Examples showing the utility of doping experiments in ^{1}H NMR analysis of mixtures

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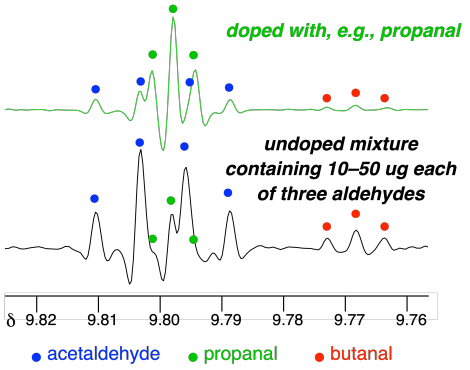
Supporting Information Placeholder

ABSTRACT: Here we provide examples that demonstrate the value of using properly designed and easily performed doping experiments to give insights about the nature of the analyte(s) present in a ^{1}H NMR sample. Two mixtures, the first quite complex and the second far less so, have been chosen: **i**) the crude pyrolysate from reaction of butyric acid in (supercritical) water at 600 °C and **ii**) a mixture of two basic amines. In the former, 13 distinct carbonyl-containing compounds, ranging in relative concentration of nearly two orders of magnitude, were positively identified. The latter highlights the advantage of using a doping experiment as opposed to merely comparing the spectra from two separate samples containing the same analyte.



■ INTRODUCTION

Chemists are often faced with the need and inherent challenge of evaluating mixtures of compounds. Crude product mixtures from chemical reactions, mixed fractions from chromatographic separations, crude extracts from natural product isolation studies, and direct NMR analysis of reagents[1] or reaction solutions[2] are examples of some more commonly encountered situations. Various chromatographic techniques of either a preparative or analytical nature are of great value in providing insight. In that context, commonly used hyphenated chromatography-mass spectrometry (GC-MS and LC-MS) approaches can be invaluable. The use of NMR spectroscopy can also play an important and complementary role in providing insight to the composition of mixtures. Indeed, a number of powerful and sophisticated strategies for doing just that have emerged;[3] some are especially prevalent and valuable in the fields of natural products chemistry[4] and metabolomics.[5]

We show here, by way of two different examples, how the addition of a component suspected to be present in the mixture being analyzed (the dopant) can be used to positively identify (or rule out) the presence of that component in the NMR sample. Having both the original mixture and the dopant in the same NMR tube assures that the resonances from all molecules are being measured under identical conditions. Many analysts and spectroscopists are well familiar with the concept of spiking an authentic sample into a mixture under analysis.[6] Similarly, the bench chemist often uses co-spotting of their starting material and/or product during tlc analysis of a reaction mixture. The two examples we have elected to demonstrate this NMR doping approach are i) a complex product mixture arising from the high temperature (e.g., 600 °C) pyrolysis of butanoic acid (BA) and ii) a mixture of basic amines (N-methylpiperidine and N-methylpiperazine).

The doping method represented by the examples here is not the only way to address the question posed by these two particular samples, nor is it necessarily the best way. The goal, however, is to give readers who might not otherwise know about (or think about) using this strategy an introduction to (or reminder of) another, complementary tool to consider for addressing analyses of compound mixtures.

■ RESULTS AND DISCUSSION

i) Product mixture from pyrolysis of butanoic acid (BA). We have been studying the pyrolytic conversion of various waste organic feedstocks comprising organic compounds into distillable biofuels.[7] The reactions are performed at high temperature in supercritical water (SCW) over a bed of solid catalyst. The reactor design is given schematically in the Supporting Information (SI). One thrust of the studies has been to use a single model compound to probe the behavior of certain classes of

substances prevalent in the waste feedstock. One such class is fatty acids, because triglycerides represent common constituents in organic waste such as yellow or brown grease.[8] To that end we have studied the reactions of octanoic, pentanoic, and, as selected for one example of a complex mixture that we are presenting here, butanoic acid. In the course of that work, we have recognized the power of direct analysis of the crude product mixtures by proton NMR spectroscopy. Doping the NMR sample of the pyrolysate with authentic samples of components potentially present in the mixture has proven to be both very convenient to carry out and very powerful. For example, this allowed for the positive identification of the presence of even a very small amount of a unique analyte from a resonance of only a single proton whose relative intensity was significantly <0.1% of those of all other protons in the sample. Thus, the example presented here also shows the ability to identify even minor constituents within highly complex mixtures.

The crude butanoic acid pyrolysate was obtained as an effluent from the SCW reactor was obtained as an aqueous product mixture, which was then extracted with $CDCl_3$ to provide what we will call here the "master" BA mixture. Although this is an imperfect method by which to accurately judge product ratios because of differential partitioning of the various low molecular weight products between water and chloroform, that fact does not otherwise detract from the ability to positively identify many of the non-gaseous products formed in this reaction. Said another way, one should be careful to not over interpret the ratios of components in any sample where prior fractionation may have occurred.

