF5OHxA; LC/MS/MS Data (Selected MRM Transitions)



Conditions for Figure 1:

LC: **Waters Acquity Ultra Performance LC**
MS: **Waters Xevo TQ-S micro MS**

Chromatographic Conditions

Column: Acquity UPLC BEH Shield RP_{18}
1.7 µm, 2.1 x 100 mm

Mobile phase: Gradient
Start: 40% (80:20 MeOH:ACN) / 60% H_2O
(both with 10 mM NH_4OAc buffer)
Ramp to 90% organic over 8 min and hold for 2 min
before returning to initial conditions in 0.75 min.
Time: 12 min

Flow: 300 µl/min

MS Parameters

Experiment: Full Scan (150 - 850 amu)

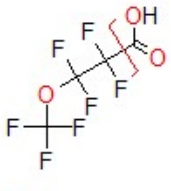
Source: Electrospray (negative)
Capillary Voltage (kV) = 0.50
Cone Voltage (V) = 14.50
Desolvation Temperature (°C) = 500
Desolvation Gas Flow (l/hr) = 1000

The information from the Wellington Certificates of Analysis indicate that it is possible to get sufficient response for two MRMs each for PFMOPrA and PFMOBA. The following procedures may allow for increased signal for PFMOPrA and PFMOBA: ensuring complete solubilization of the analyte in solution and optimizing analyte specific mass spectrometer settings (including collision energy and use of correct masses of parent and product ion to the nearest tenth of a decimal place based on current mass spectrometer calibration – ex. 279.0 vs 278.9 can make a difference in signal intensity).

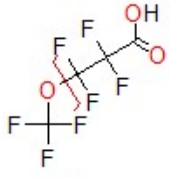
If the above recommendations do not provide adequate signal for PFMOPrA and PFMOBA, then global mass spectrometer settings (ex. ESI source conditions) may need to be optimized for the linear isomers. If source parameters are different enough between the isomers such that ionization of one or the other isomers is significantly inhibited, then two analytical methods may be needed for the purposes of this investigation (one method with source conditions optimized for linear isomers and the second method with source conditions optimized for branched isomers)—the same HPLC conditions would be used for both methods.

MRM selection: Multiple MRM transitions can be monitored for each analyte once a signal of approximately 1 x 10^6 is achieved for the predominant MRM. This level of instrument signal for the predominant MRM should allow for increased signal and detection of analytes using additional MRM transitions. Expected fragmentation patterns for each analyte is shown below:

PFMOPrA
[M-H]-: 228.9

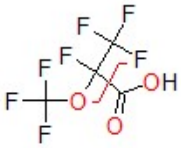


C_3F_7O
184.9

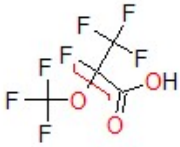


CF_3O
84.9

PMPA
[M-H]-: 228.9

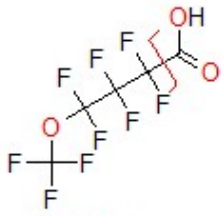


C_3F_7O
184.9

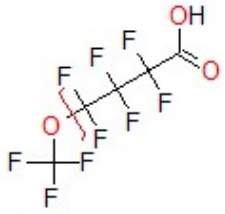


CF_3O
84.9

PFMOBA
[M-H]-: 278.9

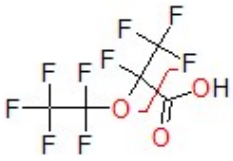


C_4F_9O
234.9

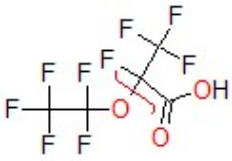


CF_3O
84.9

PEPA
[M-H]-: 278.9



C_4F_9O
234.9



C_2F_5O
134.9

Specifically, the following MRMs can be monitored for each analyte:

- PMPA: 229→185 and 229→85
- PFMOPrA: 229→185 and 229→85
- PEPA: 279→235, 279→85, and 279→135
- PFMOBA: 279→235, 279→85, and 279→135

PMPA and PFMoPrA have the same two MRM transitions. Therefore, chromatographic separation of these two isomers is necessary for unambiguous identification. PEPA and PFMOBA have unique MRM transitions that should provide spectral data for isomer identification even when the compounds coelute. The unique transitions are 279→135 for PEPA and 279→85 for PFMOBA. The MRM transition of 279→235 is common to both isomers. When both isomers are present in solution, and particularly when they coelute, both isomers will contribute to this signal. Chromatographic separation of PEPA and PFMOBA, if possible, would also provide more definitive isomer identification.

