Methodology for Identification of Phenolic Acids in Complex Phenolic Mixtures by High-Resolution Two-Dimensional Nuclear Magnetic Resonance. Application to Methanolic Extracts of Two Oregano Species

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Spectroscopic methodology in analyzing two-dimensional (2D) NMR spectra of a mixture of several phenolic compounds that occur in natural products is described. Particular emphasis has been given to the determination of scalar coupling connectivities by homonuclear 2D correlated spectroscopy (COSY), remote intraresidue connectivities by totally correlated spectroscopy (TOCSY), and spatially close but uncoupled ^{1}H nuclei by homonuclear 2D nuclear Overhauser effect spectroscopy (NOESY/ROESY). Preliminary data to identify phenolic acids in the methanolic extracts from two oregano plants are also reported.

Keywords: *Oregano; phenolic acids; ^{1}H NMR; COSY; TOCSY; NOESY/ROESY*

INTRODUCTION

Phenolic acids, in their free or bound form, are important natural antioxidants, and their impact on human health is a matter of concern among physicians and nutritionists (Ho, 1992; Vinson et al., 1995; Teissedre et al., 1996; Pearson et al., 1997). Due to the complexity of the natural mixtures of phenolic components in the various plant extracts, it is rather difficult to characterize them chemically and to assess their antioxidant potential in one step. As a rule, a certain plant extract is further fractionated and phenol characterization is achieved after chromatographic isolation of the individual components using various spectroscopic methods (Kikuzaki and Nakatani, 1989; Namiki, 1990; Finger et al., 1992; Cuvelier et al., 1994; Kanazawa et al., 1995; Madsen and Bertelsen, 1995; Schwarz and Ernst, 1996). Antioxidant activity evaluation of the extract and/or fractions is feasible through well-established accelerated oxidation tests.

The currently available high-resolution spectroscopic techniques coupled with the facilities of computerized mathematical or other treatment of the data have found interesting applications in the field of agricultural and food science. One of their merits is the minimal treatment of the sample prior to analysis.

NMR spectroscopy is increasingly used as a technique to provide insight into mixtures of various components belonging to the same or different chemical classes without previous separation of the individual components (Belton et al., 1995; Martin et al., 1995; Limiroli et al., 1996; Sacchi et al., 1996).

In this study a combined NMR methodology of two-dimensional homonuclear (2D ^{1}H–^{1}H) correlated spec-

troscopy (COSY), totally correlated spectroscopy (TOCSY), and nuclear Overhauser effect in both the laboratory frame (NOESY) and rotating frame of reference (ROESY) was utilized to analyze the spectra of a mixture of phenolic acids, derivatives of hydroxybenzoic, hydroxycinnamic, and phenylacetic acids. The study is part of a larger project aiming at evaluating the antioxidant activity of plant extracts of unknown composition without isolation of individual components. The NMR methodology was then applied to the characterization of methanolic extracts from two oregano species, botanically characterized as *Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart and *Origanum onites* L. The two extracts have been selected on the basis of their antioxidant activities and the presence of phenolic components from the above classes, which were tentatively identified using TLC and HPLC procedures. The interest in oregano plants is related to previous studies on their importance as a source of various types of natural antioxidants (Lagouri et al., 1993; Tsimidou and Boskou, 1994; Lagouri and Boskou, 1996).

MATERIALS AND METHODS

Standards and Reagents. Methanol, hexane, ethyl acetate, dichloromethane (proanalysis R.G.), hydrochloric acid, acetic acid 99–100% (glacial), methanol Chromasolv (HPLC grade), and chlorogenic acid were purchased from Riedel-de Häen AG (Seelze, Germany). Protocatechuic acid, vanillic acid, omovanillic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, syringic acid, and BHT were from Sigma (St. Louis, MO). Caffeic, *p*-hydroxybenzoic, and gallic acids were from Fluka (Buchs, Switzerland). Sinapic acid was from Aldrich (Milwaukee, WI) and ferric chloride from BDH Chemicals Ltd. (Poole, England).

Samples. *Plant Material: O. vulgare subsp. hirtum and O. onites.* Aerial parts of the flowering plants of the two oregano species were collected from the wild and characterized in the Laboratory of Systematic Botany and Phytogeography, Aristotle University of Thessaloniki.

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Preparation of Oregano Extracts. The plant material was air-dried at room temperature in the dark, and the ground oregano leaves were extracted subsequently with solvents of increasing polarity in a Soxhlet apparatus for 6 h. The sequence of the solvents was hexane, ethyl acetate, dichloromethane, and methanol. This sequence of solvents was used because they are of gradually increasing order of polarity as indicated by the polarity indices. Analogous series of solvents have been extensively used by other investigators in the study of active ingredients present in aromatic plants, seeds, and other sources (Bracco et al., 1981; Duve and White, 1991; Svoboda and Deans, 1992; Kanazawa et al., 1995). The methanolic extracts were concentrated in a rotary evaporator and kept in sealed dark flasks after a few minutes of nitrogen flushing. The extract content, expressed in grams per 100 g of dry material, was 8.9 for both plant species.

