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Article

Practical pH Measurements in Wet Organic Solvents by Gradient NMR: From Charged Amino Acid pK_a to Identification of Ionizable Natural Products in Crude Extracts

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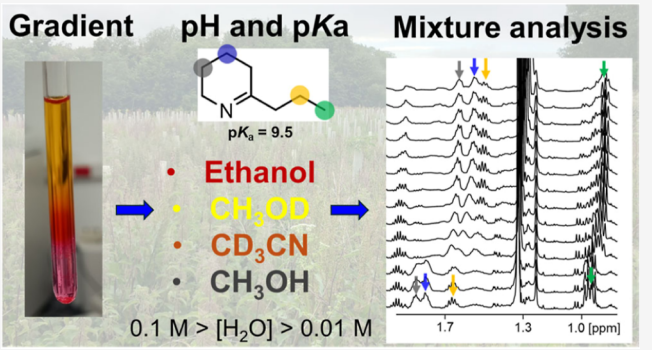
ABSTRACT: We present NMR-based pH measurements in nondried commercial methanol, methanol−OD, ethanol, and acetonitrile. Combining these measurements with pH gradients and spatially selective NMR enables determination of pK_a and provides a highly effective tool to analyze complex molecular mixtures and identify unknown organic compounds by the functional groups present. We determine all the pK_a values of alanine, glutamic acid, histidine, lysine, proline, tryptophan, and tyrosine in methanol−OD, filling a major gap in the literature for these important polyprotic molecules that exhibit zwitterionic behavior. To demonstrate the potential of our method for the identification of compounds in natural mixtures, we use pH gradients to identify alkaloids and other ionizable organic compounds in unrefined extracts of hemlock (*Conium maculatum*), orange lichen (*Xanthoria parietina*), and love-in-the-mist (*Nigella damascena*). No chromatographic separation is necessary, highlighting how our technique could provide a fast and untargeted screening of biological samples, reaction mixtures, and digestates.



INTRODUCTION

Polar organic solvents such as methanol, ethanol, and acetonitrile are widely used media for synthesis, analysis, chromatography, extraction, and formulation. These processes can be strongly pH-dependent and frequently generate highly complex mixtures of organic molecules.[1−3] In such cases, recording NMR spectra of samples at different pH values can help reveal the components present,[4,5] while knowledge of pK_a can guide process design. However, the electrochemical measurement of pH or pK_a in these solvents presents significant challenges.[1] Libraries of indicator compounds have been constructed in acetonitrile that enable determination of relative pK_a via NMR or UV−vis.[6,7] However, these scales are typically based on absolute pK_a values that have been measured at high dilution ($<10^{-4}$ M) in dry solvents that may not be representative of typical laboratory media. Furthermore, tabulated literature data are largely restricted to monoprotic compounds, leaving important classes of polyprotic molecules such as amino acids undocumented.

Here, we derive NMR-based ab initio pH scales and determine pK_a in nondried commercial methanol−OH (CH_3OH), methanol−OD (CH_3OD), ethanol−OH and acetonitrile-d_3 (CD_3CN) by adapting our method for aqueous−organic solvent mixtures.[8] We find that the level of water in the alcoholic solvents (ca. 40−100 mM) does not significantly affect absolute pK_a values relative to literature values determined in dried media,[9] as has also been reported in dimethyl sulfoxide (DMSO).[10,11] However, significant differences (>1 unit) are observed in acetonitrile, suggesting that pK_a values of indicator molecules determined in dry dilute acetonitrile cannot be used to determine absolute pH or pK_a in nondried media at higher solute concentrations (ca. 0.1 M).[7,12,13] We construct pH gradients in NMR tubes by layering a basic sample on top of either solid acid or concentrated acidic solution. Diffusion of the acid up the tube generates a continuous and predictable spatial variation in pH that can be monitored at each vertical position from the ^{1}H chemical shifts of the indicators by chemical shift imaging (CSI) using our measured pK_a values.[8,14] The high pH-resolution afforded enables us to determine all the pK_a values of tryptophan, alanine, proline, lysine, tyrosine, glutamic acid, and histidine in CH_3OD in single experiments. The carboxyl

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and amino pK_a values are determined as ca. 6 and 12 in all cases, respectively, indicating that the amino group will be uncharged only under strongly basic conditions in alcoholic solvents. The wide pH ranges afforded also enable us to assign resonances to molecules in complex mixtures[4] and determine which functional groups are present from the pH-dependence of their NMR resonances. As proof of concept, we identify pharmacologically active compounds in unrefined extracts of hemlock (*Conium maculatum*, CH_3OD), love-in-the-mist (*Nigella damascena*, CD_3CN) and common orange lichen (*Xanthoria parietina*, ethanol). As most ionizable functional groups do not present distinct NMR resonances, the combination of pH gradients and CSI is complementary to existing tools for complex mixture analysis such as diffusion ordered spectroscopy (DOSY) and other 2D NMR experiments.

