Simultaneous Quantification of H$_2$O$_2$ and Organic Hydroperoxides by $^1$H NMR Spectroscopy

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ABSTRACT: Due to similar reactivity of organic hydroperoxides (OHPs), an HPLC separation step is typically required for their indirect (chemical) quantification in mixtures. The high sensitivity of chemical shifts to chemical structure makes NMR an ideal tool for the simultaneous quantification of OHPs in mixtures, but the concentration of these analytes in the samples of interest is usually well below the sensitivity of standard NMR experiments. This sensitivity problem can be mitigated by taking advantage of the fact that the $z$ magnetization of the H$_2$O$_2$ resonance recovers at the rate of hydrogen exchange with water, which is significantly faster than longitudinal relaxation, thus enabling very fast scanning for signal-to-noise enhancement. An adaptation of the E-BURP pulse is described that suppresses the water signal by more than 4 orders of magnitude, yielding uniform excitation of peroxide signals without interference of the ca. 10$^8$-fold stronger H$_2$O$_2$ resonance. We demonstrate the method for a mixture of OHPs and report the chemical shifts for multiple OHPs that are of interest in atmospheric chemistry. As shown for hydroxymethyl hydroperoxide, the chemical decay of OHPs can be tracked directly by NMR spectroscopy.

This Letter extends the application of a recently developed NMR method for nanomolar detection of hydrogen peroxide (H$_2$O$_2$)$^{1,2}$ to organic hydroperoxides (OHPs). This class of peroxides plays key roles in many fields, ranging from synthetics and atmospheric chemistry$^3$ to human diseases$^5$, and their quantification is important from safety and product quality points of view.$^6$ In the atmosphere, OHPs can result from radical reactions initiated by hydrogen abstraction from saturated hydrocarbons$^7$ or via ozonolysis of alkenes like $\alpha$-pinene$^8,9$, followed by hydration of Criegee intermediates.$^{10}$ Iodometric and other chemical methods typically used for the quantification of OHPs do not distinguish them from each other or from H$_2$O$_2$, and, thus, only report the total peroxide concentration.$^1$ Good selectivity of the catalase enzyme for H$_2$O$_2$ vs OHPs has been utilized for the determination of total OHP concentrations in samples containing H$_2$O$_2$. Post-HPLC derivatization has been used for quantification of individual OHPs down to 20 nM.$^{12}$ Although these methods can distinguish between stable hydroperoxides, they are not suitable for hydroxalkyl hydroperoxides that decompose to their corresponding carbonyl compounds within minutes.$^5$ Mass spectroscopy is another sensitive method for the detection of OHPs without the need for HPLC purification,$^{14}$ however, it is inherently not a quantitative method.$^{15}$

The facts that NMR spectroscopy is quantitative and that chemical shifts are quite sensitive to chemical structure make it a suitable method for the quantification of OHPs in mixtures. While NMR detection of the downfield shifted H$_2$O$_2$ signal (at ca. 11.3 ppm) was first reported nearly two decades ago,$^{16}$ the limited sensitivity of NMR prevented applications to OHPs in the concentration ranges that are of most practical interest. The low sensitivity of NMR, in part, originates from long interscan delays required for $z$ magnetization recovery via longitudinal relaxation. However, for protons that exchange with water, as applies to OHPs, recovery following selective excitation of the hydroperoxide region of the spectrum (ca. 11–12.5 ppm) is dominated by hydrogen exchange (HX) with water, obviating the need for long interscan delays. Together with advances in spectrometer hardware, this now permits detection down to the nanomolar range.

EXPERIMENTAL SECTION

Validation of Commercial H$_2$O$_2$ and t-BuOOH Sources. $^1$H NMR spectra of concentrated samples were fitted to Lorentzian functions for accurate quantification. To reduce radiation damping and receiver overload due to strong resonances in concentrated samples, the probe was detuned, the receiver gain was set to its minimum value, and a single 1$\mu$s pulse, corresponding to a ca. 4° flip angle, was applied followed by a 1 s acquisition. Signal integration of the
water and solute signals confirmed all concentrations agreed to
within ±1% of the manufacturer’s specification (see Figure
S1). We note that quantification relative to the water signal is
only needed to evaluate the concentrated commercial samples
(e.g., 30% H₂O₂ and 70% t-BuOOH). Quantification of dilute
peroxide samples is easily accomplished by the standard
addition of a small known quantity of such a commercial
reference sample.

