Internal Standard and Deuterated Solvent Selection: A Crucial Step in PFAS-Based Fluorine-19 (^{19}F) NMR Research

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PURPOSE: This work is vital because it provides researchers with a framework and rationale for selecting the best internal standard and deuterated solvent for their nuclear magnetic resonance (NMR) analysis of per- and polyfluoroalkyl substances (PFAS)-based compounds. Selecting the best internal standard and deuterated solvent will help to ensure that their results are accurate, precise, and sensitive. The internal standard that is chosen can significantly affect the accuracy, precision, sensitivity, and quantification of NMR measurements. Therefore, it is essential to carefully select an internal standard and a matching deuterated solvent that are well-suited for analyzing PFAS compounds.

BACKGROUND: Internal standards play a crucial role in NMR analysis, providing a reference point for quantification and ensuring the accuracy and reproducibility of measurements (Rundlöf et al. 2010). In NMR spectroscopy, internal standards are substances that are added to the sample being analyzed, and they serve as a reference signal against which other signals can be compared (Peterson 1992). By using an internal standard, variations in sample concentration, temperature, and instrument settings can be corrected, allowing for reliable and precise NMR analysis (Westwood et al. 2019).

The selection of an appropriate internal standard is essential for perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) studies because these compounds are persistent organic pollutants that have been widely studied due to their adverse effects on human health and the environment. The presence of an internal standard is particularly crucial when quantifying these compounds in complex matrices, such as environmental samples or biological tissues.

When choosing an internal standard for PFOS and PFOA NMR studies, several factors should be considered. First and foremost, the internal standard should exhibit minimal chemical shift overlap with the analyte signals of interest. This ensures accurate peak integration and avoids signal interference that could lead to erroneous quantification. The internal standard should also have good solubility in the chosen NMR solvent to facilitate sample preparation and analysis.

Selecting an appropriate deuterated solvent for PFAS-based NMR investigations is equally vital. Solvents can affect the NMR peak shape, resolution, and position. In addition, the selection of an aqueous miscible deuterated solvent is more applicable when dealing with environment-related samples.

Unlike other spectroscopic techniques, fluorine-19 (^{19}F) NMR spectroscopy is immune to matrix effects, making it suitable for in vivo applications and quantitative analysis in complex sample

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matrices (Yu et al. 2013). The advantage of ^{19}F NMR is that it eliminates the need for extensive sample cleanup, thus minimizing the risk of analyte loss during solid-phase extraction. Moreover, ^{19}F NMR enables quantitative measurements through signal integration, even in the absence of a reference standard. The signal strength in ^{19}F NMR is directly proportional to the number of equivalent ^{19}F atoms, resulting in stronger signals with increased atom count. Therefore, it's highly desirable to use ^{19}F NMR as an analytical tool for identifying novel PFAS compounds and quantifying existing PFAS compounds (Camdzic et al. 2021). Therefore, this work investigated several solvent effects, the selection of viable internal reference standards, and the effect of recycle delay time (D1) for quantification of PFOA in a given sample. PFOA was selected because it has a higher solubility in aqueous media than PFOS.

MATERIALS: The following reagents were purchased from Sigma Aldrich and were used as received:

- deuterium oxide (D_2O) 99.9 atom % D (Chemical Abstracts Service [CAS] 7789-20-0)
- chloroform-d ($CDCl_3$) 99.8 atom % D, contains 0.03 % (v/v) tetramethylsilane (TMS; CAS 865-49-6)
- dimethyl sulfoxide-d_6 (DMSO-d_6) 99.9 atom % D, contains 0.03 % (v/v) TMS (CAS 2206-27-1)
- acetone-d_6 99.9 atom % D, contains 0.03 % (v/v) TMS (CAS 666-52-4)
- methanol-OD (MeOD) 99.5 atom % D (CAS 1455-13-6)
- acetonitrile-d_3 $\geq$99.8 atom % D (CAS 2206-26-0)
- ammonium hexafluorophosphate (NH_4PF_6) $\geq$95% (CAS 16941-11-0)
- ammonium fluoride (NH_4F) American Chemical Society (ACS) reagent, $\geq$98.0% (CAS 12125-01-8)
- tetrabutylammonium fluoride hydrate (TBAF) 98% (CAS 22206-57-1)
- hexafluorobenzene (HFB) $\geq$99.5%, NMR grade (CAS 392-56-3)

GENERAL METHODOLOGY: All ^{19}F NMR were performed on a 300 MHz[*] Bruker NMR spectrometer, and 5 mm inner diameter Wilmad-LabGlass thin-walled high throughput NMR tubes were used for the NMR experiments. The ^{19}F nuclei were tuned using an automated tuning program. The spectral width was set to be -200 to -30 ppm. All experiments were conducted at a 3 sec D1, thus increasing sample acquisition time. The number of scans was set to 100 to increase peak resolution. All ^{19}F NMR spectra were processed (i.e., phase correction and baseline correction were conducted) using MestreNova software (Santiago de Compostela, Spain). Several concentrations of PFOA (i.e., 1, 5, and 10 mM) were used for this work. The higher concentration (10 mM) was selected over the other concentrations for all studies. The total volume of the NMR tube was set between 600 and 700 µl. All PFOA solutions were prepared using the PFOA standard dissolved in Milli-Q water to obtain the desired concentration. Internal standards (i.e., 0.043 mmol) were introduced by first mixing them in a gas chromatography vial with the solvent due to the low volumes of the standards being used. All samples were vortexed for 10–15 sec before being

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[*] For a full list of the spelled-out forms of the units of measure used in this document and their conversions, please refer to *US Government Publishing Office Style Manual*, 31st ed. (Washington, DC: US Government Publishing Office, 2016), 248–252, https://www.govinfo.gov/content/pkg/GPO-STYLEMANUAL-2016/pdf/GPO-STYLEMANUAL-2016.pdf.

analyzed by NMR. Volumes were changed as needed for different internal standards to optimize the peak height of the ^{19}F NMR spectra. The numerical values of volumes and concentration are discussed in the "Results and Discussion" section.

RESULTS AND DISCUSSION

Effect of Deuterated Solvent in Relation to Fluorine-19 (^{19}F) Nuclear Magnetic Resonance (NMR) Spectra of Perfluorooctanoic Acid (PFOA): We investigated the role of deuterated solvent choice in preparing samples containing aqueous-based PFAS compounds. The deuterated solvents that follow were used: D_2O, $CDCl_3$, DMSO-d_6, acetone-d_6, MeOD, and acetonitrile-d_3. These are widely used in NMR spectroscopy. For this study, the highest concentration (i.e., 10 mM) of PFOA was used. Figure 1 shows the changes that were observed with each change of solvent. All samples were prepared by adding 300 µl of aqueous PFOA solution into 300 µl of deuterated solvent. Each solvent was locked as per solvent choice. Solvent locking is performed to ensure that the strength of the magnetic field surrounding the sample does not change or drift during an experiment or that the field is not modulated or perturbed by external disturbances (McClure 1999).
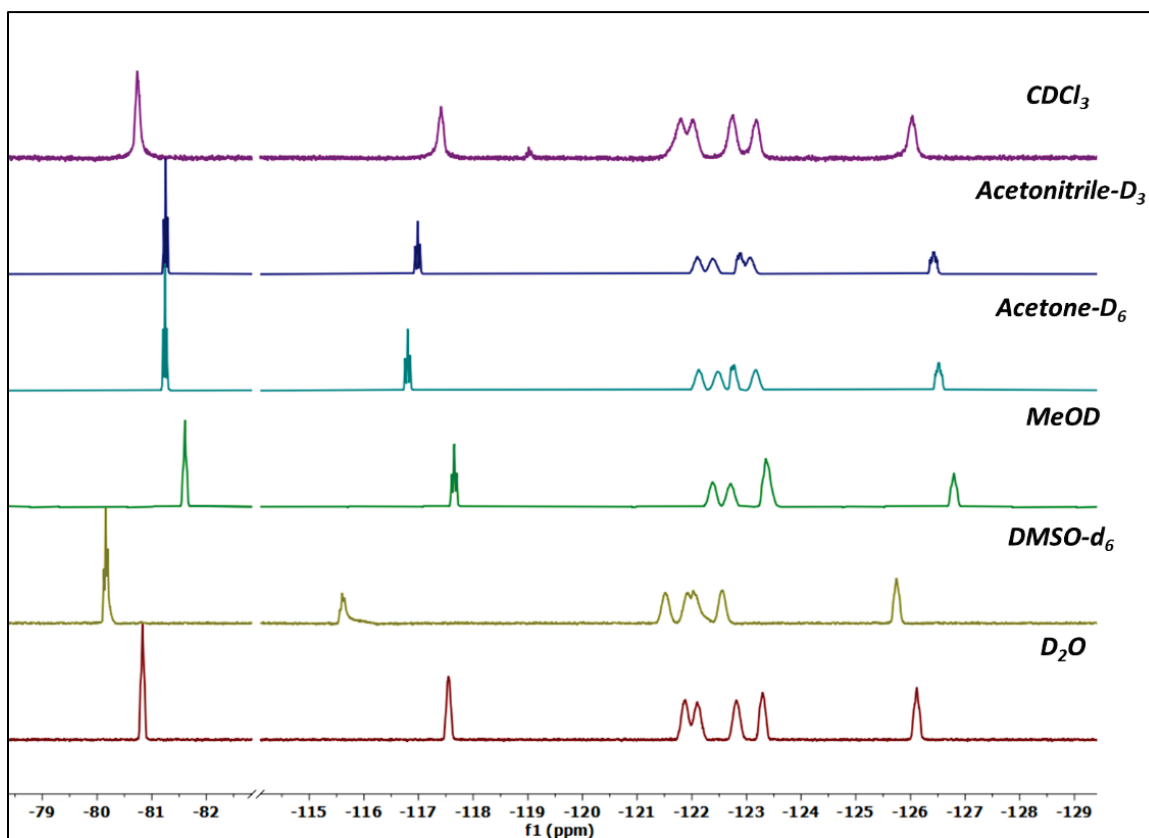