Careful analysis of the ^{1}H NMR spectrum of the master BA mixture and a knowledge of the chemistry likely to be seen under the pyrolysis reaction conditions, led us to hypothesize the presence of the ketones, aldehydes and carboxylic acid products shown in Figure 1. The reaction of aliphatic carboxylic acids having an alpha methylene group at high temperatures, including over various solid catalyst beds, are known to proceed primarily by an initial ketonization event that amounts to a net Claisen-like condensation and decarboxylation to give a ketone dimer.[9] For example, 4-heptanone can be expected as a major primary product from butanoic acid. Aliphatic ketones, in turn, are known to enter into various fragmentation reactions to produce an array of smaller molecular derivatives.[10]

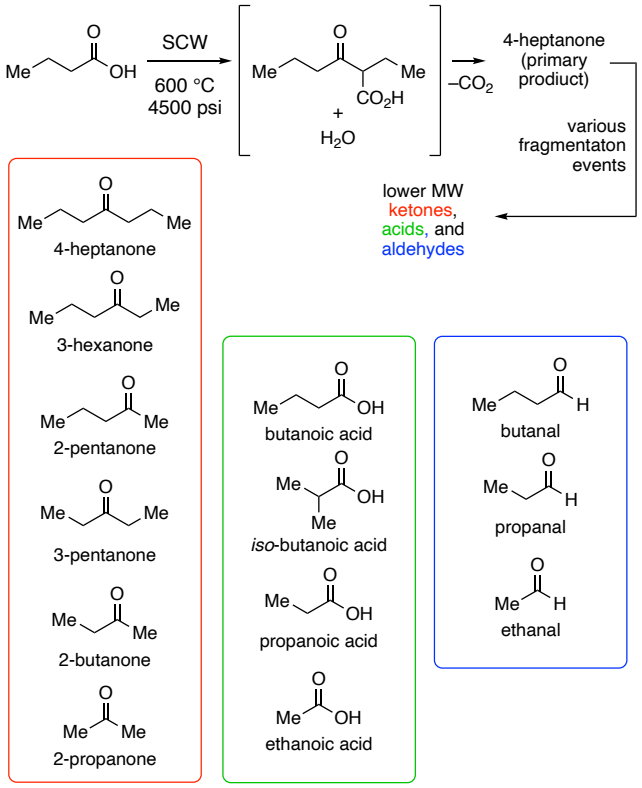


Figure 1. All of the products detected following butanoic acid degradation.

The value of resolution enhancement. Because it plays a very valuable role in analysis of 1D ^{1}H NMR spectral data, including those shown here, we remind readers about the benefit of using resolution enhancement [e.g., apodization (in MNova®) or line broadening (in TopSpin®)] of NMR raw FID data.[5a,11] Enhanced spectra were used in the doping studies of the BA pyrolysis product mixture to enable distinction between minute differences in chemical shifts for the resonances of similar substructural units present in more than one of the compounds shown in Figure 1.

The spectrum of a typical $CDCl_3$ extract of the crude product mixture from a 600 °C run of the BA pyrolysis is shown in Figure 2a. To definitively confirm which of the sets of resonances in this ^{1}H NMR spectrum are those from unconverted butanoic acid, we performed the first (of many) doping experiments. An appropriate amount (see doping guidelines, below) of BA was added to the sample of the pyrolysate and the spectrum was retaken. Expansions that include the resonances for the C2H_2, C3H_2, and C4CH_3 protons of the doped (green) vs. undoped (black) spectra are shown in Figure 2b, there as the black superimposed on top of the green spectrum. In each of these swaths, the doped spectrum has a greater intensity, of course, for the resonances unique to BA. For comparison purposes, in Figure 2c we have shown several alternative methods for viewing the doped vs. undoped spectra (see italicized descriptions for each).

