

## NMR as biphasic $pK_a$ measurement technique

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**Abstract:** NMR is a powerful technique with a wide range of applications, including measurements of acid dissociation constants ( $pK_a$  values). One such example where NMR is particularly valuable is the measurement of biphasic octanol-water  $pK_a$  ( $pK_a^{ow}$ ) values. Acid-base properties in biphasic systems (extraction systems, lipid bilayers, phase-transfer catalysis systems) are commonly estimated from single-phase (typically aqueous)  $pK_a$  values. However, acid-base properties in biphasic systems are more complex than in systems consisting of one phase and the single-phase  $pK_a$  value does not adequately describe the situation. Biphasic  $pK_a$  values (such as octanol:water biphasic  $pK_a$  values,  $pK_a^{ow}$ ) have been introduced to provide a more realistic description of behaviour acids in biphasic systems.  $^1H$  and  $^{13}C$  NMR have been used extensively for  $pK_a^{ow}$  measurements and their advantages and disadvantages have been discussed in detail. However, the suitability of  $^{31}P$  NMR has not yet been assessed in depth and  $^{19}F$  NMR has until now not been used for  $pK_a^{ow}$  measurements at all. In this study, we measure  $pK_a^{ow}$  values with  $^1H$ ,  $^{13}C$ ,  $^{19}F$  and  $^{31}P$  NMR and provide a detailed comparison of NMR with these four nuclei in terms of  $pK_a^{ow}$  measurement.

**Keywords:** NMR; acidity; biphasic systems; lipophilicity; partitioning equilibria. © 2022 ACG Publications. All rights reserved.

### 1. Introduction

NMR is a widely used technique in all areas of chemistry. Although the main application of NMR is still structure determination of organic compounds, it is increasingly being used in measurements of equilibrium constants [1,2], in particular acid dissociation constants ( $pK_a$  values) [1,3,4]. There are four main nuclei that are used in such measurements –  $^1H$ ,  $^{13}C$ ,  $^{19}F$  and  $^{31}P$ . In principle, any of these can be used for  $pK_a$  measurement, as long as the compound under question contains the respective nucleus and it is sufficiently close to the acidity centre [3]. In the case of many compounds, several nuclei can be used.

We have recently been engaged with measuring the so-called biphasic octanol:water  $pK_a$  values ( $pK_a^{ow}$  values). Their usefulness for quantifying acid-base properties in biphasic systems, especially in comparison to monophasic conditions, has been discussed in detail previously [4,5]. In many biphasic systems, solvated  $H^+$  ions reside mainly in the aqueous phase, while more lipophilic species, especially neutrals but also ions (as ion pairs), are mostly present in the low-polarity organic phase. Moreover, acid-base properties in biphasic systems involve several processes and phenomena that are either much simpler or not even relevant in a system consisting of one phase.

Therefore, the common approach of describing the behaviour of an acid or base in such systems in terms of single-phase  $pK_a$ , is unsuitable. This has been demonstrated by research as octanol:water

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biphasic  $pK_a$  values of various acids have been shown to be several units higher than the corresponding aqueous  $pK_a$  values.[4]

Biphasic  $pK_a$  values can be measured in various biphasic systems. However, octanol:water biphasic  $pK_a$  values ( $pK_a^{ow}$ ) are particularly relevant as octanol:water system is considered a suitable reference system for biological partitioning[6] and the most commonly used quantitative estimate of lipophilicity is the octanol-water partitioning coefficient,  $\log P_{ow}$ , of the compound.

NMR is the most useful technique for measuring  $pK_a^{ow}$  values. Its distinct advantage over e.g. UV-Vis spectrometry is that under fast exchange conditions the shifts of the signals are measured, not their intensities. This means that the concentration of the acid in the measured solutions does not need to be constant. This is important with  $pK_a^{ow}$  measurements because the extent of migration of the acid (in the form of anion) from octanol (where it mainly resides) into the aqueous phase strongly depends on pH. Thus, the spectra of the octanol phase measured at different pH values correspond to different concentrations of the acid.