Figure 1. The fluorine-19 (^{19}F) nuclear magnetic resonance (NMR) stacked spectra for various deuterated solvents mixed with constant amounts of perfluorooctanoic acid (PFOA).

As shown in Figure 1, the solvent is crucial to obtaining a more defined peak splitting for the relevant PFOA sample. Deuterated acetone and acetonitrile showed the best peak splitting and most defined peak shape. Solvents such as $CDCl_3$ were not ideal because of their large spectra

broadening and immiscibility with aqueous solutions. In previous literature, PFAS-based NMR was mostly acquired using MeOD or D_2O as the preferred deuterated solvent system (Heerah et al. 2020); however, this work showed that it was not as ideal as acetone-d_6 or acetonitrile-d_3 regarding peak splitting and shape definition. This can be further visualized in Figure 2, where these identical spectra were reported in a superimposed image. In this figure, various sections of the PFOA molecule were zoomed in on so the difference in the spectra observed with the change in the deuterated solvent could be clearly visualized.
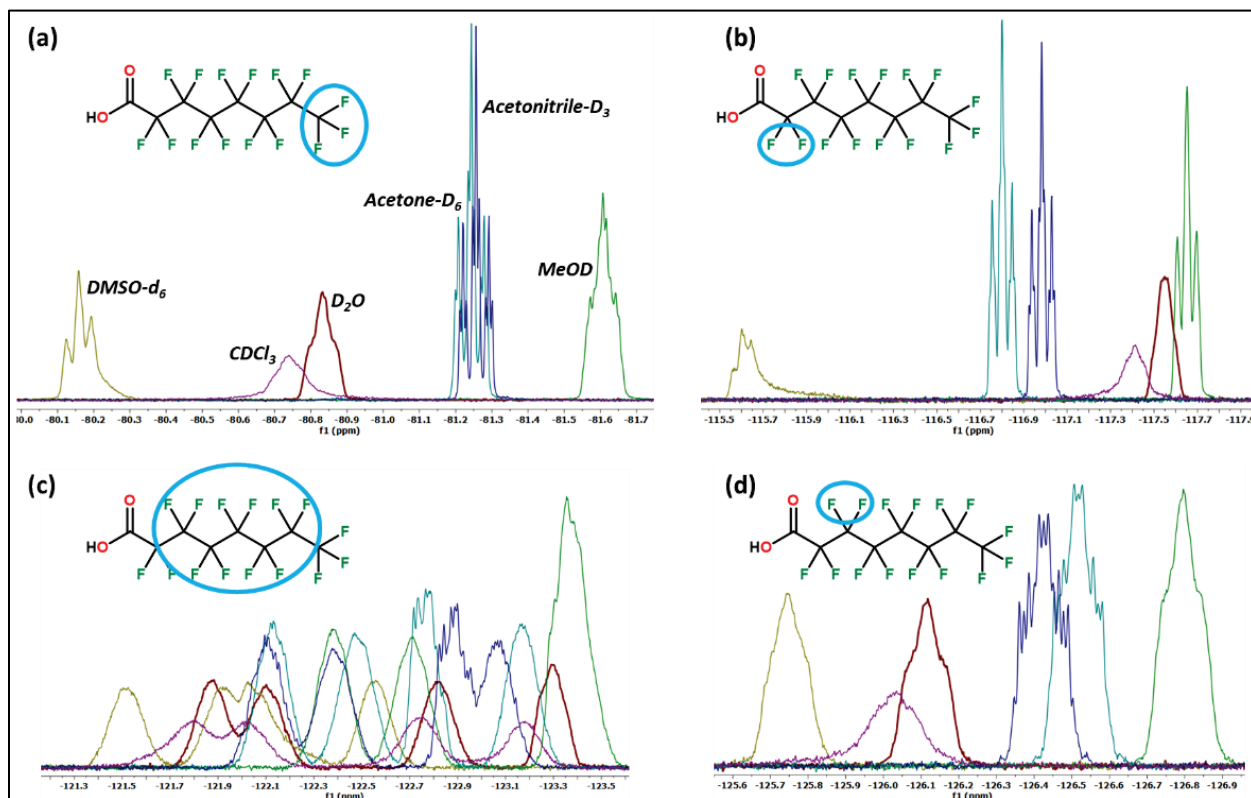
Figure 2. The ^{19}F NMR of various solvents, including (*a*) terminal CF_3, (*b*) CF_2 adjacent to the carboxylic acid group, (*c*) the bulk of the middle CF_2 groups, and (*d*) the CF_2 group, which is one carbon away from the carboxylic acid group (each area is circled in *blue*).

The solvent study allowed us to identify the ideal deuterated solvent to use for PFOA-based ^{19}F NMR experiments. Based on the results shown in Figure 2, acetone and acetonitrile were selected for the next set of experiments.

Effect of Various Internal Standards: For this work, we investigated five different internal standards (Figure 3). Each internal standard is discussed in a separate subsection.
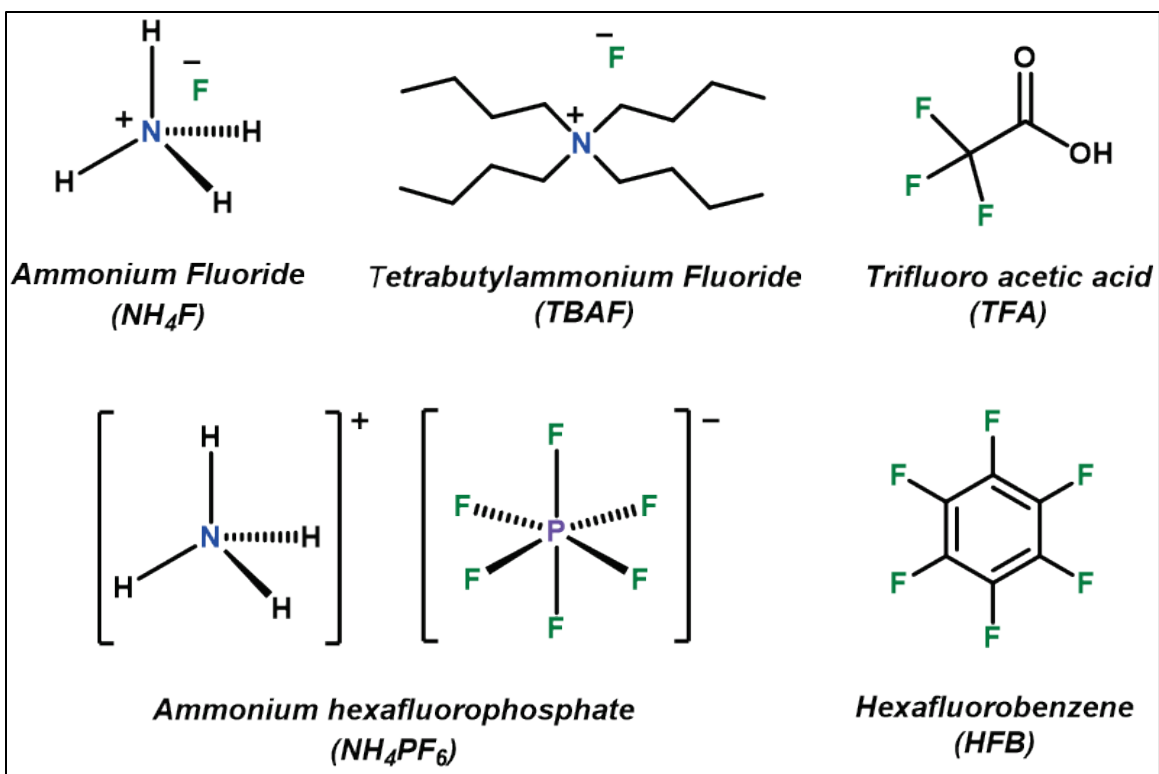
Figure 3. Chemical structures of the internal standards used in this work.