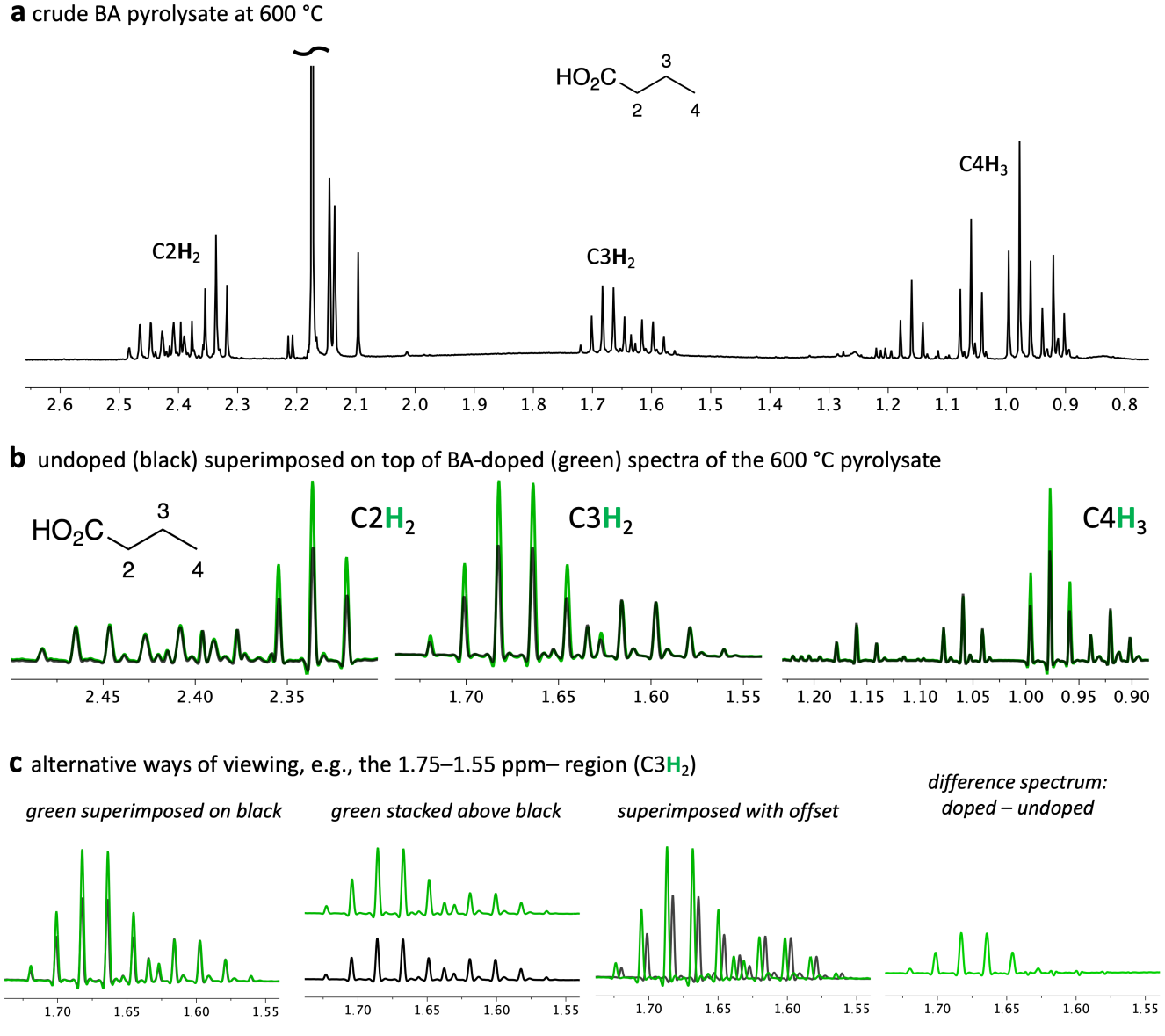


Figure 2. a) ^{1}H NMR spectrum (δ 0.75–2.65 ppm) of the $CDCl_3$ extracts of butanoic acid pyrolysis products at 600 °C. **b**) Superimposed expansions of the relevant portions of the spectra of the undoped (black) on top of the samples doped with an appropriately small amount of butanoic acid (green). **c**) Four alternative ways (see italics) of visualizing the spectra before and after doping, here for the δ 1.54–1.74 ppm region. [Exponential and Gaussian weighting of –1.0 and +1.0, respectively (in the Apodization feature of MNova®), was used to enhance the data shown in panels b and c; no enhancement was used for the spectrum shown in panel a.]

Doping guidelines. Some discussion about aspects of the doping experiment itself is in order. Especially for mixtures that have many components with resonances of similar chemical shifts and multiplicities, it is often not sufficient to merely look up reference values in the literature for a given compound and impose those numbers onto the spectral data at hand in an attempt to make a positive identification. Nor is it even sufficient to have taken a separate spectrum of an authentic sample on one's own instrument and compare the shifts. A different method for comparing chemical shifts is to add – i.e., dope in – an appropriately small amount (see below) – emphasis on "small" – of the authentic material to the same sample tube and then compare the spectra of the pre- vs. the post-doped samples. If too much of the dopant is used, it can easily swamp out minute features of the undoped spectrum[5a] critical to the key comparison because the new resonance can overwhelm subtle important spectral features – and there is often no quick recourse, if any, for recovery.

Using too much dopant is a mistake easily made by first-time users as they attempt to implement this strategy. For chloroform solutions, one convenient way to judge the amount of dopant to be added is to take advantage of the information provided by the residual $CHCl_3$ proton resonance in the spectrum of the mixture. When using 99.8% $CDCl_3$, the residual solvent proton resonance corresponds to ca. 2 mg of $CHCl_3$ in a sample volume of

ca. 0.6 mL. Estimating the amount of a component in the mixture by comparing the relative integration of one of its expected resonances to that of the $CHCl_3$ allows for a wise first choice of the amount of dopant to add. It is recommended to always undershoot rather than overshoot the amount that is initially added. For convenience, making an appropriately dilute stock solution of the dopant in the NMR solvent and adding a small, measured volume of that to the spectrum of the master mixture allows one to quickly arrive at a proper level of dopant., adding a second bolus if necessary. The targeted range for the amount of dopant to be introduced is fairly broad; anywhere from, say, a 25% to, even as high as, a 500% enhancement of the component's initial intensity is typically quite manageable. Also worth noting, when estimating the amount of very low concentrations of a suspected component in a mixture, it is convenient to use the ^{13}C satellite peak from $^{13}C^{1}HCl_3$, which corresponds to ca. 10 µg (in 0.6 mL) of that isotopomer, as the internal reference.

To emphasize a point made earlier, a distinct advantage of this protocol is that in doped samples there can be no doubt that all of the solutes are being observed under identical conditions. This concept is analogous to comparing the results from chromatographic analysis of a sample both prior and subsequent to doping of a known analyte to discern identical vs. slightly different retention behavior—the same concept as co-spotting in tlc analysis as mentioned earlier. Again, this assures that the known compound is subjected to the identical chromatographic conditions as that of the unknown entity one is trying to positively identify.

Example: Doping the butanoic acid pyrolysate with 3-hexanone. As a representative example of doping with a minor constituent whose presence is suspected in the master mixture, consider the spectra shown in Figure 3. 3-Hexanone (100 uL of a stock solution containing ca. 1.5 mg in 1 mL of $CDCl_3$) was added to an NMR sample containing ca. 5 mg of total analytes in the master BA mixture.

