Supporting info

Quantifying acidity in heterogeneous systems: biphasic pK_a values

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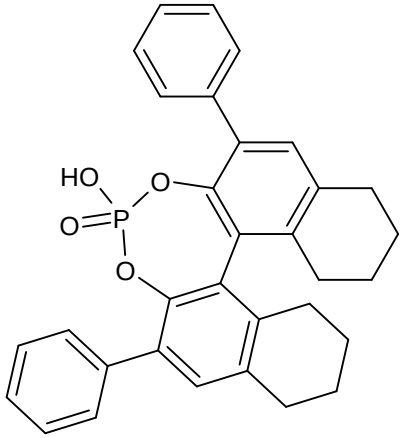
Experimental

Chemicals

In this work, the pK_a^{ow} values of 35 acids (see Table 1 in the main text) were measured. (4-NC_5F_4)(C_6F_5)NH, (4-CN-C_6F_4)(C_6F_5)NH, (4-NC_5F_4)(C_6F_5)CHCN, (4-NC_5F_4)(2-$C_{10}F_7$)CHCN, 3-CF_3-C_6H_4CH$(CN)_2$, (2-$C_{10}F_7$)CH(CN)COOEt, C_6F_5CH$(CN)_2$, sorbic acid, 2-perfluoronaphthol, 2,4,6-tribromophenol, pentabromophenol, pentachlorophenol, benzoic acid, 3-NO_2-$C_6H_4SO_2$NHCOC_6H_4-3-Cl, 4-NO_2-$C_6H_4SO_2$NHCOC_6H_4-3-Cl, 4-NO_2-$C_6H_4SO_2$NHCOC_6H_4-4-CH_3, [Ph]-BPA-[H_8] (see Scheme 1), 4-NO_2-C_6H_4-SO_2-NH_2 and Tos_2NH are same as in ref. [1]. 1-pyrenecarboxylic acid (BLD Pharmatech, ACS reagent, 97%), salicylic acid (Sigma, pharmaceutical secondary standard), hexanoic acid (Aldrich, ACS reagent, ≥99%), 2,4-dinitrobenzoic acid (Lancaster, 98%), lauric acid (C. A. F. Kahlbaum, reagent grade), N-hydroxyphthalimide (Fluka, purum >98%), naproxen (Aldrich, ACS reagent, 98%), (S)-(+)-ibuprofen (Aldrich, ReagentPlus, 99%), stearic acid (Sigma-Aldrich, Grade I, ≥98%), cinnamic acid (a kind gift from Prof. Tullio Ilomets), 4-nitrobenzoic acid (Alfa Aesar, ACS reagent, 99%), Tos-NH-Boc is same as in ref. [2], 2-CN-C_6H_4CH$(CN)_2$ and 2,4-$(CH_3O)_2$-C_6H_3CH$(CN)_2$ (a kind gift from Dr. Toomas Rodima). Acids 4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-3-OCH_3 and 4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-2-F were synthesized as described below. The rest of the acids and other used chemicals were of commercial origin: octan-1-ol (Sigma-Aldrich, ReagentPlus grade), glycine (Reanal, analytical reagent), HEPES (Sigma, ACS reagent, >99.5%), tetraethylammonium chloride (Alfa Aesar, ACS reagent), tetraethylammonium hydroxide (Sigma-Aldrich, 20% w/w in H_2O, ACS reagent). Water was prepared using a MilliQ Advantage A10 setup.



Scheme S1. Structure of [Ph]-BPA-[H_8]

Instruments

For the NMR measurements, Bruker Avance-III 700 NMR spectrometer was used (16.4 T, ^{1}H resonance frequency 700.1 MHz, ^{13}C resonance frequency 176.0 MHz, ^{31}P resonance frequency 283.4 MHz). Measurements were carried out in water-saturated 1-octanol at 25.0 ± 0.1 ^{o}C, using TopSpin 3.2 software. Since deuterated solvents were not used, the most intense and broadest peak of the spectrum

(corresponding to -CH_2 hydrogens of carbons 3-7 in 1-octanol) was used for shimming to correct any inhomogeneities in the applied magnetic field during the NMR measurements. ^{1}H and ^{13}C NMR spectra of the samples were calibrated internally, using the 1-octanol -CH_3 peak (^{1}H δ 0.88 ppm; ^{13}C δ 14.11 ppm). ^{31}P NMR spectra were calibrated externally with 4-NO_2-Ph-N=N-Ph-NPPh(pyrr)$_2$ solution in $CDCl_3$ (^{31}P δ set at 15 ppm).

UV-Vis spectrometric measurements were carried out at room temperature (23.0 ± 2.0 ^{o}C) on a Thermo Spectronic Evolution 300 double-beam spectrophotometer, using 10.00 mm quartz cuvettes. In the measurements where the concentration of the acid of interest was in the sub-mM range, the wavelength for absorbance measurements was chosen so that the difference in molar absorptivities of the neutral acid and its conjugate base was maximal. At higher concentrations the absorbances at these wavelengths were significantly above the usually recommended maximum absorbance value of 1 absorbance unit (AU) and therefore, a longer wavelength (at which the maximum absorbance was below 1 AU) was chosen. 1-octanol was used as the reference solution.

pH was measured using a Mettler Toledo InLab Micro pH-sensor, which was calibrated using pH 4.00 and pH 7.00 Hydrion® buffers. pH 10.00 Hydrion® 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, typically for 5 minutes at 7800 rpm, using an Eppendorf 5430R Centrifuge.

Workflow

The measured samples consisted of 2 mL of the aqueous phase with measured pH and 2 mL of 1-octanol solution of the acid(s) of interest and were prepared into 4 mL vials.

Aqueous phases with different pH values were generated by combining the following solutions:

Solution 1: 0.1 M Et_4NCl + 0.01 M zwitterionic buffering agent (HEPES or glycine);

Solution 2: 0.1 M HCl + 0.1 M Et_4NCl;

Solution 3: 0.1 M Et_4NOH.

The pH of pure Solution 1 is in the 5.3-5.4 range. Solution 2 was used for reducing the pH while Solution 3 was used for increasing the pH. This way the ionic strengths of all the solutions and their combinations were approximately 0.1 M. HEPES and glycine were used as zwitterionic buffering agents. For most measurements, the buffer concentration was kept at a 0.01 M to minimize the effect of buffer on the ionic strength in the aqueous phase, especially outside the zwitterionic pH range.

Once the samples were prepared, they were shaken thoroughly and then centrifuged. Next, the 1-octanol phase was removed from the sample and one or more of UV-Vis,

^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra of the 1-octanol phase was recorded as soon as possible. The pH of the aqueous phase was measured both before it was added to the 1-octanol phase and right after the 1-octanol phase was removed from the sample. In cases of UV-Vis measurements where we suspected that a significant amount of the conjugate base (acid anion) might migrate from the 1-octanol phase to the aqueous phase, the UV-Vis spectrum of the most basic aqueous phase was also obtained.

For most of the UV-Vis measurements, an approximately 1 mM solution of the acid(s) of interest in 1-octanol was made. This was diluted to approximately 10-100 µM in order to achieve suitable absorbances. For the NMR measurements 1-40 mM solutions were used. Due to difficulties with accurate pipetting of 1-octanol solutions, the concentrations of the acids in the solutions were determined gravimetrically.

Calculation methods

pK_a^{ow} of a single measurement

The calculation method is virtually identical for both UV-Vis and NMR methods. Simply put, the calculation method fits a sigmoid curve – absorbance or chemical shift vs pH – to the data points, using a least-squares method (see Figure S1). Absorbance (UV-Vis method) and chemical shift values (NMR method), both denoted below as signal *A*, can directly be related to the degree of dissociation (*α*). The detailed description of the calculation method is given below.

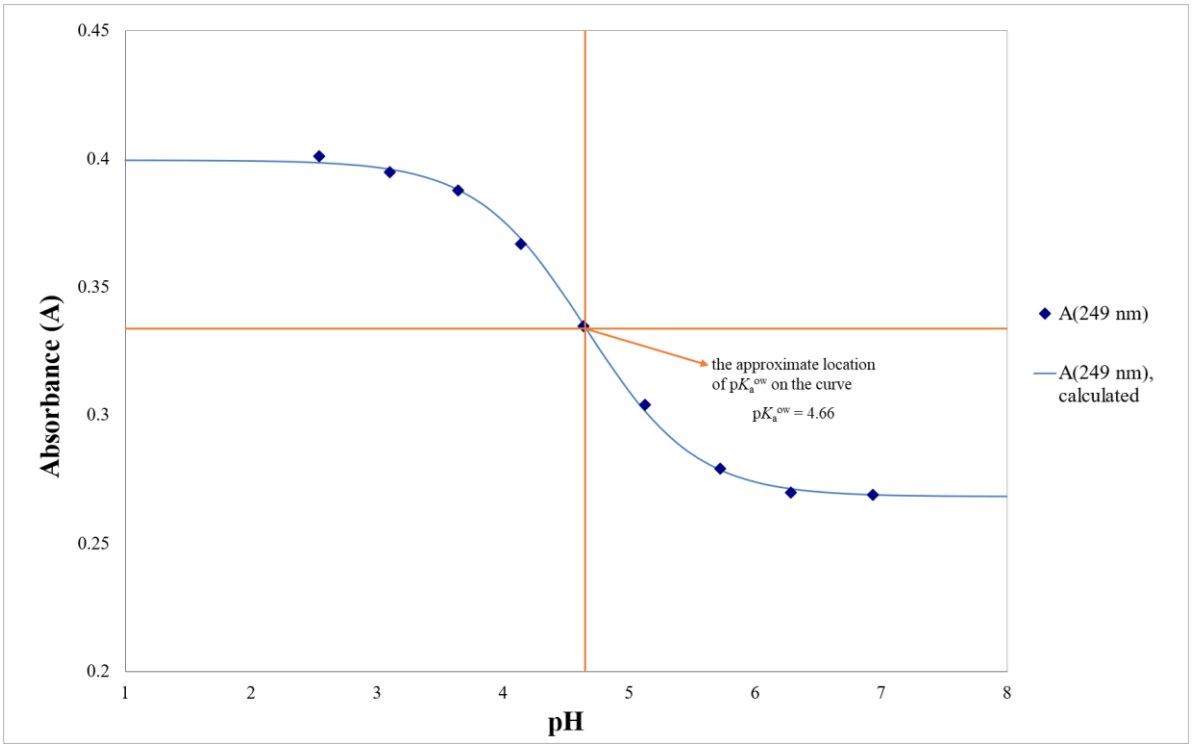


Figure S1. Example of data collected during a single measurement

For every data point i with experimental signal value A_i the corresponding calculated value was expressed as follows:

$$A_{\mathrm{calc,i}} = \alpha_{\mathrm{i}} \cdot A(A^{-})_{\mathrm{calc}} + (1 - \alpha_{\mathrm{i}}) \cdot A(AH)_{\mathrm{calc}} \quad (1)$$