Ammonium Fluoride (NH₄F): Figure 4 shows that the NH₄F ^{19}F peak corresponds to the peak that can be observed at 117.65 ppm. The addition of the internal standard did not affect the overall peak shape of the terminal CF_3 group or the other CF_2 groups present in PFOA. Both solvents gave similar integration values with respect to the NH₄F peak. Also, NH₄F is readily soluble in aqueous-based media, making this internal standard a desirable candidate for PFAS-based aqueous NMR experiments.
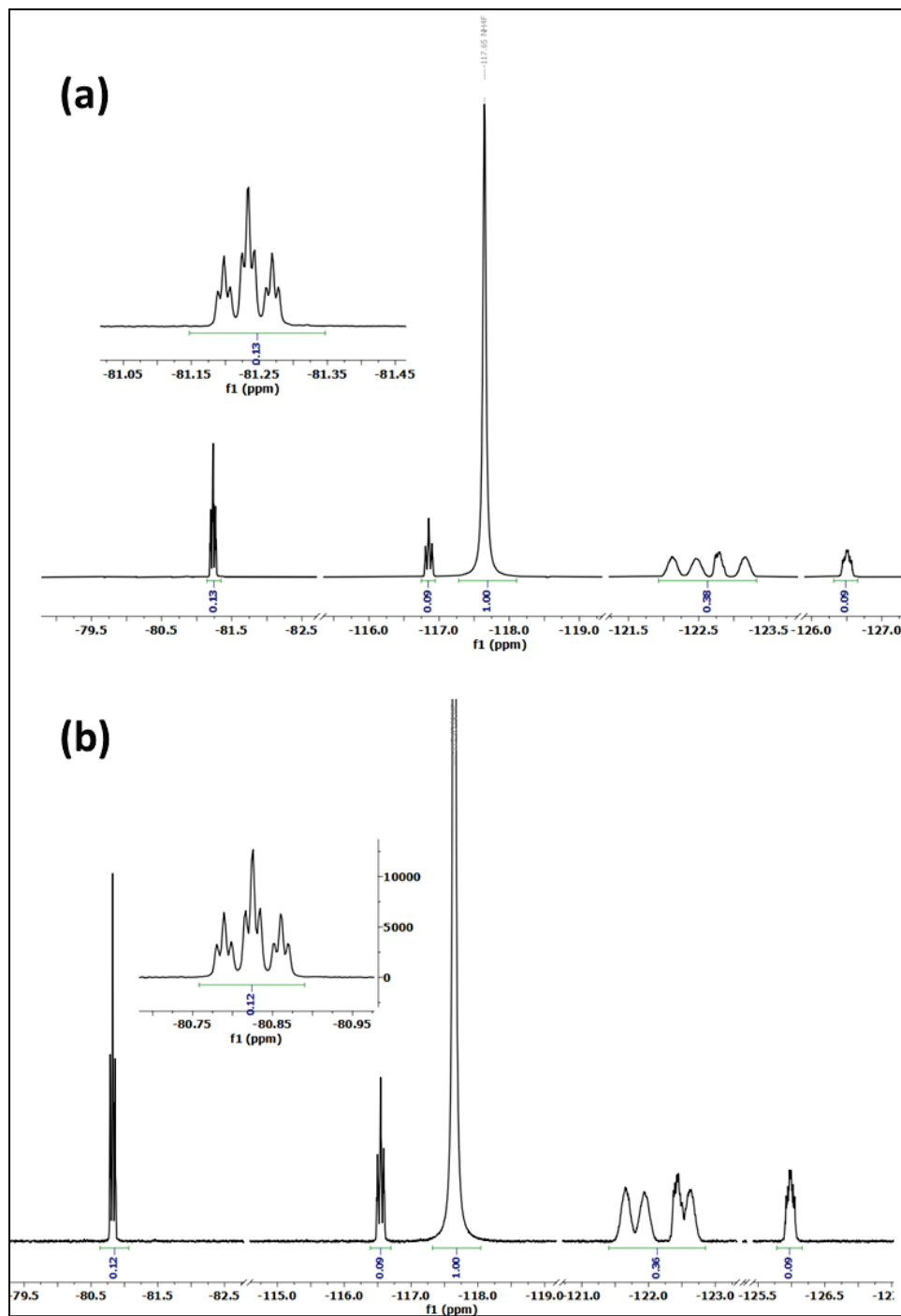
Figure 4. The ^{19}F NMR of PFOA with ammonium fluoride (NH$_4$F) in (*a*) acetone-d$_6$ and (*b*) acetonitrile-d$_3$ (*inset* shows a zoomed-in section of the terminal CF$_3$ group).

***Tetrabutylammonium Fluoride Hydrate (TBAF)*:** TBAF contains two prominent peaks between −73.6 and −71.0 ppm (Figure 5). However, during the preparation step, TBAF highly precipitated with acetone solvent, making it an undesirable internal standard. In addition, peak definition was better in the acetonitrile-d$_3$ solvent because of its better solubility. However, as seen

from the spectra with acetonitrile-d_3, there seemed to be more noise, making it an undesirable internal standard. Furthermore, the integration changed significantly with the solvent change, making this internal standard undesirable for ^{19}F NMR-based PFOA studies.
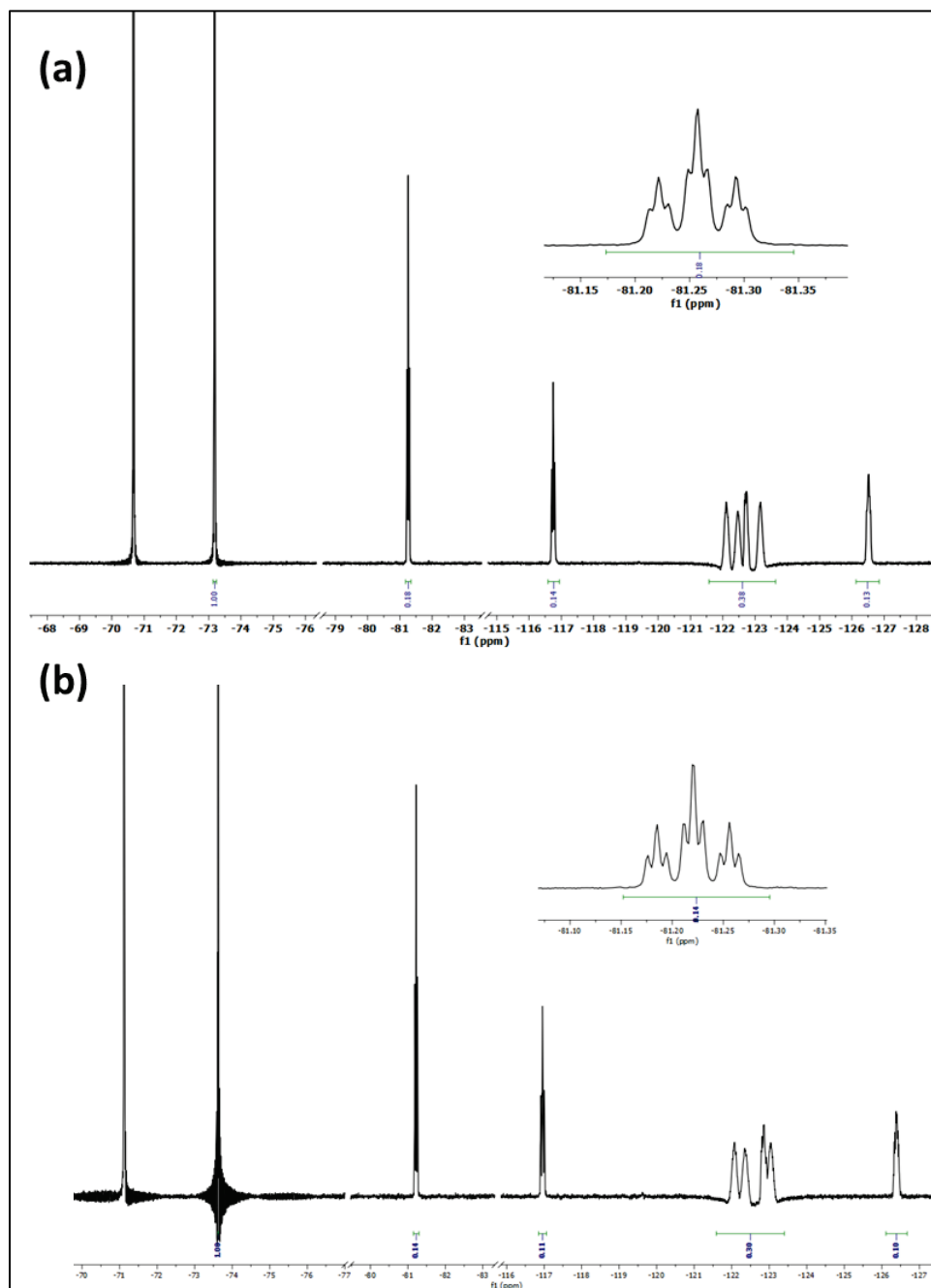