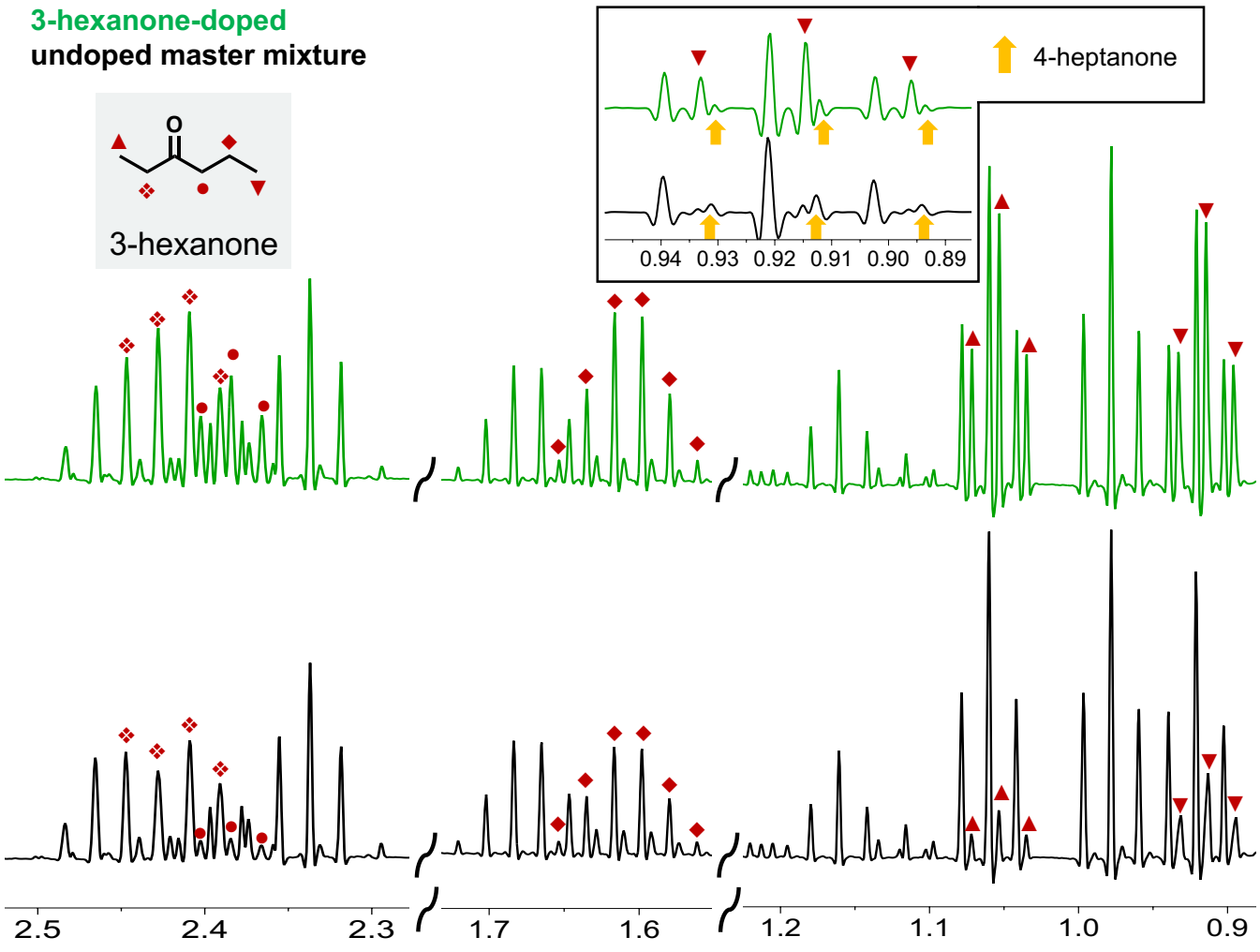


Figure 3. Portions of the ^{1}H NMR spectrum of the BA pyrolysate mixture (black, pre-doping) doped with 3-hexanone (green, post-doping), a product comprising only ca. 1% of the total analytes (and present in ca. 1/3rd the amount of another minor component, 4-heptanone). [Main spectra: exponential and Gaussian multipliers of -1.0 and +1.0; Boxed inset: exponential and Gaussian multipliers of -2.2 and +1.0.]

Both of the doped and undoped spectra shown in Figure 3 were weighted with an exponential = -1.0 and Gaussian = +1.0. These values are empirically chosen as a convenient compromise between line width and negative

distortion of the multiplets. It is helpful to use software that allows observation of effect of adjusting the weighting on the spectrum in real time (e.g., "interactive" in MNova®). The resonance for the C2 methylene protons (chevron, δ 2.42 ppm) is superimposed on a multiplet from a more major component. However, the shift of the C4 methylene protons resonance (circle, δ 2.38 ppm) is uniquely discernable, as is the C5 methylene 'sextet' (diamond, δ 1.61 ppm). The C1 and C6 methyl triplets (δ 1.06 and 0.91 ppm, respectively) were, at first glance, distinguishable; however, notice that they are not of equal intensity. Closer scrutiny, gained with the use of a greater amount of resolution enhancement, showed that the more upfield resonance was two resolvable triplets of nearly identical chemical shift. More specifically, the boxed inset in the upper right shows an expansion of the resonance for the C6 methyl protons (down triangle, δ 0.915 ppm). A larger exponential weighting (-2.2) was used to resolve the lines of the triplets for C6 of 3-hexanone and C7 of what, in a subsequent doping experiment, was established to be 4-heptanone (gold arrows). Note that the doped spectrum allows definitive distinction between the triplets at 0.915 and 0.912 ppm.

By using a series of analogous doping experiments to the one just detailed for 3-hexanone, now with each of the additional products shown in Figure 1 (six ketones, three aldehydes, and three acids), we were able to positively identify virtually every resonance in the original spectrum of the master BA mixture (i.e., the $CDCl_3$ extract of the pyrolysate). This is indicated by the set of assignments shown in the spectrum and insets in Figure 4. Once the first dopant level was determined (the butanoic acid in Figure 2), all of the other amounts of dopants were easily and correctly identified upon the first addition. In other words, there was not a lot of trial and error required to achieve a proper "appropriately small amount" of the dopant in each case.