EXPERIMENTAL SECTION

Materials

All reagents were purchased from commercial suppliers and used as received. 5 mm 7″ Wilmad 528 PP NMR tubes were used in all experiments. Samples were prepared under air on the open bench or fume cupboard and stored in glass vials with PTFE-lined rubber lids. All glassware had been dried under air at ambient temperature. No further precautions were taken to protect the samples from atmospheric water vapor. Methanol and ethanol samples contained 10 vol % CH_3OD and ethanol−OD, respectively, for locking and shimming. Hexamethyldisilane (HMDS, 10 mM) was used as the integral and chemical shift reference (0 ppm) for all spectra. The CH_3OH, CH_3OD (99.8% D enrichment), and ethanol used to determine pK_a (Tables 1 and 2) were purchased from Fisher Scientific and had stated water contents of 0.03%, 0.067%, and 0.012%, respectively. Water contents in the experiments in CH_3OD were determined as 40−100 mM by integration of the OH resonance (Section S4), correcting for the stated deuteration level of the solvent and exchangeable protons. Similar contents are assumed in samples prepared in CH_3OH and ethanol−OH. The water content in CD_3CN (W, eq 7) was determined from the integral of the H_2O resonance. Green fruits of *C. maculatum* (hemlock) were collected in July 2024 from a roadside in Norwich, UK, dried in air for 2 days and ground in a mortar and pestle (Figure S1) with CH_3OD (4 mL). The mixture was transferred to 2 mL polypropylene centrifuge tubes and centrifuged at 12,000 rpm for 5 min on a Thermo Scientific mySPIN 12 centrifuge and the clear supernatant collected for analysis. The concentrations of H_2O and γ−coniceine were determined as 1.3 M and 15 mM, respectively, by integration against HMDS. Whole *X. parietina* thallus was collected from fallen ash tree (*Fraxinus excelsior*) branches from Trimingham, UK, in March 2025 and dried in air for 2 weeks. Dried thallus (ca. 200 mg) was placed inside 2 mL polypropylene centrifuge tubes and ground with ethanol with a stainless steel spatula. Following centrifugation (12,000 rpm, 5 min), the supernatant was combined with fresh ethanol and ground with another 200 mg portion of lichen. This procedure was repeated twice, using the supernatant as the extraction solvent. The concentration of parietin in the supernatant used for analysis (Figure 3b) was determined as 0.3 mM by integration. Mature (black) seeds of *N. damascena* were collected in August 2024 in Normandy (France) and dried in air. Seeds (500 mg) were ground with ca. 2−3 mL CD_3CN in a mortar and pestle and transferred to 2 mL polypropylene centrifuge tubes for centrifugation (12,000 rpm for 5 min). The concentrations of water and damascenine in the supernatant analyzed (Figure 3c) were determined as 670 mM and 25 mM, respectively, by integration against HMDS.

Table 1. Parameters of Indicators in CH_3OH and CH_3OD[a]

CH_3OH	δ_H/ppm	δ_L/ppm	p$K_{a,0}$	p$K_{a,0Lit}$[16]
1,2,4-triazole	9.2919	8.2540	3.46 ± 0.22	-
2,6-dihydroxybenzoic acid (3,5-position)	6.3586	6.1615	4.93 ± 0.07	-
3,5-dimethylpyrazole	2.3584	2.1503	4.98 ± 0.08	-
3-picoline	2.5027	2.3128	5.57 ± 0.08	5.82
2,6-lutidine	2.6634	2.4340	6.81 ± 0.09	6.86
formic acid	8.0330	8.4999	8.18 ± 0.11	-
1,4-dimethylpiperazine	2.5627	2.2400	8.74 ± 0.12	-
acetic acid	1.9436	1.8467	9.40 ± 0.13	9.63
trimethylamine	2.9249	2.1914	9.64 ± 0.14	9.80
tert-butylamine	1.3107	1.0963	11.51 ± 0.14	-
1,2,4-triazole	8.2540	7.8547	12.86 ± 0.15	-
CH_3OD	**δ_H/ppm**	**δ_L/ppm**	**p$K_{a,0}$**	**p$K_{a,0\ Lit}$**
1,2,4-triazole	9.3489	8.2512	3.43 ± 0.27	-
2,6-dihydroxybenzoic acid (3,5-position)	6.3683	6.1601	5.09 ± 0.21	-
3,5-dimethylpyrazole	2.3646	2.1506	4.92 ± 0.26	-
3-picoline	2.5404	2.3113	5.64 ± 0.26	-
2,6-lutidine	2.7118	2.4320	6.70 ± 0.26	-
formic acid	8.0227	8.5025	8.11 ± 0.27	-
1,4-dimethylpiperazine	2.5762	2.2366	8.80 ± 0.27	-
acetic acid	1.9429	1.8440	9.42 ± 0.27	-
trimethylamine	2.8733	2.1880	9.87 ± 0.27	-
tert-butylamine	1.3133	1.0910	11.65 ± 0.28	-
1,2,4-triazole	8.2512	7.8482	13.06 ± 0.28	-

[a]Methyl resonances of indicators were observed.