Figure 5. The ^{19}F NMR of PFOA with tetrabutylammonium fluoride hydrate (TBAF) in (*a*) acetone-d_6 and (*b*) acetonitrile-d_3.

***Ammonium Hexafluorophosphate (NH₄PF₆)*:** Similar to TBAF, NH$_4$PF$_6$ has two large peaks (at -73.7 and -71.2 ppm) that correspond to the fluoride present in PF$_6$, where one of the fluorides differs from the others due to the molecular structure of PF$_6$. The internal standard peak

intensity of NH_4PF_6 was higher than that of NH_4F due to the more significant number of fluorides. In addition, the peak shape was not as defined as when NH_4F was used a standard, as shown in the inset, which relates to the terminal CF_3 group (Figure 6). The solvents' integration values were almost equal, indicating that either solvent will work.
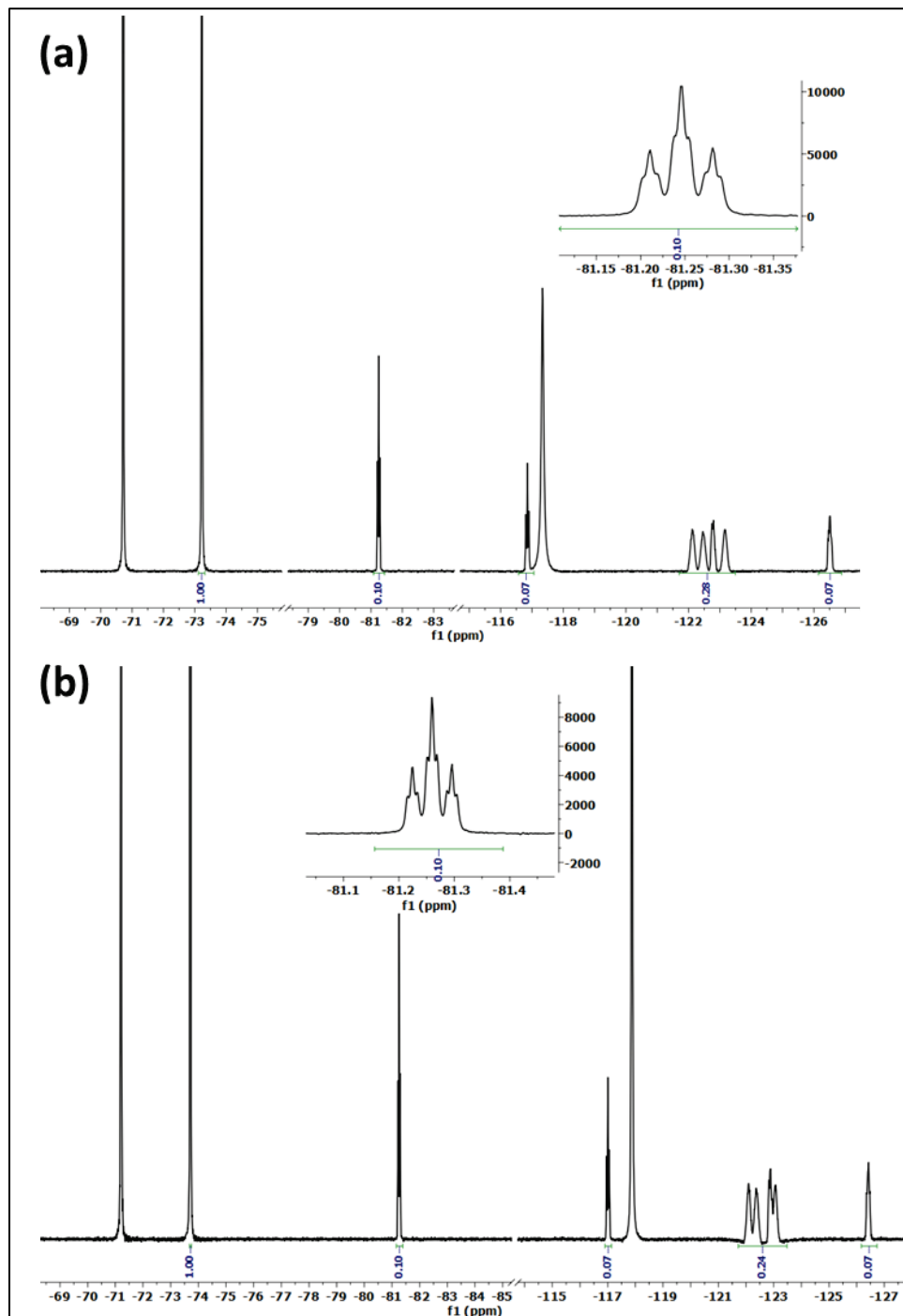

Figure 6. The ^{19}F NMR of PFOA with ammonium hexafluorophosphate
(NH_4PF_6) in (*a*) acetone-d_6 and (*b*) acetonitrile-d_3.

***Trifluoro Acetic Acid (TFA)*:** The reference peak related to trifluoro acetic acid (TFA) is seen around −76 ppm; in both solvents, there were significant amounts of noise related to solvent peak domination (compared to PFOA peaks; Figure 7). Also, TFA highly reacted with water, which can be a safety issue. Reaction with water can also cause side reactions, which are unattractive in an internal standard. Furthermore, the peak integration also significantly changed with the solvent changes. Hence, this internal standard is unsatisfactory for ^{19}F NMR-based PFAS studies.
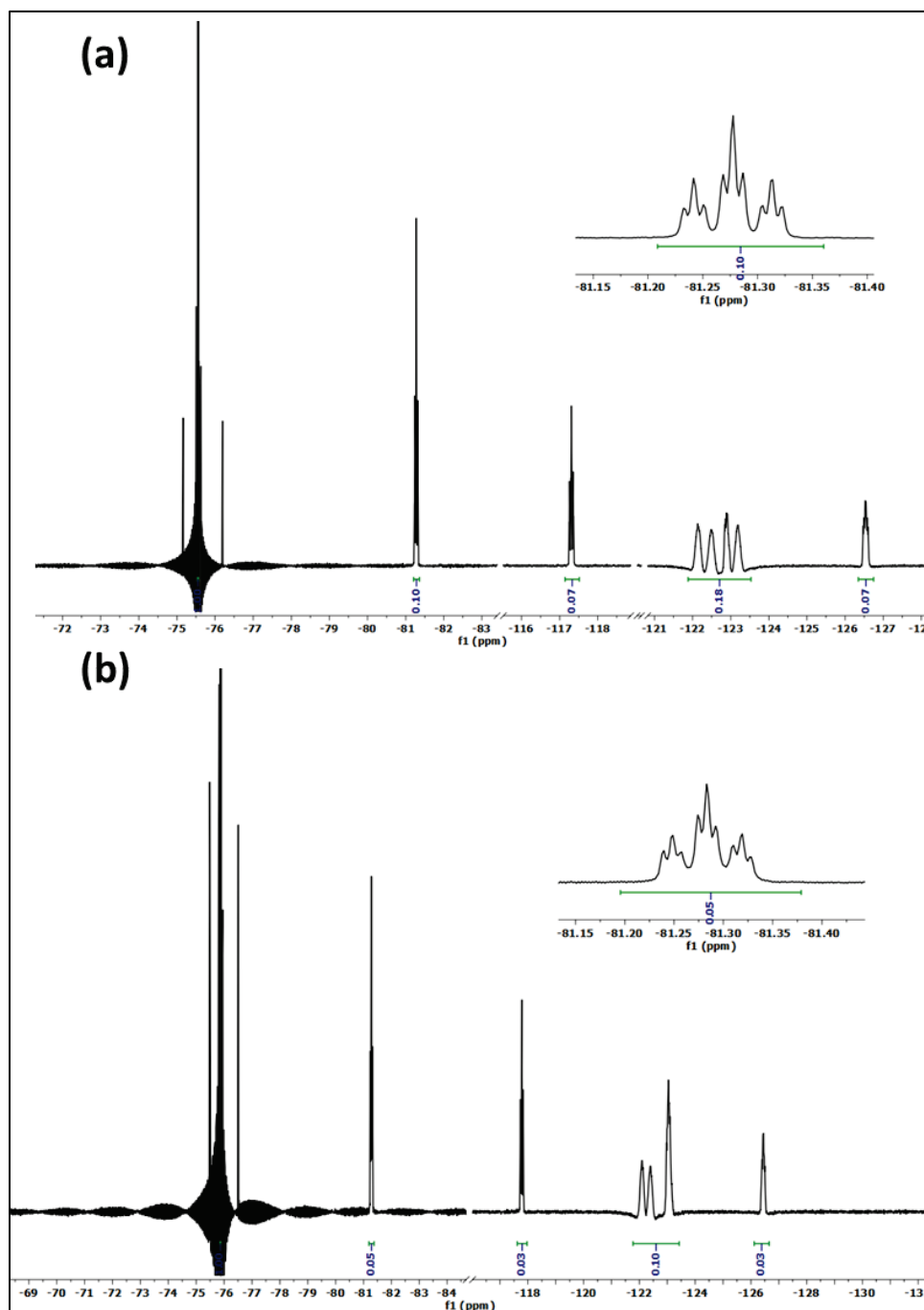