We have previously used  $^1H$ ,  $^{13}C$  (and  $^{31}P$  NMR in limited scope) for these measurements.[4] Although many acids of significant importance contain fluoro substituents, the suitability of  $^{19}F$  NMR has not yet been investigated at all. In this work, we use  $^{19}F$  NMR for  $pK_a^{ow}$  measurements along with the remaining three nuclei and provide a comprehensive comparison of the  $^1H$ ,  $^{13}C$ ,  $^{19}F$  and  $^{31}P$  NMR methods.

## 2. Experimental

### 2.1 Materials and Instruments

The following chemicals and solvents were used: 1-octanol (Sigma-Aldrich, ReagentPlus grade), HEPES (Sigma, ACS reagent, >99.5%), tetraethylammonium chloride (Alfa Aesar, ACS reagent), tetraethylammonium hydroxide (Sigma-Aldrich, 20% w/w in H<sub>2</sub>O, ACS reagent), 4-trifluoromethylbenzoic acid (Alfa Aesar, 98+%), diheptylphosphinic acid (Reakhim "Pure") and hydrochloric acid (Sigma-Aldrich, >36%). Water was prepared using a MilliQ Advantage A10 setup.

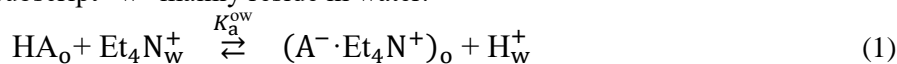
NMR measurements were performed on a Bruker Avance-III 700 NMR spectrometer (16.4 T,  $^1H$  resonance frequency 700.1 MHz,  $^{13}C$  resonance frequency 176.0 MHz,  $^{19}F$  resonance frequency 658.7 MHz,  $^{31}P$  resonance frequency 283.4 MHz). Measurements were carried out in water-saturated octanol at  $25.0 \pm 0.1$  °C, using TopSpin 3.2.7 software. The largest peak of the spectrum (corresponding to -CH<sub>2</sub> hydrogens of carbons 3-7 in 1-octanol) was used for shimming.  $^1H$  and  $^{13}C$  NMR spectra of the samples were calibrated internally, using the octanol -CH<sub>3</sub> peak ( $^1H$   $\delta$  0.88 ppm;  $^{13}C$   $\delta$  14.11 ppm).  $^{19}F$  and  $^{31}P$  NMR spectra were calibrated using capillary tubes with calibration solution submerged into the sample. For calibration of  $^{19}F$  NMR spectra, tubes with potassium fluoride ( $^{19}F$   $\delta$  set at -125.3 ppm) solution in D<sub>2</sub>O was used,  $^{31}P$  NMR spectra were calibrated using phosphoric acid ( $^{31}P$   $\delta$  set at 0 ppm) solution in D<sub>2</sub>O.

Mettler Toledo InLab Micro pH-sensor was used for pH measurements. It was calibrated using pH 4.01 and pH 7.01 Mettler Toledo buffers. pH 10.04 Mettler Toledo buffer was used to verify the calibration (discrepancies usually did not exceed 0.03 pH units and never 0.05 pH units).

Samples were centrifuged using an Eppendorf 5430R Centrifuge (5 minutes at 7800 rpm).

### 2.2. Measurement Method

In  $pK_a^{ow}$  measurement experiments, we monitor the dissociation reaction of the acid of interest under fast proton exchange conditions in the octanol:water biphasic system in which the phases are in equilibrium. The measured quantity is the equilibrium constant of the dissociation reaction of the acid HA (denoted as  $K_a^{ow}$ ), according to equilibrium (1). Subscript "o" denotes that the species mainly reside in octanol, while species with subscript "w" mainly reside in water.