NMR

Experiments were performed at 298 K on a Bruker Avance III 500 MHz spectrometer operating at 500.21 MHz for ^1H, locking to the ^2H signal of CH_3OD, ethanol-OD or CD_3CN. The $\pi/2$ pulse was 12.75 μs. ^1H CSI experiments were recorded with suppression of the OH (ethanol, CH_3OH) or CH_3 resonance (CH_3OD) using a perfect-echo sequence (based on Bruker library, zgesgppe).[8] 4 ms Gaussian pulses were used for suppression. Experiments to determine the parameters of indicators (Table 1) were acquired with a relaxation delay of 1 s, acquisition time of 1.17 s. The encoding gradient pulse (172 μs) was ramped from −16.5 to 16.5 G/cm in 64 steps with the vertical range of the CSI experiment set to 3.0 cm. Only rows 14−57 of the data sets were used to avoid off-coil effects. Eight scans were acquired at each step, with 16 dummy scans acquired before the CSI experiment, giving a total acquisition time of 19 min 38 s. To determine pK_a values of glutamic acid, histidine and proline, experiments were acquired with 128 gradient increments, ramping from −41 to 41 G/cm with an encoding gradient pulse of 141 μs, acquisition and relaxation time of 1.2 and 0.1 s, respectively. Other amino acids were analyzed with 64 steps. CSI experiments on hemlock extract (Figure 3a) were acquired with 32 gradient steps from −10.4 to 10.4 G/cm, 144 μs encoding gradient pulse, with 16 scans at each increment and a signal acquisition and relaxation delay of 2.15 and 1 s, respectively. CSI experiments on lichen extract (Figure 3b) were acquired with 16 gradient steps from −6 to 6 G/cm, 64 scans at each increment, and an acquisition and relaxation delay of 3.3 and 2 s. Sixteen dummy scans were acquired before the CSI experiments. An exponential line broadening of 1 Hz was applied. The vertical range was set to 2.8 cm. ^1H 1D spectra to determine the pK_a of 2,6-DHB and 3,5-DMP (Figure 1a,b) were recorded with perfect-echo suppression of the solvent, as described for CSI. ^1H 1D spectra to determine the pK_a of 4ClNAn in CD_3CN were recorded with a 30-degree pulse and a relaxation delay of 60 s (Figure S5). CSI spectra in CD_3CN were recorded using a spin−echo sequence ($\pi/2$−τ−π−gp acquire), where gp is a ramped gradient pulse[15] varying from −16.5 to 16.5 G/cm in 64 steps and τ is a delay equal to the sum of the gradient pulse (172 μs) and recovery delay (200 μs).[8] Four scans were acquired with an acquisition time and relaxation delay of 2.15 and 3 s, respectively.

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Anal. Chem. 2026, 98, 24921−24928

Table 2. Parameters of Indicators in Ethanol and CD_3CN[a]

Ethanol	δ_H/ppm	δ_L/ppm	p$K_{a,0}$	p$K_{a,0\ Lit}$
1,2,4-triazole	9.4273	8.1846	3.04 ± 0.25	-
2,6-dihydroxybenzoic acid (3,5-position)	6.3395	6.1356	5.30 ± 0.19	-
3,5-dimethylpyrazole	2.3789	2.1425	4.49 ± 0.25	-
2,6-lutidine	2.6976	2.4336	6.03 ± 0.26	6.37[2]
imidazole	7.5525	6.9579	7.32 ± 0.26	-
1,4-dimethylpiperazine	2.5803	2.2040	8.23 ± 0.27	-
trimethylamine	2.8684	2.1611	9.15 ± 0.28	-
N,*N*-diisopropylethylamine	3.1868	2.4578	10.49 ± 0.28	-
acetic acid	1.9028	1.8196	10.09 ± 0.29	10.26[9]10.58 ± 0.08[2]
1,2,4-triazole	8.1846	7.7952	13.53 ± 0.53	
CD_3CN	**δH/ppm**	**δL/ppm**	**p$K_{a,0}$**	**p$K_{a,0\ Lit}$**
4-chloro-*N*-methyl-2-nitroaniline (4ClNAn)[b]	3.1856	2.9442	3.17 ± 0.21	2.96[b,13]
2-nitroaniline	7.8855	7.3369	4.17 ± 0.21	4.80[6]
4-nitroaniline	8.3002	7.9409	5.70 ± 0.21	6.22[6]
pyrazine	9.0688	8.5293	7.05 ± 0.21	7.74[6]
1,2,4-triazole	8.9861	8.0597	9.37 ± 0.28	-
3,5-dimethylpyrazole	2.3408	2.1197	10.48 ± 0.31	-
3-picoline	2.4879	2.2643	11.68 ± 0.31	-
2,6-lutidine	2.6600	2.3860	12.71 ± 0.31	14.16[6]
imidazole	8.4949	7.5193	13.48 ± 0.31	15.07[6]
2-methylimidazole	2.5626	2.2497	14.14 ± 0.37	-
1,4-dimethylpiperazine	2.5134	2.1225	14.72 ± 0.47	17.38[6]
tert-butylamine	1.3184	1.0301	15.82 ± 0.51	18.14[17]

[a]Methyl resonances of indicators observed where present. [b]The literature value was reported in the presence of 17 mM H_2O.[13] Other values were reported under anhydrous conditions.