Figure 7. The ^{19}F NMR of PFOA with trifluoro acetic acid (TFA) in (*a*) acetone-d$_6$ and (*b*) acetonitrile-d$_3$.

Hexafluorobenzene (HFB): Unlike the other internal standards, HFB is an aromatic compound; therefore, the fluorine peaks are located on the far right, around -165 ppm. Interestingly, the HFB peak shape was far more pronounced when acetone was used as the solvent; when acetonitrile was used, large amounts of noise were observed, which in turn caused errors during integration (Figure 8). Furthermore, with acetonitrile integration, values were much smaller, making HFB in acetonitrile an unattractive choice. HFB in acetone was comparable to NH_4F in acetone. Hence, we proceeded with these two combinations for concentration dependence studies to investigate if these combinations could be used for quantitative NMR (qNMR).
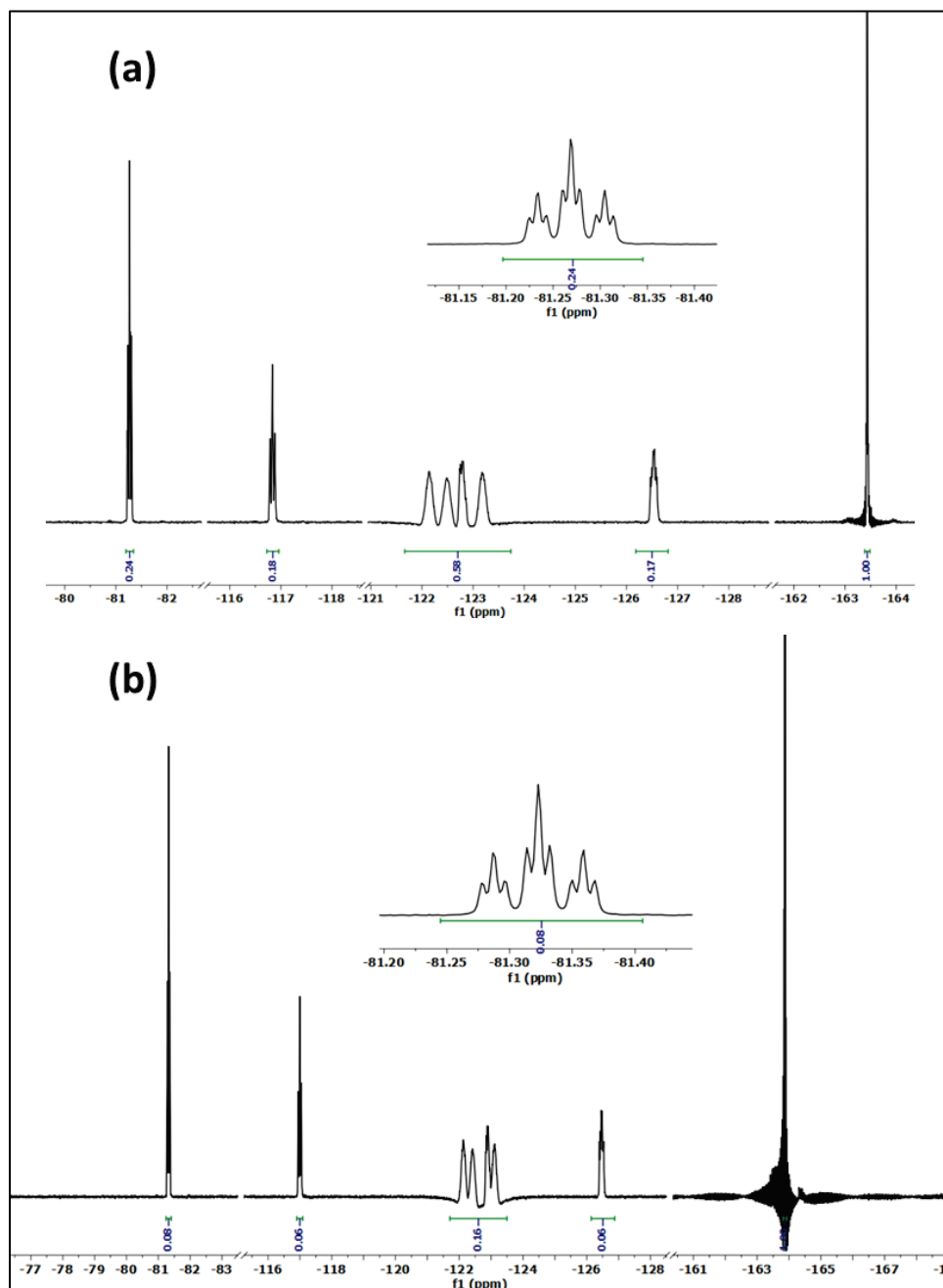

Figure 8. The ^{19}F NMR of PFOA with hexafluorobenzene (HFB) in
(*a*) acetone-d_6 and (*b*) acetonitrile-d_3.

