# ARTICLES

## Identification and Quantification of Caffeic and Rosmarinic Acid in Complex Plant Extracts by the Use of Variable-Temperature Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

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A combination of advanced nuclear magnetic resonance (NMR) methodologies for the analysis of complex phenolic mixtures that occur in natural products is described, with particular emphasis on caffeic acid and its ester derivative, rosmarinic acid. The combination of variable-temperature two-dimensional proton−proton double quantum filter correlation spectroscopy ($^1$H−$^1$H DQF COSY) and proton−carbon heteronuclear multiple quantum coherence ($^1$H−$^{13}$C HMQC) gradient NMR spectroscopy allows the identification and tentative quantification of caffeic and rosmarinic acids at 243 K in extracts from plants of the Lamiaceae family, without resorting to previous chromatographic separation of the components. The use of proton−carbon heteronuclear multiple bond correlation ($^1$H−$^{13}$C HMBC) gradient NMR spectroscopy leads to the complete assignment of the correlations of the spins of $H_{2a}$ and $H_{3a}$ with the ester and carboxyl carbons of rosmarinic and caffeic acid, even at room temperature, and confirms the results of the above methodology. Quantitative results are in reasonable agreement with reverse phase HPLC measurements.

**Keywords:** *Antioxidants; Lamiaceae plants; caffeic acid; rosmarinic acid; DQF-COSY; HMQC; HMBC; NMR spectroscopy; RP-HPLC*

### INTRODUCTION

Phenolic acids are important constituents of the human diet, and their antioxidant activity is well established. Polyphenols and phenolic acids are thought to play a positive role in the prevention of chronic diseases (Ho, 1992; Vinson et al., 1995; Teissedre et al., 1996; Pearson et al., 1997). Plants belonging to the Lamiaceae family have been studied as sources of different classes of natural antioxidants (Tsimidou and Boskou, 1994; Madsen and Bertelsen, 1995). These antioxidants are usually obtained as complex extracts. For the characterization of individual components of such extracts, fractionation is first needed. Identification and quantification are based on liquid chromatographic separation and isolation of the individual components.

High-resolution spectroscopic techniques and particularly NMR spectroscopy have found interesting applications in the analysis of complex mixtures of various plant extracts (Colquhoun and Goodfellow, 1994; Martin, 1995; Martin et al., 1995; Kanazawa et al. 1995; Rabenstein et al., 1997; Lindon et al., 1996; Bringmann et al., 1998). As a part of a larger project on natural antioxidants, we have previously reported the results of a two-dimensional NMR study of methanolic extracts from two oregano species. These extracts were analyzed for derivatives of hydroxybenzoic, hydroxycinnamic, and phenylacetic acid, without prior chromatographic separation of the components (Gerothanassis et al., 1998). During the course of that study, the NMR spectra indicated that phenolic acids may exist in their free as well as bound forms. Identification of these compounds with classical NMR techniques is extremely difficult due to the similar $^1$H and $^{13}$C shieldings. This problem is particularly acute in the case of caffeic acid and its ester derivative, rosmarinic acid (Figure 1). These two compounds are important natural antioxidants (Kikuzaki and Nakatani, 1989; Nardini et al., 1995, 1998). Rosmarinic acid has also been reported to be a potent active substance against human immunodeficiency virus type 1 (HIV-1) (Mazumder et al., 1997). It is, therefore, of interest to try to apply a combination of advanced NMR methodologies that could lead to the identification of certain natural bioactive compounds without previous chromatographic separation of the components.

The strategy employed in the present study is based on variable-temperature two-dimensional $^1$H−$^1$H double-quantum filter correlation spectroscopy (DQF-COSY), $^1$H−$^{13}$C heteronuclear multiple-quantum coherence (HMQC), and $^1$H−$^{13}$C heteronuclear multiple-bond correlation (HMBC) gradient NMR spectroscopy. Identification and quantification of caffeic acid and rosmarinic acid were achieved in methanolic and ethanolic extracts

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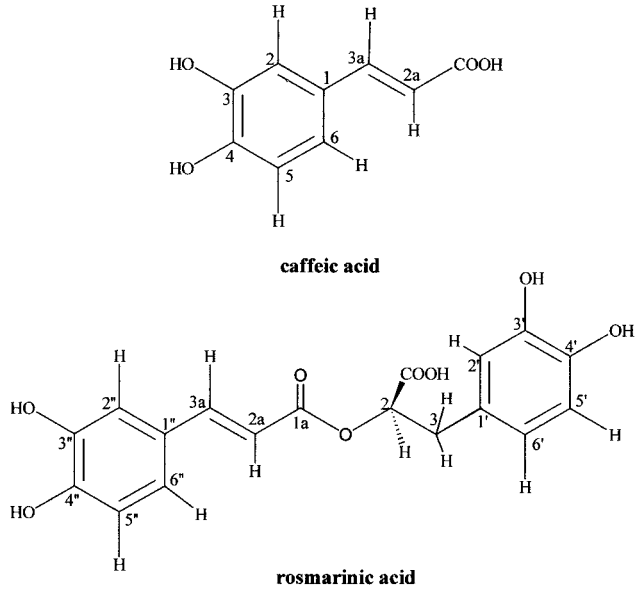



**Figure 1.** Chemical structures of caffeic and rosmarinic acid.

from plants of the Lamiaceae family. The results were confirmed with RP-HPLC measurements for selective extracts.