Here, $A(A^-)_{calc}$, $A(AH)_{calc,}$ and $A_{calc,i}$ refer to the calculated signal of the neutral acid, its conjugate base and the weighted sum of the former two in the sample i with a certain pH value, respectively. α_i is the degree of dissociation of the compound in the sample i with a certain pH value and is found as follows (Eq. 2):

$$\alpha_{\mathrm{i}} = \frac{1}{1+10^{(\mathrm{p}K_{\mathrm{a}}^{\mathrm{ow}} - \mathrm{pH_i})}} \quad (2)$$

The sum of squares of differences Δ_i between the experimentally observed values (A_i) and the calculated (from eq 1) values at the same pH value as in sample i ($A_{calc,\,i}$) is then minimized (similarly to ref [3]) by varying three parameters, $A(A^-)_{calc}$, $A(AH)_{calc}$ and pK_a^{ow} (Eq. 3 and 4):

$$\Delta_{\mathrm{i}} = A_{\mathrm{i}} - A_{\mathrm{calc,i}} \quad (3)$$

$$\sum_{\mathrm{i}=1}^{n} \Delta_{\mathrm{i}}^2 \rightarrow min \quad (4)$$

The obtained pK_a^{ow} value is used as the quantitative estimate of the acidity of the compound of interest at the concentration it was measured

ΔpK_a^{ow}

The relative pK_a^{ow} value of two acids at a given concentration (ΔpK_a^{ow}) was determined for some acid pairs. If the obtained ΔpK_a^{ow} agreed well with the difference of individual pK_a^{ow} values at the same concentrations, this was taken as an additional piece of evidence that the method works well. In particular, this was used to verify the correctness of pH measurements – the ΔpK_a^{ow} values do not depend on pH metry, while the individual pK_a^{ow} measurements do. The agreement, thus, indicates that pH measurements were correct. The relative pK_a^{ow} from the solution i can be expressed through the observed degrees of dissociation of both compounds ($\alpha_{1,i}$ and $\alpha_{2,i}$) in the same solution (Eq. 5):

$$\Delta \mathrm{p}K_{\mathrm{a,i}}^{\mathrm{ow}} = \log \frac{(1-\alpha_{1,\mathrm{i}})\alpha_{2,\mathrm{i}}}{(1-\alpha_{2,\mathrm{i}})\alpha_{1,\mathrm{i}}} \quad (5)$$

The α values were expressed from eq. 1 as follows:

$$\alpha_{\mathrm{i}} = \frac{A_{\mathrm{i}} - A(\mathrm{AH})_{\mathrm{calc}}}{A(\mathrm{A^-})_{\mathrm{calc}} - A(\mathrm{AH})_{\mathrm{calc}}} \quad (6)$$

At α values below 0.05 or above 0.95 the accuracy of ΔpK_a^{ow} value decreases. Thus, when calculating the ΔpK_a^{ow}, we included only the samples in which the degree of

dissociation of both compounds was between 0.05 and 0.95. We found these cutoff values appropriate based on our experimental data. Degrees of dissociation of 0.05 and 0.95 for the two compounds would correspond to a ΔpK_a^{ow} of 2.56. However, such difference in acidity could be measured only at a very narrow pH range. Because fine-tuning the pH values of samples in advance is complicated with our experiments (the reasons are explained below), we estimate that this method is not suitable for measurement of ΔpK_a^{ow} values higher than 1.

pK_a^{ow} extrapolated to zero concentration

While the activity of H^+ is measured directly in the aqueous phase with a pH-electrode, the ratios of the neutral acid and its anion were calculated from absorbances at a certain wavelength or chemical shifts. This means that the measured quantity is the ratio of equilibrium concentrations, not the ratio of activities.

Every compound was measured at several different concentrations, and it was apparent from the data that the observed pK_a^{ow} values are dependent on the concentration of the compounds. Generally, the higher was the concentration of the compound, the higher was the observed pK_a^{ow} value. As this effect is likely due to ionic strength effects in the 1-octanol phase, the second approximation of the Debye-Hückel theory was used to model this dependence. We do realise that the Debye-Hückel theory is a crude approximation under the experimental conditions of our study. However, as is demonstrated below, the theory describes the situation surprisingly well.

The ratio of activities in Eq. 4 in main text can be expressed as ratio of molar concentrations as follows:

$$pK_a^{ow} = pH - \log\frac{[A^-\cdot Et_4N^+{}_o]\cdot f_o}{[HA_o]} = pH - \log\frac{[A^-\cdot Et_4N^+{}_o]}{[HA_o]} - \log f_o \quad (7)$$

In Eq. 7, f_o denotes the activity coefficient of the anion in the 1-octanol phase (the activity coefficient of the neutral acid can be assumed 1). According to the Debye-Hückel equation, f_o can be expressed in the following way (Eq. 2):

$$\log f_o = \frac{-A_o\sqrt{I_o}}{1+B_o a\sqrt{I_o}} \quad (8)$$

In Eq. 8, I_o is the ionic strength of the 1-octanol phase, a is the mean effective distance of closest approach of other ions and A_o and B_o are constants dependent on the solvent and temperature.

Based on eqs. 7 and 8, the difference between an observed pK_a^{ow} value (here denoted as pK_{ac}^{ow}) and the "concentration-independent" pK_a^{ow} (here denoted as pK_a^{ow}) can be expressed as follows:

$$pK_a^{ow} = pK_{ac}^{ow} - \log f_o = pK_{ac}^{ow} + \frac{A_o\sqrt{I_o}}{1+B_o a\sqrt{I_o}} \quad (9)$$

We assumed that I_o is approximately equal to the concentration of the anion in 1-octanol at half-neutralization point if no significant migration to the aqueous phase

occurs during measurement. In other words, equal to half of the concentration of the compound in the 1-octanol phase.

Some of the NMR measurements were performed with compounds which migrate significantly into the aqueous phase. This results in reduction of the areas of the peaks which correspond to the compound of interest. As the conjugate bases of the neutral acids of interest are more hydrophilic than the acids themselves, the migration is more pronounced in samples of higher pH. Due to this, the peak areas within the same measurement series generally decrease as the pH increases. To account for this, the ionic strength was corrected for the decrease of concentration of the compound in 1-octanol phase. First, the areas of the compound's peaks in each sample were normalized by diving the area with the area of a suitable 1-octanol peak measured from the same sample. As the migration to the aqueous phase was insignificant in samples of the lowest pH (typically around 3 units lower than the pK_a^{ow} of the compound), even with the most hydrophilic compound measured in this study (N-hydroxyphthalimide), the normalized peak area from the sample with the lowest pH was considered corresponding to the concentration of the compound in 1-octanol solution which was used to prepare the sample. This normalized peak area was then divided by the normalized area of the peak of the analyte from the sample with the closest pH to the observed pK_{ac}^{ow} value to obtain the factor by which the concentration of the analyte in 1-octanol phase was lower than when the sample was prepared. The reduced concentration was then used to calculate ionic strength as explained previously.

The accuracy of this method of estimating the concentration was not thoroughly assessed but is likely in the range of ±20%. However, it is worth nothing that even if the final concentration was over- or underestimated by 100%, the difference in the resulting pK_{ac}^{ow} value, based on our model would be no greater than 0.05. For comparison, we have estimated the combined standard uncertainty of the pK_a^{ow} values in this study to be on the order of 0.1.

The concentration dependence of apparent pK_a^{ow} values was more closely investigated and modelled using two reference compounds, with which a larger number of measurements across a wide concentration range were made. The compounds and principles of their selection are described below.

The model was created using a two-step least-squares minimization. First, the constants A_o, B_oa and the pK_a^{ow} values of the two reference compounds were optimized based on the results of the 29 successful measurements with these two compounds so that the sum of the squared differences between the result of measurement *j* with reference compound *i* (p$K_{ac,i,j}^{ow}$) and the "concentration-independent" pK_a^{ow} value of reference compound *i* (p$K_{a,i}^{ow}$) would be minimal (Eqs. 10-12).

$$\mathrm{p}K_{\mathrm{a},i}^{\mathrm{ow}} = \mathrm{p}K_{\mathrm{ac},i,j}^{\mathrm{ow}} + \frac{A_o\sqrt{I_{o,i,j}}}{1+B_o a\sqrt{I_{o,i,j}}} \tag{10}$$

$$\Delta_{\mathrm{i}} = \mathrm{p}K_{\mathrm{ac},i,j}^{\mathrm{ow}} + \frac{A_o\sqrt{I_{o,i,j}}}{1+B_o a\sqrt{I_{o,i,j}}} - \mathrm{p}K_{\mathrm{a},i}^{\mathrm{ow}} \tag{11}$$

$$\sum_{i=1}^{n} \Delta_i^2 \rightarrow min \quad (12)$$

A_o and B_oa values from this optimization were -48 and 82, respectively. The resulting Debye-Hückel model along with the data points of the references is presented on Figures S2 and S3.