Quantitative NMR (qNMR)-Based Experiments Related to Varying Concentrations of PFOA with the Down Selected Internal Standards and Deuterated Solvent: For this section, three different concentrations were used (Figure 9). The idea behind this set of experiments was to investigate if HFB and deuterated acetone, along with an aqueous solution of PFOA, could be used, along with a corresponding calibration curve, to quantify the amount of PFOA present in the NMR sample. As seen in Figure 9, with increasing PFOA concentration, the integration value also increased. A global analysis was performed for all but the middle CF_2 peaks, which all tended to be present together, making those more challenging to integrate separately than the rest of the ^{19}F peaks. As for the integration, the internal standard was set to 1.00. The global analysis showed that all three peaks gave a linear calibration curve with R^2 around 0.99, which is highly acceptable for qNMR-based experiments (Figure 10).
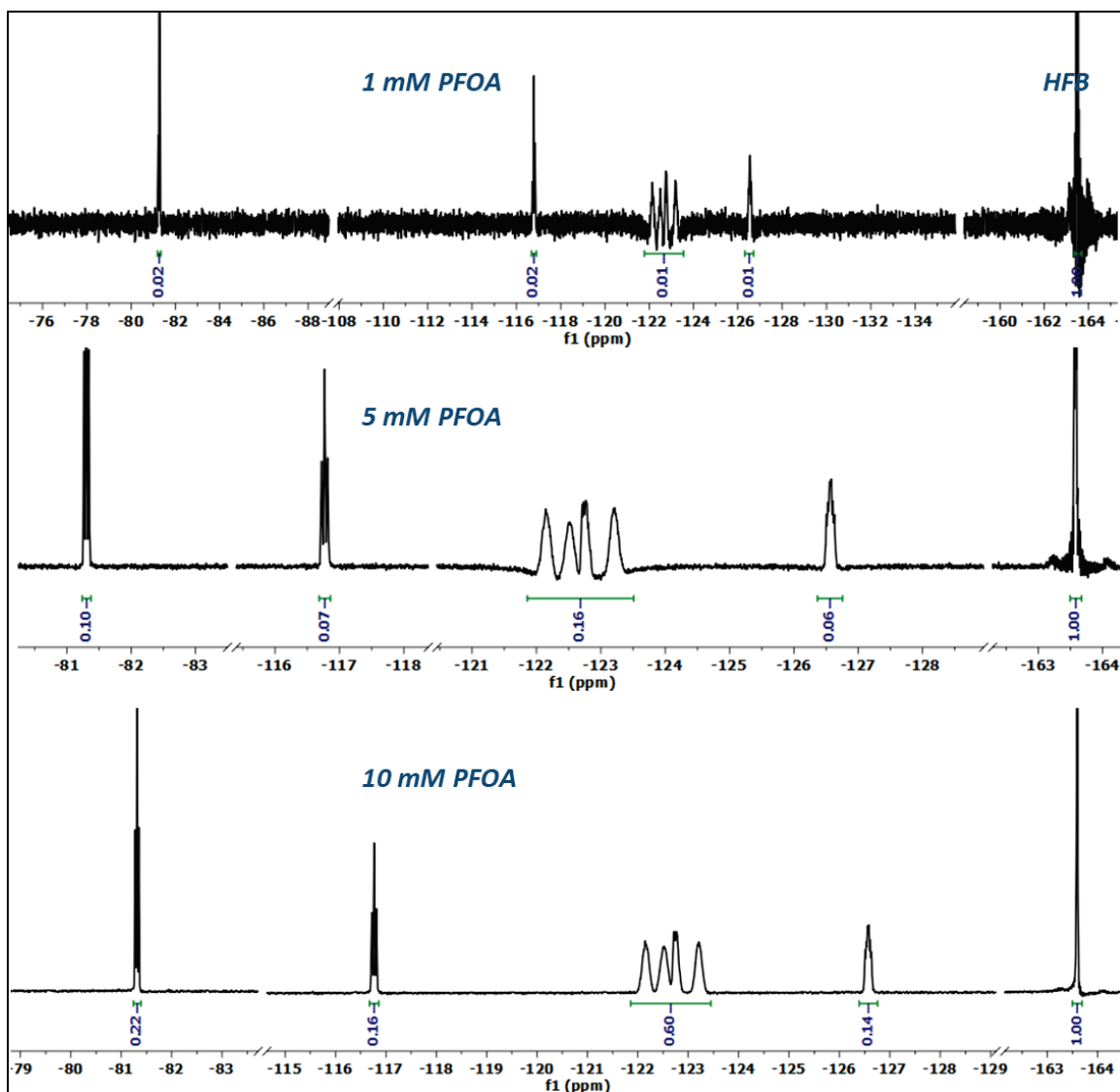

Figure 9. *Top* to *bottom*: Increasing concentrations of PFOA with added internal standard HFB.
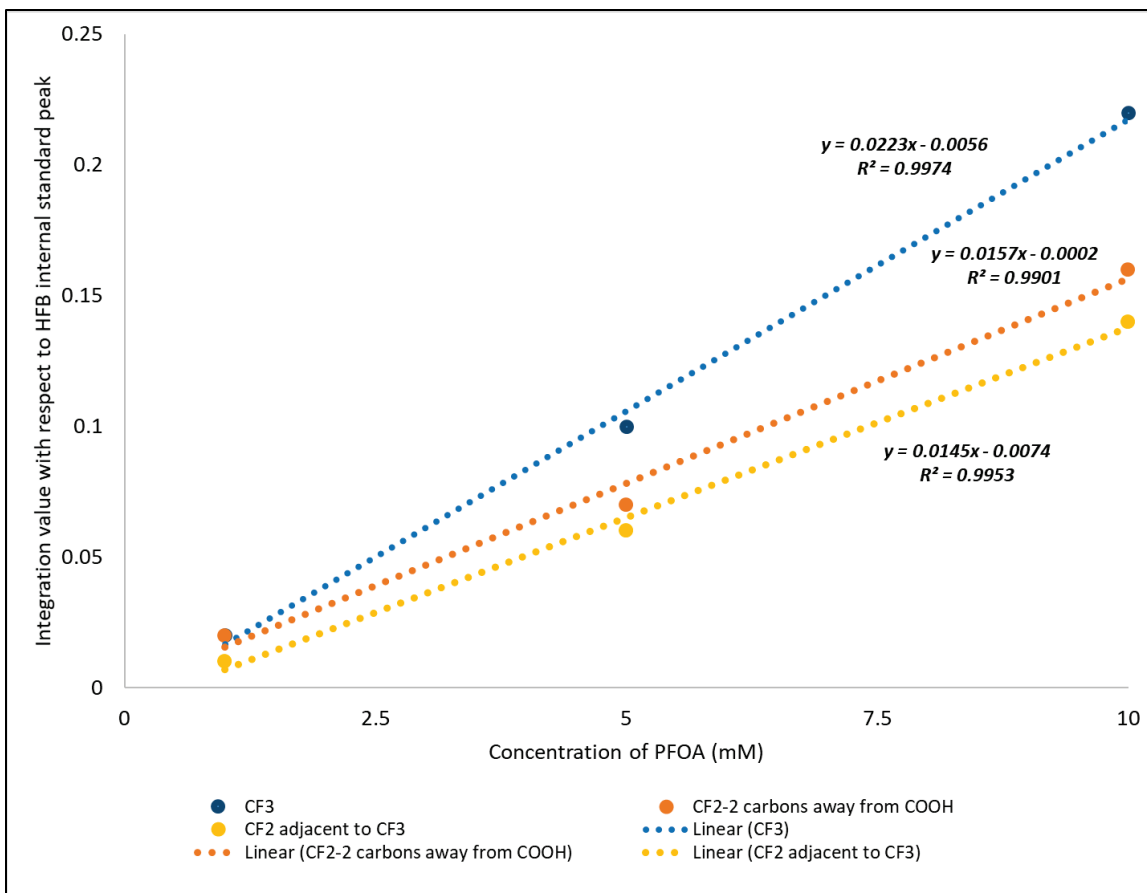
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Figure 10. Global analysis of quantitative NMR (qNMR) for PFOA in the presence of HFB as an internal standard.

As can be seen in Figure 11, NH$_4$F also showed increasing integration values with the increase of PFOA concentration. However, the integration numbers were higher than those for HFB because the NH$_4$F internal peak was not as high as that for HFB due to the presence of fewer fluoride nuclei. Furthermore, in this section, a lower amount of the internal standard was used to increase the peak intensity of PFOA compared to the internal standard. Large amounts of internal standards cause the PFOA peak heights to be extremely small, making it challenging to integrate them. Also, the peak shape loses its definition due to the presence of excess amounts of the internal standard.