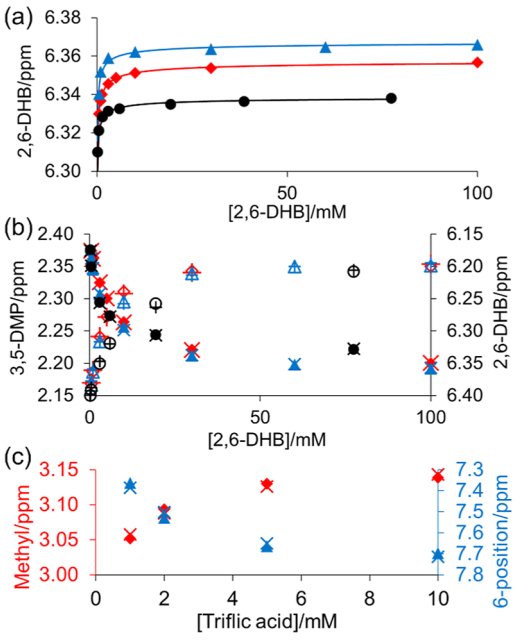

Figure 1. (a) Plot of ^{1}H chemical shift of 2,6-DHB (CH, 3,5-position) versus concentration in CH_3OH (red diamond), CH_3OD (blue triangle), and ethanol (black circle). Fits to eqs 1 and 2 to determine p$K_{a,0}$ (solid lines). (b) Plot of ^{1}H chemical shifts of 3,5-DMP (methyl, open symbols) and 2,6-DHB (3,5-position, closed symbols) versus concentration of 2,6-DHB. Fits to eq 5 to determine p$K_{a,0}$ of 3,5-DMP (vertical cross), and eq 6 (cross): methanol (red diamond), CH_3OD (blue triangle), and ethanol (black circle). (c) Plots of methyl (red diamond) and 6-position (blue triangle) resonances of 4ClNAn versus triflic acid concentration. Fit to eq 5 (crosses).

Pulse programs and automation scripts are provided in Section S9. Processing of CSI data sets is discussed in our previous work.[8]

NMR Sample Preparation

When using 2,6-DHB as the acidic diffusant, solid acid was weighed into the NMR tubes by difference using a Pasteur pipet and the analyte solution layered on top (Figure S2). When using triflic or methanesulfonic acid, solutions (0.8 M, 30−50 μL) were placed at the bottom of 5 mm NMR tubes using extended length 200 μL polypropylene pipet tips (Starlab UK) and the tube spun on a Hettich H1011 hand-wound centrifuge to drive the liquid to the bottom of the tube. The solution of indicators was then pipetted slowly down the tube which was held at a 45° angle to vertical. The higher density of the acid solution ensured that the analyte solution floated on top. Following preparation, samples were left to stand in the autosampler rack of the spectrometer at ambient temperature (ca. 21 °C) before analysis. δ_L of the indicators was determined in the solution of indicators mentioned, prior to placement on top of the acid. The compositions of the samples used to determine the pK_a values of the indicators (Tables 1 and 2) are provided in Section S1 of the Supporting Information.

To determine the pK_a values of amino acids, solutions were prepared containing 20 mM *tert*-butylamine, 1,2,4-triazole, 2,6-lutidine, 3,5-DMP, sodium acetate, 15 mM NaOH, and 5 mM amino acid with one (alanine, histidine, tryptophan, lysine, proline) or two (tyrosine, glutamic acid) equivalents of NaOH to ensure the amino group was uncharged. 500 μL of this solution was placed on top of 50 μL of 0.8 M triflic acid and the samples stood between 14 and 21 h, or for 30 h (glutamic acid). The time period over which the sample should be analyzed was predicted from the desired pH range using the spreadsheet accompanying this work (Figure S32). Chromium(III) acetylacetonate (2 mM) was added to reduce the relaxation delay and enable the number of vertical slices in the CSI experiment to be increased from 64 to 128, keeping the overall experimental time within 25 min and without affecting pK_a (Section S6.3). This agent was included in the determination of the pK_a values of histidine, glutamic acid, and proline.

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Anal. Chem. 2026, 98, 24921−24928

Hemlock extract was combined with *tert*-butylamine (20 mM), sodium formate (40 mM), NaOH (50 mM), and 3,5-DMP (40 mM) and 500 μL of this solution layered on top of 40 μL of 0.8 M methanesulfonic acid and the sample stood for 30 h in the autosampler rack for a pH gradient to develop. Lichen extract was combined with 1,2,4-triazole (20 mM), NaOH (30 mM), 3,5-DMP (30 mM), *N*,*N*-diisopropylethylamine (30 mM), and trimethylamine (30 mM). 500 μL of this solution was placed on top of 50 μL of 0.8 M triflic acid and the sample stood for 19 h before analysis. Extract of *N. damascena* was combined with *tert*-butylamine (20 mM), 2-methylimidazole (20 mM) and 3,5-DMP (20 mM), and 440 μL layered on top of 50 μL of 0.8 M triflic acid. The sample was stood for 7 h before analysis.