Assessment of Antioxidant Activity. The antioxidant activity of the oregano extracts was screened with the coupled oxidation of β-carotene and linoleic acid method (Taga et al., 1984) and then tested using cottonseed oil as a substrate. Samples of the oil (1 g each) containing 1000 mg/kg of the extract from the oregano plants and samples of the oil containing 200 mg/kg BHT were transferred to a series of glass bottles (cross section = 12 cm², volume = 36 cm³). The bottles were stored in an oven at 63 °C, in the dark. Periodical determinations of peroxide values were carried out according to the IUPAC standard method (IUPAC, 1987). Initial peroxide value of the substrate was 0.8 mequiv of O_2/kg of oil.

TLC Screening of Phenolic Acids. Silica plates (0.25 mm) were used for the thin-layer chromatography of the oregano methanolic extracts. A mixture of dichloromethane/water/acetic acid (100:50:50, v/v/v) was used as the developing system. Phenolic acids were viewed using a spray of 1% FeCl₃ solution in methanol (Schulz and Herrmann, 1980).

HPLC Separation of Phenolic Acids. Chromatographic separation was performed on an RP-C₁₈ column (250 mm × 4 mm i.d., 10 μm) placed in a 505 LC column oven set at 30 °C. The HPLC system consisted of a Marathon IV pump and a Shimadzu UV detector. Data acquisition and processing were performed by the computer software EZChrom (EZChrom data system, Scientific Software, Inc.). A gradient elution consisting of H₂O/2% CH₃COOH, as solvent A, and MeOH/2% CH₃COOH, as solvent B, at a flow rate of 1.2 mL/min was used. UV detection was at 280 nm. The gradient consisted of the following steps: $t = 0$ min, 6% B; $t = 60$ min, 37% B; $t = 70$ min, 100% B; $t = 90$ min, 100% B; $t = 105$ min, 6% B; $t = 130$ min, 6%B. Peak identification was based on relative retention time and spiking with standards.

NMR Methods. ¹H NMR spectra of a mixture of standard phenolic acids were obtained on a Bruker AMX-400 spectrometer (20 mM solution in CD₃OD). ¹H NMR spectra of the methanolic extracts of the two oregano species were obtained from 30 mg of material in 0.5 mL of solution in CD₃OD. When not being studied, the solutions were kept at −20 °C. The state of the samples did not change to any significant degree over a period of several months while the various NMR studies were being carried out.

The parameters for the ¹H−¹H COSY spectrum were as follows: spectral width = 2809 Hz; acquisition time = 0.36 s; relaxation delay = 1 s; 48 transients were acquired (2 dummy scans) for each of 256 increments. The FIDs were zero-filled to 512 data points prior to FT, sine- bell window function for resolution enhancement. The parameters of the 400 MHz phase sensitive (TPPI mode) ¹H−¹H TOCSY spectra were as follows: spectral width = 2809 Hz; acquisition time = 0.36 s; relaxation delay = 1 s; 48 transients (4 dummy scans) for each of the 256 increments; spin-locking pulse = 40−100 ms. NOESY and ROESY spectra were acquired using a spectral width of 2809 Hz, acquisition time of 0.18 s, relaxation delay of 1 s, mixing time of 200−750 ms, and 48 transients (4 dummy scans) for each of the 256 increments.

RESULTS AND DISCUSSION

Development of Methodology for the Analysis of NMR Spectra. The NMR study was carried out on a mixture of 12 phenolic acids shown in Figure 1. The acids were chosen on the basis of (a) chemical structure and (b) previously reported antioxidant activity.

In an initial attempt to assign the one-dimensional ¹H NMR spectrum of the mixture of the 12 phenolic compounds of Figure 1, several difficulties were encountered. Particulary, in the region of 6.00−8.00 ppm, the precise assignment of the chemical shift of each proton in the molecules under investigation could not readily be achieved by the use of the classical selective spin decoupling experiments, although, in some cases, double-irradiation effects could be improved by difference spectroscopy. Figure 2 shows the improved peak separation relative to the 1D NMR spectrum in the COSY aromatic fingerprint region. Evidently, two-dimensional (2D) methods are the best way to tackle a resolution problem of such complexity, offering advantages in terms of both efficiency and resolution over 1D approaches.

The strategic step, following the acquisition of the 2D spectra, is to connect the spins together (Wüthrich, 1986; Sanders and Hunder, 1993; Derome, 1987) in addition to detailed chemical shift arguments.