Chromatographic separation: Retention times of isomers run by the Table 3 and Table 6 method in the report are as follows:

PMPA: 6.3 min (Table 3), 6.5 min (Table 6)

PFMOPrA: 6.8 min (Table 3), 6.3 min (Table 6)

PEPA: 8 min (Table 3), 7.13 min (Table 6)

PFMOBA: 8 min (Table 3), 7.14 min (Table 6)

Once sufficient signal is obtained for each analyte in multiple MRM transitions, chromatographic separation of the isomer pairs should be optimized, with baseline resolution of the isomers preferred. Separation of PMPA and PFMoPrA is the most important because both share the same two MRM transitions and therefore are not able to be distinguished spectrally. The Table 3 and Table 6 methods do not provide baseline resolution between these isomers. Identification of this isomer pair cannot be made using retention times alone because the isomers are not well enough resolved and retention times can shift, particularly in different sample matrices. Three chromatographic methods that may provide better separation (from Strynar et al 2015, EPA Method 533, and Song et al 2018) are shown

below along with representative chromatograms. The Strynar et al 2015 and Song et al 2018 HPLC conditions in particular show chromatographic separation for both isomer pairs.

Strynar et al 2015 – the second chromatogram shows likely PFMOPrA and PMPA separation

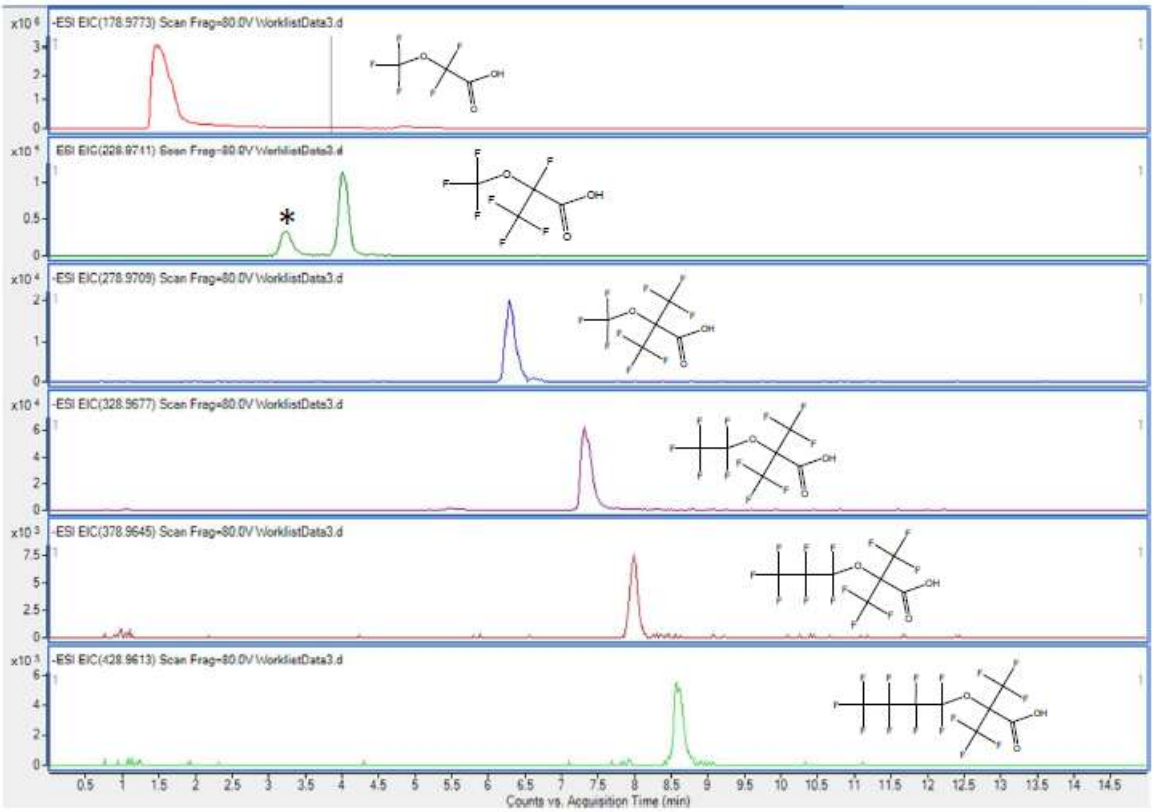
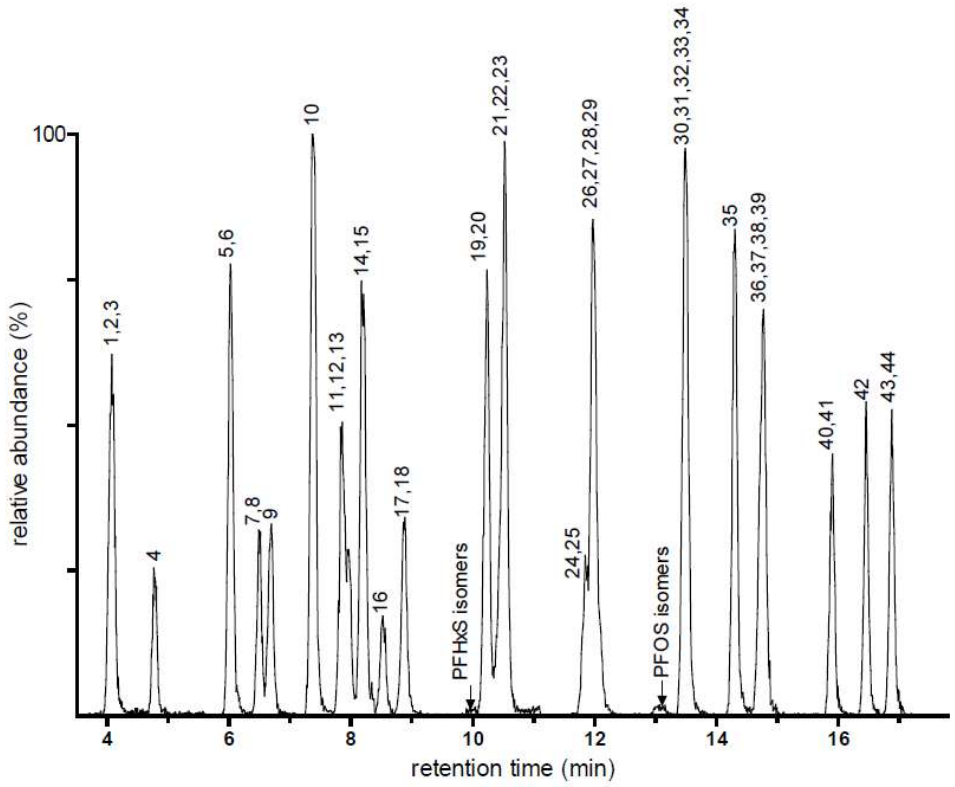


Figure S5. Extracted Ion Chromatogram (EIC) of a suspected homologous series of perfluorinated ether carboxylic acids. Note: The chromatographic peak indicated by an * is the substance associated with this homologous series based on the H^+ and Na^+ dimer co-elution at this retention time. The second later eluting peak in this chromatogram is a similar m/z, possibly an isomer.

Chromatographic separation was accomplished using an Eclipse Plus C8 column (2.1 × 50 mm, 3.5 μm; Agilent). The method consisted of the following conditions: 0.2 mL/min flow rate; column at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water/methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol/DI water (95:5 v/v); gradient: 0−15 min a linear gradient from 75:25 A/B to 15:85 A/B; with a 4 min post time for equilibration.