A similar global analysis was conducted. Figure 12 shows the data from the integration with respect to the NH$_4$F reference peak. When NH$_4$F was used, even the middle CF$_2$ section could be easily integrated, and all regions of PFOA showed a linear response toward increased amounts of PFOA.
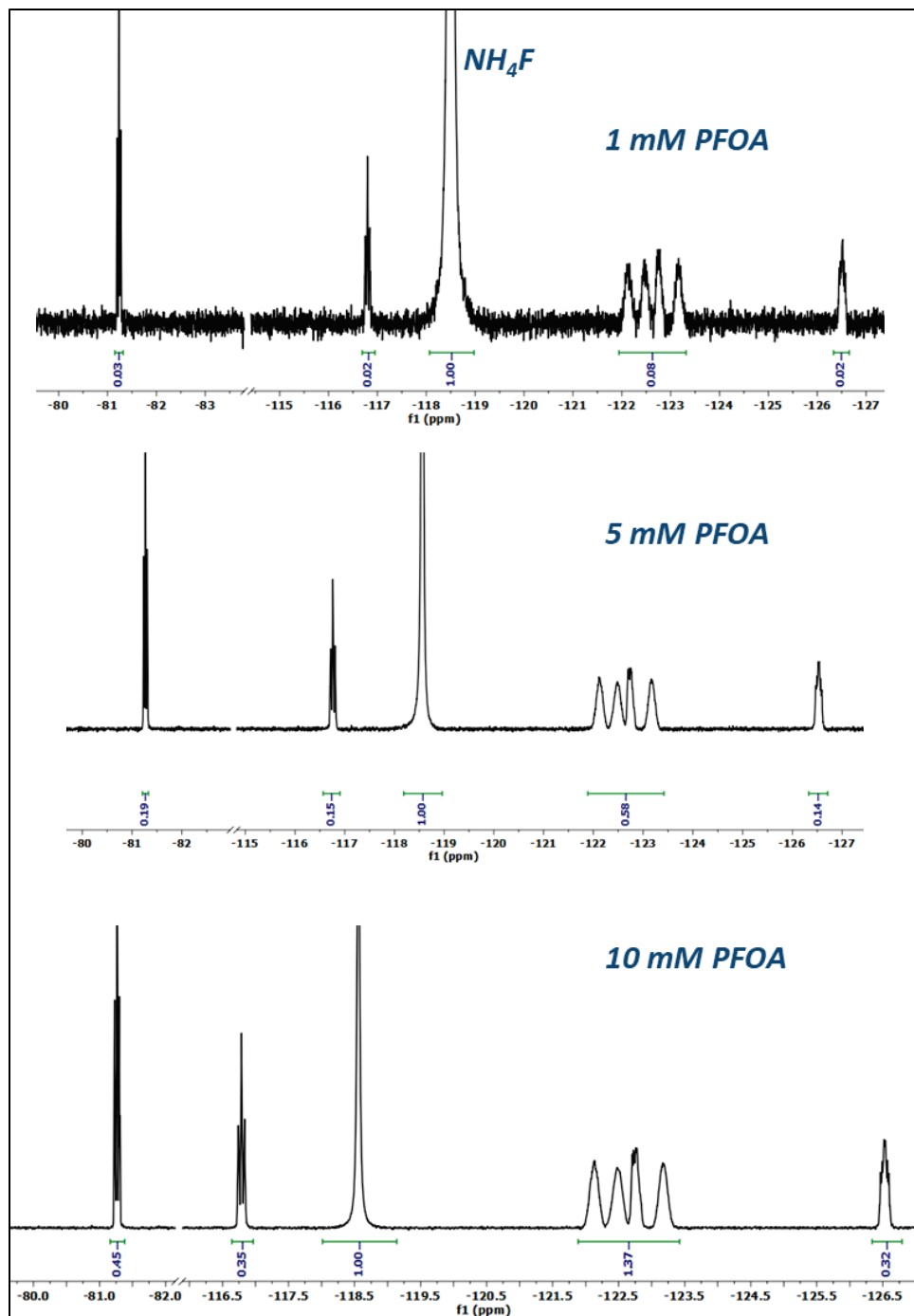
Figure 11. *Top* to *bottom*: Increasing concentrations of PFOA with added internal standard NH$_4$F.
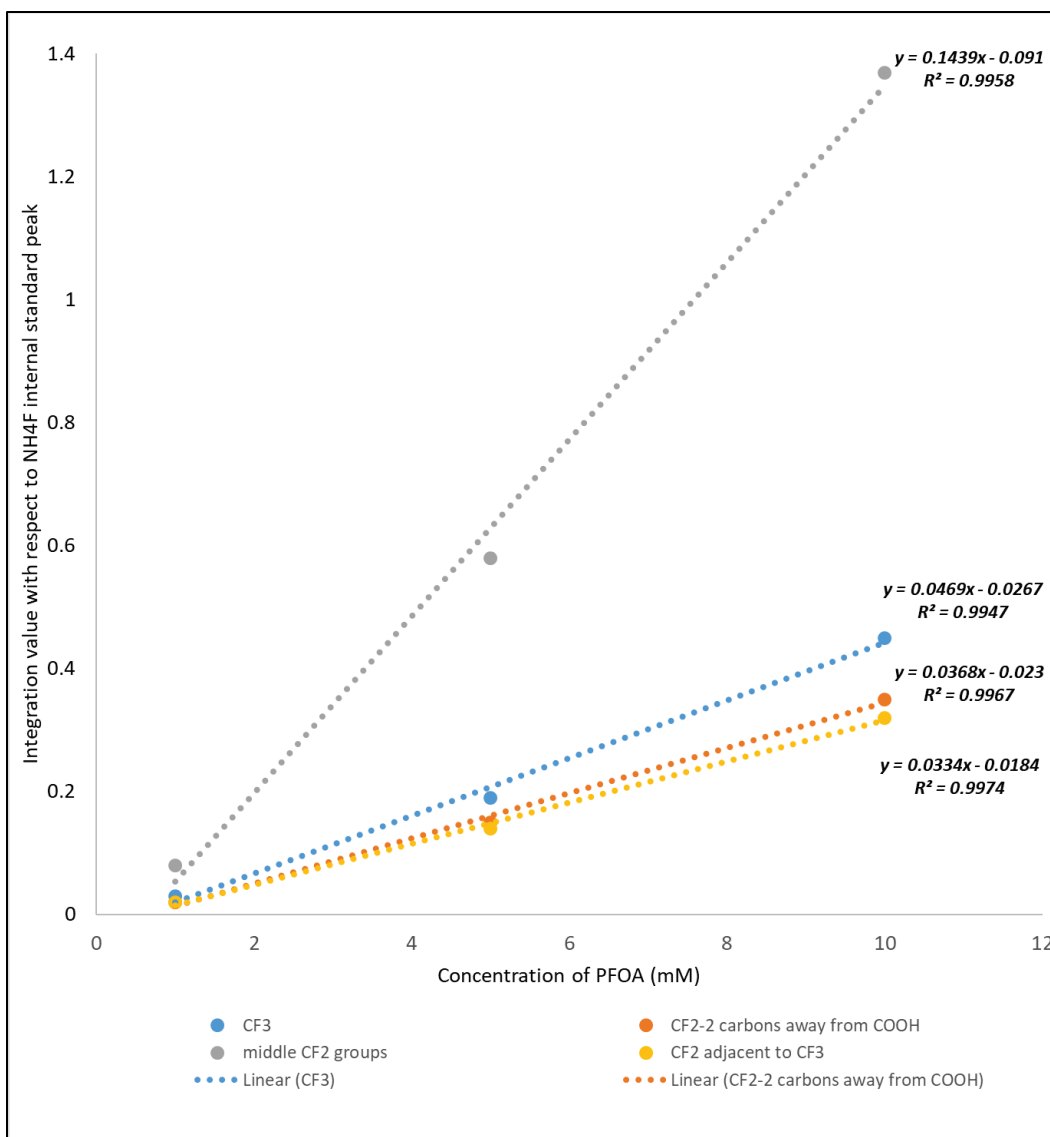
Figure 12. Global analysis of qNMR for PFOA in the presence of NH$_4$F as an internal standard.

Based on our results, both NH$_4$F and HFB can be used as internal standards with acetone-d$_6$ mixed in with aqueous solutions of PFOA. In addition to the preceding work, several other experiments were conducted to further understand the correct parameters required for ^{19}F NMR-based experiments for PFAS analysis. One factor that is as vital as the previously mentioned factors is recycle delay time, which is also represented as D1 in the Bruker NMR software. In preparation, this delay should be thought of as a necessary factor after the acquisition time. Hence, it is an important parameter and plays a vital role in obtaining accurate integration for qNMR-based experiments. We investigated the D1 parameter for 10 mM PFOA with HFB and acetone-d$_6$. D1 was changed from 1 sec to all the way up to 20 sec.

From the data obtained, we deduced that D1 plays a small role in obtaining sharper peaks. However, the peak height and the area under the curve were not immensely changed (Figure 13).