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The acids under question are relatively lipophilic molecules and mainly reside in octanol. Upon dissociation the highly hydrophilic  $H^+$  ions are formed that migrate (almost entirely) from octanol into water.  $Et_4N^+$  (tetraethylammonium) cation acts as the counter-ion for the anions  $A^-$  (Eq. 1). Generally, ions in octanol are poorly solvated and they exist in octanol as ion pairs. Due to this, as a part of the dissociation reaction,  $Et_4N^+$  migrates from the aqueous phase into the octanol phase and there forms an ion-pair ( $A^- \cdot Et_4N^+$ ). This migration also balances the charge of the hydrogen ions that migrated from octanol into water. As the properties of the counter-cation and its activity influence the acid-base properties of compounds in such system, the activity of  $Et_4N^+$  is kept approximately constant (0.1 M) throughout the measurements and included in the standard state definition. Based on the above,  $K_a^{ow}$  and  $pK_a^{ow}$  can be expressed as follows (Eqs. 2-4):

$$K_a^{ow} = \frac{a(H^+)_w \cdot a(A^- \cdot Et_4N^+)_o}{a(HA)_o} \quad (2)$$

$$pK_a^{ow} = -\log K_a^{ow} = pH - \log \frac{a(A^- \cdot Et_4N^+)_o}{a(HA)_o} \quad (3)$$

$$pK_a^{ow} = pH - \log \frac{[A^- \cdot Et_4N^+]_o \cdot f_o}{[HA]_o} = pH - \log \frac{[A^- \cdot Et_4N^+]_o}{[HA]_o} - \log f_o \quad (4)$$

We assume that the activity coefficient of the neutral acid HA is 1. However, the activity coefficient of the ion pair is different from 1 and equal to  $f_o$ .

For each sample, 2 mL of 1-octanol solution of the acid of interest was prepared into 4 mL vials and 2 mL of aqueous phase with measured pH was added. Aqueous phases with different pH values were generated by combining the following aqueous solutions in various ratios: (1) 0.1 M  $Et_4NCl$  + 0.01 M zwitterionic buffering agent HEPES; (2) 0.1 M  $HCl$  + 0.1 M  $Et_4NCl$ ; (3) 0.1 M  $Et_4NOH$ . After preparation, the samples were equilibrated by shaking. Subsequently, the phases were separated by centrifuging the samples. Thereafter the pH of the samples was measured from the aqueous phase while the ratio of the equilibrium concentrations of acid and its anion (conjugate base) at that pH was measured from the octanol phase, using NMR spectrometry.

As the octanol phase and the aqueous phase are in equilibrium, the thermodynamic activities of the species in both phases are equal. This means that, in terms of the unified pH ( $pH_{abs}$ ) [7], the acidities of the phases are equal as well. Therefore, as the activity of  $H^+$  is measured in the aqueous phase, it is not necessary to measure it in the octanol phase.

$pK_a^{ow}$  is calculated by using the least-squares method described in ref [4]. Under fast proton exchange conditions, chemical shift values of the compound at different pH can be directly related to the ratio of the neutral acid and its conjugate base through degree of dissociation ( $\alpha$ ). Therefore, chemical shift values are plotted against pH at which each chemical shift value was measured. A sigmoid curve is fitted to the data points so that the sum of squared distances on the chemical shift axis would be minimal. The apparent  $pK_a^{ow}$  value is one of the parameters varied in the least squares optimization process. The obtained apparent  $pK_a^{ow}$  values depend on the concentration they are measured at. Concentration-independent  $pK_a^{ow}$  values were calculated via extrapolation to zero concentration using the previously reported approach based on Debye-Hückel model [4].

The measurement and calculation methods used in this study are essentially identical to the previously reported methods [4], the only significant exception is the inclusion of  $^{19}F$  NMR for the analysis of octanol phases of the samples. A more detailed overview of the methods used is given in ref [4].