Next, the pK_a^{ow} values of every other compound were minimized while keeping the constants A_o and B_oa from previous minimization. These values are presented in Table 1 in the main text. The mean root square difference of the pK_{ac}^{ow} values and the estimated pK_a^{ow} values using this minimization is 0.06

Additionally, we created a model, where the power of $I_{o,i,j}$, below denoted as n, was introduced as one of the minimized parameters, in addition to A_o and B_oa and the pK_a^{ow} values of the reference compounds, as shown in Eq. 13.

$$pK_{a,i}^{ow} = pK_{ac,i,j}^{ow} - \frac{A_o I_{o,i,j}^n}{1+B_o a \cdot I_{o,i,j}^n} \quad (13)$$

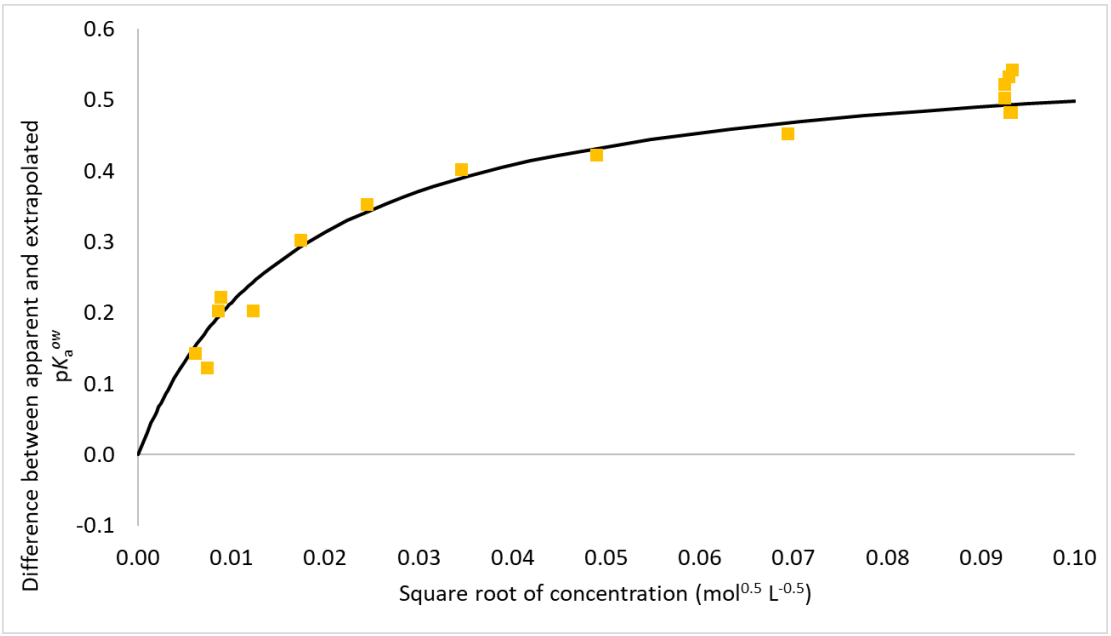


Figure S2. Debye-Hückel plot with the results of measurements with pentabromophenol

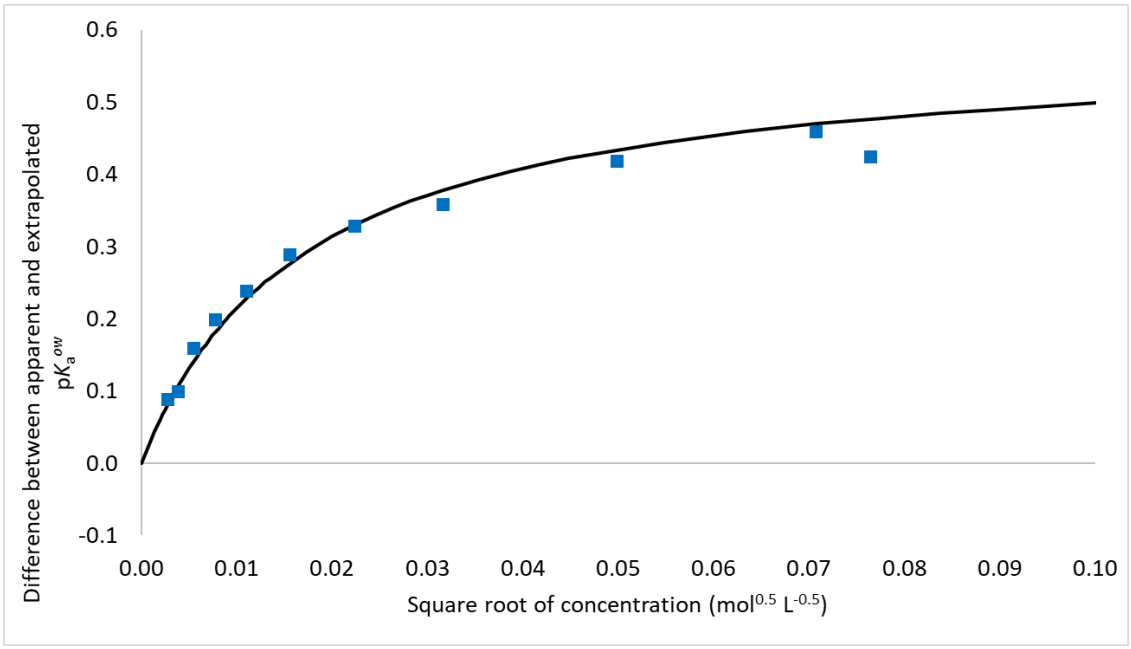


Figure S3. Debye-Hückel plot with the results of measurements with 3-trifluoromethylphenylmalononitrile

This minimization yielded n, A_o and $B_o a$ values of 0.56, 73 and 136, respectively. As the obtained n value is close to 0.5 (which corresponds to square root) and the root mean square difference of the pK_{ac}^{ow} values and the estimated pK_a^{ow} values using this minimization is 0.06, just as with the minimization based on Eq. 10, we decided that allowing the change in the variable n is not justified.

Measurement data

Table S1. General measurement data from every successful absolute pK_a^{ow} measurement in this study. Background color in the column „Result“ indicates the measurement method used for the measurement: yellow – measured with ^{1}H NMR; gray – measured with UV-Vis; orange – measured with ^{13}C NMR, purple – measured with 31P NMR. Double borders indicate that the same samples were used for measurements with both methods.

Compound	Initial concentration (M)	Result (pK_a^{ow})
Br_5-phenol	1.50E-04	**8.18**
	1.50E-04	**8.21**
	3.40E-05	**7.66**
	6.80E-05	**7.67**
	1.40E-04	**7.86**
	3.69E-05	**7.13**
	7.30E-05	**7.19**
	1.50E-04	**7.19**
	3.02E-04	**7.29**
	5.99E-04	**7.34**
	1.19E-03	**7.39**
	2.39E-03	**7.41**
	4.81E-03	**7.44**
	5.39E-05	**7.11**
	8.64E-03	**7.52**
	8.66E-03	**7.47**
	8.69E-03	**7.48**
	8.69E-03	**7.46**
	1.01E-02	**7.46**
	7.85E-05	**7.21**
	8.70E-03	**7.54**
	8.70E-03	**7.52**
	8.56E-03	**7.51**
	8.56E-03	**7.50**
	8.56E-03	**7.48**
Cl_5-phenol	1.00E-04	**7.34**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	2.55E-05	**7.33**
	5.27E-05	**7.32**
2,4,6-Br_3-phenol	1.73E-04	**8.78**
	1.17E-04	**8.78**
	6.82E-05	**8.79**
	2.32E-05	**8.73**
	2.33E-04	**8.89**
	1.10E-04	**8.67**
	1.03E-02	**8.94**
	1.03E-02	**8.95**
	1.08E-04	**8.66**
	5.71E-05	**8.76**
2-perfluoronaphthol	9.72E-05	**7.17**
	1.81E-04	**7.21**
	5.38E-05	**7.19**
	3.18E-05	**7.17**
N-OH-phthalimide	9.97E-03	**9.22**
	9.99E-03	**9.19**
	1.01E-02	**8.99**
(4-NC_5F_4)(C_6F_5)CHCN	2.53E-05	**6.17**
	2.56E-05	**6.15**
	1.27E-05	**6.12**
	3.60E-05	**6.11**
	3.45E-05	**6.34**
	9.22E-06	**6.22**
	4.08E-06	**6.00**
(4-NC_5F_4)(2-$C_{10}F_7$)CHCN	1.77E-05	**5.60**
	1.14E-05	**5.62**
	2.21E-05	**5.71**
	3.28E-05	**5.70**
3-CF_3-C_6H_4CH$(CN)_2$	1.01E-02	**4.49**
	1.01E-02	**4.50**
	5.84E-03	**4.45**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	5.84E-03	**4.42**
	5.00E-03	**4.47**
	2.48E-03	**4.43**
	1.00E-03	**4.37**
	4.98E-04	**4.34**
	2.42E-04	**4.30**
	1.21E-04	**4.25**
	6.00E-05	**4.21**
	3.04E-05	**4.17**
	1.50E-05	**4.11**
	7.53E-06	**4.10**
$C_6F_5CH(CN)_2$	1.18E-04	**2.42**
	4.70E-05	**2.30**
	2.52E-05	**2.35**
2-CN-$C_6H_4CH(CN)_2$	1.64E-04	**3.06**
	3.21E-05	**3.01**
	6.76E-05	**2.93**
	1.17E-05	**2.99**
	1.09E-05	**2.98**
2,4-$(MeO)_2$-$C_6H_3CH(CN)_2$	8.27E-05	**9.26**
	2.82E-05	**9.23**
	5.10E-05	**9.27**
ethylcyano(F_7-2-naphthyl)acetate	6.52E-05	**6.36**
	3.18E-05	**6.38**
	4.64E-05	**6.21**
	3.06E-05	**6.21**
(4-CN-C_6F_4)(C_6F_5)NH	2.24E-05	**11.63**
	1.52E-05	**11.71**
	9.37E-06	**11.89**
(4-NC_5F_4)(C_6F_5)NH	2.03E-05	**11.67**
	3.97E-05	**11.63**
	3.34E-05	**12.03**
$(Tos)_2NH$	9.92E-03	**2.01**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	2.86E-03	**2.01**
	5.60E-03	**2.04**
	5.32E-03	**1.93**
Tos-NH-Boc	1.49E-03	**7.89**
	1.14E-03	**7.90**
	1.97E-03	**8.11**
4-NO_2-C_6H_4-SO_2-NH_2	4.66E-03	**11.92**
	5.87E-03	**12.17**
	8.16E-03	**11.99**
4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-Cl	4.41E-05	**4.25**
	2.19E-05	**3.97**
	4.95E-05	**4.14**
	1.12E-03	**4.42**
	6.83E-04	**4.40**
	3.54E-05	**4.02**
3-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-Cl	5.07E-05	**4.32**
	3.90E-05	**4.34**
	2.97E-05	**4.29**
	2.60E-05	**4.31**
4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-OCH_3	3.98E-05	**4.64**
	2.27E-05	**4.66**
	5.03E-03	**5.07**
	1.96E-03	**5.07**
	5.42E-05	**4.77**
4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-2-F	4.91E-05	**4.42**
	1.49E-05	**4.44**
	2.13E-05	**4.44**
	1.06E-03	**4.90**
	6.82E-03	**4.91**
	3.28E-03	**4.87**
	3.16E-05	**4.47**
4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-4-CH_3	3.90E-05	**5.01**
	1.15E-03	**5.15**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	6.87E-04	**5.17**
	2.86E-05	**4.93**
Salicylic acid	1.01E-02	**6.24**
	5.05E-03	**6.26**
	4.89E-03	**6.22**
	1.54E-02	**6.34**
	1.05E-02	**6.32**
sorbic acid	2.01E-02	**8.05**
	1.50E-02	**8.04**
	1.01E-02	**7.99**
cinnamic acid	2.01E-02	**7.83**
	2.01E-02	**7.84**
	1.06E-02	**7.84**
	2.01E-02	**7.81**
	1.50E-02	**7.83**
	9.94E-03	**7.87**
	1.00E-02	**7.79**
benzoic acid	1.00E-02	**7.85**
	1.05E-02	**7.85**
	9.92E-03	**7.98**
	5.01E-03	**7.77**
	1.46E-02	**7.87**
1-pyrenecarboxylic acid	5.03E-03	**7.65**
	5.03E-03	**7.63**
	3.79E-03	**7.61**
	3.79E-03	**7.55**
	3.26E-03	**7.56**
	3.26E-03	**7.46**
	3.15E-03	**7.55**
	3.15E-03	**7.52**
	2.49E-03	**7.51**
	1.55E-03	**7.62**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	1.55E-03	**7.53**
	1.55E-03	**7.65**
	1.55E-03	**7.57**
	9.95E-04	**7.47**
	9.95E-04	**7.46**
	5.00E-04	**7.46**
	5.01E-04	**7.46**
	2.52E-04	**7.44**
	1.26E-04	**7.36**
	8.67E-05	**7.41**
	6.04E-05	**7.32**
	3.01E-05	**7.31**
	1.52E-05	**7.30**
	9.14E-06	**7.30**
4-nitrobenzoic acid	1.99E-02	**6.62**
	1.51E-02	**6.60**
	1.01E-02	**6.49**
2,4-dinitrobenzoic acid	1.01E-02	**4.35**
	2.02E-02	**4.45**
	1.51E-02	**4.43**
stearic acid	7.89E-03	**8.47**
	1.28E-02	**8.54**
	1.13E-02	**8.52**
	1.02E-02	**8.52**
	8.34E-03	**8.43**
hexanoic acid	4.05E-02	**8.41**
	2.03E-02	**8.37**
	2.00E-02	**8.38**
	1.02E-02	**8.27**
lauric acid	9.98E-03	**8.43**
	9.98E-03	**8.48**
	1.01E-02	**8.48**