**Organic Peroxide Synthesis.** Details regarding the
preparation of hydroxymethyl hydroperoxide (HMHP), benzyl
hydroperoxide (PhCH₂OOH), isopropyl hydroperoxide (i-
PrOOH), methyl hydroperoxide (MeOOH), ethyl hydro-
peroxide (EtOOH), and tert-amyl hydroperoxide (t-Amyl-
OOH) are included in the Supporting Information. tert-Butyl
hydroperoxide (t-BuOOH) was purchased from Sigma-
Aldrich.

**NMR.** All NMR spectra were collected on a 600 MHz
Bruker NEO spectrometer equipped with a cryogenic probe.
Measurements were analogous to those in our earlier report,1
but in order to prevent large frequency-dependent phase
errors that interfered with baseline correction, the excitation pulse
was replaced by a 2.5 ms E-BURP2 pulse17 that covers a
bandwidth of ca. 2 ppm at 600 MHz and an offset parameter
that centers its excitation at 11.8 ppm. This pulse results in a
weak, spurious excitation of the on-resonance water signal,
interfering with the detection of this weak signal. This spurious
water signal was reduced more than 100-fold by application of
a rectangular 5 µs pulse whose RF-field strength and phase
were empirically fine-tuned to negate water excitation by the
E-BURP2 pulse. The initial parameters for this rectangular pulse
were first adjusted in a separate measurement that used only
this pulse to yield the same amplitude and phase of the water
signal as the E-BURP2 excitation. Then, after inverting its
phase, it was appended immediately after the E-BURP2 pulse
(Supporting Information). Following additional fine-tuning of
the power and phase of this water flip-back pulse, an additional
ca. 100-fold suppression of the water signal was obtained
relative to that of just the E-BURP2 pulse. Even after this
optimization, the suppressed H₂O signal can remain several
orders of magnitude more intense than low-concentration
peroxide signals. To avoid truncation wiggles from the water
signal in the Fourier transformed spectrum, the time domain
data must be apodized to smoothly decrease to zero at the end
of the free induction decay (FID). The commonly used π/2-
shifted sine bell function, or its squared variant, is well suited
for this purpose. Because such an apodization window does not
scale the first data point of the time domain, application of the
window does not alter the integrated signal intensity.

Simulated excitation profiles show that the added, small flip
angle pulse applied to suppress the water peak has minimal
impact on the uniformity of excitation of the hydroperoxide
region. The uniformity of excitation across the hydroperoxide
region was confirmed experimentally by measuring the
peroxide resonance intensity of a 10 mM t-BuOOH sample
while varying the offset frequency (Figure S2).

**Chemical Shift Calculations.** For each compound, a
systematic conformer search was performed at the oB97X-D/
6-31G* level of theory using Spartan20,21 The free energies
for each conformer were then calculated using the G4(MP2)
composite method19 from which the Boltzmann distribution of
conformer populations was calculated using

\[ P_i = \frac{e^{-G_i/RT}}{\sum_i e^{-G_i/RT}} \]

where \( p_i \) and \( G_i \) are the population and calculated relative
free energy of the \( i \)th conformer, respectively; \( R \) is the universal
gas constant; \( T \) is the absolute temperature. Next, the
optimized geometries obtained from the G4(MP2) calcula-
tions were checked for duplicate geometries and imaginary
frequencies before being used to calculate the NMR isotropic
shielding values (\( \sigma_i \)) using the Gauge-Independent Atomic
 Orbital (GIAO) method. The chemical shielding calculations
were performed at the oB97X-D/6-31G** level of theory. Boltzmann-weighted shielding values (\( \sigma_{BW} \)) were then
derived using

\[ \sigma_{BW} = \sum_i p_i \times \sigma_i \]

in which \( i \) runs over all conformers. Finally, the \( \sigma_{BW} \) values
were converted to chemical shifts (\( \delta_{calc} \)) using

\[ \delta_{calc} = \sigma_{BW}(DSS) - \sigma_{BW} \]

where \( \sigma_{BW}(DSS) \) is the Boltzmann-weighted isotropic
shielding value for the 2,2-dimethylsilapentane-5-sulfonate
(DSS) anion calculated at the same level of theory. Gaussian
1622 was utilized for all the calculations after the conforma-
tional search, and water was simulated by SMD23 for both
thermochemical and NMR calculations. For details, see the
Supporting Information.