RESULTS AND DISCUSSION

Based on our previous work,[8] we first determine the pK_a of compounds which are partially dissociated when present in millimolar concentrations in the solvent under investigation. For CH_3OH, CH_3OD, and ethanol, we measured the ^{1}H chemical shift, δ_{obs}, of 2,6-dihydroxybenzoic acid (2,6-DHB) in homogeneous samples as a function of concentration, C, and fitted to eqs 1 and 2 to obtain a thermodynamic pK_a, pK_{a0}:

$$f = \frac{\frac{-10^{-pK_{a,0}}}{\gamma^2} + \sqrt{\left(\frac{10^{-pK_{a,0}}}{\gamma^2}\right)^2 + 4\left(\frac{10^{-pK_{a,0}}}{\gamma^2}\right)C}}{2C} \quad (1)$$

$$\delta_{obs} = f\delta_L + (1 - f)\delta_H \quad (2)$$

where δ_H and δ_L are the chemical shits of the compound when fully protonated and deprotonated, respectively. The activity coefficient, γ, is calculated from the ionic strength, I, using eq 3

$$\log_{10}(\gamma) = \frac{-A\sqrt{I}}{1 + B\sqrt{I}} \quad (3)$$

A and *B* are parameters calculated from the dielectric constant of the solvent as 1.90 and 2.04 (CH_3OH), 1.99 and 2.07 (CH_3OD), 3.00 and 2.37 (ethanol), and 1.54 and 1.90 (CD_3CN), respectively (Section S2, Supporting Information). The experiments were repeated in the presence of 3,5-dimethylpyrazole (3,5-DMP, 5 mM) and the pH of the samples calculated from the ^{1}H chemical shift of 2,6-DHB using eq 4

$$pH = pK_{a,0} + \log_{10}\left(\frac{\delta_H - \delta_{obs}}{\delta_{obs} - \delta_L}\right) + z\log_{10}(\gamma) \quad (4)$$

where $z = 1$. The p$K_{a,0}$ of 3,5-DMP was obtained by fitting the ^{1}H chemical shift of this compound to eq 5

$$\delta_{obs} = \frac{\delta_L + \delta_H 10^{pK_{a,0} + z\log_{10}(\gamma) - pH}}{1 + 10^{pK_{a,0} + z\log_{10}(\gamma) - pH}} \quad (5)$$

where $z = -1$. The ^{1}H chemical shift of 2,6-DHB was fitted simultaneously to eqs 2 and 6 to provide an estimate of the uncertainty in the p$K_{a,0}$ of 3,5-DMP (Section S3, Supporting Information).[8]

$$f = \left\{q(P + C + [H^+]) - [H^+] - \sqrt{[[H^+] - q(P + C + [H^+])]^2 - 4q(q-1)C(P + [H^+])}\right\} / \{2(q-1)C\} \quad (6)$$

where $[H^+] = \gamma^{-1}10^{-pH}$, $q = \gamma^{-2}10^{pK_{a,0DMP} - pK^*_{a,0DHB}}$, p$K_{a,0}$* is a fitting parameter used in the calculation of uncertainty in p$K_{a,0}$[8] and P and C are the concentrations of 3,5-DMP and 2,6-DHB, respectively. For CD_3CN, the low acidity of neutral acids[7] precludes determination of pK_a via acid dissociation. Instead, we measured the ^{1}H chemical shift of 4-chloro-2-nitro-*N*-methylaniline (4ClNAn, 1 mM) as a function of the concentration of trifluoromethanesulfonic (triflic) acid (T). Assuming complete dissociation of triflic acid and equal activity coefficients of H^+, H_3O^+, and protonated 4ClNAn (N^+), the concentration of H_3O^+ is obtained using eq 7

$$[H_3O^+] = \left\{(T - [N^+] + W + K_H) - \sqrt{(T - [N^+] + W + K_H)^2 - 4W(T - [N^+])}\right\}/2 \quad (7)$$

The molar concentration of water, *W*, is obtained by integration of the water resonance against hexamethyldisilane (HMDS, Section S4). K_H is the dissociation constant of H_3O^+, $K_H = [H^+][H_2O]/[H_3O^+]$, from Kolthoff and Ikeda (Section S3).[12] $[N^+]$ is calculated from eq 8

$$[N^+] = N\left(\frac{\delta_{obs} - \delta_L}{\delta_H - \delta_L}\right) \quad (8)$$

where *N* is the total concentration of 4ClNAn. The concentration-based pH, p_cH, is calculated as $p_cH = -\log_{10}(T-[N^+]-[H_3O^+])$. The p$K_{a,0}$ of 4ClNAn is obtained by fitting to eq 5, setting $\gamma = 1$ and $pH = p_cH$. δ_L was measured in a solution in the absence of triflic acid, δ_H was a free parameter. The water content was determined as 12−19 mM in CD_3CN (Section S4). Good fits are obtained in all solvents (Figure 1).