Compound	Initial concentration (M)	Result (pK_a^{ow})
	7.61E-03	**8.53**
	1.99E-02	**8.47**
ibuprofen	5.04E-03	**8.16**
	1.03E-02	**8.20**
	4.93E-03	**8.22**
	2.03E-02	**8.22**
naproxen	4.99E-03	**7.98**
	9.99E-03	**8.06**
	1.01E-02	**8.09**
	4.94E-03	**8.05**
	2.02E-02	**8.13**
[Ph]-BPA-[H_8]	1.00E-03	**2.72**
	1.00E-03	**2.73**
	6.44E-04	**2.67**
	1.70E-03	**2.70**
	1.70E-03	**2.71**

Table S2. General measurement data from every successful relative pK_a^{ow} measurement (all with NMR) in this study.

acid 1	acid 2	concentration of acid 1 (mM)	concentration of acid 2 (mM)	**ΔpK_a^{ow}**
naproxen	Br_5-phenol	10.1	10.1	**0.64**
naproxen	ibuprofen	4.9	4.9	**0.17**
lauric acid	N-OH-phthalimide	10.0	10.0	**0.77**
lauric acid	N-OH-phthalimide	10.1	10.0	**0.71**
stearic acid	cinnamic acid	10.2	10.6	**0.65**
benzoic acid	cinnamic acid	9.9	9.9	**0.10**

Practical considerations

Selection of compounds and measurement technique

Absolute pK_a^{ow} measurement methods are generally suitable for compounds which have a pK_a^{ow} value between 1.5 and 12.5, as samples with pH below 1 and above 13 cannot be prepared under the experimental conditions described above. Compounds of interest must also be suitable for either UV-Vis or NMR measurement method.

The UV-Vis method is applicable for highly lipophilic compounds that exhibit UV-Vis spectral changes upon changes in its degree of dissociation. As absorbance is proportional to concentration (Lambert-Beer law), a significant change in concentration would result in changes in absorbance even if α remains same. Although correcting for the changes in concentration was attempted, the obtained results could not be considered reliable. Therefore, in case of UV-Vis measurement of pK_a^{ow}, high lipophilicity of the acid (e.g pentabromophenol) of interest is a requirement. For most compounds measured with the UV-Vis method in this study, the UV-Vis spectral changes were reliably observed at concentrations at around 10 µM and higher.

Acids that are not suitable for the UV-Vis method can often be analysed using the NMR method as most acids have at least one hydrogen atom in the vicinity of the acidity center so that changes in chemical shift at different degrees of dissociation can reliably be observed. Serious overlaps between the peaks of the compound of interest and 1-octanol peaks are possible but with all acids investigated in this work it was possible to find suitable peaks that did not overlap with 1-octanol peaks. ^{13}C NMR can also be considered in cases ^{1}H NMR is not applicable (e.g pentabromophenol). However, ^{13}C NMR is significantly less sensitive than ^{1}H NMR, which in turn is significantly less sensitive than UV-Vis. Thus, ^{1}H NMR requires typically at least 1 mM concentration when measuring highly lipophilic compounds. In the case of compounds that partition into the aqueous phase to a large extent, several times higher concentrations are needed. As ^{13}C NMR is still less sensitive than ^{1}H NMR, it is suitable only for highly lipophilic compounds and typically requires concentrations at least 5 mM. For comparison, the sensitivity of ^{31}P NMR, based on measurements with [Ph]-BPA-[H_8], was similar to ^{1}H NMR. In NMR measurements with two CH-acids, 3-trifluoromethylphenylmalononitrile and 2,4-dimethoxyphenylmalononitrile, the NMR peaks of the compounds at pH values within ± 2 units of the pK_a^{ow} value broadened to such extent that reliable determination of the peak position was not reasonably possible. This issue occurs presumably due to slow proton exchange kinetics, a common feature of CH-acids. While it is remarkable that the results with 3-trifluoromethylphenylmalononitrile were consistent with the results of the UV-Vis measurement of the same samples, the NMR method should be used cautiously for measuring the pK_a^{ow} of CH-acids.

Selection of reference compounds

Reference compounds must be suitable for measurements across a wide concentration range, including the low concentrations, accessible for the UV-Vis method only. Therefore, the acid must meet all the UV-Vis method requirements. In order to ensure higher reliability of the results, the pK_a^{ow} value should be between 3 and 11 and the solubility of the compound in 1-octanol should be high enough to allow preparation of up to at least 1 mM solutions. Additionally, the reference compound should be suitable for the NMR method as well.

For this study, we initially chose pentabromophenol, 3-trifluoromethylphenylmalononitrile and 1-pyrenecarboxylic acid as the reference compounds. With 1-pyrenecarboxylic acid, unfortunately, the results at higher

concentrations were inconsistent. With all these compounds we performed some measurements in which the same samples were analyzed with both UV-Vis and NMR methods. 1-pyrenecarboxylic acid was the only one with which the discrepancy between the results obtained with UV-Vis and NMR method varied by more than 0.03 units. Therefore, we decided to exclude 1-pyrenecarboxylic acid from the reference compounds. The suboptimal performance of 1-pyrenecarboxylic acid is also visible in Figure S4.