**RESULTS AND DISCUSSION**

Since OHPs have \( p_K \) values very close to that of \( H_2O_2 \),24 they
are expected to have resonances that are similar in HX rate,
\( R_{ex} \), and chemical shift. Indeed, a 1 mM sample of
commercially available t-BuOOH shows a \( ^1H \) NMR resonance
at 11.66 ppm, close to but readily distinguishable from that of
\( H_2O_2 \). \( R_{ex} \) can be derived from transverse or longitudinal
relaxation measurements,1 but due to the absence of \( J_{HH} \) or
\( J_{CH} \) couplings, \( R_{ex} \) is also directly reflected in the resonance
line width, which for a sufficiently long acquisition time, equals
\( R_{ex}/\pi \) Hz in the absence of apodization. At 2 °C and 1 mM
MES buffer, the \( R_{ex} \) minimum of t-BuOOH is at pH 6.3
(Figure 1) and is the lowest (27 s⁻¹) of the OHPs investigated
here, only moderately slower than that of \( H_2O_2 \) (41 s⁻¹).24
With the \( R_{ex} \) rates and their pH dependence being comparable
for OHPs and \( H_2O_2 \), their NMR intensities therefore can be
quantified simultaneously on a single sample.

With multiple peroxide resonances in a single spectrum, the
strong linearly offset-dependent phase correction associated
with the originally used Gaussian-shaped pulse1 resulted in
severe baseline undulations, and an E-BURP2 pulse followed
by a very weak water flip-back pulse was used instead (see
Experimental Section). The excitation profile of this pulse
pulse is flat to within ±2% over a 2 ppm bandwidth (Figure S2),
thus providing full excitation of all OHP resonances.

All observed OHP signals resonate downfield of \( H_2O_2 \) and
have unique chemical shifts (Figure 2). Their chemical shifts
relative to 2,2-dimethylsilapentane-5-sulfonic acid (DSS) are
listed in Table 1. The comparison of \( H_2O_2 \) and MeOOH
shows that the addition of one methyl group results in a
substantial chemical shift change (11.29 vs 12.37 ppm). This
SMD free energies and ω calculations.

The pH of the sample was adjusted to 6.0 at 20 °C. The pH values at 2 °C were calculated via two-point extrapolation from pH measurements at 20 and 5 °C. The dashed line represents the best fit to \( k = k_{tH_2O} + k_{tMES}[MES] + k_H10^{-pH} + k_{tOH}10^{-pOH} \), in which \( k_{tH_2O} \), \( k_{tMES} \), \( k_H \), and \( k_{tOH} \) are water-, MES-, acid-, and base-catalyzed rate constants, respectively, and their best-fitted values are \( k_{tH_2O} + k_{tMES}[MES] = 5.7 \pm 0.6 \text{ M}^{-1}\text{s}^{-1}; \ k_H = (2.2 \pm 0.1) \times 10^7 \text{ M}^{-1}\text{s}^{-1}; \ k_{tOH} = (3.6 \pm 0.1) \times 10^8 \text{ M}^{-1}\text{s}^{-1} \).

Table 1. Calculated vs Experimental 1H Chemical Shifts

| compound       | exp δ (ppm)\(^a\) | calc'd δ (ppm)\(^b\) |
|----------------|---------------------|----------------------|
| \( \text{H}_2\text{O}_2 \) | 11.29               | 7.68                 |
| HMHP           | 12.42               | 8.60                 |
| \( \text{MeOOH} \)  | 12.37               | 8.29                 |
| \( \text{PhCH}_2\text{OOH} \) | 12.31              | 8.18                 |
| \( \text{EtOOH} \)  | 12.22               | 8.12                 |
| \( \text{i-PrOOH} \) | 12.04               | 7.84                 |
| \( \text{t-BuOOH} \) | 11.66               | 7.43                 |
| \( \text{t-AmylOOH} \) | 11.54               | 7.32                 |

\(^a\)Referenced vs DSS. \(^b\)Boltzmann-weighted values using G4(MP2)/SMD free energies and \( \omega \text{B97X-D} / \text{aug-cc-pVDZ} / \text{SMD} \) NMR calculations.