EPA Method 533 – Peaks 4 and 9 are PFMOPrA and PFMOBA

Figure 1. Example Chromatogram for Reagent Water Fortified with Method Analytes at 80 ng/L[a]



a. Numbered peaks are identified in **Table 3**, **Table 4**, and **Table 5**.

Table 1. HPLC Method Conditions[a]

Time (min)	% 20 mM ammonium acetate	% Methanol
Initial	95.0	5.0
0.5	95.0	5.0
3.0	60.0	40.0
16.0	20.0	80.0
18.0	20.0	80.0
20.0	5.0	95.0
22.0	5.0	95.0
25.0	95.0	5.0
35.0	95.0	5.0

a. Phenomenex Gemini® C18, 2 x 50 mm, 3.0 µm silica with TMS end-capping. Flow rate of 0.25 mL/min; run time 35 minutes; 10 µL injection into a 50 µL loop. The chromatogram in **Figure 1** was obtained under these conditions.

Table 5. Method Analytes, Retention Times and Suggested Isotope Dilution Analogue References

Analyte	Peak # (Figure 1)	RT (min)	Isotope Dilution Analogue
PFBA	3	4.15	$^{13}C_4$-PFBA
PFMPA	4	4.84	$^{13}C_4$-PFBA
PFPeA	6	6.13	$^{13}C_5$-PFPeA
PFBS	8	6.62	$^{13}C_3$-PFBS
PFMBA	9	6.81	$^{13}C_5$-PFPeA
PFEESA	10	7.53	$^{13}C_3$-PFBS
NFDHA	11	8.01	$^{13}C_5$-PFHxA
4:2FTS	13	8.12	$^{13}C_2$-4:2FTS
PFHxA	15	8.36	$^{13}C_5$-PFHxA
PFPeS	16	8.69	$^{13}C_3$-PFHxS
HFPO-DA	18	9.06	$^{13}C_3$-HFPO-DA
PFHpA	20	10.42	$^{13}C_4$-PFHpA
PFHxS	22	10.62	$^{13}C_3$-PFHxS
ADONA	23	10.73	$^{13}C_4$-PFHpA
6:2FTS	25	12.04	$^{13}C_2$-6:2FTS
PFOA	28	12.19	$^{13}C_8$-PFOA
PFHpS	29	12.28	$^{13}C_8$-PFOS
PFNA	31	13.70	$^{13}C_9$-PFNA
PFOS	34	13.74	$^{13}C_8$-PFOS
9Cl-PF3ONS	35	14.53	$^{13}C_8$-PFOS
8:2 FTS	37	14.94	$^{13}C_2$-8:2FTS
PFDA	39	15.00	$^{13}C_6$-PFDA
PFUnA	41	16.14	$^{13}C_7$-PFUnA
11Cl-PF3OUdS	42	16.70	$^{13}C_8$-PFOS
PFDoA	44	17.13	$^{13}C_2$-PFDoA

Song et al 2018 – Second and third chromatograms in the surface water portion of figure below show separation of PFMOPrA/PMPA and PFMOBA/PEPA using an Acquity HSS PFP HPLC Column