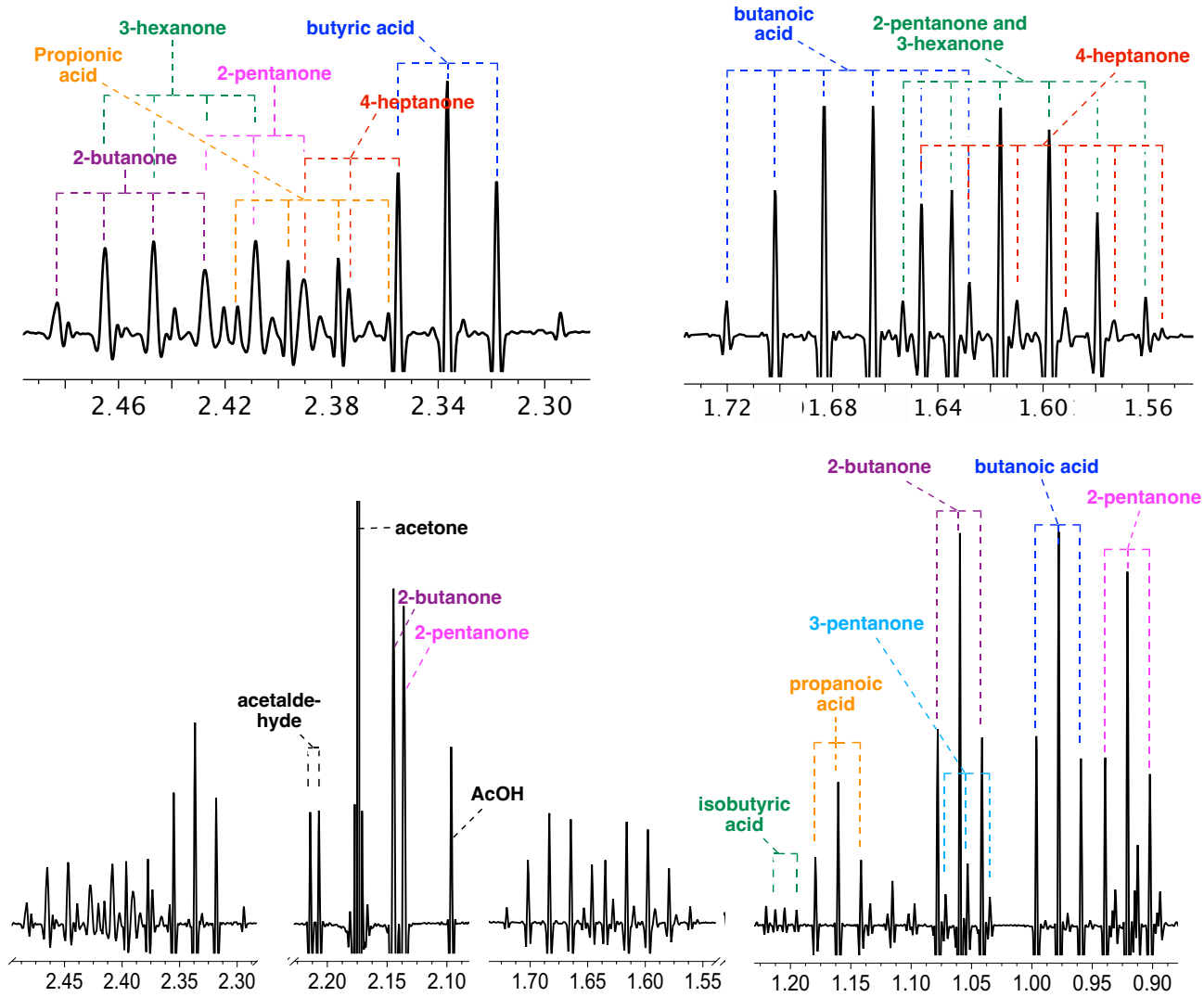


Figure 4. Assignment of nearly all resonances in the master spectrum of the 600 °C BA pyrolysate, verified by individual doping with each of the components (Figure 1). The following chemical shift ranges correspond to the resonances of the types of protons indicated in parentheses: δ 2.5–2.3 (methylene protons alpha to carbonyl groups), 2.25–2.10 (methyl protons alpha to carbonyl groups), 1.75–1.55 (methylene protons beta to carbonyl groups), and 1.25–0.85 (methyl protons beta and gamma to carbonyl groups). [Exponential and Gaussian multipliers of -1.0 and +1.0, respectively.]

Detection of (the most) minor components: Doping with aldehydes. We also observed in the δ 9.8 ppm region of the spectrum an additional set of resonances arising from very minor amounts of aliphatic aldehydes (bottom spectrum in Figure 5). Collectively, these amounted to ca. 1 wt% of the extracted pyrolysate mixture. Using analogous doping experiments to those already described we were able to identify the presence of each of the aldehydes acetaldehyde (ethanal, green), propanal (blue), and butanal (red) as shown in the top three spectra in Figure 5. This demonstrates the ability to positively identify individual members of a mixture present in much lower proportion than that of the major components.