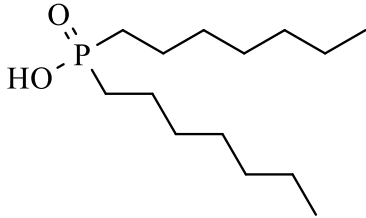
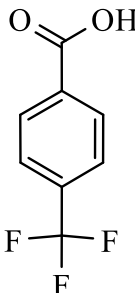
## 3. Results and Discussion

### 3.1. Measurement Results

In order to evaluate and compare the applicability of  $^1H$ ,  $^{13}C$ ,  $^{19}F$  and  $^{31}P$  NMR methods, we measured  $pK_a^{ow}$  values of diheptylphosphinic acid (using  $^{13}C$  and  $^{31}P$  NMR) and 4-trifluoromethylbenzoic

acid (using  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{19}\text{F}$  NMR). Each of the compounds was measured separately at three different concentrations and the concentration-independent values were also calculated (Table 1).

**Table 1.** Results of  $\text{p}K_{\text{a}}^{\text{ow}}$  measurements with diheptylphosphinic acid and 4-trifluoromethylbenzoic acid

Compound	Concentration	$\text{p}K_{\text{a}}^{\text{ow } a}$	Method	
	10.0 mM	6.53	$^{13}\text{C}$ NMR	
		6.53	$^{31}\text{P}$ NMR	
	15.0 mM	6.58	$^{13}\text{C}$ NMR	
		6.58	$^{31}\text{P}$ NMR	
	20.1 mM	6.61	$^{13}\text{C}$ NMR	
		6.60	$^{31}\text{P}$ NMR	
	Extrapolated to zero concentration	6.17 <sup>b</sup>	$^{13}\text{C}$ NMR	
		6.17 <sup>b</sup>	$^{31}\text{P}$ NMR	
		10.0 mM	7.09	$^1\text{H}$ NMR
			7.08	$^{13}\text{C}$ NMR
7.07			$^{19}\text{F}$ NMR	
15.0 mM		7.12	$^1\text{H}$ NMR	
		7.12	$^{13}\text{C}$ NMR	
		7.11	$^{19}\text{F}$ NMR	
20.0 mM		7.11	$^1\text{H}$ NMR	
		7.10	$^{13}\text{C}$ NMR	
		7.09	$^{19}\text{F}$ NMR	
Extrapolated to zero concentration		6.77 <sup>b</sup>	$^1\text{H}$ NMR	
	6.76 <sup>b</sup>	$^{13}\text{C}$ NMR		
	6.75 <sup>b</sup>	$^{19}\text{F}$ NMR		

<sup>a</sup> Apparent  $\text{p}K_{\text{a}}^{\text{ow}}$  values at given concentration unless indicated otherwise. Standard uncertainties for the apparent and extrapolated values are 0.03 and 0.10, respectively, the same as in ref [4]. <sup>b</sup> Debye-Hückel constants used for extrapolation,  $A$  and  $Ba$ , were -48 and 82, respectively, the same as in ref [4].

As expected, NMR measurements with different nuclei yield near-identical results, indicating the suitability of all the methods for measurement of  $\text{p}K_{\text{a}}^{\text{ow}}$  values. Based on the previously reported Debye-Hückel model[4], concentration-independent  $\text{p}K_{\text{a}}^{\text{ow}}$  values of diheptylphosphinic acid and 4-trifluoromethylbenzoic acid are 6.17 and 6.76, respectively. The latter value is consistent with the reported aqueous  $\text{p}K_{\text{a}}$  value (3.67 [8]), as  $\text{p}K_{\text{a}}^{\text{ow}}$  values have shown to be roughly 3 units higher than corresponding aqueous  $\text{p}K_{\text{a}}$  values on average.[4]

### 3.2 Comparison of the NMR methods for $\text{p}K_{\text{a}}^{\text{ow}}$ measurements

As our results show (Table 1), all 4 nuclei are suitable for measuring  $\text{p}K_{\text{a}}^{\text{ow}}$  values. Their advantages and disadvantages are often closely related with each other.  $^{19}\text{F}$  and  $^{31}\text{P}$  NMR are not as universally applicable as  $^1\text{H}$  and  $^{13}\text{C}$  NMR, as compounds of interest are much more likely to contain