Mapping Intraresidue Spin-Coupled Networks Determining Scalar Coupling Connectivities by Homonuclear ¹H−¹H Shift-Correlated Spectroscopy. The general use of the two-dimensional homonuclear correlation spectroscopy (2D ¹H-¹H COSY) is to determine which spins are coupled by scalar interactions (Sanders and Hunder, 1993), thereby revealing subspectra such as those arising from a given compound or from different phenolic compounds in the mixture. Generally, two COSY cross-peaks coincide only when both pairs of coupled resonances have identical chemical shifts; this greatly reduces the probability of degeneracy. For example, the connectivity of the $H_{2,6}$ and the $H_{3,5}$ spin system for *p*-hydroxybenzoic acid, **b**, can unequivocally be derived from its COSY spectrum shown in Figure 2. For caffeic acid, **c**, and *o*-coumaric acid, **c**₀, the cross-peak connectivities of H_{1a} and H_{2a} and the phenolic spin systems can readily be assigned (Figure 2).

Generally, a limit is imposed on the size of coupling when the line width is of the order of or exceeds the splitting. In this case cross-peaks rapidly lose intensity (Turner, 1985). Connections through small couplings may be emphasized by inserting fixed delays in the COSY pulse sequence, immediately after each pulse; this allows modulation due to small couplings to develop and an increase in the relative intensity of signals, due to small couplings, although it may also lead to alleviation of cross-peaks due to large couplings. An alternative method is the use of relayed correlation spectroscopy.

Multiple Relayed Correlation Spectroscopy: Direct Assignment of Remote Intraresidue Connectivities by the Use of TOCSY. Ambiguities associated with assignments from COSY data can be resolved by the use of TOCSY (Eich et al., 1982), also known as HOHAHA (Davis and Bax, 1985), which reveals remote connectivities by the use of a spin-locking pulse. In this experiment, cross-peaks are observed between spins i and k, which are not directly coupled but share a mutual coupling partner j, or between spins that are *J*-coupled with a magnitude less than the line widths of the

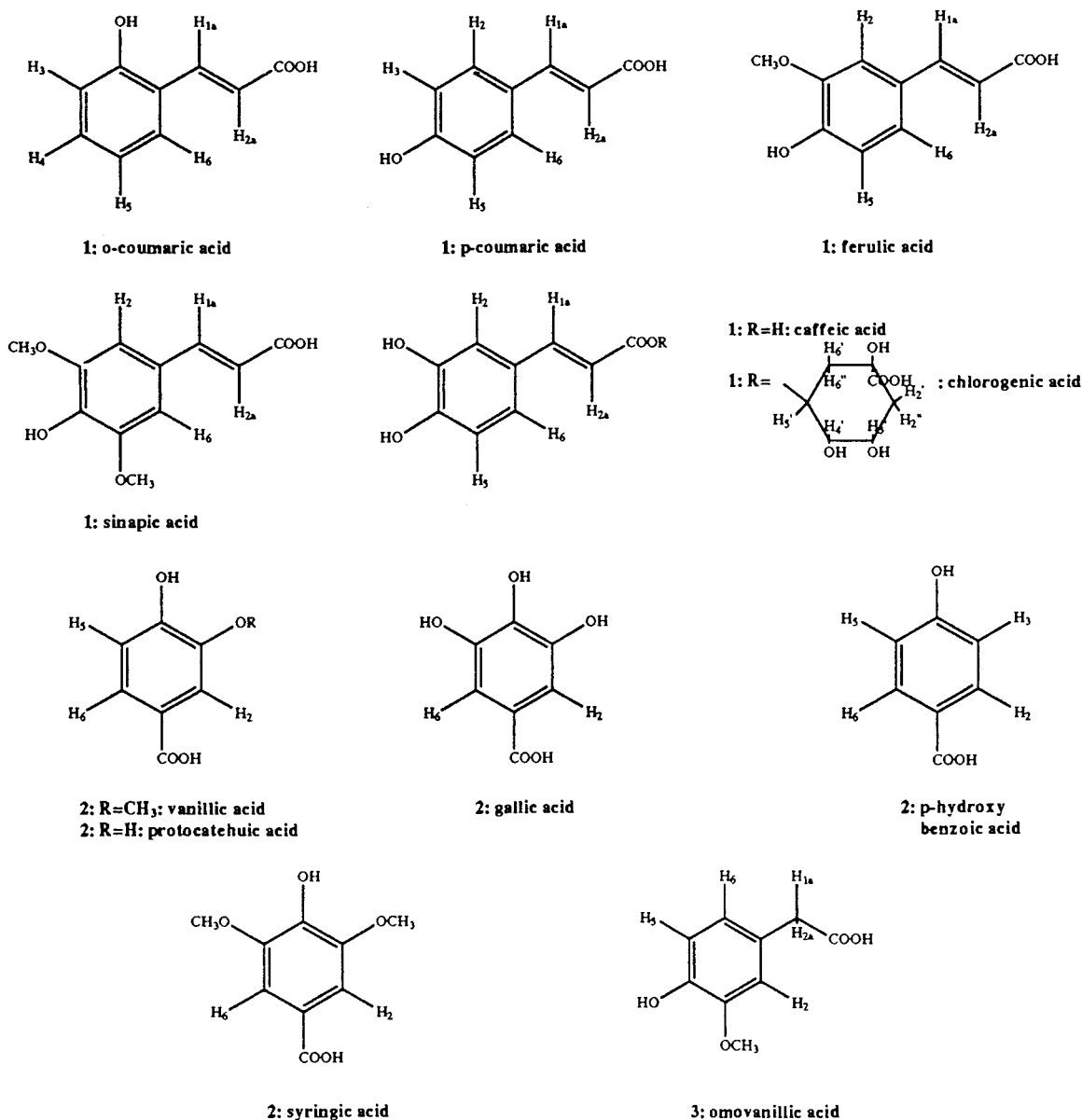Identification of Phenolic Acids in Oregano by HRTD-NMR