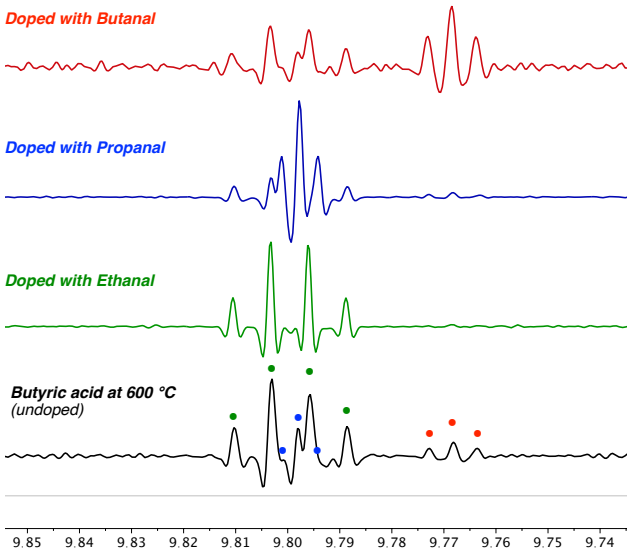


Figure 5. Doped spectra (top three) demonstrating the presence of three aldehydes among the BA pyrolysis products. [Exponential and Gaussian multipliers of -2.0 and +1.0, respectively.]

Summation of the BA pyrolysate example. NMR analysis is , of course, not the only way to address this problem. At the outset or our work with the BA pyrolysate product mixture, we also examined the GC-MS data of the master BA mixture (chloroform extract). This allowed identification of the higher molecular weight, C5-C7 ketones (Figure 1). However, some of the smaller components were not discernable because of the interference from the large proportion of chloroform molecules. Moreover, the GC chromatogram was further complicated by the fact that residual butanoic acid showed poor chromatographic behavior and eluted as a very broad peak, at least partially masking other components. Could we have developed a superior GC-MS analysis protocol for this analysis? Perhaps, but not without considerable investment of time and there likely would remain the challenge of seeing low MW components. Different analytical strengths and expertises in different research settings should always be considered in choosing the best way to answer questions. Awareness of complementary approaches provides more options of how best to address each specific question.

We also briefly explored the approach of consecutive doping. That is, more than one dopant can be added serially to the initial NMR sample (i.e., A then A+B then A+B+C, etc.). We mention this here because it could be of value in situations where, for example, there is only a limited amount of an initial master mixture.

ii) A mixture of two basic amines: 1-methylpiperazine (1) and 1-methylpiperidine (2). As a second instructive example, we have chosen to show a situation sometimes encountered in which different ^{1}H NMR samples of the same amine are observed to have inconsistent chemical shifts. This is an indication of the likely presence of varying amounts of Brønsted acid in the NMR samples, the exact percentage of which is nearly impossible to know. Sometimes this is recognized as an issue and other times not. A consequence is that chemical shift data reported in the literature for the same amine can be inconsistent from one study, researcher, or era to another. $CDCl_3$ used as the NMR solvent, which lacks an inhibitor, is particularly notorious for having low, variable concentrations of HCl present (via autoxidation to phosgene and hydrolysis[12]), depending upon its history (e.g., age and storage conditions). As a result, varying portions of the basic amine will be present as its HCl salt, giving rise typically to a rapidly exchanging free-base/ammonium ion mixture that shows a single set of time-averaged NMR resonances.[13] The extent of protonation is a function of the relative number of amine vs. HCl molecules in the sample. This problem can be accentuated when working with small quantities of the amine, as can be the case in studies of alkaloid natural products.[14]

For the master mixture in this second example, we used a 1:2 ratio of 1-methylpiperazine (**1**) and 1-methylpiperidine (**2**) (Figure 6). The spectrum of this master (undoped) mixture was recorded as a solution in $CDCl_3$ containing an unknown quantity of HCl contaminant. Three sets of proton resonances for the spectrum of this mixture are shown in Figure 6 (panel a, bottom). Shown in panels b and c are spectra for each of the pure free bases **2** and **1**, respectively, recorded in $CDCl_3$ that had been pre-treated with potassium carbonate to remove the HCl.

The vertical black dashed lines in panels a)-e) are centered on the resonances in panels b) and c) for each of the free bases. Comparison of the stacked spectra in panels b) vs a) clearly shows an inconsistent chemical shift for compound **2** for both the methyl protons at δ 2.24 and the C3 (and C5) methylene protons at 1.59 ppm. Notice that the resonances for **1** in panels c) vs. a) for both the methyl protons at δ 2.27 and the C3 (and C5) methylene protons at 2.90 ppm also show a (very slight) difference in the shifts of the free base vs. the master mixture. Incidentally, the relative magnitude of the Δδs for **2** vs. **1** can be explained by the expected greater basicity of the former (i.e., the piperidine vs. the piperazine). Thus, the comparison of the precise chemical

shifts of the spectra of two different samples would lead one to an incorrect conclusion – that is, that neither **2** nor **1** was present in the mixture.

In contrast, when the sample of the master mixture was doped by adding either of the free bases **2** [panel d)] or **1** [panel e)], only a single set of resonances was observed in the resulting spectrum, even upon applying a severe degree of exponential weighting (not shown). This example demonstrates that a doping experiment can lead to a definitive identification of an analyte under circumstances where stacking vs. a second, external sample of the analyte would not.

Finally, while on the topic of strategies for dealing with variable spectroscopic behavior of basic amines (including N-heterocycles), it is worth noting that the practice of adding an excess of trifluoroacetic acid (TFA) to the NMR sample has been recommended.[15] This assures essentially full conversion to the ammonium ion and gives a more reproducible set of chemical shifts for reporting in the primary literature for newly characterized amines. In other words, the (unknown) amount of Brønsted acid impurity becomes irrelevant.