A larger D1 increases the time required for the acquisition. Hence, we recommend using a D1 between 3 sec and 7 sec to achieve a better peak shape without compromising the time factor.
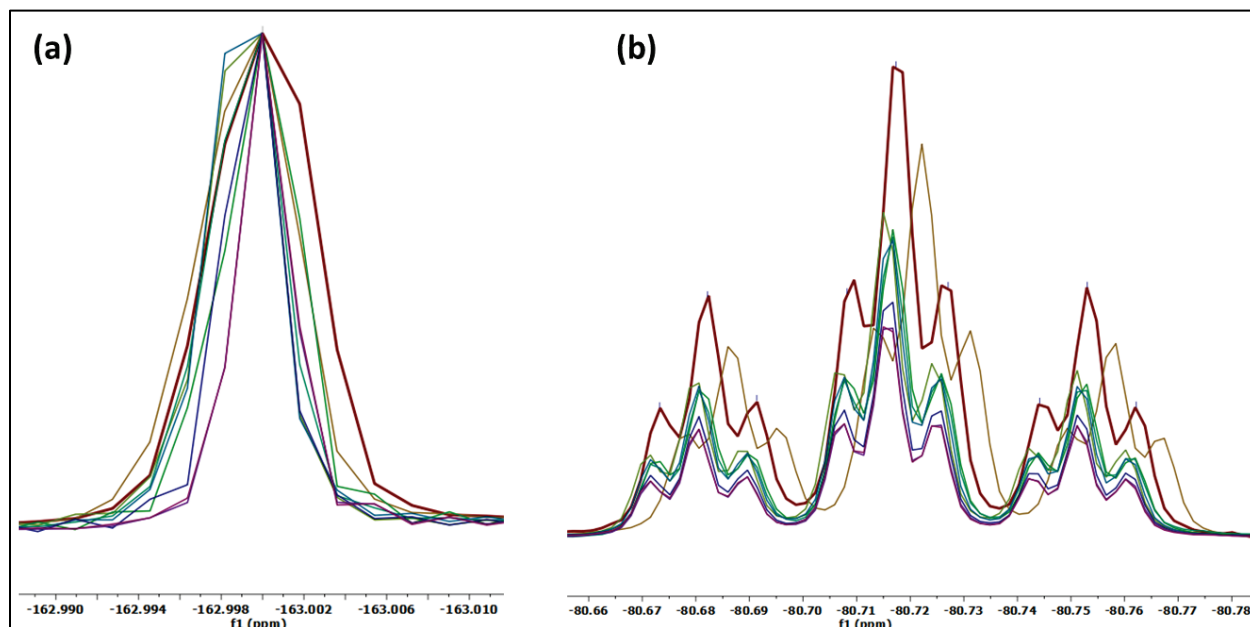

Figure 13. Change in recycle delay time (D1) from 1 sec to 20 sec for (*a*) the HFB reference peak and (*b*) the terminal CF_3 peak.

Two experiments were performed to evaluate the stability of the added internal standards over time. These studies were conducted for as long as 24 hr. Both HFB and NH_4F were investigated. All experimental conditions were kept the same; the only variable factor was time. Figure 14 showcases the CF_3 terminal peak area for both the HFB and NH_4F internal standards. In HFB, the CF_3 middle-triplet peak only changed by 0.014 ppm after 24 hr. Peak height remained the same in all PFOA-related peaks. A slight change was observed in the HFB reference peak, where peak height slightly increased until 11 hr and then showed a slight gradual decrease at 24 hr. However, between the sample at 2 hr and the sample at 24 hr, the integration value increased by 18%, which is significant. However, the sample integration only increased by 8% after 12 hr. This suggests that it is vital to obtain data within a few hours of adding the internal standard HFB.

Unlike with HFB, integration that was obtained through NH_4F did not fluctuate with time. The integration value remained the same from the beginning to hour 24. The peak position varied slightly, up to 0.067 ppm, but this was relatively minimal movement for 24 hr. Peak height remained constant throughout the 24 hr (Figure 14). All changes are shown in Figure 14.
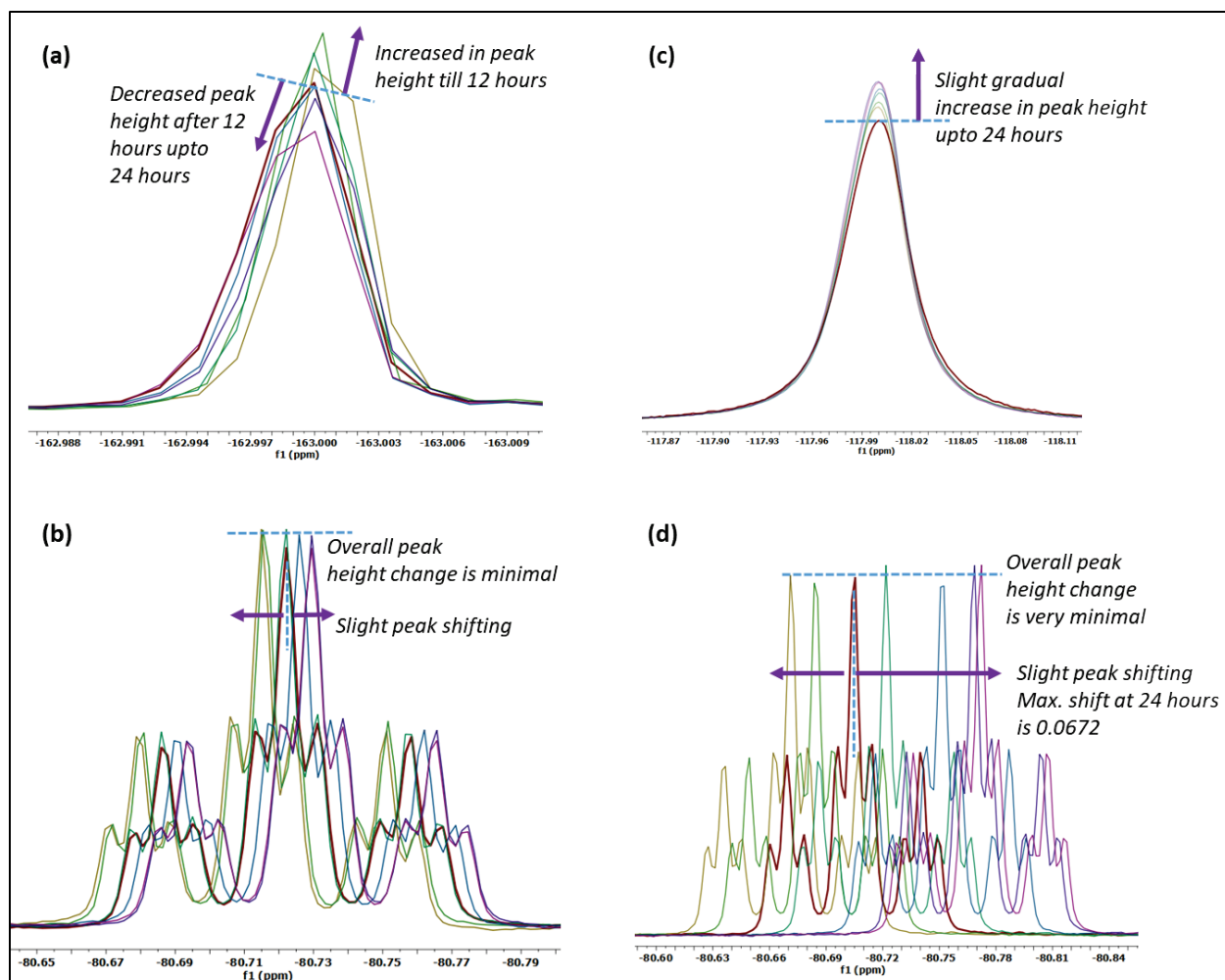
Figure 14. The ^{19}F NMR of the (*a*) reference HFB peak with time, (*b*) terminal CF$_3$ PFOA peak with time, (*c*) reference NH$_4$F peak change with time, and (*d*) terminal CF$_3$ peak with increasing time.

SUMMARY: To perform accurate qNMR studies, it is essential to identify the correct deuterated solvent and internal standards. Based on our results, acetone-d$_6$ and NH$_4$F were the best choices. Furthermore, it is essential to investigate the PFOA's peak shape, resolution, and height compared to the internal standard. Therefore, when preparing samples, we highly recommend using the minimum amount of internal standard that will be adequate to integrate peaks without sacrificing peak height. Furthermore, acquisition parameters, such as D1, should be adjusted to an optimum value to obtain better quality ^{19}F NMR data. Finally, once the internal standard is added, it is essential to monitor the change in peak height or shape with respect to time (Figure 14). In future experiments, we plan to dive deep into understanding the effect of relaxation time (typically referred to as T1 and T2) in these experiments to further optimize ^{19}F NMR toward PFOA-based analysis.

ADDITIONAL INFORMATION: This work was performed by researchers from the US Army Engineer Research and Development Center, Environmental Laboratory (ERDC-EL). This technical note was prepared by Dr. P. U. Ashvin Iresh Fernando, who also designed the

experiments, interpreted and analyzed the data, and prepared the figures. Ms. Samantha Sullivan performed the experiments, prepared the sample, and made minor manuscript edits. Drs. Edith Martinez-Guerra and Jared S. Cobb acted as project supervisors. For additional information, please contact Dr. P. U. Ashvin Iresh Fernando (Payagala.a.fernando@erdc.dren.mil), Dr. Edith Martinez-Guerra (Edith.L.Martinez-Guerra@usace.army.mil), and Dr. Jared S. Cobb (Jared.S.Cobb@erdc.dren.mil). This technical note should be cited as follows:

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