Figure 1. pH dependence of the t-BuOOH HX rate with water in samples containing 1 mM MES and 1 mM t-BuOOH. The pH of the samples was adjusted at 20 °C, and the rates were measured at 2 °C. The pH values at 2 °C were calculated via two-point extrapolation from pH measurements at 20 and 5 °C. The dashed line represents the best fit to \( k = k_{tH_2O} + k_{tMES}[MES] + k_H10^{-pH} + k_{tOH}10^{-pOH} \), in which \( k_{tH_2O} \), \( k_{tMES} \), \( k_H \), and \( k_{tOH} \) are water-, MES-, acid-, and base-catalyzed rate constants, respectively, and their best-fitted values are \( k_{tH_2O} + k_{tMES}[MES] = 5.7 \pm 0.6 \text{ M}^{-1}\text{s}^{-1}; \ k_H = (2.2 \pm 0.1) \times 10^7 \text{ M}^{-1}\text{s}^{-1}; \ k_{tOH} = (3.6 \pm 0.1) \times 10^8 \text{ M}^{-1}\text{s}^{-1} \).

Figure 2. Downfield region of the 600 MHz 1H NMR spectrum for a mixture of \( \text{H}_2\text{O}_2 \) and six OHPs, all at ca. 1 µM concentration in 1 mM MES. The pH of the sample was adjusted to 6.0 at 20 °C using a glass electrode, and the spectra were collected at 2 °C. The spectrum was collected using 4096 scans (4 min), an acquisition time of 50 ms, and an interscan delay of 1 ms. The FID was apodized by a π/2-shifted sine bell function. Resonances correspond to (A) MeOOH; (B) PhCH\(_2\)OOH; (C) EtOOH; (D) i-PrOOH; (E) t-BuOOH; (F) t-AmylOOH; (G) \( \text{H}_2\text{O}_2 \). For spectra of the individual OHPs, see Figure S3.

Figure 3. Correlation between experimental and calculated chemical shifts. The slope and intercept are 0.74 ± 0.07 and 6.2 ± 0.6 ppm, respectively.

Hydroxylalkyl hydroperoxides such as HMHP are of interest in atmospheric chemistry. HMHP is thought to be mostly produced in the atmosphere through ozonolysis of terminal alkenes, followed by hydration of a Criegee intermediate.\(^{25}\)

Once in the condensed phase, however, these compounds will decompose to their corresponding carbonyl compounds and \( \text{H}_2\text{O}_2 \). This reaction can be readily tracked by NMR (Figure 4), showing a decomposition rate of HMHP at pH 6 and 25 °C that is about 10-fold slower than prior values measured at pH 7.07 and 22 °C.\(^{26}\) The decay rate is further decreased by more than 20-fold (Figure 3) when the temperature is lowered to 2 °C, indicative of a high activation energy of ca. 26 kcal/mol for this reaction at pH 6, ignoring the small sample pH change upon cooling.

CONCLUSIONS

Although NMR spectroscopy typically is not considered to be a sensitive method, when one takes advantage of the fast HX of OHPs with water, the quantification of their presence down to submicromolar concentrations within minutes is straightforward. The advantage of NMR over other methods is that chemical shifts are quite sensitive to covalent structure, resulting in unique shifts that simultaneously enable the identification of OHPs and their approximate quantification in aqueous mixtures, eliminating the need for separation associated with other detection methods. However, if precise quantitation is of the essence, we recommend that the acquisition time is increased to 100 ms, such that HX with water is essentially complete (Figure S4). If an OHP were to
have a much slower HX rate than H₂O₂, then this would be immediately apparent by its narrower line width and the measurement may need to be repeated with an even longer interscan delay. However, this did not apply for any of the OHPs in our study. We also note that the method requires H₂O as the solvent because it relies on fast HX. Rapid HX was observed for all OHPs evaluated in our work, and both the adjustment of the pH to ca. 6 and lowering the temperature to just above the freezing point of water were needed to slow down the HX rates to values that yielded high resolution ¹H NMR spectra.

The above procedure therefore permits quantitative OHP analysis by comparison with the intensity of a known H₂O₂ reference intensity (Figure 5). We also find that the NMR peak intensity correlates well with the t-BuOOH concentration over a concentration range that spans 6 orders of magnitude (Figure S5).

The simple NMR pulse sequence introduced here to suppress the water signal, without requiring pulsed field gradients or echo delays, reduces excitation of the water signal by more than 4 orders of magnitude while retaining full intensity for the resonances selected by the band-selective excitation pulse. The good correlation between experimental peroxide ¹H chemical shifts and quantum—mechanical calculations will aid in the identification of unknown compounds.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c00264.

Experimental details of the OHP syntheses; NMR spectra of individual OHPs and the excitation profile of the E-BURP2/flip-back pulse pair; Cartesian coordinates of the optimized structures; calculated thermodynamic parameters and isotropic shielding values; NMR pulse program (PDF)

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#### Notes

The authors declare no competing financial interest.

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