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Figure 1. Chemical structures of miscellaneous phenolic compounds reported to occur in natural products: **1**, hydroxycinnamic acid derivatives; **2**, hydroxybenzoic derivatives; **3**, phenylacetic derivatives.

individual resonances. Because the coupling constants along the phenol spin system and its substituents differ significantly, optimization for all nuclei simultaneously is impossible. Nevertheless, in the TOCSY contour plot of the mixture of the 12 phenolic compounds of Figure 1, several remote intraresidue connectivities have been observed for a spin-locking pulse of 100 ms. Figure 3 illustrates the complete connectivity pattern of the $H_{5'}$, $H_{3'}$, $H_{4'}$, $H_{2',''}$, and $H_{6',''}$ spin system of the cyclic moiety of chlorogenic acid, which is clearly visible in the f_1 cross section of the TOCSY spectrum; on the contrary, the corresponding f_1 cross section of the classical 2D-COSY indicates spin connectivities only between the $H_{5'}$, $H_{4'}$, and $H_{6',''}$ protons. Spin correlations in other phenolic compounds have been found in a similar manner.

Identifying Spatially Close but Uncoupled ^1H Nuclei by Homonuclear $^1H–^1H$ NOESY and ROESY. The next step would be to assemble the fragments of the spin systems into the complete molecule using the NOE method. Nuclear Overhauser enhancements and the rates at which they grow and decay are measures of the strength of the dipole–dipole interaction between two

spins and are, therefore, dependent on through-space interatomic distances and correlation times for molecular tumbling. As indicated below, these small through-space interactions are most useful because they carry information not found in the COSY and TOCSY spectra (Noggle and Schrimer, 1971; Neuhaus and Williamson, 1989; Williamson, 1996).