We use the p$K_{a,0}$ of 3,5-DMP and 4ClNAn to determine the p$K_{a,0}$ values of other indicators that enable measurement of pH across wide ranges using eq 4 (Tables 1 and 2). The fitting procedures, constraints, and uncertainty calculations used are described in Section S3 of the Supporting Information. Determinations of p$K_{a,0}$ were performed using pH gradients, created by the diffusion of either 2,6-DHB, methanesulfonic, or triflic acid. The ^{1}H chemical shifts of the indicators were measured as a function of pH by ^{1}H CSI and fitted to eq 5.[8] The difference in charge of the compound between the nonprotonated and protonated states (z, eqs 4 and 5) was set to 1 for carboxylic acids and 1,2,4-triazole (deprotonation step) and −1 for nitrogen bases. We note that the chemical shift of ionizable compounds may depend on factors other than pH, such as ion pairing; however, in all our experiments the chemical shifts closely followed eq 5 with the largest deviations observed in CD_3CN. Plots and stacked spectra are provided in Section S5, Supporting Information. In alcoholic solvents, good agreement between fitted and literature pK_a values is obtained, confirming that traces of water have only a small effect on pK_a and providing a validation of our NMR-derived pH and pK_a values.[9] No significant differences are observed between CH_3OH and CH_3OD, suggesting that the use of deuterated alcohols for NMR does not significantly affect pK_a.

Data for additional analytes in these solvents are presented in Section S6.1 of the Supporting Information.

In CD_3CN (Table 2), the p$K_{a,0}$ of 4ClNAn determined by titration with triflic acid (Figure 1c) is in agreement with the value of 2.96 reported by Coetzee and McGuire in acetonitrile containing 17 mM H_2O, compared to the value of 3.77 reported by these authors in the pure solvent.[13] To confirm the

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Anal. Chem. 2026, 98, 24921−24928

validity of the parameters of 4ClNAn (Figure 1c) at the higher concentrations of acid and base employed in the CSI experiments, a sample was prepared containing 4ClNAn (0.1 M) and triflic acid (0.05 M). The pH calculated using eqs 3, 7 and 8 agrees with the pH calculated from the chemical shift of 4ClNAn using eq 4 and the parameters in Table 2, confirming the validity of the $pK_{a,0}$ values of the other indicators under the conditions of our experiments (Section S3). For these compounds, the measured (24−80 mM H_2O) pK_a values are consistently lower than the literature (anhydrous) values, with the difference increasing substantially with basicity (Table 2). Repeating the determination of the $pK_{a,0}$ of imidazole, 2-methylimidazole, 1,4-dimethylpiperazine, and *tert*-butylamine at 170 mM H_2O, using the parameters of 2,6-lutidine (Table 2) as a reference, gives values of 13.49 ± 0.31, 14.17 ± 0.35, 14.77 ± 0.44, and 15.81 ± 0.46, respectively, suggesting that additional H_2O does not significantly affect the determined $pK_{a,0}$ (Figure S25).

Kolthoff and Ikeda demonstrated that the pK_a of 4-chloro-2-nitroaniline in acetonitrile decreased by less than 1 unit as the water content was increased from 1 to 56 mM H_2O, if basicity of water was taken into account.[12] However, for stronger bases, studies by infrared spectroscopy have demonstrated a strong interaction of water with *n*-butylamine and pyrrolidine.[18] Homoconjugation constants of nitrogen bases have been determined as negligible for aniline, moderate for pyridine, and high for primary amines.[17] In our experiments, we hypothesize that the presence of water and high concentrations of NH groups (ca. 0.1 M) to act as H-bond donors renders the nitrogen lone pairs less available to coordinate to H^+ relative to dilute anhydrous acetonitrile. The basicity of our strongest bases (e.g., *tert*-butylamine) is thus lowered relative to the weaker bases that are used to determine their pK_a by CSI. To investigate the effects of water on carboxylic acids, the pK_a values of dichloroacetic acid and 2,6-DHB were determined as 11.44 ± 0.32 and 10.09 ± 0.32 (100 mM H_2O), compared to the literature values of 18.02[19] and 12.6,[20] respectively (Section S6.1). The large difference for dichloroacetic acid is attributable to stabilization of the carboxylate anion by H_2O and other H-bond donors present in the sample,[19] while the closer agreement for 2,6-DHB is attributable to its strong intramolecular hydrogen bonding that renders it less sensitive to the presence of external donors.[20]

We then explored the use of CSI to reveal the dissociation behavior of amino acids (Figure 2). The proximity of the amino and carboxyl groups and tendency to form zwitterions can be expected to lead to a different solvent dependence of pK_a compared with monocarboxylic acids and monoamines. Their dissociation behavior has been reported in DMSO[21] and mixed aqueous−organic media.[22] However, despite their importance in synthesis and in the study of biological systems, to the best of our knowledge, the dissociation constants of amino acids in alcohols have not been reported to date, except for the amino and carboxyl pK_a values of tyrosine in CH_3OH.[23]

Selecting CH_3OD as the alcoholic solvent most amenable to study by NMR, we designed samples to have wide-range pH gradients spanning from approximately pH 3 to pH 14.[14] This wide pH range enables us to extract all of the pK_a values (Table 3). The carboxyl and amino pK_a values are ca. 6 and 11−12, respectively, for all compounds studied, contrasting markedly with their values in water.

