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Rapid and Direct Low Micromolar NMR Method for the Simultaneous Detection of Hydrogen Peroxide and Phenolics in Plant Extracts

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Supporting Information

ABSTRACT: A rapid and direct low micromolar ^{1}H NMR method for the simultaneous identification and quantification of hydrogen peroxide and phenolic compounds in plant extracts was developed. The method is based on the highly deshielded ^{1}H NMR signal of H_2O_2 at ∼10.30 ppm in DMSO-d_6 and the combined use of picric acid and low temperature, near the freezing point of the solution, in order to achieve the minimum proton exchange rate. Line widths of H_2O_2 below 3.8 Hz were obtained for several Greek oregano extracts which resulted in a detection limit of 0.7 μmol L^{-1}. Application of an array of NMR experiments, including 2D ^{1}H−^{13}C HMBC, spiking of the samples with H_2O_2, and variable temperature experiments, resulted in the unequivocal assignment of H_2O_2 precluding any confusion with interferences from intrinsic phenolics in the extract.

KEYWORDS: *hydrogen peroxide, ^{1}H−^{13}C HMBC, NMR, phenolics, spiking, DETAPAC*

INTRODUCTION

Hydrogen peroxide plays an important role in fermentation and food industry, as a bleaching agent for textiles, as an oxidizer for vat dyes, and as an active reagent in chemical synthesis and in detoxicating organic pollutants.[1,2] Hydrogen peroxide is also involved in oxidative processes required in the initiation and the promotion of ripening of fruits,[3] and in a number of signaling cascades in plants[4,5] in response to both biotic and abiotic stress.[6] Plants possess very efficient enzymatic and non-enzymatic antioxidant defense systems that allow scavenging of H_2O_2 and protect plant cells from oxidative damage.[7]

As a consequence of the increasing importance of H_2O_2 in plant metabolism, adequate and efficient methods are necessary to determine its concentration in plant tissue and organs. Several analytical techniques have been used for determination of hydrogen peroxide. These include titrimetry,[8] a chromatographic method based on the conversion of methylphenyl sulfide to methylphenyl sulfoxide and methylphenyl sulfone,[9] infrared spectroscopy,[10] Raman spectroscopy,[11] chemiluminescence,[12] electrochemical methods,[13,14] and biosensors.[15,16] Electrochemical and chemiluminescence detection of H_2O_2 is distinctive for its low detection limit as well as low costs.[17,18] However, phenolics and ascorbate, which are normally present at high concentrations in plant tissue and extracts, quench luminescence and affect electrochemical detection. Therefore, additions of insoluble polyvinylpolypyrrolidone (PVPP) and ascorbate oxidase are usually required in order to remove phenols and ascorbate from extracts.[19,20] Furthermore, several sensors based on proteins may result in limited lifetime, a stability problem, and complex procedures in the fabrication process. Recently, a technique utilizing ^{1}H NMR has been developed to measure the concentration of hydrogen peroxide from 10^{-3} to 10 mol L^{-1}.[21] Line widths, however, of over 250 Hz were observed which limit the wide application of the method.

Despite the numerous analytical techniques available, few methods are applicable for quantitative measurements of hydrogen peroxide in the micromolar to millimolar concentration range for natural product extracts. Furthermore, the origin of the great variation in the determined H_2O_2 concentrations in leaves and extracts from various plant species that spanned more than 3 orders of magnitude was ascribed by Cheesman[22] to be due to methodology and experimental uncertainties rather than biological variation. Thus, continued generation or degradation of H_2O_2 during analysis is potentially as important as other types of interference by ascorbate and phenolics with the hydrogen peroxide assay itself.[22]

In the present work, we demonstrate that a rapid and direct micromolar identification and quantification of hydrogen peroxide can be achieved in plant extracts using one-dimensional ^{1}H NMR spectroscopy. The determination of H_2O_2 is based on the highly deshielded ^{1}H NMR signal at ∼10.30 ppm and the extremely sharp ($\Delta\nu_{1/2} \approx 1.2-3.8$ Hz) resonance which can be achieved with the use of DMSO-d_6 as solvent, picric acid, and temperatures near the freezing point of the solution which result in a minimum of intermolecular proton exchange rate. Unequivocal assignment of H_2O_2 was achieved with the combined use of spiking with H_2O_2 and 2D ^{1}H−^{13}C HMBC. Moreover, the simultaneous determination of phenol −OH containing compounds (Scheme 1) is reported herein.