To our knowledge, applications of the 2D NOESY method in the analysis of mixtures of natural products have not appeared in the literature. This is probably due to the poor detectability of weak NOEs in rapidly tumbling small molecules because of unfavorable relaxation properties. Furthermore, for molecules of intermediate size (500−2000 Da), the product of spectrometer angular frequency (ω_0) and molecular rotational correlation time (τ_c) is approximately equal to unity, resulting in unobservable NOEs. The effect of unfavorable relaxation properties can be alleviated by recording the NOESY spectra at lower temperatures. The problem of unobservable NOEs for molecules of intermediate size can be circumvented by the use of a transient NOE experiment in the rotating frame, that
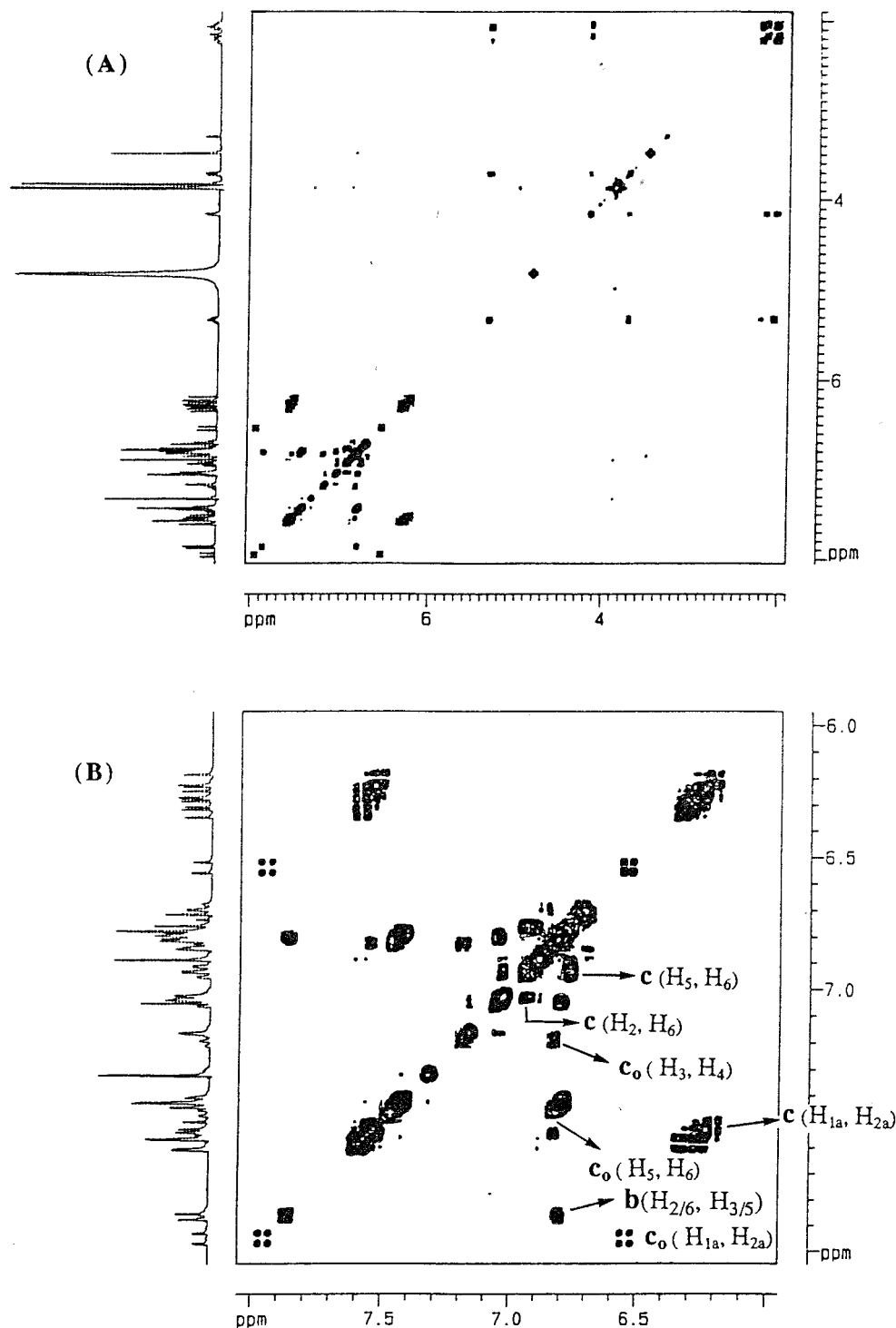
Figure 2. (A) 400 MHz ^{1}H$-^1$H COSY spectrum of the 12 phenolic compounds of Figure 1 together with the corresponding 1D spectrum; (B) expansion of the aromatic region. The arrows denote the cross-peaks of the spin system of *p*-hydroxybenzoic acid (**b**), caffeic acid (**c**), and *o*-coumaric acid (**c$_o$**).

is, by applying a spin-locking pulse (Bothner-By et al., 1984; Bax and Grzesiek, 1996). In the rotating frame the maximum transient NOE increases from 38.5% for the extreme-narrowing condition, $\omega_0\tau_c \ll 1$, to 67.5% for $\omega_0\tau_c \gg 1$; that is, it does not vanish for any value of $\omega_0\tau_c$.

Phenol compounds bearing $-CH=CHCOOH$ and $-OCH_3$ substituents (Figure 1) are an ideal class of molecules for illustrating the revelatory powers of the NOE/ROE experiment, particularly across the spectroscopically silent quartenary carbon. The crucial use of NOEs/ROEs is to make the connection of the $-CH=CH-$ COOH or $-OCH_3$ spin system with the spin system of

the protons of the phenyl group, which is otherwise impossible. The structures of caffeic acid and syringic acid (Figure 4) illustrate the type of connection that NOEs/ROEs can make for molecules of this type. Figure 5 illustrates the expected ROE cross-peak of the $H_{2,6}$ and $-OCH_3$ spin system of syringic acid in the corresponding f_1 cross section of the 400 MHz ^{1}H$-^1$H ROESY spectrum. In practice, both NOESY/ROESY and COSY/TOCSY are needed and also suitable combination and visual display of information on through-space and through-bond connectivities. Thus, a continuous pathway along the backbone of, for example, caffeic acid is
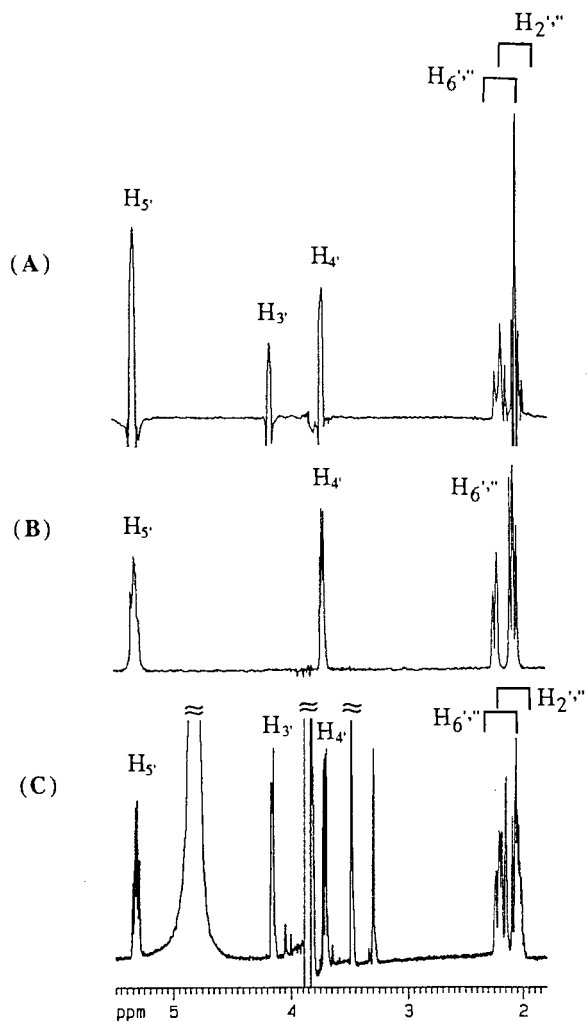
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Figure 3. (A) Selected f_1 cross section (together with the corresponding 1D spectrum) of the 400 MHz 2D ^{1}H$-^1$H TOCSY spectrum (100 ms spin-locking pulse) of the 12 phenolic compounds of Figure 1 that indicates the complete connectivity pattern (correlations) between the $H_{5'}$, $H_{3'}$, $H_{4'}$, $H_{2',''}$, and $H_{6',''}$ protons of the cyclic moiety of chlorogenic acid; (B) corresponding f_1 cross section of the cyclic moiety of chlorogenic acid in the 2D ^{1}H$-^1$H COSY spectrum of Figure 2; (C) 1D spectrum.