Such information will likely prove to be invaluable in the design of analytical or synthetic procedures where an anionic

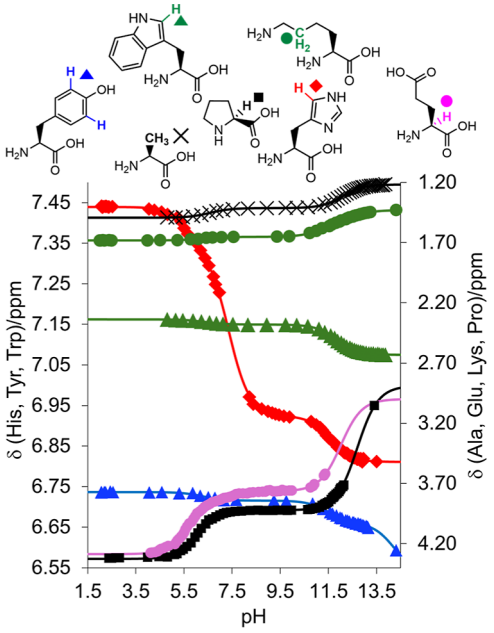

Figure 2. Plot of observed ^1H chemical shifts of amino acids versus pH in CH_3OD: alanine (black cross), glutamic acid (pink circle), histidine (red diamond), lysine (green circle), proline (black square), tryptophan (green triangle) and tyrosine (blue triangle). Fits to eq S14 are solid lines.[14]

Table 3. Fitted pK_a Values[a] of Amino Acids in CH_3OD

amino acid[a]	pK_{a1}	pK_{a2}	pK_{a3}
alanine	6.57 ± 0.29	12.08 ± 0.29	-
glutamic acid	5.70 ± 0.30	7.73 ± 0.27	12.02 ± 0.27
histidine	5.60 ± 0.31	7.37 ± 0.27	11.59 ± 0.32
lysine[b]	6.15 ± 0.32	11.21 ± 0.36	12.52 ± 0.29
proline[b]	6.05 ± 0.30	12.70 ± 0.29	-
tryptophan	6.38 ± 0.28	11.84 ± 0.28	-
tyrosine[b,c]	6.29 ± 0.32	11.51 ± 0.31	14.26 ± 0.48

[a]Measured pK_a values not corrected for ionic strengths of 0.1 M (pK_a ≈ 6), 0.07 M (pK_a ≈ 8), 0.06 M (pK_a ≈ 12). [b]Fitted using additional data point measured in the absence of triflic acid. [c]Ogston and Brown reported values of 6.1 and 11.7 for pK_{a1} and pK_{a2} in CH_3OH.[23]

state is required.[24,25] We note that the limited experimental data can hinder prediction of the pK_a in nonaqueous solvents,[26] while closely spaced pK_a values (e.g., lys, glu) can be difficult to model.[14] Though the alpha proton resonance was often obscured by other compounds and overlapped with the suppressed methyl resonance of the solvent (Figure S32), other resonances remained sufficiently clear across the full pH range to enable fitting to eq S14 (Section S6.2). We also note that data points at high pH (>13) are difficult to achieve with pH gradients due to the absence of buffering effects above the pK_a of the highest indicator used (1,2,4-triazole), while the resonances of proline and glutamic acid overlap with other compounds at higher pH (Figures S32 and S33). The chemical shifts of lysine, proline, and tyrosine measured in a solution prior to addition to triflic acid were used to provide an additional data point at high pH in the fitting of these compounds.

To explore the potential of concentration gradients and quantitative pH measurements to identify components in complex molecular mixtures, we prepared extracts of botanical

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Anal. Chem. 2026, 98, 24921−24928

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samples. A CSI experiment of hemlock extract (Figure 3a) revealed that the resonances of a propyl chain could be fitted to the same pK_a value as two multiplets that could be assigned as the piperideine ring of γ-coniceine (Section S7.1), which has been reported to be an abundant alkaloid in green fruits with levels of approximately 20 μg/fruit.[3] The fitted value (9.5) is consistent with the lower basicity of imines relative to amines (H_2O: cyclohexylimine, 9.15;[27] cyclohexylamine, 10.65[28]). Resonances belonging to other molecules do not move with pH or can be fitted to different pK_a values (e.g., red cross, Figure 3a). Individual resonances in this highly complex sample can be conveniently tracked and matched based on pK_a (eq 5) as a visual overlay of a 2D plot using the spreadsheet accompanying this work (Section S8).