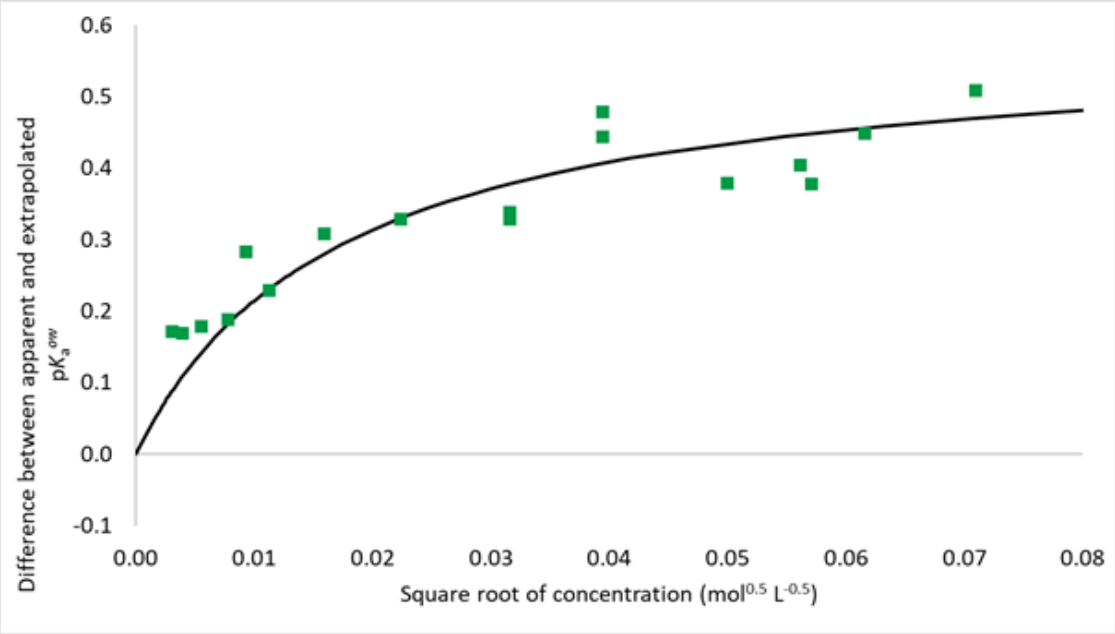


Figure S4. Debye-Hückel plot with the results of measurements with 1-pyrenecarboxylic acid

Measurements of ΔpK_a^{ow}

In addition to measuring pK_a^{ow} of individual acids, we also performed measurements with solutions containing up to two acids (Table S2). The result of a measurement with one compound is the absolute pK_a^{ow} of that compound. A measurement with two compounds simultaneously in the same solution yields the absolute pK_a^{ow} values of both compounds, as well as their relative acidity, ΔpK_a^{ow}. It is worth noting that ΔpK_a^{ow} does not depend on the accuracy of pH measurement, because pH is equal for both compounds in the same solution.

Measuring solutions of two or more acids has some additional limitations. It is difficult (but possible[1]) to measure multiple compounds simultaneously using the UV-Vis method, as spectral overlaps are virtually guaranteed. In the case of NMR, for every compound, there needs to be at least one peak in the NMR spectrum that can clearly be attributed to that compound and does not overlap with any of the peaks of the other compounds in the solution (including 1-octanol itself) at any relevant aqueous phase pH. This is usually possible, unless the measured compounds are very similar. Generally, the concentration of each compound needs to be at least 1 mM for ^{1}H and

^{31}P NMR and 5 mM for ^{13}C NMR, to ensure sufficient intensity of its signal. It is worth nothing that ^{31}P NMR was used to determine the pK_a^{ow} value of just one compound, [Ph]-BPA-[H_8]. However, the result was consistent with the corresponding ^{1}H result, proving that ^{31}P NMR is applicable for measuring pK_a^{ow}.

The agreement between the ΔpK_a^{ow} values and the respective pK_a^{ow} differences can be characterised by root mean square deviation, equal to 0.11. This can be considered good, when taking into account that the ΔpK_a^{ow} values are the ones directly found at initial acid concentrations in the range of 5-10 mM, while the individual pK_a values are the ones extrapolated to zero concentration.

Choice of pH

Absorbance and chemical shift values at different solution pH values are directly influenced by the degree of dissociation (Eq. 1), as they all similarly form a sigmoid with two plateaus: one at pH values significantly higher than the pK_a^{ow} of the compound (corresponding to α of close to 1) and one at significantly lower pH values than the pK_a^{ow} of the compound (corresponding to α of close to 0), as shown on Figure S1. Significant changes in the degree of dissociation and therefore, chemical shift and absorbance values, can be observed at pH values generally within 2 units of the pK_a^{ow} values. In order to calculate the pK_a^{ow}, it is necessary to determine the chemical shift or absorbance, jointly referred to as signal, values of the plateaus and some signal values at pH within ±2 units from the expected pK_a^{ow} value. In the case of more extreme pK_a^{ow} values (such as below 3 and above 11), the chemical shift or absorbance of one of the plateaus cannot be determined directly as pH values of less than 1 and more than 13 cannot be prepared with the set of solutions defined above. Therefore, the number of absorbance or chemical shift values at various α values should be higher (preferably more than 6), so that the part of the sigmoid curve that cannot be directly measured could instead be reliably extrapolated from these data points. In any case, the larger the number of measured signal values at pH within ±2 units from the pK_a^{ow}, the higher the reliability of the result by increasing the number of data points as well as providing means to discover possible inconsistencies in the measurement. The pH values should be chosen so that the degrees of dissociation represented across the samples would be as different as possible.

We observed that in the 1-100 µM concentration range, typically used in the UV-Vis spectrophotometric measurements, the difference between the pH values obtained before and after adding the 1-octanol phase (containing the acid) was negligible. However, in the 1-10 mM range, typically used in NMR measurements, the measured pH after adding the 1-octanol phase was sometimes by up to several pH units lower. This is due to the larger amount of acid used, so that its partial dissociation in the 1-octanol phase and migration of the H^+ ions into aqueous phase in larger amount than the buffer system can compensate. This effect becomes more evident as the concentration of the acid is increased. This also means that in case the concentration of the acid of interest is higher than 100 µM, the initial pH of the aqueous phase is only relevant for approximately estimating the equilibrium pH of the sample, but not for calculating the pK_a^{ow} value.

The extent, to which the pH of the aqueous phase decreased, was in most cases difficult to predict, as it is apparently dependent on various experimental properties, such as concentration and acidity of the acid, as well as the buffering capacity of the used buffer system in the specific pH region. Because of this, the preparation of samples of various degrees of dissociation of the acid, especially between 0.2 and 0.8, was often achieved through trial-and-error. We noticed that while this can be performed reasonably well at 20 mM and lower acid concentrations, it can easily become very time-consuming at 40 mM and above. In some cases, in order to obtain samples with moderate degree of dissociation (for example, this concerns pH values roughly between 7 and 8 for an acid with pK_a^{ow} value of 7.5) at such acid concentration, initial pH values of the aqueous phases (that is, before adding the 1-octanol phase) above 12 were required. In such instances, a change of pH by 0.02 units resulted in a difference of more than 2 pH units in post-extraction pH. Therefore, we recommend avoiding concentrations above 20 mM in pK_a^{ow} measurements.

The pH could also be measured with the 1-octanol and aqueous phases still at equilibrium. However, the measurements are performed with relatively low volumes of solution (2 mL of the 1-octanol phase and aqueous phase, each) and an accurate measurement of pH would require stirring the aqueous phase. This would result in significant amount of excess aqueous phase being emulsified into the 1-octanol phase, complicating the analysis of the 1-octanol phase. We compared the measured pH value of a sample with the phases at equilibrium and the measured pH of the same sample with the 1-octanol phase extracted from it. The differences were no larger than 0.02 units and can therefore be considered negligible.

Separation of the phases

In most of the measurements of this study, the prepared samples were allowed to stand for 30 minutes after being thoroughly shaken, so that the separation of the phases could take place. However, when measuring relatively hydrophilic compounds, such as benzoic acid, with the NMR method, the consistency of the data points was significantly worse than in the case of more lipophilic compounds, resulting in decreased consistency of the results. This was apparently due to some of the aqueous phase being emulsified in the 1-octanol phase in some of the samples, typically those of higher pH. As the aqueous pK_a values for compounds studied are lower than the pK_a^{ow} values, the portion of the compound in the aqueous droplets is significantly more dissociated, leading to the measured average chemical shift value of the affected sample shifting towards a more dissociated acid. This resulted in the observed chemical shift range across different aqueous phase pH values being narrower. The issue was eliminated by centrifuging the samples and once this was introduced, all samples were centrifuged from there on. In order to assess whether any of the previously obtained results were erroneous, we performed at least one confirmatory measurement with each compound in which the samples were centrifuged. We compared the chemical shift ranges and all measurements with significantly narrower chemical shift ranges were deemed unreliable and left out of the results.

Summary of measurement methods used

In this work, three techniques were used to measure the ratio of the neutral and the anion (conjugate base) of the acid of interest – UV-Vis spectrophotometry[4], ^{1}H NMR spectrometry and ^{13}C NMR spectrometry. A comparison of these techniques is presented in Table 2. ^{31}P NMR is not included in this comparison as this method was used with just one compound.

The most important difference arises from the way how acid concentration, or more specifically, its change during measurement affects the measurement. According to the Lambert-Beer law, absorbance (UV-Vis method) is proportional to concentration. Therefore, in case of UV-Vis measurement of pK_a^{ow}, constant concentration of the acid of interest is a requirement. In contrast, the chemical shift (NMR) is independent of the concentration of the compound. Thus, reliable analysis is possible with part of the compound partitioning into water, unless the partitioning is so extensive that it is impossible to obtain NMR signals in 1-octanol. The signal-to-noise ratio of an NMR measurement can be improved by collecting the spectrum during a longer time period, thereby increasing the clock time (but not the operator time).

NMR methods are suitable for relative pK_a^{ow} measurements as the likelihood of spectral overlap between compounds of interest and 1-octanol is relatively low. In case of UV-Vis method, spectral overlaps are likely and therefore, relative pK_a^{ow} measurements are very complicated.

Table 2. Comparison of the most important features of the UV-Vis spectrometry and NMR spectrometry for measuring pK_a^{ow} values.