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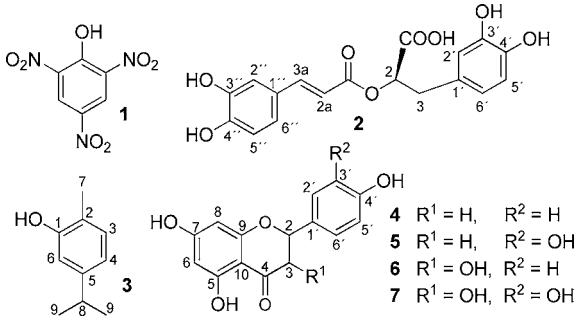
Scheme 1. Chemical Structures of Picric Acid (1) and Major Phenol −OH Containing Compounds Present in Greek Oregano Extracts [Rosmarinic Acid (2), Carvacrol (3), Naringenin (4), Eriodictyol (5), Aromadendrin (6), Taxifolin (7)]



MATERIALS AND METHODS

Chemicals. DMSO-d_6 and CD_3CN (NMR quality) were purchased from Deutero (Kastellaun, Germany), H_2O_2 was from Carlo Erba Reagents (Milan, Italy), and 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP-d_4) was from Cambridge Isotope Laboratories Inc. (Cambridge, MA). Hexane and methanol were of analytical grade from Scharlau (Barcelona, Spain), and ethyl acetate and acetone were from Lab-Scan (Dublin, Ireland). Diethylenetriaminepentaacetic acid (DETAPAC) was purchased from Sigma-Aldrich.

Plant Material. Aerial parts of oregano (*Origanum vulgare*) were collected in August 2008 from the region of Epirus, Greece. Reference specimens are retained in the University of Ioannina with voucher accession number UOI080811.

Sample Preparation. The plant material was air-dried at room temperature in the dark, and stored at −20 °C. In order to prepare the ethyl acetate and methanol extracts ground oregano leaves (∼5 g) were extracted sequentially with 200 mL of three solvents of gradually increasing polarity in a Soxhlet apparatus for 6 h with each solvent. The sequence of the solvents was the following: hexane (in order to remove chlorophylls), ethyl acetate, and methanol. The ethyl acetate and methanolic extracts were concentrated using a rotary evaporator and kept in sealed dark flasks after a few minutes of nitrogen flushing. Approximately 0.3 and 0.4 g of residue was obtained from the ethyl acetate and methanol extracts, respectively. In order to prepare the crude acetone extract, dried material (5 g) was extracted with 200 mL of acetone in a Soxhlet apparatus for 6 h, as previously described. Approximately 0.4 g of residue was obtained from the acetone extract. In order to investigate the effect of metal chelating agent (DETAPAC) and neutral pH buffer on the hydrogen peroxide content, three different aqueous extracts were prepared as follows: dried material (2 g) was placed in (a) 100 mL of distilled water, (b) 100 mL of 10 mM sodium phosphate buffer pH 6.2 with 1.4×10^{-5} M DETAPAC, and (c) 100 mL of distilled water with 1.4×10^{-5} M DETAPAC. The samples were heated under stirring for 1 h in water bath at 40 °C and then filtrated. The water was removed with freeze-dryer. Approximately 0.5 g of solid residue for extracts a and c and 0.6 g for extract b were obtained. All solid residues were stored at −20 °C.

NMR Instrumentation. NMR experiments were performed on a Bruker AV500 spectrometer equipped with a TXI cryoprobe (Bruker Biospin, Rheinstetten, Germany). Samples were dissolved in 0.6 mL of DMSO-d_6 and transferred to 5 mm NMR tubes. All chemical shifts were measured with reference to the internal standard, TSP-d_4 (δ_H = 0.000 ppm), of a certain concentration (0.02 mmol L^{-1}). The amount of added picric acid (**1**) was 40−80 μL from a stock DMSO-d_6 solution of 0.1 mol L^{-1} of (**1**) for the aqueous and methanol solid residues and 10−20 μL from a stock DMSO-d_6 solution of 0.01 mol L^{-1} of (**1**) for the acetone solid residue. The NMR spectrometer was controlled by the software TopSpin 2.1. All spectra were acquired with an acquisition time of 2.499 s, 64 K data points, and 90° pulse length. The 2D $^1H-^{13}C$ HSQC and HMBC experiments were carried out using standard Brüker software, and parameters were optimized for coupling constants of 145.0 and 8.0 to 2.5 Hz, respectively. To ensure adequate relaxation of the protons that were used for quantification, their T_1 was measured using the inversion recovery pulse sequence. T_1 was found to be 1.27 s for H_2O_2 and 2.51−2.97 s for the phenolic compounds and flavonoids of interest. The pulse repetition time was set at $4T_1$ of the longest T_1 value.