Figure 4. Schematic NOE ($-$) and COSY ($\rightarrow$) spin connectivities in (A) caffeic acid and (B) syringic acid.

obtained using alternatively COSY and NOESY connectivities. Analysis of the NOESY$-$COSY connectivity diagram starts with the **c**(H_{1a},H_{2a}) COSY cross-peak. A vertical line through the COSY cross-peak indicates the **c**(H_{2a},H_6) NOESY cross-peak. This procedure makes use of the inherent symmetry of COSY and NOESY spectra with respect to the diagonal.

The results of the complete ^{1}H NMR analysis of the mixture of the 12 phenolic compounds of Figure 1 are given in Table 1. Resonances are detailed with splitting pattern and chemical shifts and assigned to the appropriate molecular moiety.

Application to Methanolic Extracts of Two Oregano Species. The two extracts had already been tested for their antioxidant activity on both emulsions and oil systems. The presence of phenolic components in the extracts has been checked using chemical and spectrometric data after chromatographic separation either on TLC plates or on RP-HPLC using well-established procedures (Tsimidou et al., 1992). These extracts presented antioxidant activity and were found to contain phenolic acids of the three mentioned classes.
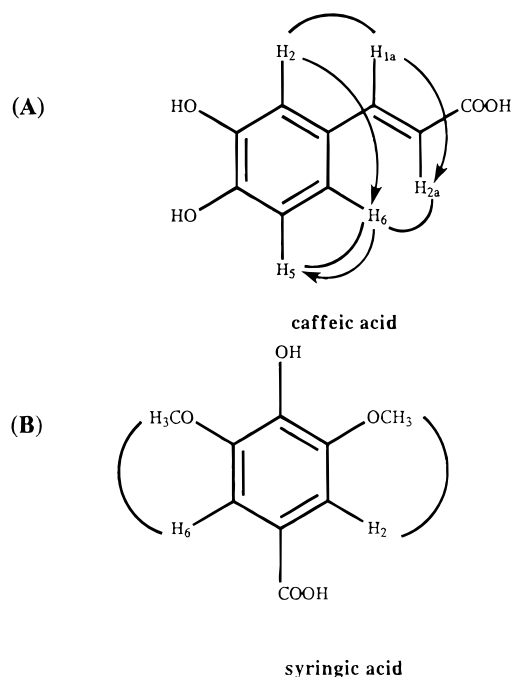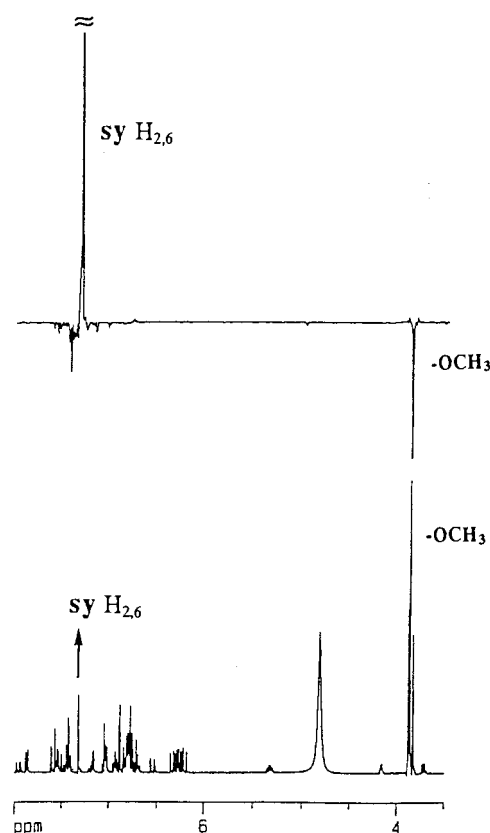