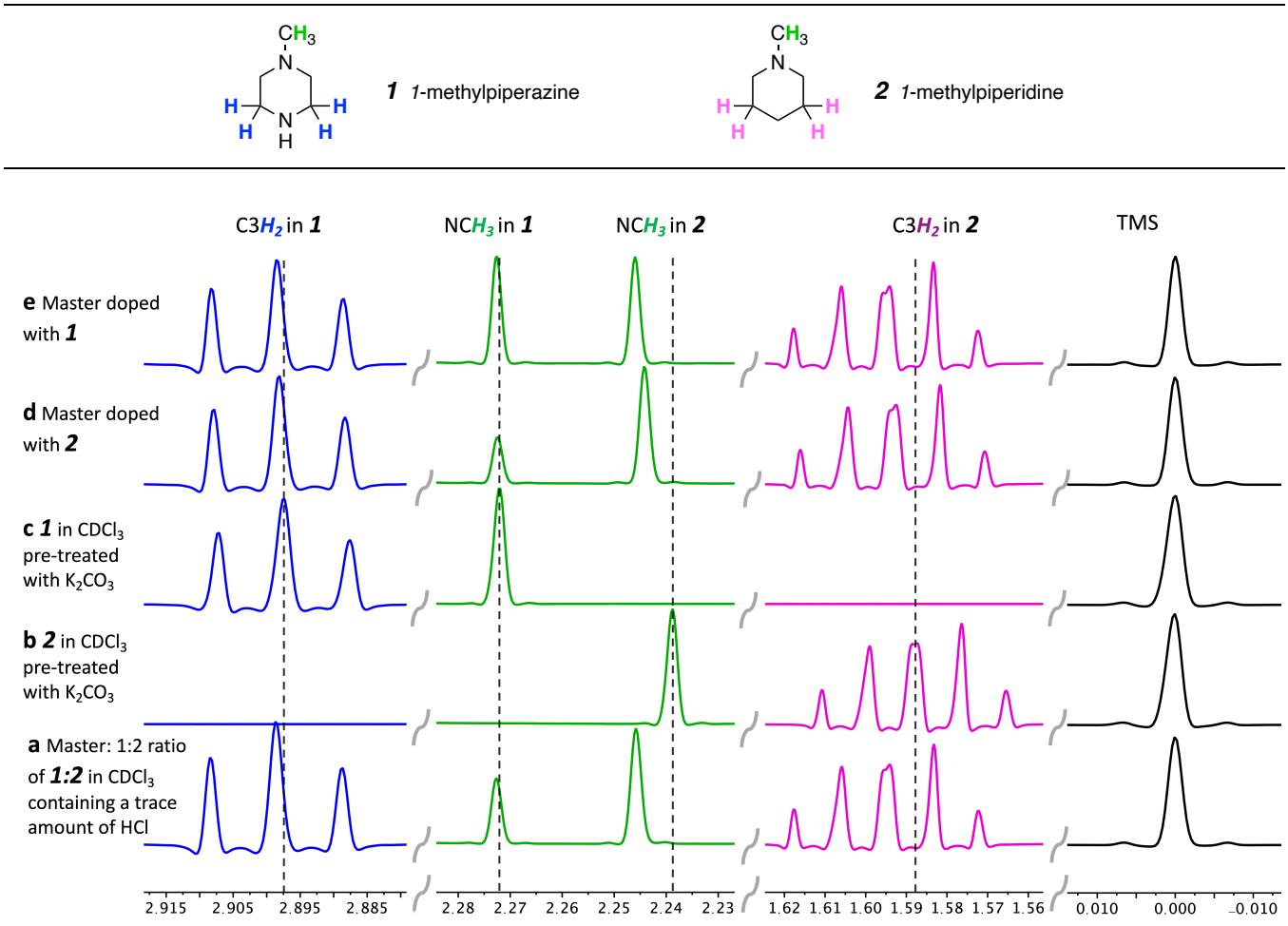


Figure 6. ^{1}H NMR spectra (in $CDCl_3$) of **a**) the master mixture of a 1:2 ratio of **1**:**2** in $CDCl_3$ containing an unknown amount of HCl, **b**) an authentic sample of **2** in chloroform that had been treated with solid K_2CO_3, **c**) an authentic sample of **1** in chloroform that had been treated with solid K_2CO_3, **d**) the master mixture doped with a small amount of the panel b) sample of **2**, and **e**) the master mixture doped with a small amount of the panel c) sample of **1**. [Exponential and Gaussian multipliers of -0.6 and +1.0, respectively, used for the methyl proton singlets and -1.8 and +1.0 for the methylene proton multiplets.]

■ CONCLUSIONS

Well-designed doping experiments can be invaluable in definitively determining the presence or absence of a substance in a ^{1}H NMR sample. Two examples are presented to demonstrate this strategy. Guidelines (best practices) are provided for how to avoid a common pitfall of using too much dopant. With a properly designed experiment, the protocol is quite easy to implement. Readers are reminded of the power of resolution enhancement, a simple technique for maximizing the information content of their 1D proton spectral data.

In the first example, a highly complex mixture of, principally, thirteen components (ketones, acids, and aldehydes) produced during high-temperature pyrolysis of butanoic acid were each positively identified by a series of doping experiments with an (appropriately small

amount of) authentic sample of each substance. The second example is of a mixture of two, structurally simple basic amines. It demonstrates the advantage of a doping strategy vs. comparison of the spectrum of the mixture with that of a separate spectrum of any one potential component of the mixture. Although, of course, no single method for answering questions will ever be universally best, we hope that readers will find this practical guide of value in approaching some of the analytical challenges they encounter.