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hydrogen or carbon than fluorine or phosphorus. However, fluoro substituents are relatively popular in acids as they often enhance the acidity of the compound. Using  $^{19}\text{F}$  NMR or  $^{31}\text{P}$  NMR requires external calibration as no component in either of the phases contains F or P atoms. This also means that the spectra contain signals only from the calibrant and the sample (Scheme 1). Therefore, by choosing suitable calibrants, peak overlapping can be completely avoided. In addition, differently from  $^1\text{H}$  and  $^{13}\text{C}$  NMR, the signal intensity of the measured compounds is not suppressed by the solvent. With  $^1\text{H}$  NMR (and to a much smaller extent,  $^{13}\text{C}$  NMR as well), there is a considerable likelihood of all analyte peaks being covered by the solvent peaks if the compound of interest contains CH moieties that are chemically similar to the CH moieties in the solvent (Scheme 2). For example,  $pK_a^{\text{ow}}$  of diheptylphosphinic acid could not be measured using  $^1\text{H}$  NMR, as all of its peaks in  $^1\text{H}$  NMR spectrum were hidden under much larger octanol peaks. There are various perfluorinated acids, which do not have a  $^1\text{H}$  NMR spectrum but could be measured almost as fast by using  $^{19}\text{F}$  NMR. The sensitivity of these methods[9] decreases in the following order:  $^1\text{H}$  NMR  $\sim$   $^{19}\text{F}$  NMR  $>$   $^{31}\text{P}$  NMR  $\gggg$   $^{13}\text{C}$  NMR. In order to illustrate this, for more lipophilic compounds such as the ones used in this study, spectra with sufficient quality can be obtained within a few minutes ( $^1\text{H}$  NMR,  $^{19}\text{F}$  NMR and  $^{31}\text{P}$  NMR) or 1.5 to 2.5 hours ( $^{13}\text{C}$  NMR). It is worth noting that  $^{31}\text{P}$  NMR is rarely an alternative to  $^{19}\text{F}$  NMR as very few compounds of interest contain both P and F atoms. Based on the above, the NMR methods used in this study can often complement each other in the measurement of  $pK_a^{\text{ow}}$  values.

**Table 2.** Comparison of the NMR techniques used in this study

	$^1\text{H}$ NMR	$^{13}\text{C}$ NMR	$^{19}\text{F}$ NMR	$^{31}\text{P}$ NMR
Sufficient measurement time of 1 sample <sup>a</sup>	3 minutes	2 hours 15 minutes	5 minutes	10 minutes
Signal-to-noise ratio of the most intensive peak of the analyte <sup>a</sup>	65 <sup>b</sup>	3.5 <sup>b</sup> 5.5 <sup>c</sup>	110 <sup>b</sup>	98 <sup>c</sup>
Target compounds <sup>d</sup>	Moderately lipophilic compounds ( $\log P > 1$ )	Highly lipophilic compounds ( $\log P > 2.5$ )	Moderately lipophilic compounds ( $\log P > 1$ )	Lipophilic compounds ( $\log P > 1.5$ )
Recommended minimum concentration of acid in 1-octanol	0.5 mM	10 mM	1 mM	2.5 mM
Likelihood of solvent peaks overlapping with analyte peaks <sup>e</sup>	Moderate	Low	None	None
Calibration of spectra	Solvent peak	Solvent peak	External	External