Figure 5. Selected f_1 cross section (together with the corresponding 1D spectrum) of the 400 MHz ^{1}H$-^1$H ROESY spectrum of the 12 phenolic compounds of Figure 1, illustrating the ROE cross-peak of the $H_{2,6}$ and $-OCH_3$ spin system of syringic acid.

This was indicated by thin-layer chromatography, specific color reaction, and RP-HPLC.

The above combined methodology of 2D-COSY, TOCSY, NOESY, and ROESY NMR techniques was applied to extracts of the two oregano plants. Figure 6 illustrates the expected COSY connectivity pattern of
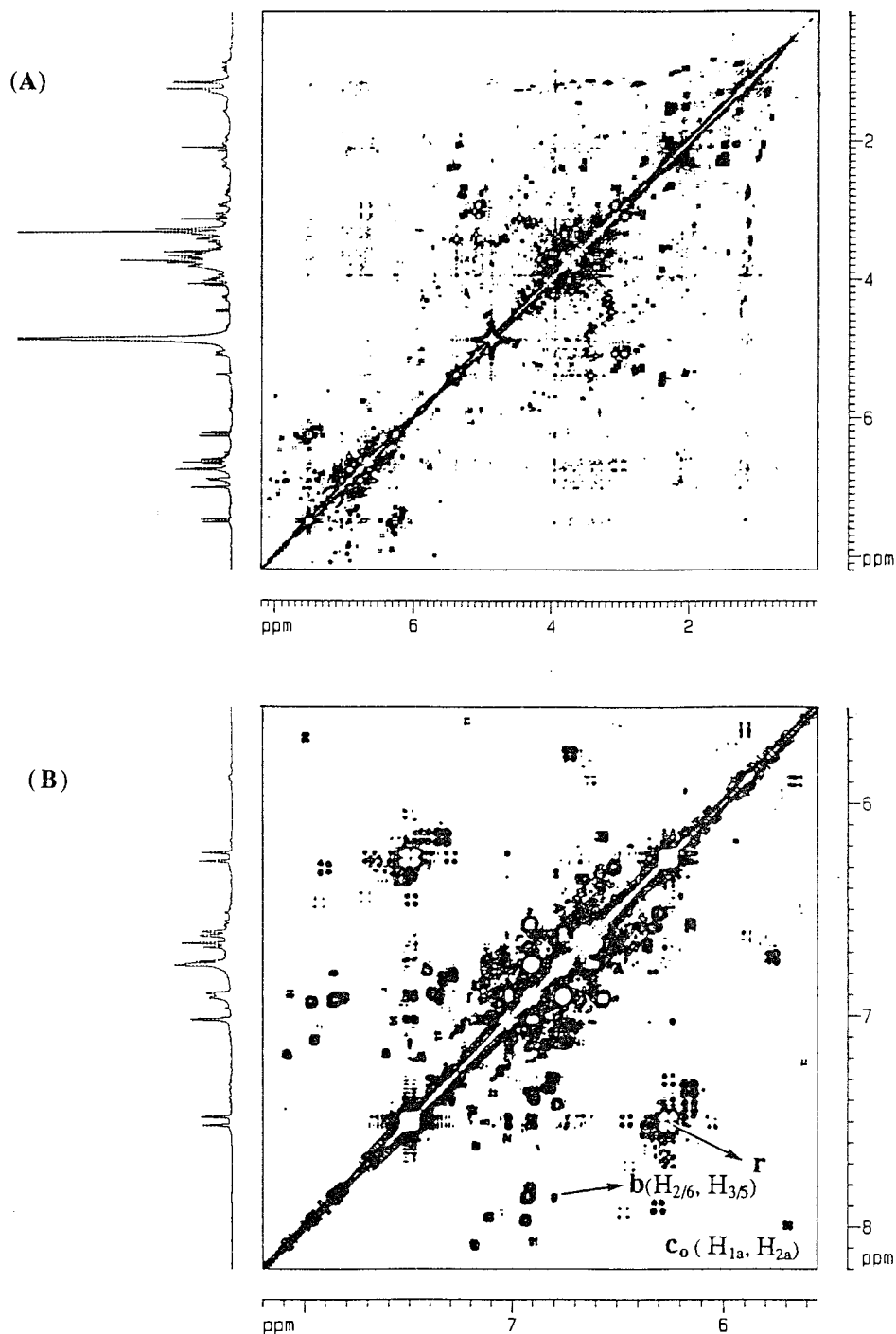
Figure 6. (A) 400 MHz ^{1}H$-^1$H COSY spectrum of the methanolic fraction of the extract of *O. vulgare*; (B) expansion of the aromatic region. The arrows denote selected cross-peaks of the spin systems of *p*-hydroxybenzoic acid (**b**), *o*-coumaric acid (**c$_o$**), and rosmarinic acid (**r**).