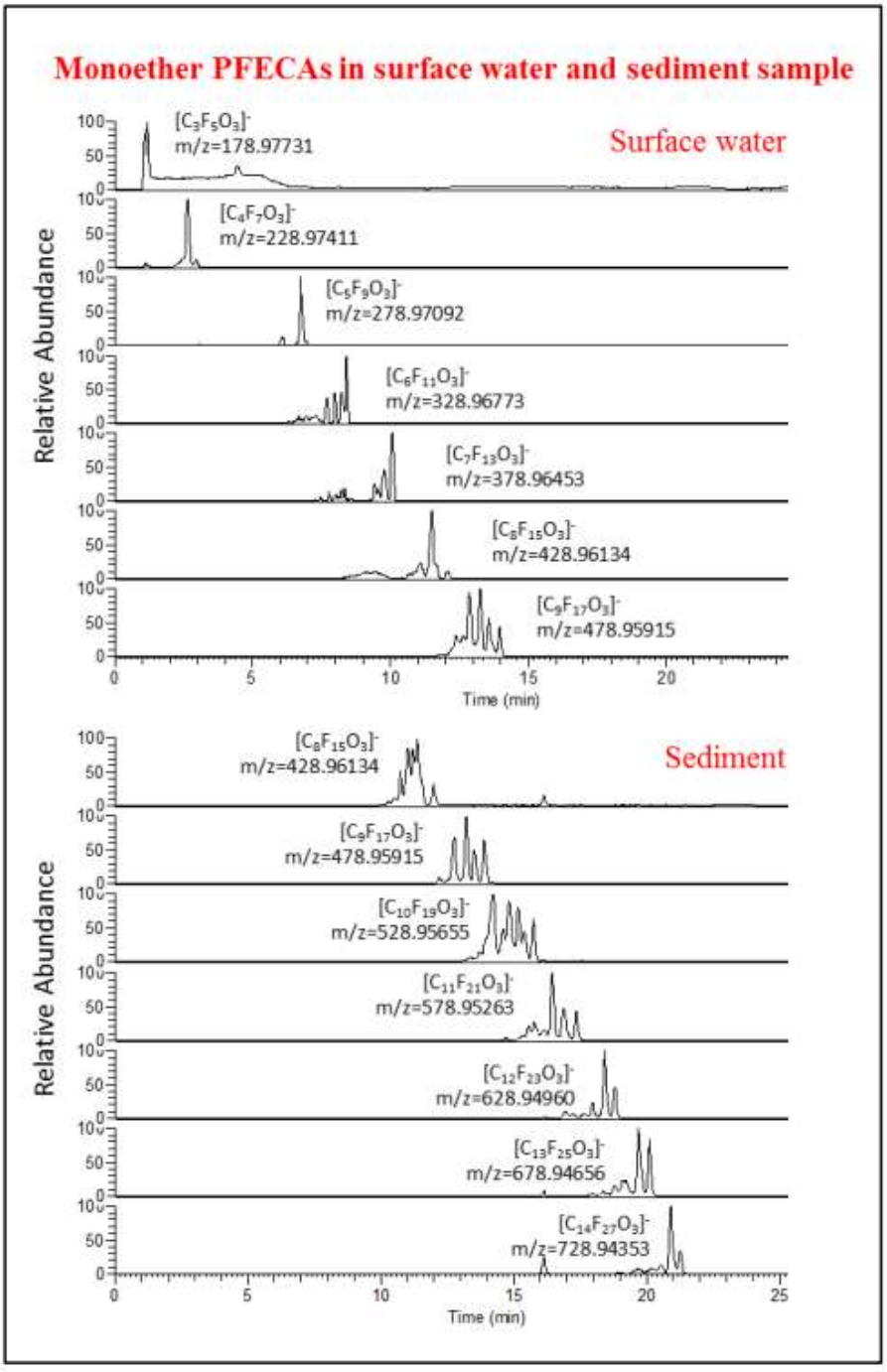


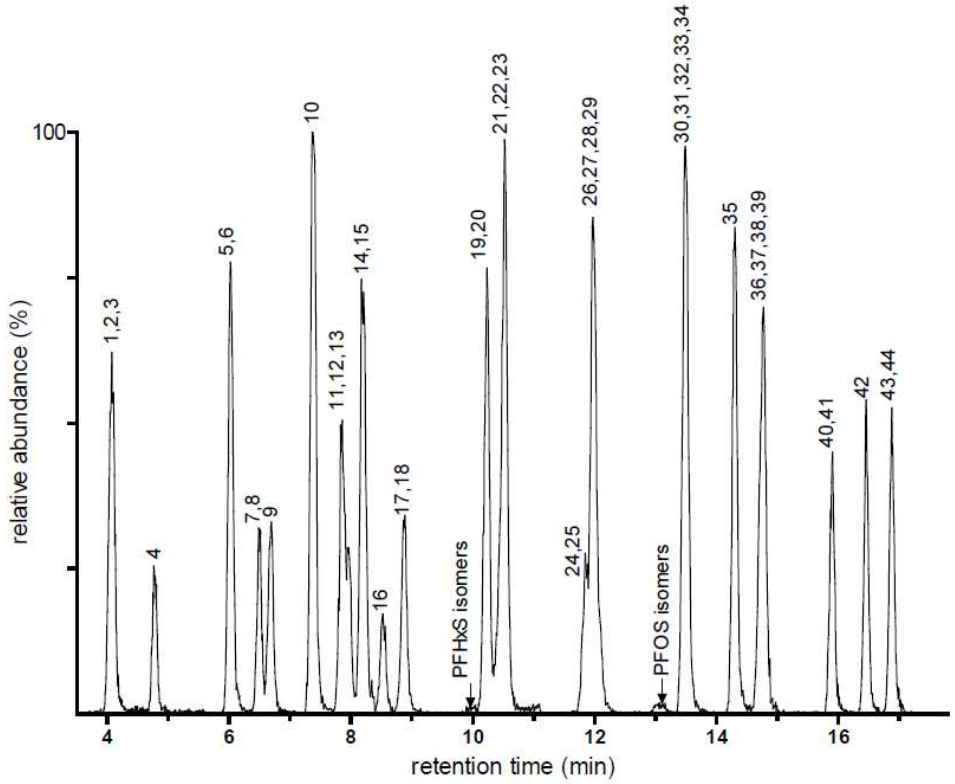
Figure S19. Extracted ion chromatogram (PFP Column) of monoether PFECAs in surface water and sediment extract with mass windows of theoretical mass ±5 ppm.

Suspect Screening Analysis. The identification of suspect PFASs was performed using the HPLC-Orbitrap MS (Thermo Fisher Scientific Inc., Waltham, MA), which was operated using negative electrospray ionization (ESI^-). Data were acquired in the full scan mode (60−1000 m/z) with a resolution of 120 000. The chromatographic separation of PFASs was achieved using both a high-resolution ACQUITY HSS PFP Column (1.8 μm, 100 Å, 50 mm × 2.1 mm, Waters Co.) and an Acclaim 120 C18 column (5 μm, 4.6 mm × 150 mm, Thermo Fisher Scientific Co.) in suspect screening. Additional details regarding the instrumentation for ESI-MS/MS and Orbitrap MS are provided in the SI.

The above chromatographic methods are examples that may be used. Any chromatographic method that provides baseline resolution of PMPA and PFMOPrA would be sufficient. Resolution of PEPA and PFMOBA is desired, however this isomer pair may be more difficult to resolve chromatographically and has unique MRMs that can be used for identification.