RESULTS AND DISCUSSION

The ^1H NMR chemical shift of the model compound H_2O_2 in various solvents was found to be strongly dependent on the hydrogen bonding strength and solvation ability of the solvent (δ_H = 10.27 ppm in DMSO-d_6 and δ_H = 8.77 ppm in CD_3CN). Furthermore, δ_H of H_2O_2 in DMSO-d_6 was found to be practically concentration independent in the region of 5 mmol L^{-1} (δ_H = 10.25 ppm) to 50 mmol L^{-1} (δ_H = 10.30 ppm). δ_H of H_2O_2 in DMSO-d_6, therefore, is well outside the overcrowded aromatic region present in natural product mixtures.[23]

Figure 1A illustrates a selected region of the ^1H NMR spectrum of a methanol extract of Greek oregano in DMSO-d_6

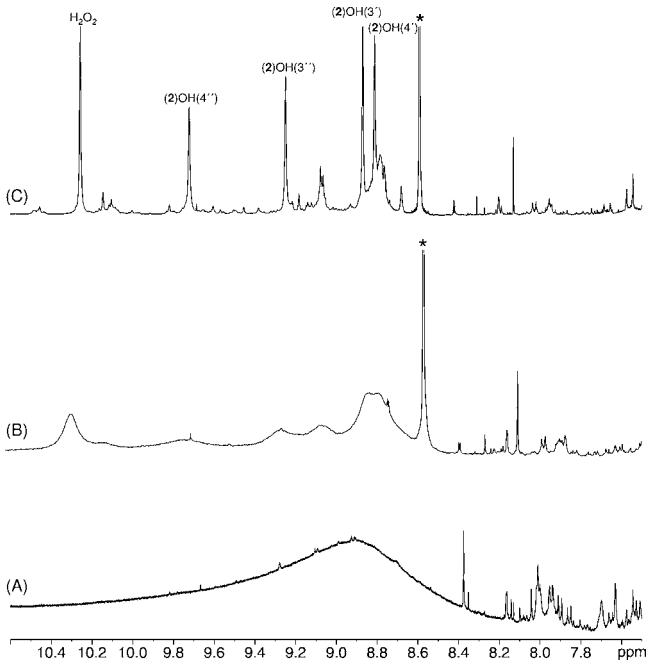


Figure 1. Selected regions of the 500 MHz 1D ^1H NMR spectra of 21.3 mg methanol extract of Greek oregano in 0.6 mL of DMSO-d_6 (T = 288 K, number of scans = 512, experimental time = 1 h and 8 min). (A) Without the addition of picric acid (**1**). (B and C) The same solution as in part A with a dilution factor of 2 and with mass ratio of [**1**]/[extract] = 158.5×10^{-3} and 225.7×10^{-3}, respectively. The asterisk denotes the resonance of **1**. The assignment of the resonances of the −OH groups of rosmarinic acid **2** is indicated.

which was chosen because of its enhanced antioxidant activity[23] and its chemical complexity.[24] In the region δ_H 8.1−10.4 ppm a strong and broad resonance ($\Delta\nu_{1/2}$ = 280 Hz) was observed, which can be attributed to the composite signal of the −OH groups of the main constituents of the extract. The −OH groups appear as broad signals due to intermolecular exchange of the −OH protons with the residual H_2O in DMSO-d_6 and the various −OH and −COOH groups.[25] It has been recently illustrated that ultra high-resolution can be achieved in the ^1H NMR spectroscopic region (8−14 ppm) where the phenolic hydroxyl resonances appear with the addition of picric acid

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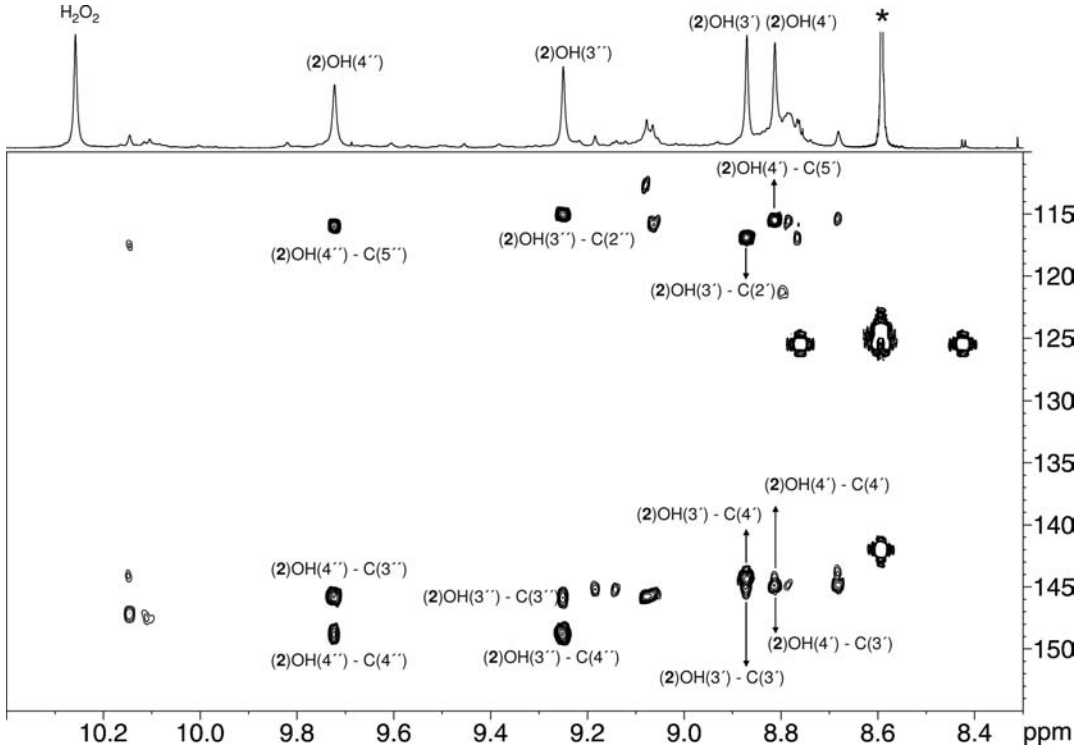