p-hydroxybenzoic and *o*-coumaric acid present in *O. vulgare*. It is interesting to note that very significant cross-peaks are observed for, for example, *p*-hydroxybenzoic acid, although the S/N ratio of the respective 1D resonances appears very small. If for each t_1 value in a 2D experiment a spectrum with poor S/N ratio is obtained, then the second Fourier transformation with respect to t_1 combines the signal energy of a particular resonance from all spectra obtained for different t_1 values (Bax, 1985). The strongest cross-peaks in the aromatic region are diagnostic of the H$_{1a}$, H$_{2a}$, H$_2$, H$_5$, and H$_6$ protons of the caffeic acid moiety. However, the existence of very strong cross-peaks at (5.12, 3.11), (5.12, 2.97), and (2.97, 3.11) ppm indicates that this coupling

network should be attributed not only to caffeic acid but also to its derivatives. This is in agreement with NMR data previously reported by Kikuzaki and Nakatani (1989), who isolated rosmarinic acid, **r**, an ester derivative of caffeic acid, as an essential component in methanolic extracts of *O. vulgare*.

Some of the phenolic compounds shown in Figure 1 were identified in methanolic extracts of the two oregano species. The presence of these components was also checked using RP-HPLC. *O. vulgare* contained *o*-coumaric acid, ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, and vanillic acid. *O. onites* contained ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, and vanillic acid.

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Table 1. ^{1}H Chemical Shifts^a and Assignments of the Phenolic Compounds of Figure 1

compound	notation	proton	multiplicity^b	δ
o-coumaric acid	**c$_o$**	H$_3$	d	7.45
		H$_4$	t	6.85
		H$_5$	t	6.73
		H$_6$	d	6.73
		H$_{1a}$	d	7.95
		H$_{2a}$	d	6.54
p-coumaric acid	**c$_p$**	H$_2$	d	7.42
		H$_3$	d	6.84
		H$_5$	d	6.84
		H$_6$	d	7.42
		H$_{1a}$	d	7.54
		H$_{2a}$	d	6.25
ferulic acid	**f**	H$_2$	s	7.15
		H$_5$	d	6.84
		H$_6$	d	7.05
		H$_{1a}$	d	7.60
		H$_{2a}$	d	6.30
sinapic acid	**s**	H$_2$	s	6.88
		H$_6$	s	6.88
		H$_{1a}$	d	7.59
		H$_{2a}$	d	6.33
caffeic acid	**c**	H$_2$	s	7.04
		H$_5$	d	6.80
		H$_6$	d	6.95
		H$_{1a}$	d	7.52
		H$_{2a}$	d	6.20
chlorogenic acid	**ch**	H$_2$	s	7.03
		H$_5$	d	6.80
		H$_6$	d	6.96
		H$_{1a}$	d	7.54
		H$_{2a}$	d	6.24
		H$_{2'}$	m	2.15
		H$_{2''}$	m	2.05
		H$_{3'}$	m	4.15
		H$_{4'}$	dd	3.60
		H$_{5'}$	m	5.33
		H$_{6'}$	m	2.20
		H$_{6''}$	m	2.06
vanillic acid	**v**	H$_2$	s	7.55
		H$_5$	d	6.84
		H$_6$	d	7.55
protocatechuic acid	**p**	H$_2$	s	7.48
		H$_5$	d	6.80
		H$_6$	d	7.48
gallic acid	**g**	H$_{2,6}$	s	7.00
p-hydroxybenzoic acid	**b**	H$_{2,6}$	d	7.86
		H$_{3,5}$	d	6.82
syringic acid	**sy**	H$_{2,6}$	s	7.32
omovanillic acid	**o**	H$_2$	s	6.84
		H$_5$	d	6.71
		H$_6$	d	6.71
		H$_{1a}$	s	3.48
		H$_{2a}$	s	3.48

^{a} Chemical shifts in ppm to high frequency of d$_4$-TSPA. ^{b} s, singlet; d, doublet; m, multiplet; t, triplet; dd, two doublets.

The combined 2D NMR methodology with emphasis on scalar connectivities (COSY), remote intraresidue connectivities by totally correlated spectroscopy (TOCSY), and spatially close but uncoupled ^{1}H nuclei by homonuclear NOE and ROE experiments seems to be very promising to elucidate the structure of the phenol content of a plant matrix. It should be emphasized that the above methodology is not limited to phenolic acids but can be extended to other important classes of phenolic compounds (e.g., flavonoids) for which the spin systems likely to be present are known. Further research in this direction is currently in progress. Quantitative information concerning these mixtures is also under investigation.

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