Surrogates: After MRM signal and chromatographic separation have been optimized and PMPA, PEPA, PFMOPrA, and PFMOBA have each been run with the optimized method, a surrogate can be introduced into the method. The surrogate serves as a reference point to monitor any retention time shifts that occur in samples. Good surrogate candidates are perfluoropentanoic acid (PFPeA) or labeled perfluoropentanoic acid ($^{13}C_5$- PFPeA) because these compounds elute at a retention time in between that of the isomer pairs (see figure below- PFMOPrA, PFPeA, $^{13}C_5$- PFPeA, and PFMOBA are analytes 4,5,6, and 9 in the chromatogram, respectively).

Figure 1. Example Chromatogram for Reagent Water Fortified with Method Analytes at 80 ng/L[a]



[a] Numbered peaks are identified in **Table 3**, **Table 4**, and **Table 5**.

Surrogate should be added to each individual analyte standard solution and analyzed by LC/MS/MS to establish a relative retention time for each analyte. Environmental samples analyzed should have surrogate added prior to analysis to provide additional confidence in the identification of isomers using retention time information. Surrogates should be optimized in the mass spectrometer to determine two suitable MRMs transitions (PFPeA has a transition of 263→219 and $^{13}C_5$- PFPeA has a transition of 268→223 in EPA Method 533).