Figure 2. Selected regions of the 500 MHz 2D $^{1}H-^{13}C$ HMBC NMR spectrum of the solution of Figure 1 (number of scans = 102, experimental time = 13 h and 20 min). The common cross-peaks of the OH groups of rosmarinic acid (**2**) are indicated. The asterisk denotes the resonance of **1**.

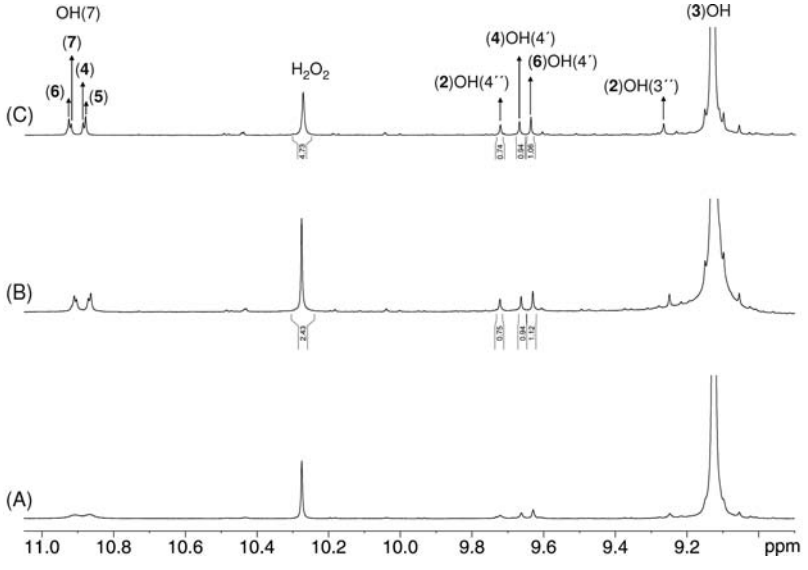

Figure 3. Selected regions of the 500 MHz 1D ^{1}H NMR spectra of 20 mg of a Greek oregano acetone extract in 0.6 mL of DMSO-d_6 (T = 292 K, number of scans = 64, experimental time = 8 min). (A) Without the addition of picric acid (**1**). (B) With mass ratio of [**1**]/[extract] = 1.68×10^{-3}. (C) The same solution as in part B with the addition of 0.77 mmol L^{-1} H_2O_2. The assignment of the −OH resonances of the major species carvacrol (**3**), rosmarinic acid (**2**), naringenin (**4**), taxifolin (**7**), eriodictyol (**5**), and aromadendrin (**6**) is indicated.

(**1**).[26] The chemical exchange rate of the hydroxyl protons exhibits a U-shaped curve with a minimum in aqueous solutions at pH ≈ 4.5.[27,28] A similar minimum in the exchange rate of the relevant protons has been observed in a mixture of H_2O and organic solvents.[27] The pH values of aqueous solutions of the studied Greek oregano extracts were found to be in the range 5.2−6.5. Addition of **1** (Figure 1B) resulted in excellent resolution of all the phenol −OH resonances. Specifically, four major peaks of equal integral at 9.72 ppm ($\Delta\nu_{1/2}$ = 4.2 Hz), 9.25 ppm ($\Delta\nu_{1/2}$ = 3.5 Hz), 8.87 ppm ($\Delta\nu_{1/2}$ = 3.3 Hz), and 8.81 ppm ($\Delta\nu_{1/2}$ = 3.9 Hz) can be attributed to the OH(4″), OH(3″), OH(3′), and OH(4′) of rosmarinic acid (**2**), which is one of the main constituents of the extract. Application of 2D $^{1}H-^{13}C$ HMBC (Figure 2) in the same solution resulted in a significant number of $^{n}J(^{1}H,^{13}C)$ cross-peaks of the OH(4″), OH(3″), OH(3′), and OH(4′) of rosmarinic acid and of other minor constituents of the extract. On the contrary, the resonance at 10.30 ppm did not present any $^{n}J(^{1}H,^{13}C)$ cross-peaks. Moreover, spiking of the solution with 0.77 mmol L^{-1} of

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H_2O_2 in DMSO-d_6 resulted in an increase of the resonance at 10.30 ppm ($\Delta\nu_{1/2}$ = 3.3 Hz) (Figure 3C) which should be attributed to H_2O_2. The results of the spiking experiments were quantitative, and recovery values of 95−98% were obtained for H_2O_2 on the basis of the NMR integration data (Figure 3C).