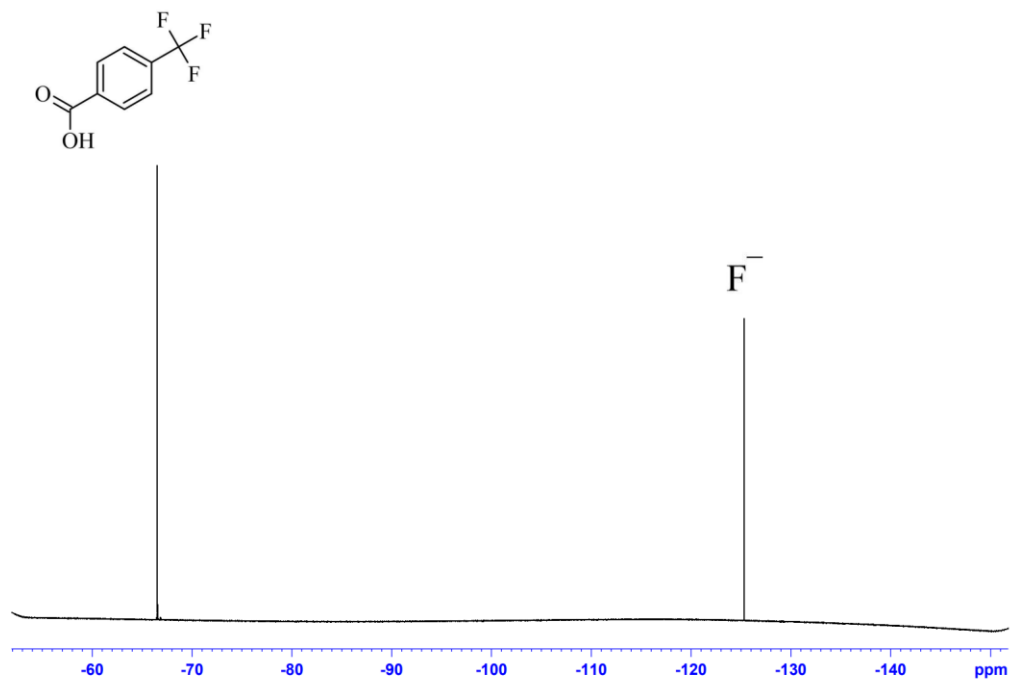
<sup>a</sup> Based on experimental conditions used in this study, outlined in the Experimental section.

<sup>b</sup> Estimated from pH 11.79 sample at 10 mM concentration with 4-trifluoromethylbenzoic acid.

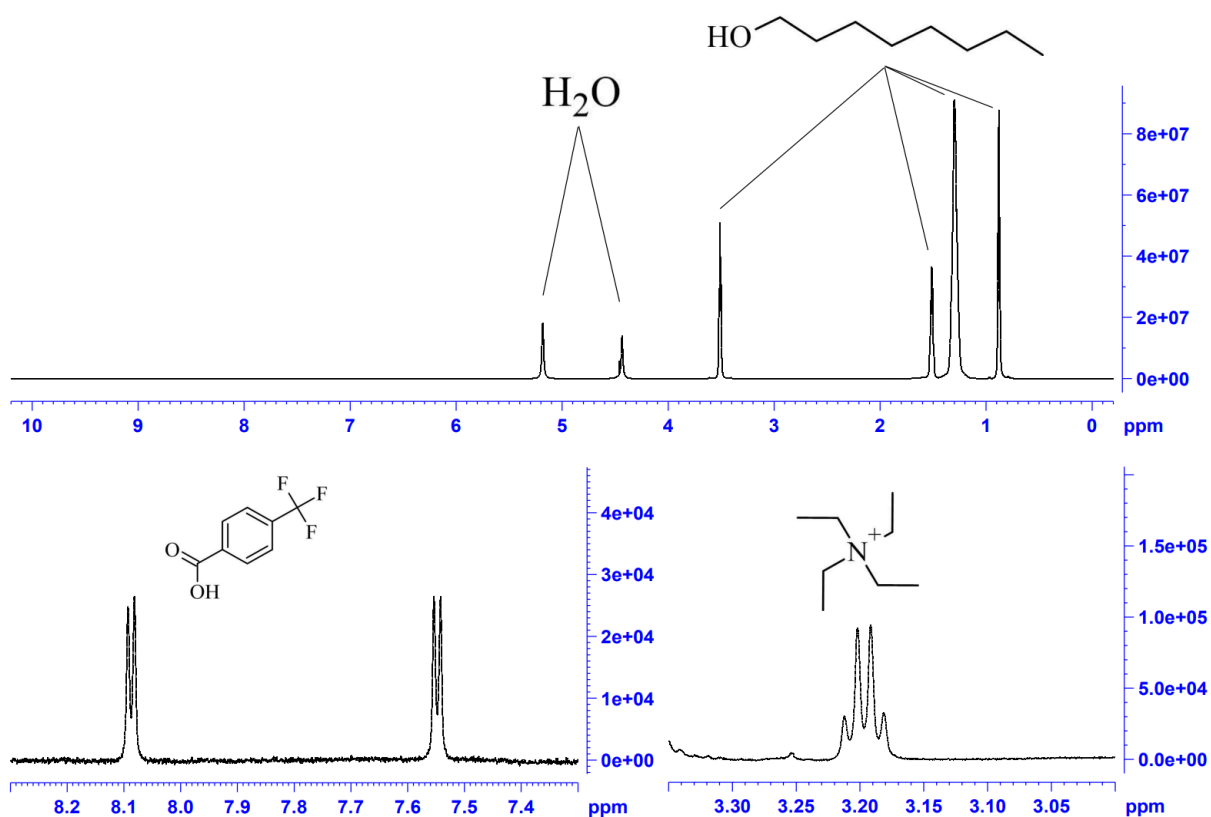
<sup>c</sup> Estimated from pH 11.51 sample at 10 mM concentration with diheptylphosphinic acid.