Environmental Samples: Environmental samples should be freshly collected, preserved, and stored refrigerated within the holding time of the method rather than from archived samples. Analysis for all analytes should occur at the same time using the following MRMs for each analyte:

- PMPA: 229→185 and 229→85
- PFMOPrA: 229→185 and 229→85
- PEPA: 279→235, 279→85, and 279→135
- PFMOBA: 279→235, 279→85, and 279→135

The chromatographic method should achieve as much separation as possible between PMPA and PFMOPrA. A surrogate should be added to samples in order to better track shifts in retention times that may occur in environmental samples analyzed by LC/MS/MS.

Report figures: This section contains specific comments about figures contained in the report.

- Figure 3 has no explanation regarding the hump on the back side of the peak in the 229→185 transition. The 229→85 may have more signal with a more concentrated standard.
- Figure 4 PFMOPrA signal for both MRMs likely will be better with mass spectrometer optimized conditions
- Figure 5 has no explanation of peak at 7.336 minutes. More signal from a more concentrated standard would allow for observations in MRM 229→85 as well
- Figure 6 signal likely will be better with optimized mass spectrometer conditions
- Figure 7 zooming to a narrower scale on the y axis for the 279→85 transition would allow the viewer to see whether there is any signal in the 10^2 to 10^3 range. The transition 279→135 should also be monitored
- Figure 8 expected retention time is at 8 minutes. Please expand the chromatogram in this area to see any 10^2 to 10^3 intensity peaks present there. Signal is present at 8 minutes for the 279→85. Please adjust the range of the y-axis to better show this response. The transition 279→135 should also be monitored
- Figure 9 PEPA data should be collected at the MRMs 279→235, 279→85, and 279→135 in a higher concentration standard (to obtain a signal of 1 x 10^6 response for the MRM shown)
- Figure 10 PFMOBA data should be collected at the MRMs 279→235, 279→85, and 279→135 with optimized mass spectrometer conditions and a more concentrated standard solution
- Figure 11 Demonstrates good signal for PMPA in 229→185 transition. Should also monitor 229→85 transition. Chromatographic resolution of isomers needed.
- Figure 12 Figure 11 Demonstrates good signal for PMPA in 279→235 transition. Should also monitor 279→85 and 279→135 transitions. Chromatographic resolution of isomers needed.

- Each analyte should be monitored using the Table 3 and Table 6 methods, even when weak signal was observed previously because this is work is investigative in nature and the analytes may show up unexpectedly.

Comments on Report Conclusions:

- **Conclusion stated in Isomer Report**: Additionally, both the lack of peak broadening or shoulder peaks for PMPA and PEPA and lack of spectral response characteristic of PFMOPrA and PFMOBA under Table 6 conditions indicate that Chemours's PMPA and PEPA standards consist primarily or entirely of the branched isomer.
    - **Reviewer Responses**:

- Figure 3 does show a shoulder on the peak for PMPA in the 229→185 transition.

- Figure 5 additionally shows a peak at 7.336 minutes in the 229→185 transition for PMPA.

- There is not enough signal in the MRM transitions for either PFMOPrA or PFMOBA to assess spectral response. Method optimization (mass spectrometer conditions) for these compounds would likely allow for increased signal and collection of data for these compounds, from which then conclusions can be drawn. Two different mass spectrometer methods may need to be run, one each with MS conditions optimized for linear and branched isomers.

- The branched isomer is likely to be the predominant form of the isomers; however more data needs to be collected, especially regarding the linear isomers to support this statement and the purity assessment.

- **Conclusion stated in Isomer Report**: Both the Table 3 and Table 6 Methods are likely to be able to resolve the PMPA/PFMoPrA and PEPA/PFMOBA isomer pairs when chromatographic and spectral data are considered together.

- **Reviewer Responses**:

    - PEPA/PFMOBA cannot be resolved with the chromatographic and spectral data collected in this report. The compounds are not chromatographically resolved using either the Table 3 or Table 6 method. The MRM transitions that could identify the compounds are either not monitored (279→135 for PEPA) or appear to not be optimized (279→85, PFMOBA). The MRM transition (279→235) is used but is common to both PEPA and PFMOBA and cannot be used to distinguish between the two isomers.