It is important to define the temperature at which the ^{1}H NMR spectra of the extracts must be carried out to avoid undesirable broadening of the −OH groups.[29] Therefore, detailed variable temperature ^{1}H NMR spectra of the Greek oregano ethyl acetate extract were recorded (Figure 4). A linear

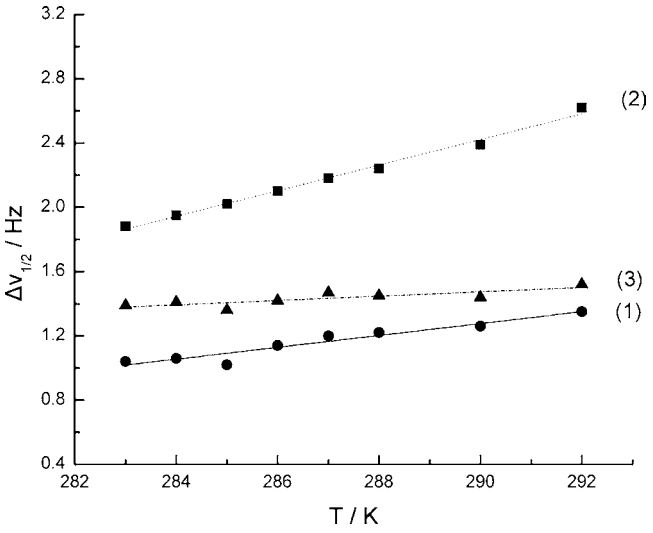

Figure 4. Temperature dependencies of the line widths ($\Delta\nu_{1/2}$) of the −OH protons of H_2O_2 (**1**), rosmarinic acid (**2**) OH (4″), and carvacrol (**3**) OH, in 0.6 mL DMSO-d_6 of the Greek oregano ethyl acetate extract.

dependence of the line width, $\Delta\nu_{1/2}$, of H_2O_2, OH(4″) of rosmarinic acid and carvacrol was obtained in the temperature range 292−283 K. The minimum line width was obtained at 283 K which is approximately 2 °C above the freezing point of the solution (281 K) and was found to be up to 11 °C below the freezing point of the neat solvent (292 K) and depended upon the concentration of the solution and the amount of the residual H_2O. This demonstrates that in the region 292−283 K the effect of the decrease in proton exchange upon lowering the temperature on the −OH line widths more than compensates for the effect of the increase in solvent viscosity.

In order to evaluate the efficiency of this analytical method to natural product extracts, it was applied to four different solvent extracted Greek oregano samples (Tables 1 and 2). Since the spectral region at 10−11 ppm is not crowed (Figures 1 and 3), contrary to the case of the aromatic or aliphatic region, the analysis of H_2O_2 in complex plant extracts can be obtained directly by the use of one-dimensional ^{1}H NMR spectra. It

Table 1. Chemical Shifts, δ (ppm), and Line Widths, $\Delta\nu_{1/2}$ (Hz), of H_2O_2 in Greek Oregano Extracts at T = 288 K

extract	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)
aqueous	10.30	3.8[a]
methanol	10.30	3.3[a]
acetone	10.29	2.9[a]
ethyl acetate	10.29	1.2

[a]After the addition of picric acid (**1**).

should be emphasized that the ^{1}H NMR chemical shift variation of H_2O_2 in the different Greek oregano extracts was found to be below 0.01 ppm (Table 1). This demonstrates that δ_H in DMSO-d_6 can be of high diagnostic value for identifying H_2O_2 in complex plant extracts. Even in the rare case of interference from −OH containing compounds in this spectral region, the combined use of spiking with H_2O_2 (Figure 3C) and variable temperature ^{1}H NMR acquisition (Figure S1 in the Supporting Information) can ensure the presence of H_2O_2. Interestingly, the chemical shift temperature coefficient of H_2O_2 (−4.5 ppb K^{-1}) is distinctly different from those of the phenolic groups which are exposed in the solvent[29] (−6 to −7 ppb K^{-1}) (Table 3, Figure S2).

The quantitative content of H_2O_2 and phenol containing compounds in crude extracts was determined by comparing the integrals of the signal of H_2O_2 and selected −OH groups of phenols with the integral of a given quantity of the internal standard TSP-d_4 (δ_H = 0.000 ppm). It is evident that identification and quantification is not limited in cases where H_2O_2 is a major species, e.g., 2.30 ± 0.08 mg g^{-1} in the ethyl acetate extract after storage of the NMR tube in the dark and at room temperature for 12 days, but also in the case of freshly prepared NMR sample of the ethyl acetate extract where H_2O_2 is a minor species (0.08 ± 0.005 mg g^{-1}). Linear responses were observed over a range of 1 μmol L^{-1} to 5 mmol L^{-1} for H_2O_2 with correlation coefficients for the calibration curves of 0.988. The limit of detection (for S/N = 3) was found to be 0.7 μmol L^{-1}, and the limit of quantification was (for S/N = 10) ≈2.2 μmol L^{-1} (number of scans 256, experimental time 51 min).