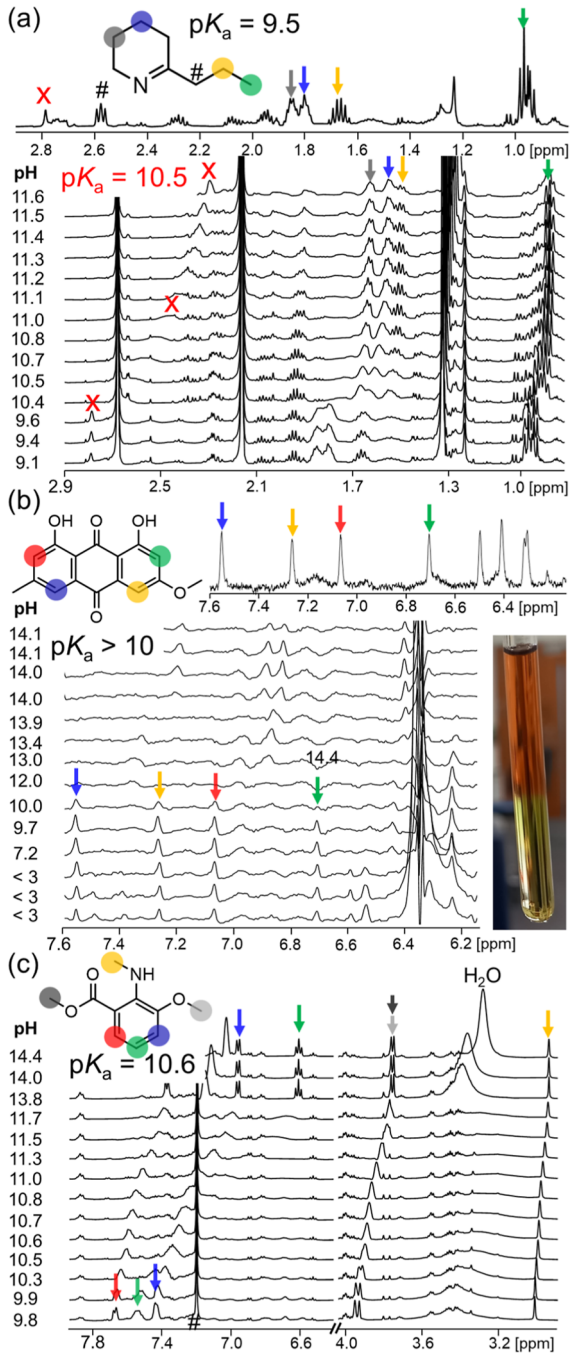

Figure 3. (a) Partial ^{1}H spectra of extract of green hemlock fruit (CH_3OD) with assignment of γ-coniceine and spectra extracted from CSI data set along gradient at pH values indicated. The singlet resonance (X) can be assigned to a different molecule based on pK_a. The resonance marked # is not visible due to deuterium exchange with CH_3OD (Figure S35). (b) Parietin in extract of *Xanthoria parietina* (ethanol). The aromatic resonances have the same pH-dependence. Movement of these resonances corresponds with a color change in the sample. (c) Damascenine in extract of *Nigella damascena* seed (CD_3CN). Other resonances in the sample do not move with pH except for H_2O and 2-methylimidazole (#).

The four aromatic resonances of parietin can be discerned in the extract of *X. parietina* (Figure 3b) from their pH dependence (pK_a > 10), in comparison to other phenolic compounds (Table S2), confirmed by COSY and DOSY (Section S7.2). A color change from yellow (acidic) to red (basic) was observed in the sample that matched the change in chemical shift observed by CSI. We note that the color change from orange to red with addition of base is a diagnostic feature for this species of lichen that can be attributed to the presence of parietin.[29] Analysis of the seed extract of *N. damascena* in CD_3CN by ^{1}H CSI reveals resonances of damascenine as a major component,[30] fitting to a pK_a value of 10.6 ± 0.4 (Figure 3c), consistent with the reported pK_a of similar anilines.[6] By contrast, no significant pH-responsive resonances are observed in extracts from mature seeds of *Nigella sativa* (Figure S44), which does not contain significant levels of aniline alkaloids and has culinary uses.[31]

CONCLUSIONS

We have investigated the dissociation behavior of acids and bases in organic solvents containing typical levels of water found in nondried commercial laboratory media. Knowledge of dissociation constants paves the way for the informed design of synthetic or analytical procedures where a certain charge state of the compound is required but prior knowledge of pK_a is sparse or absent. Our method efficiently spans wide pH ranges, bypassing the inconvenience of methods such as potentiometry and the uncertainty of using literature pK_a values as "anchor" points. Taking advantage of the diagnostic pK_a values of ionizable functional groups, we use pH gradients to detect, identify, and quantify potentially bioactive natural products in botanical extracts directly by NMR, without chromatographic separation. Compounds can be identified at submillimolar concentrations in nondeuterated solvents (parietin, Figure 3b). Compared to other methods such as LC−MS for interrogating complex mixtures, NMR provides more structural information in terms of chemical shift and multiplicity and can distinguish between isomers. The method successfully detects natural products that are <1% abundance in the plant source. In addition, our technique could potentially be applied to determine the composition of reaction mixtures in organic media and thus optimize the reaction conditions considering the pK_a values of the components. We could also assess complex biological matrices such as digestates.

ASSOCIATED CONTENT

Data Availability Statement

Data sets will be openly available at: https://research-portal.uea.ac.uk/en/datasets/.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.6c02161.

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Spreadsheet for processing of datasets (XLSX)

Additional experimental details, fitting procedures, and stacked NMR spectra (PDF)

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All authors have given approval to the final version of the manuscript. Conceptualization: MW, SM, CFJ, and AG; Data collection: MW, JD, SM, SD, HS, KA, DL, AO, and CFJ; Data processing: MW, SM, and CFJ.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CSI	chemical shift imaging
3,5-DMP	3,5-dimethylpyrazole
4ClNAn	4-chloro-*N*-methyl-2-nitroaniline
2,6-DHB	2,6-dihydroxybenzoic acid
HMDS	hexamethyldisilane.

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