Feature	**UV-Vis**	**^{1}H NMR**	**^{13}C NMR**
Approximate minimal concentration	1 µM	1 mM	5 mM
Approximate maximal concentration	20 mM	20 mM	20 mM
Amount of compound (M = 200 g mol^{-1}) required for a measurement	5 mg	5 mg	25 mg
Reasonable application range of compounds	Very lipophilic compounds which exhibit UV-Vis spectral changes upon protonation	Moderately lipophilic compounds with a H atom and within 10 atoms of the acidity center	Very lipophilic compounds with a C atom within 10 atoms of the acidity center
Is the method suitable for ΔpK_a^{ow} measurements?	No	Yes	Yes
Is reliable measurement of compounds partially partitioning into the aqueous phase feasible?	No[a]	Yes	No
Typical operator time required to perform one measurement series[b]	3 hours	3 hours	3 hours
Sensitivity to possible impurities, due to spectral overlap	High	Low	Very low
Within-lab reproducibility (interim precision) in the case of well-behaving compounds (in pK_a^{ow} units)	0.03	0.03	0.03

[a] In principle it is possible to correct for the decrease of concentration and this was tried but led to so unstable and uncertain results that this approach was abandoned. [b] The NMR spectrometer used in this work allows automation of the measurements whereas the UV-Vis spectrometer does not. Therefore, recording of the UV-Vis spectra needs to be supervised. The required clock time depends on the concentration and how much the compound leaks into the aqueous phase, ranging from roughly 1 to 16 hours for ^{1}H NMR. ^{13}C NMR is not recommended for compounds that significantly leak into the aqueous phase, as the minimal analysis clock time is roughly 12 hours.

As the likelihood of the signal of a possible impurity affecting the signal of the analyte is much lower in case of NMR (especially ^{13}C NMR), the NMR methods can be considered markedly less sensitive for impurities.

The main drawback of NMR (especially ^{13}C NMR) is the significantly higher required concentration and therefore, the amount of compound required, as NMR is generally significantly less sensitive than UV-Vis.

^{19}F and ^{31}P NMR can be considered for pK_a^{ow} measurements as an alternative to ^{1}H and ^{13}C. However, as none of the components of the experimental system contains a suitable reference, the spectra would either need to be calibrated externally using a sealed capillary containing a suitable reference compound, or another ingredient would need to be introduced to the solutions, adding another layer of difficulty to the measurements. For this reason and because of no urgent need, ^{19}F and ^{31}P NMR were not used extensively in this study. The suitability of ^{31}P NMR for pK_a^{ow} measurements was briefly tested with [Ph]-BPA-[H_8], using external calibration. The results at concentrations above 1 mM were consistent with the corresponding ^{1}H NMR results. Based on this, ^{31}P NMR can be considered applicable for measuring pK_a^{ow} values.

The NMR method occasionally has difficulties with measuring the pK_a^{ow} of CH-acids, as slow proton exchange kinetics results in seriously complicated determination of peak positions in the spectra of solutions that have mixture of anion and neutral.

Literature sources of pK_a values in H_2O

Sorbic acid 4.62,[5] cinnamic acid 4.44,[6] benzoic acid 4.20,[7] hexanoic acid 4.88,[8] lauric acid 4.85,[9] ibuprofen 4.31,[10] naproxen 4.18,[11] 4-nitrobenzoic acid 3.44,[12] 2,4-dinitrobenzoic acid 1.42,[13] Br_5-phenol 4.82,[14] Cl_5-phenol 4.75,[15] 2,4,6-Br_3-phenol 6.17,[16] 4-phenylazophenol 8.30,[17,18] 4-NO_2-C_6H_4-SO_2-NH_2 9.04,[19] $(Tos)_2NH$ 1.7,[20] Tos-NH-Boc 5.05.[21]

Synthesis and characterization data

4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-3-OCH_3
3-methoxy-N-((4-nitrophenyl)sulfonyl)benzamide

Dry acetonitrile (15 ml) and triethylamine (2.25 g) was added to 4-nitrobenzenesulfonamide (0.28 g, 1.4 mmol) that were weighed into 50 ml round bottom flask. 3-methoxybenzoyl chloride (0.24 g, 1.4 mmol), dissolved in dry acetonitrile (10 ml), was added dropwise to the solution of sulfonamide. The reaction was carried out under nitrogen atmosphere. The solution was then refluxed for 3 hours. After the completion of reaction (monitored by TLC), the reaction mixture was concentrated under reduced pressure to get the slightly oily product. The oily residue was treated with water (slightly acidic), the precipitated white solid was filtered off. The crude compound was recrystallized from ethanol to get the pure compound (0.31 g, 1.4 mmol, yield 65%) as white crystals.

^{1}H NMR (700.1 MHz, DMSO, 25 °C): δ = 8.45 (m, 2H), 8.25 (m, 2H), 7.70 (m, 2H), 7.31 (m, 2H), 2.36 (m, 3H) ppm.

^{13}C{^{1}H} NMR (176.0 MHz, DMSO, 25 °C): δ = 165.6, 159.2, 150.2, 145.0, 132.7, 129.8, 129.3, 124.5, 120.8, 119.8, 113.0, 55.4 ppm.

ESI-FT-ICR-MS: *m/z* calc for $[C_{14}H_{11}N_2O_6S]^-$: 335.03433 $[M-H]^-$; found: 335.03400.

m.p. 182.9 – 183.5 °C.

4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-2-F
2-fluoro-N-((4-nitrophenyl)sulfonyl)benzamide

Dry acetonitrile (15 ml) and triethylamine (2.5 g) was added to 4-nitrobenzenesulfonamide (0.28 g, 1.4 mmol) that were weighed into 50 ml round bottom flask. 2-fluorobenzoyl chloride (0.22 g, 1.4 mmol), dissolved in dry acetonitrile (10 ml), was added dropwise to the solution of sulfonamide. The reaction was carried out under nitrogen atmosphere. The solution was stirred for 24 hours at room temperature. After the completion of reaction (monitored by TLC), the reaction mixture was concentrated under reduced pressure to get the white solid product. The crude compound was recrystallized from ethanol to get the pure compound (0.22 g, 0.69 mmol, yield 50%) as white powder.

^{1}H NMR (700.1 MHz, DMSO, 25 °C): δ = 8.45 (m, 2H), 8.25 (m, 2H), 7.61 (m, 2H), 7.32 (m, 2H) ppm.

^{13}C{^{1}H} NMR (176.0 MHz, DMSO, 25 °C): δ = 163.2, 160.2 (d, J_{CP}=253 Hz), 150.2, 144.4, 134.5, 130.3, 129.2, 124.6, 124.5 (d, J_{CP}=3.2 Hz), 121.2 (d, J_{CP}=12.9 Hz), 116.4 (d, J_{CP}=21.8 Hz) ppm.

ESI-FT-ICR-MS: *m/z* calc for $[C_{13}H_8N_2O_5FS]^{-}$: 323.01435 $[M-H]^{-}$; found: 323.01437.

m.p. 159.2 – 159.9°C.