Although H_2O_2 is a mild oxidant, being the least reactive of reactive oxygen species (ROS),[30] the measured quantity of hydrogen peroxide can be variable even for a given plant due to aging, fermentation, extraction protocol, and fluctuation in pH. The presence of metal ions such as Fe^{2+} or Cu^{2+} can promote Fenton reaction, enzymatic activity for consumption or production of H_2O_2 might continue,[22] and the presence of native plant diphenols may affect H_2O_2, especially during water extraction. Several experiments, therefore, were performed in order to investigate the effect of the extraction protocol, and the effect of storage of the solution on the amount and stability of the hydrogen peroxide content. Furthermore, the effect of diethylenetriaminepentaacetic acid (DETAPAC), which is a metal chelating agent that was introduced as a tool to mediate the catalytic activity of redox active metals in superoxide generating systems,[31,32] and the effect of pH were additionally studied for the aqueous residue.[32,33]

For all Greek oregano extracts, measurements on three different aliquots from the same extract exhibited high reproducibility of the concentration level of H_2O_2 with standard deviation below 10% (Table 2) depending on the concentration and the achievable line width of H_2O_2. However, the amount of H_2O_2 was found to be strongly dependent upon storing the solution in the NMR tube in the dark and at room temperature for a period of several days. Thus, the amount of H_2O_2 for the ethyl acetate extract progressively increased from 0.08 mg g^{-1} for a freshly prepared NMR sample to 2.30 mg g^{-1} after 12 days of storage. Similar results were obtained for the other residues.

The effect of lyophilization, metal chelation, and neutral pH buffer on the measured quantity of H_2O_2 of the aqueous residue was investigated in detail. Since H_2O_2 has a boiling point of 150 °C, lyophilization would remove any H_2O_2 present

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Table 2. Comparison of Concentration[a] Levels of H_2O_2 and Major Phenolic Compounds in Greek Oregano Extracts in DMSO-d_6, at T = 288 K

extract	H_2O_2			carvacrol	rosmarinic acid	naringenin	eriodictyol
ethyl acetate[b]		0.08 ± 0.005[h]		143.19 ± 3.34	6.36 ± 0.18	4.88 ± 0.73	6.41 ± 0.56
		2.30 ± 0.08[i]					
methanol[b]		0.16 ± 0.01[h]		ND[c]	31.19 ± 0.36	0.24 ± 0.03	0.36 ± 0.02
		0.49 ± 0.01[i]					
acetone[b]		ND[c,h]		422.21 ± 9.23	6.30 ± 0.37	5.73 ± 0.25	8.93 ± 0.51
		1.62 ± 0.09[i]					
aqueous[b]	ND[c,d,e]	ND[c,d,f]	ND[c,d,g]	9.34 ± 0.41	0.42 ± 0.08	0.52 ± 0.08	0.97 ± 0.09
	2.19 ± 0.21[e,i]	2.48 ± 0.23[f,i]	1.19 ± 0.10[g,i]				

[a]mg g^{-1} extract. [b]Mean value of three NMR measurements on three different aliquots from the same solid residue ±SD (standard deviation). [c]Not detected. [d]The three samples were measured immediately after lyophilization. [e]The sample from aqueous extract using distilled water. [f]The sample from aqueous extract using distilled water with DETAPAC. [g]The sample from aqueous extract using phosphate buffer with DETAPAC (see Material and Methods section). [h]Measured immediately after preparation. [i]The same sample after 12 days of storage in the dark and at RT.

Table 3. Chemical Shift Temperature Coefficients ($\Delta\delta/\Delta T$) of the −OH Protons of H_2O_2, Rosmarinic Acid, (2)OH(4″), (2)OH(3″), (2)OH(3′), and (2)OH(4′), and Carvacrol, (3)OH, in 0.6 mL of DMSO-d_6 of the Greek Oregano Ethyl Acetate Extract of Figure S1

compound	$\Delta\delta/\Delta T$[a]
H_2O_2	−4.5
(2)OH(4″)	−6.9
(2)OH(3″)	−5.9
(2)OH(3′)	−6.6
(2)OH(4′)	−6.6
(3)OH	−6.2

[a]Expressed in parts per 10^9 (ppb) K^{-1}.