<sup>d</sup>  $\log P$  evaluations are rough estimates based on our experience with  $pK_a^{\text{ow}}$  measurements.

<sup>e</sup> Peaks from octanol and water are very large relative to the peaks of the analyte. For a reliable analysis, it is important that at least one of the peaks of the analyte is not significantly distorted or covered by the solvent peaks at any relevant pH. Potential peak overlaps in  $^{19}\text{F}$  and  $^{31}\text{P}$  NMR can be avoided completely by choosing suitable calibrants.



**Figure 1.** Example of a <sup>19</sup>F NMR spectrum collected during measurement. 4-trifluoromethylbenzoic acid, pH 4.49, 20 mM



**Figure 2.** Example of a <sup>1</sup>H NMR spectrum collected during measurement. 4-trifluoromethylbenzoic acid, pH 4.49, 20 mM

## 4. Conclusions

NMR with all four commonly used nuclei –  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{19}\text{F}$  and  $^{31}\text{P}$  – can be used for  $pK_a^{\text{ow}}$  measurements.

$^1\text{H}$  has two main advantages. It is the most sensitive nucleus, i.e. low concentrations can be used, extensive migration of the anion into the aqueous phase can be tolerated and measurement is fast. Hydrogen atoms are also almost universally present in molecules (i.e.  $^1\text{H}$  NMR is applicable to almost all compounds). The main disadvantage is that hydrogen atoms are also almost universally present in solvents, leading to possible signal overlaps (unless deuterated solvents are used).  $^{13}\text{C}$  is by far the least sensitive of the four nuclei. Thus, high concentrations and long measurement times are required. Even more importantly, only highly lipophilic compounds can be measured.

$^{19}\text{F}$  and  $^{31}\text{P}$  are relatively sensitive nuclei, so that they have essentially the same advantages as  $^1\text{H}$ , although to a lesser extent. Their distinct advantage against the remaining two nuclei is that there is no overlap with the solvent peaks. This is linked to their biggest disadvantage – applicability only to compounds containing F or P.

Although this work specifically addressed the measurement of  $pK_a^{\text{ow}}$  values, the main conclusions are applicable also to  $pK_a$  measurements commonly carried out in a single solvent medium.

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