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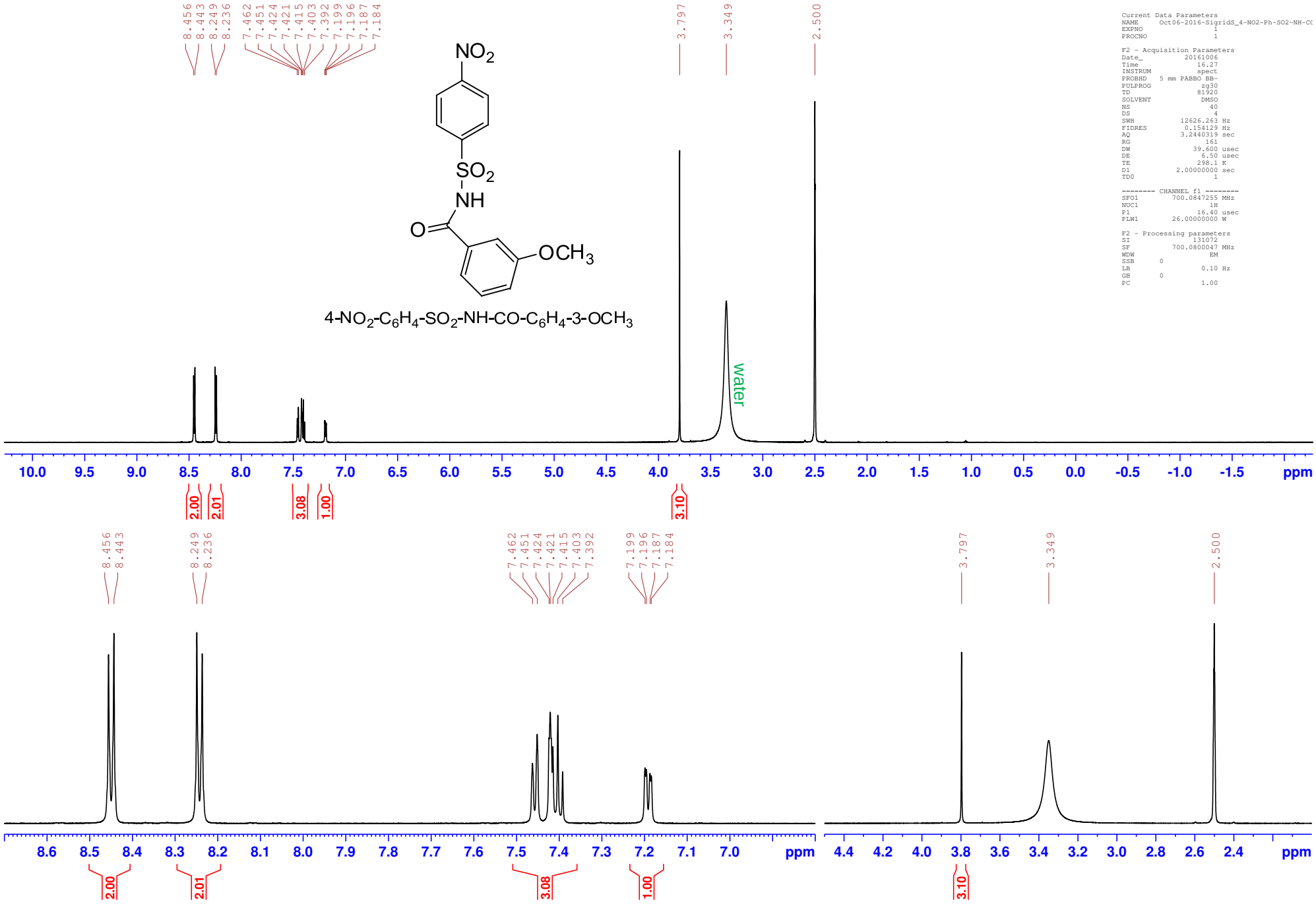
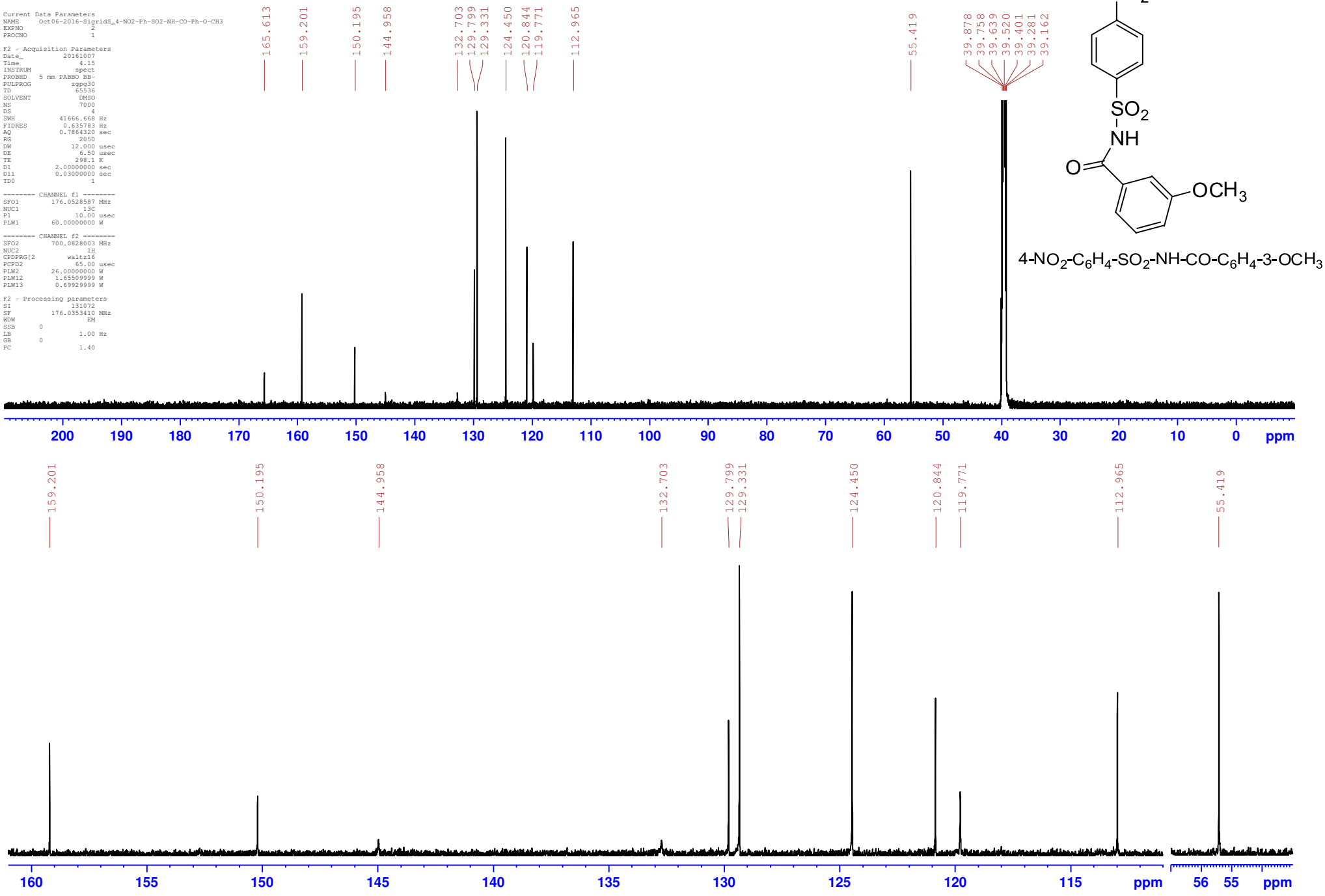
NMR and HRMS spectra

^{1}H NMR spectrum (700.1 MHz) of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-OCH_3, DMSO



^{13}C NMR spectrum (176.0 MHz) of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-OCH_3, DMSO



HRMS spectrum of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-3-OCH_3

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Varian MS

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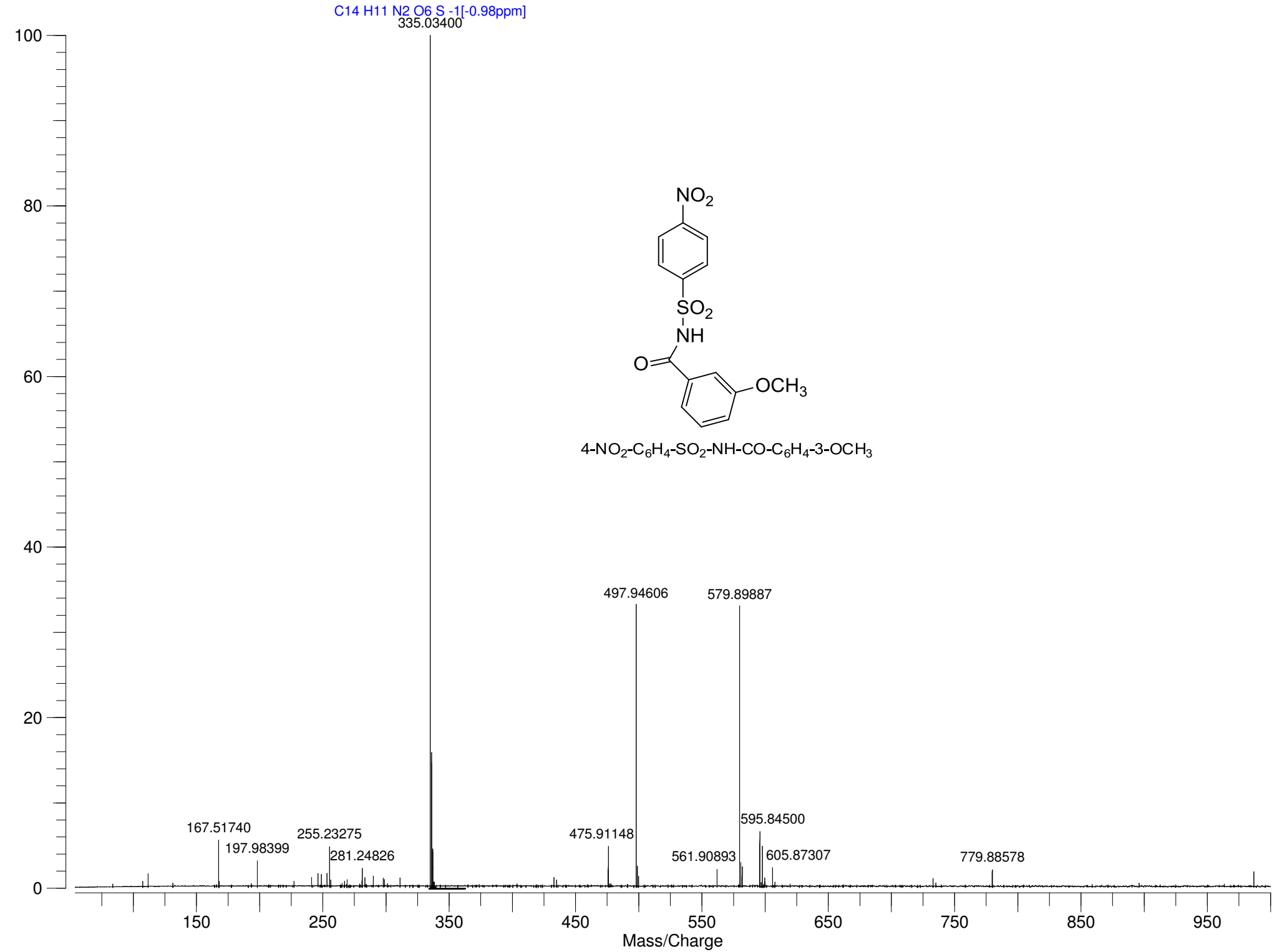
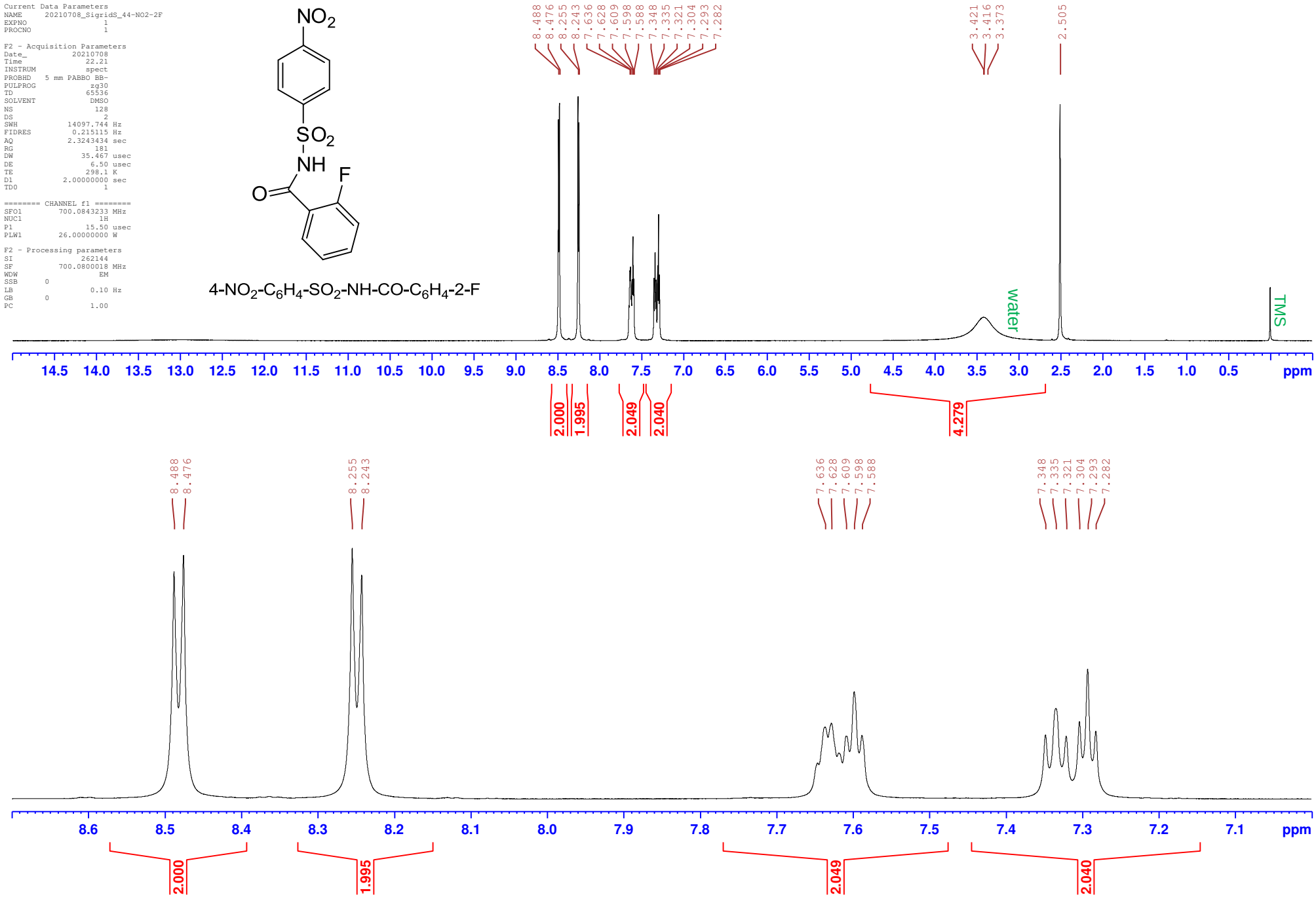
C14 H11 N2 O6 S -1[-0.98ppm]