■ EXPERIMENTAL SECTION

NMR spectra were recorded at 400 MHz on a Bruker instrument (Avance III HD AX-400). Spectra taken in $CDCl_3$ are referenced to the protons in internal TMS (d = 0.00 ppm).[16]

Pyrolysis of butanoic acid in supercritical water.

The initial solution of butanoic acid (BA) "feedstock" was prepared by dissolving 5 g of BA in 100 mL of distilled water. Pure water was pumped (2 mL min^{-1}) into the reactor[7] [10 cm l x 0.42 cm ID, packed with a titanium dioxide catalyst provided by the SarTec Corporation,[7] against a back-pressure regulator (BPR) set at 4000 psi] until the reactor had reached the targeted temperature of 600 °C. The homogeneous feedstock solution of BA was pumped into the reactor. The flow rate of 2 mL min^{-1} equates to a residence time inside the reactor of ca. 30 sec. The effluent was passed through an external cooling bath before exiting the BPR, and the resulting crude product mixture was collected. Its total volume was typically ca. 90 mL. A schematic of the reactor is provided in the SI.

Typical doping analysis of the BA pyrolysate product mixture.

Each doping experiment was carried out by placing a 1 mL aliquot of the BA effluent into a 4 mL glass vial. To this solution was added 0.8 mL of $CDCl_3$ (99.8% level of deuteration). This mixture was shaken and the $CDCl_3$ removed to give the undoped sample of the master BA pyrolysate mixture for each of the doping experiments. The concentration of all analytes in this type of extract was estimated to be ca. 5 mg mL^{-1} by comparing the integration intensity of the residual $CHCl_3$ resonance to the sum of all analyte proton resonances and making approximation of the molecular weights and numbers of protons averaged across all analytes. Each dopant was added by first preparing a stock solution of known titer, from which an appropriately small aliquot (see the discussion above in the doping guidelines section) was removed for adding to the master mixture.

■ ASSOCIATED CONTENT

Supporting Information.

"The Supporting Information (SI) is available free of charge on the ACS Publications website."

A schematic of the pyrolysis reactor and several ancillary spectra of amines vs. ammonium ions (PDF).

FAIR Data (FID for Publication.zip) of the raw data for each NMR spectrum shown in Figures 2–6 (26 files) in individual folders; this .zip file also contains a master metadata file (.docx) showing the folder names and compound structures.

An SI folder (.zip) containing .mnova files of resolution enhanced and/or stacked versions of the NMR spectra from which the graphics in Figures 2-6 were created.

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Notes

The authors have no competing financial interests to declare.

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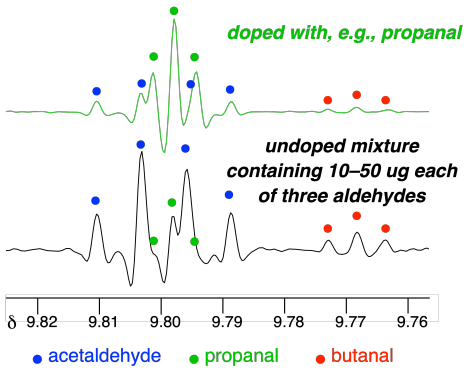

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3 (a) For an example of a recent new development (CORDY), see Yuan, B.; Zhou, Z.; Jiang, B.; Kamal, G-M.; Zhang, X.; Li, C.; Zhou, X.; Liu, M. NMR for mixture analysis: Concentration-ordered spectroscopy. *Anal. Chem*. **2021**, *93*, 9697−9703. (b) For a review of diffusion-ordered spectroscopy (DOSY) analysis of mixtures, see: Pages, G.; Gilard, V.; Martino, R.; Malet-Martino, M. Pulsed-field gradient nuclear magnetic resonance measurements (PFG NMR) for diffusion ordered spectroscopy (DOSY) mapping. *Analyst* **2017**, *142*, 3771−3796.

4 (a) For an example of a recent new development, see: Reher, R.; Kim, H. W.; Zhang, C.; Mao, H. H.; Wang, M.; Nothias, L. F.; Caraballo-Rodriguez, A. M.; Glukhov, E.; Teke, B.; Leao, T.; Alexander, K. L.; Duggan, B. M.; Van Everbroeck, E. L.; Dorrestein, P. C.; Cottrell, G. W.; Gerwick, W. H. A Convolutional neural network-based approach for the rapid annotation of molecularly diverse natural products. *J. Am. Chem. Soc.* ***2020***, *142*, 4114−4120. (b) For a review, see: Novoa-Carballal, R.; Fernandez-Megia, E.; Jimenez, C.; Riguera, R. NMR methods for unravelling the spectra of complex mixtures. *Nat. Prod. Rep.* **2011**, *28*, 78-98.

5 The use of NMR spectroscopy as a tool in metabolomics studies is a rapidly growing area of investigation. Dozens of reviews touching on this topic have appeared in the last five years alone. Some cover the field broadly while others deal with subsets of studies focusing on selected areas of biology and medicine. For examples of the former, see: (a) Dona, A. C.; Kyriakides, M.; Scott, F.; Shephard, E. A.; Varshavi, D.; Veselkov, K.; Everett, J. R. A guide to the identification of metabolites in NMR-based metabolomics/metabolomics experiments. *Comput. Struct. Biotechnol. J*. **2016**, *14*, 135−153; and (b) Emwas, A.-H.; Roy, R.; McKay, R. T.; Tenori, L.; Saccenti, E.; Gowda, G. A. N.; Raftery, D.; Alahmari, F.; Jaremko, L.; Jaremko, M.; Wishart, D. S. NMR spectroscopy for metabolomics research. *Metabolites* **2019**, *9*, 123 doi:10.3390/metabo9070123.

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