in the aqueous extract. Indeed, whereas H_2O_2 could not be determined in a freshly prepared aqueous solid residue after lyophilization (see Sample Preparation section), H_2O_2 was found in a relatively high amount after 12 days of storage of the solution in the dark and at RT. The presence of the complexation reagent DETAPAC during the aqueous extraction seems not to affect significantly the amount of H_2O_2 (Table 2); however, in the presence of neutral phosphate buffer (pH ≈ 6.2) the amount of the produced H_2O_2 was significantly reduced. This might be attributed to the fact that the plant tissue and the solid residues naturally contain multiple diphenolic compounds. Many diphenols are subject to autoxidation in air to give quinines through semiquinone radical intermediates.[34] The semiquinone radicals during the extraction process and/or upon storage of the solution can reduce molecular oxygen to the superoxide radical that can undergo dismutation either by metals or by catalase from the plant to produce H_2O_2. The minor quantity of H_2O_2 in freshly prepared samples and the significant increase in the content of H_2O_2 on storage of all the NMR samples, which is practically independent upon the presence of DETAPAC, clearly demonstrate that noncatalytic autoxidation of hydroquinones to corresponding quinines is the primary factor. This autoxidation results in the stoichiometric production of H_2O_2 which is pH dependent, and the rate increases as the pH increases.[35]

From the above it might be concluded that the proposed method based on the strongly deshielded ^{1}H NMR chemical shift of H_2O_2 can be used as a probe for its identification and quantification in complex phytochemical mixtures. Line widths below 3.8 Hz were achieved by the combined use of DMSO-d_6 as solvent, picric acid, and temperatures near the freezing point of the solution, which eliminates proton exchange rate and, thus, line broadening. Even in the rare case of interferences from −OH containing compounds in this shielding region, the combined use of spiking with H_2O_2 and variable temperature ^{1}H NMR acquisition can validate the presence of H_2O_2. The great advantage, therefore, of the above simple and rapid NMR approach as a nondestructive tool for simultaneous structure assignment and quantification of H_2O_2 and phenolics prevails over the disadvantage of the high cost of instrumentation, although it is commonly available in many research laboratories. Furthermore, since the concentration of H_2O_2 is related with aging and storage conditions of plant tissues, plant metabolism, extraction protocol, and storage of the NMR samples, its determination should be based on a direct, rapid, and easily applied methodology, as the one suggested herein.

■ ASSOCIATED CONTENT

Supporting Information

Selected regions of the 500 MHz 1D ^{1}H NMR spectra of 30.5 mg of a Greek oregano ethyl acetate extract in 0.6 mL DMSO-d_6 at various temperatures. Temperature dependencies of the −OH protons of H_2O_2, rosmarinic acid, (2)OH(4″), and carvacrol, (3)OH, of a Greek oregano ethyl acetate extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Matsubara, C.; Kawamoto, N.; Takamura, K. Oxo[5, 10, 15, 20-tetra(4-pyridyl)porphyrinato]titanium(IV): an ultra-high sensitivity