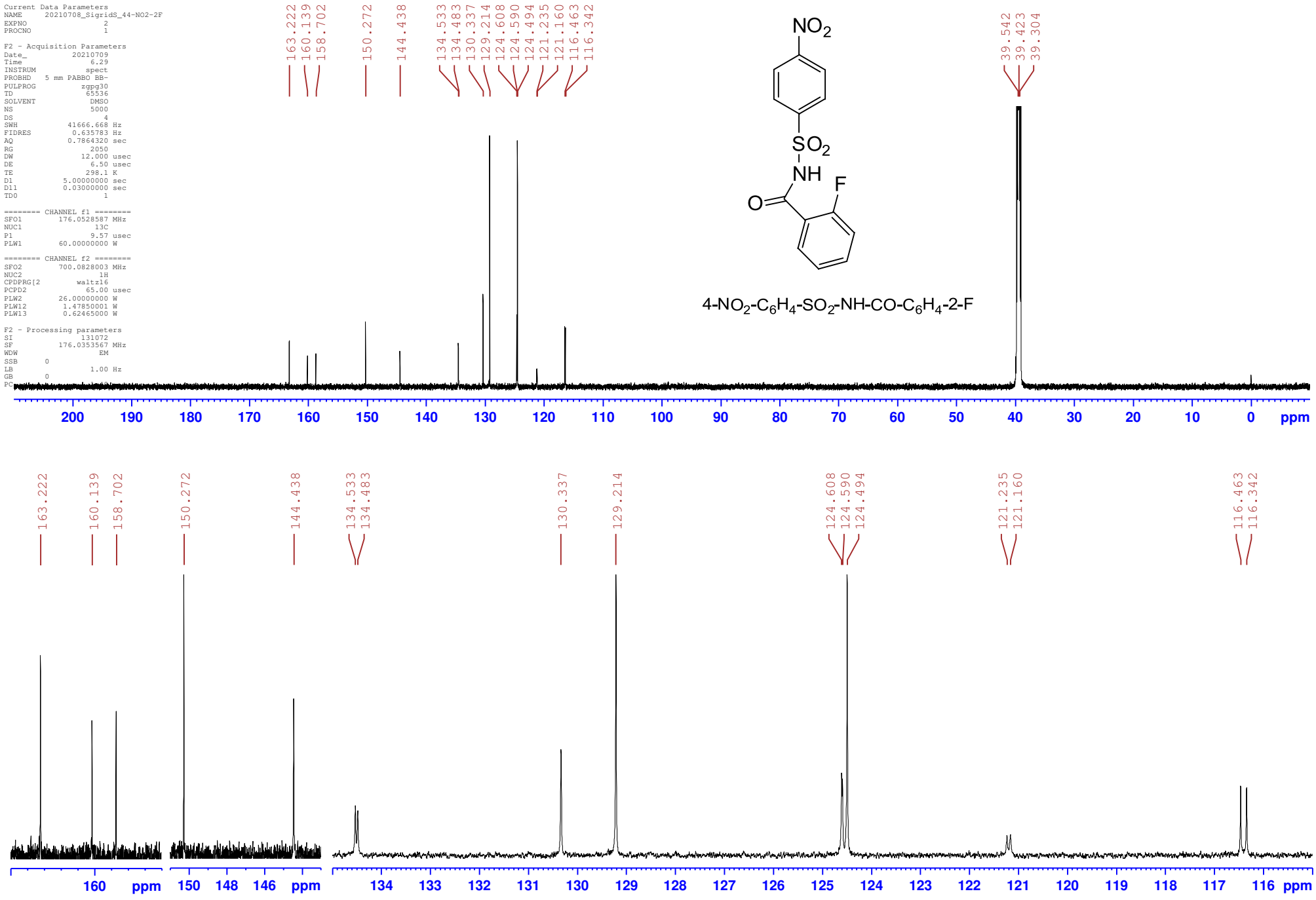


4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-3-OCH_3

^{1}H NMR spectrum (700.1 MHz) of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-2-F, DMSO



^{13}C NMR spectrum (176.0 MHz) of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-2-F, DMSO



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HRMS spectrum of compound 4-NO_2-$C_6H_4SO_2NHCOC_6H_4$-2-F

Varian MS
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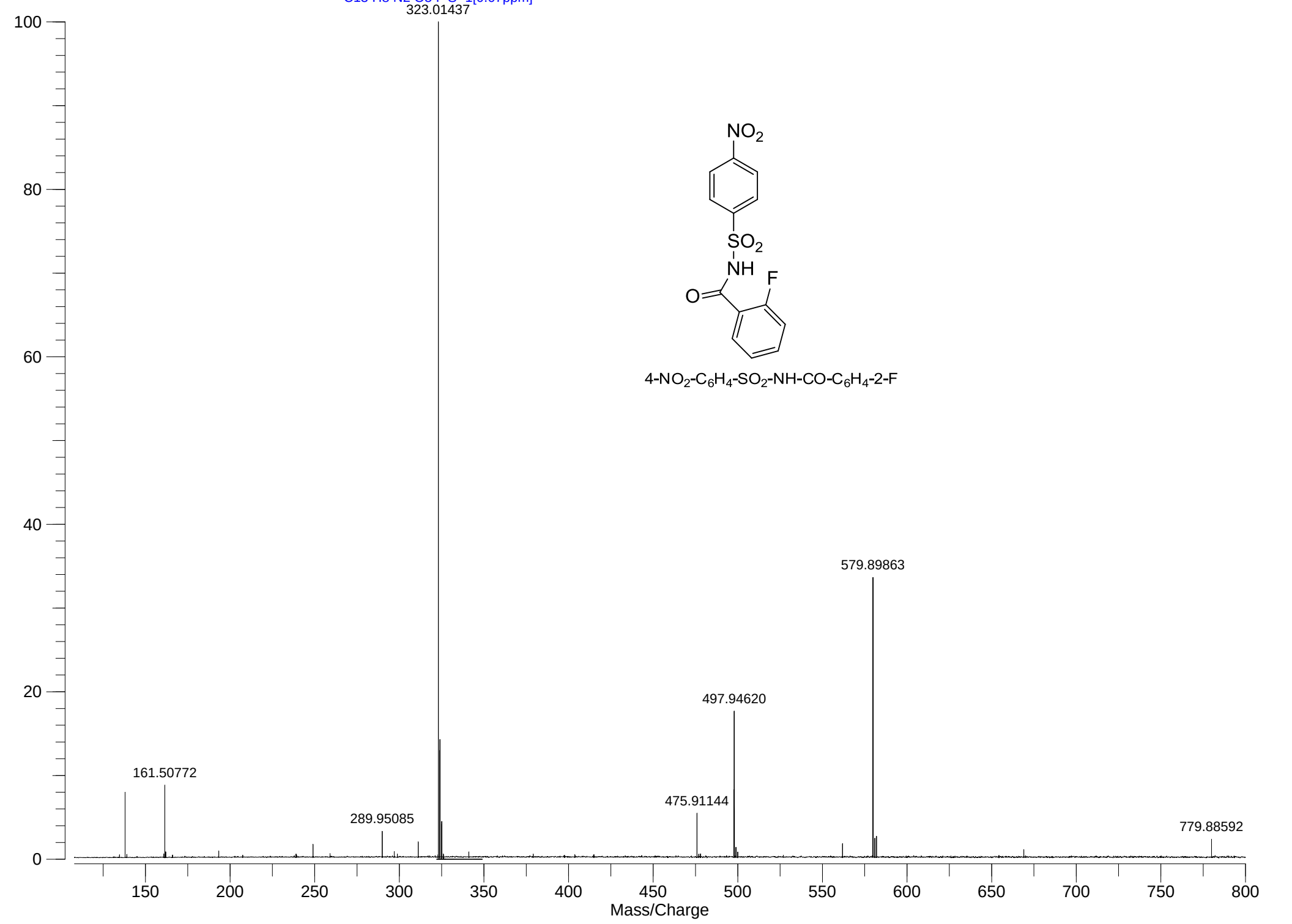

4-NO_2-C_6H_4-SO_2-NH-CO-C_6H_4-2-F