    - PMPA/PFMoPrA cannot be resolved with the chromatographic and spectral data collected in this report. These isomers share the same two MRM transitions (229→185 and 229→85) and cannot be distinguished spectrally when the peaks coelute. Section 3.2 of the report further states the following: The peak width for PMPA in both methods is wide, and the peak for PFMOPrA overlaps, indicating that PMPA and PFMOPrA will not likely be fully chromatographically resolved in both are present in a sample.

- **Conclusion stated in Isomer Report**: Review of a Site sample containing PMPA and PEPA and analyzed by the Table 6 method shows PFMOPrA and PFMOBA are unlikely to be present, as indicated by the lack of peak broadening or peak shoulders.

- **Reviewer Responses**:

    - PFMOBA elutes at the same retention time PEPA and would not likely be present as a peak shoulder, but within the center of the PEPA peak.

    - The MRM transition monitored (279→235) is common to both PFMOBA and PEPA and would therefore not distinguish between the two isomers. Additionally, the signal for PFMOBA in this MRM does not appear to be optimized. Additional MRMs unique to PEPA (279→135) and PFMOBA (279→85) under optimized MS conditions would provide data for isomer identification. Two analytical methods may have to be run – one each containing optimized MS parameters for linear and branched isomers.

    - PFMOPrA and PMPA may not be chromatographically resolved in the Table 6 method and have the same MRM transitions (229→85 and 229→185). Analysis of a sample with baseline resolution of the isomer pair would provide more definitive results.

Proposed Path Forward: Repeat the experiments as outlined below (additional information about each aspect is covered in a previous section of this document):

- Step 1: Optimize the method

    - Prepare four individual analyte standard solutions, one each for PFMOPrA, PMPA, PFMOBA, and PEPA
    - Ensure that PFMOPrA and PFMOBA standards are completely solubilized in the standard solution
    - Ensure analyte specific mass spectrometer settings are optimized for PFMOPrA and PFMOBA.
    - Optimize global mass spectrometer settings for PFMOPrA and PFMOBA (if these parameters are significantly different between linear and branched isomers may need one method each for linear and branched isomers-one each with optimized ESI source conditions)
    - Run each analyte standard by LC/MS/MS using full scan mode over the range 50 to 300 m/z in order to confirm correct selection of MRM transitions for each analyte and provide the full scan chromatograms

- Optimize MRM signal for predominant transition so that the response is approximately 1 x 10^6 using an existing chromatography method. Once this is achieved, add in additional MRMs for each analyte
- Use a different chromatographic method to baseline resolve PFMOPrA and PMPA. Ideally PFMOBA and PEPA would have better separation than in the Table 3 and Table 6 methods, but separation is less important for these isomers.
- Run each of the four individual analyte standard solutions using additional MRMs, optimized mass spectrometer settings for the linear isomers, and a chromatography method that baseline resolves PFMOPrA and PMPA
- Perform LC/MS/MS analysis of two solutions, one containing PFMOPrA and PMPA and the other containing PFMOBA and PEPA to show resolution of isomer pairs together in a solution

- Step 2: Add in surrogates
    - Prepare a standard solution of surrogate standard
    - Optimize two MRMs for the surrogate
    - Add surrogate to an aliquot of each standard solution (one each PFMOPrA, PMPA, PFMOBA, and PEPA)
    - Run each of the four individual analyte standard solutions containing surrogate using additional MRMs, optimized mass spectrometer settings for the linear isomers and surrogate, and a chromatography method that baseline resolves PFMOPrA and PMPA
    - Establish relative retention times for each analyte to the surrogate

- Step 3: Analyze Environmental/Site Samples
    - Collect samples fresh, add appropriate preservative, store refrigerated
    - Add surrogate to sample
    - Analyze within refrigerated holding time for sample
    - Perform analysis for all compounds at once using the optimized LC/MS/MS method containing additional MRMs and chromatography method capable of baseline resolving PFMOPrA and PMPA
    - Any quantitation of linear isomers should be done with linear isomer calibration standards
    - Any quantitation of branched isomers should be done with branched calibration standards