spectrophotometric reagent for hydrogen peroxide. *Analyst* **1992**, *117*, 1781−1784.
(2) Wang, J.; Freiha, B.; Naser, N.; Romero, E. G.; Wollenberger, U.; Ozsoz, M.; Evans, O. Amperometric biosensing of organic peroxides with peroxidase-modified electrodes. *Anal. Chim. Acta* **1991**, *254*, 81−88.
(3) Brennan, T.; Frenkel, C. Involvement of hydrogen peroxide in the regulation of senescence in pear. *Plant Physiol.* **1977**, *59*, 411−416.
(4) Levine, A.; Tenhaken, R.; Dixon, R. A.; Lamb, C. J. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **1994**, *79*, 583−593.
(5) Neill, S. J.; Desikan, R.; Clarke, A.; Hurst, R. D.; Hancock, J. T. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* **2002**, *53*, 1237−1247.
(6) Chen, Z.; Silva, H.; Klessig, D. F. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* **1993**, *262*, 1883−1886.
(7) Asada, K. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Ann. Rev. Plant Biol.* **1999**, *50*, 601−639.
(8) Hurdis, E. C.; Romeyn, H. Accuracy of determination of hydrogen peroxide by cerate oxidimetry. *Anal. Chem.* **1954**, *26*, 320−325.
(9) Moore, D. B.; Argyropoulos, D. S. Determination of peroxygen species present in pulp fiber matrixes. *Anal. Chem.* **1999**, *71* (1), 109−114.
(10) Voraberger, H.; Ribitsch, V.; Janotta, M.; Mizaikoff, B. Application of mid-infrared spectroscopy: measuring hydrogen peroxide concentrations in bleaching baths. *Appl. Spectrosc.* **2003**, *57*, 574−579.
(11) Vacque, V.; Dupuy, N.; Sombret, B.; Huvenne, J. P.; Legrand, P. In situ quantitative and kinetic study by Fourier transform Raman spectroscopy of reaction between nitriles and hydroperoxides. *J. Mol. Struct.* **1997**, *410*−*411*, 555−558.
(12) Nakashima, K.; Maki, K.; Kawagushi, S.; Akiyama, S.; Tsukamoto, Y.; Kazuhiro, I. Peroxyoxalate chemiluminescence assay of hydrogen peroxide and glucose using 2,4,6,8-tetrathiomorpholinopyrimido[5,4-d]-pyrimidine as a fluorescent component. *Anal. Sci.* **1991**, *7*, 709−714.
(13) Gao, Z.; Ivaska, A.; Li, P.; Lui, K.; Yang, J. Electrocatalysis and flow-injection analysis of hydrogen peroxide at a chemically modified electrode. *Anal. Chim. Acta* **1992**, *259*, 211−218.
(14) Tsiafoulis, C. G.; Trikalitis, P. N.; Prodromidis, M. I. Synthesis, characterization and performance of vanadium hexacyanoferrate as electrocatalyst of H_2O_2. *Electrochem. Commun.* **2005**, *7*, 1398−1404.
(15) Navaratne, A.; Rechnitz, G. A. Improved plant tissue-based biosensor using in vitro cultured tobacco callus tissue. *Anal. Chim. Acta* **1992**, *257*, 59−66.
(16) Oungpipat, W.; Alexander, P. W.; Southwell-Keely, P. A reagentless amperometric biosensor for hydrogen peroxide determination based on asparagus tissue and ferrocene mediation. *Anal. Chim. Acta* **1995**, *309*, 35−45.
(17) Xu, S.; Peng, B.; Han, X. A third-generation H_2O_2 biosensor based on horseradish peroxidase-labeled Au nanoparticles self-assembled to hollow porous polymeric nanopheres. *Biosens. Bioelectron.* **2007**, *22*, 1807−1810.
(18) Lin, K.-C.; Jian, X.-C.; Chen, S.-M. A bifunctional biosensor for determination of H_2O_2 and NADH using polyaniline, silicomolybdate, and MWCNT hybrid composites. *Int. J. Electrochem. Sci.* **2011**, *6*, 3427−3437.
(19) Veljovic-Jovanovic, S.; Noctor, G.; Foyer, C. H. Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate. *Plant Phys. Biochem.* **2002**, *40*, 501−507.
(20) Warm, E.; Laties, G. G. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. *Phytochemistry* **1982**, *21*, 827−831.
(21) Stephenson, N. A.; Bell, A. T. Quantitative analysis of hydrogen peroxide by ^{1}H NMR spectroscopy. *Anal. Bioanal. Chem.* **2005**, *381*, 1289−1293.
(22) Cheeseman, J. M. Hydrogen peroxide concentrations in leaves under natural conditions. *J. Exp. Bot.* **2006**, *57*, 2435−2444.
(23) Exarchou, V.; Nenadis, N.; Tsimidou, M.; Gerothanassis, I. P.; Troganis, A.; Boskou, D. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. *J. Agric. Food Chem.* **2002**, *50*, 5294−5299.
(24) Exarchou, V.; Godejohann, M.; van Beek, T. A.; Gerothanassis, I. P.; Vervoort, J. LC-UV-solid-phase extraction-NMR-MS combined with a cryogenic flow probe and its application to the identification of compounds present in Greek oregano. *Anal. Chem.* **2003**, *75*, 6288−6294.
(25) Charisiadis, P.; Exarchou, V.; Troganis, A. N.; Gerothanssis, I. P. Exploring the "forgotten" −OH NMR spectral region in natural products. *Chem. Commun.* **2010**, *46*, 3589−3591.
(26) Charisiadis, P.; Primikyri, A.; Exarchou, V.; Tzakos, A.; Gerothanassis, I. P. Unprecedented ultra-high-resolution hydroxy group ^{1}H NMR spectroscopic analysis of plant extracts. *J. Nat. Prod.* **2011**, *74*, 2462−2466.
(27) Englander, S. W.; Kallenbach, N. R. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* **1983**, *16*, 521−655.
(28) Wüthrich, K. In *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
(29) Exarchou, V.; Troganis, A.; Gerothanassis, I. P.; Tsimidou, M.; Boskou, D. Do strong intramolecular hydrogen bonds persist in aqueous solution? Variable temperature gradient ^{1}H, ^{1}H -^{13}C GE-HSQC and GE-HMBC NMR studies of flavonols and flavones in organic and aqueous mixtures. *Tetrahedron* **2002**, *58*, 7423−7429.
(30) Rhee, S. G.; Chang, T.-S.; Jeong, W.; Kang, D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. *Mol. Cells* **2010**, *29*, 539−549.
(31) Buettner, G. R.; Oberley, L. W.; Leuthauser, S. W. H. C. The effect of iron on the distribution of superoxide and hydroxyl radicals as seen by spin trapping and on superoxide dismutase assay. *Photochem. Photobiol.* **1978**, *28*, 693−695.
(32) Buettner, G. R. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: Ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Methods* **1988**, *16*, 27−40.
(33) Buettner, G. R.; Jurkiewicz, B. A. Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat. Res.* **1996**, *145*, 532−541.
(34) Squadrito, G. L.; Cueto, R.; Dellinger, B.; Pryor, W. A. Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled particulate matter. *Free Radical Biol. Med.* **2001**, *31*, 1132−1138.
(35) Song, Y.; Buettner, G. R. Thermodynamic and kinetic considerations for the reaction of semiquinone radicals to form superoxide and hydrogen peroxide. *Free Radical Biol. Med.* **2010**, *49*, 919−962.