## MATERIALS AND METHODS

**Solvents and Standards.** Caffeic acid was purchased from Fluka (Buchs, Switzerland) and rosmarinic acid from Czech Moravian & Slovac Chemicals (Oxford, U.K.). Methanol-$d_4$ and 3-trimethylsilyl-3,3,2,2-tetradeuteriopropionic acid sodium salt ($d_4$-TMSP) were obtained from Cambridge Isotope Laboratories Inc. (Cambridge, MA). Acetonitrile was of HPLC grade from Baker (Deventer, The Netherlands), and formic acid was obtained from Riedel de Haën GmbH & Co. KG (Seelze, Germany).

**Plant Material.** *Satureja hortensis* was collected at the Lithuania Institute of Horticulture, Babtai; *Origanum vulgare*, *Origanum onites*, and *Satureja thymbra* were from Crete (Chania); and *Salvia triloba* was a commercial sample. All of the plant species were botanically characterized before use.

**Preparation of the Plant Extracts.** The plant material was air-dried at room temperature in the dark, and the ground oregano leaves were extracted. Two types of extracts were used. Crude ethanolic extracts from *O. vulgare* subsp. *hirtum*, *Sat. hortensis*, *Sat. thymbra*, and *Sal. triloba* were prepared in a Soxhlet apparatus (6 h) without further purification. A methanolic extract from *O. onites* was prepared using a sequence of nonpolar and polar solvents (Gerothanassis et al., 1998).

**Thin Layer Chromatography (TLC).** Silica plates (0.25 mm) were used for the TLC of the plant extracts. Two developing systems were used: (a) $CHCl_3$/$CH_3COOEt$/HCOOH (50:40:10, v/v/v) and (b) $CH_3COOEt$/MeOH/$H_2O$ (77:13:10, v/v/v). $R_f$ values of caffeic and rosmarinic acids were compared to those of the standards. Visualization was carried out using (a) $FeCl_3$ (2% in ethanol) and (b) $AlCl_3$ (1% in ethanol) (Schulz and Hermann, 1980).

**HPLC Apparatus and Measurements.** A P4000 Finnigan MAT spectra system and spectra series pump equipped with a diode array Fasma 406 scanning detector (HPLC/DAD) were employed (Rigas Laboratories, Thessaloniki, Greece). The data from the diode array detector were stored and processed with chromatographic software EZChrom (Scientific Software Inc., San Ramon, CA). For the HPLC/DAD analysis a Nucleosil 188-5 $C_{18}$ column (125 mm × 4 mm i.d., 5 $\mu$m) from Macherey-Nagel (Düren, Germany) was used. Solvent A was 1% formic acid, and solvent B was acetonitrile. The elution system was as follows: 0−10 min, 10−13% B in A; 10−25 min, 13−70% of B in A; 25−29 min, 70% B in A; 29−30 min, 70−10% of B in A; 30−40 min, 10% of B in A (Häkkinen et al., 1999). The flow rate was 1 mL/min, and the injection volume was 20 $\mu$L. Quantitative analysis was achieved by applying eight-point calibration curves for rosmarinic and caffeic acid. The regression coefficient values ($R^2$) were 0.982 and 0.984 for caffeic and rosmarinic acid, respectively.

**NMR Methods.** All NMR experiments were performed on a Bruker AMX-400 spectrometer equipped with a z-gradient unit (Hurd and John, 1991; Norwood, 1994). The suppression of the residual OH of the solvent resonance was achieved with the use of the WATERGATE pulse sequence for gradient (Liu et al., 1998). Chemical shifts and integrals were measured with reference to internal $d_4$-TMSP, ($\delta$ = 0.000) of known concentration. Temperature calibration was achieved by the use of 4% methanol in methanol-$d_4$. $^1H-^1H$ DQF-COSY was used for spin system assignment and the suppression of the single-quantum transitions such as the methyl resonance of the solvent. Data were processed by using UXNMR (Brüker) software. The $t_1$ dimension was zero-filled to 1K or 2K real data points, and a 0° sine bell window function was applied in both dimensions. Other parameters for the $^1H-^1H$ DQF-COSY spectra were as follows: spectral width, 4032 Hz; acquisition time, 0.25 s; relaxation delay, 1 s; 24 transients were acquired for each of 128 increments. $^1H-^{13}C$ HMQC experiments were acquired with a spectral width of 4132 Hz in the $F_2$ ($^1H$) dimension and 18403 Hz in the $F_1$ ($^{13}C$) dimension; acquisition time was 0.25 s, relaxation delay was 1 s, and there were 102 scans per increment. The $t_1$ dimension was zero-filled to 1K real data points, and a $\pi/2$ square sine bell window function was applied in both dimensions. The $^1H-^{13}C$ HMBC spectra were acquired with spectral widths of 4132 Hz in the $F_2$ ($^1H$) dimension and 13000 Hz in the $F_1$ ($^{13}C$) dimension; acquisition time was 0.37 s, relaxation delay was 1 s, and there were 316 scans per increment.

## RESULTS AND DISCUSSION

**Variable-Temperature Two-Dimensional $^1H-^1H$ DQF-COSY.** An initial attempt was made to identify caffeic and rosmarinic acid with classical 1D and 2D NMR spectra in a methanolic extract from the oregano species. Several difficulties were encountered because $^1H$ and $^{13}C$ NMR chemical shifts of caffeic and rosmarinic acid are almost identical at room temperature

**Table 1. $^1H$ and $^{13}C$ Chemical Shifts, $\delta$ (ppm), and Temperature Coefficients of Selected Proton Resonances, $\Delta\delta/\Delta T$, of Caffeic and Rosmarinic Acid**

| caffeic acid | | | | | rosmarinic acid | | | | |
|---|---|---|---|---|---|---|---|---|---|
| proton | $\delta$ | $\Delta\delta/\Delta T^a$ | carbon | $\delta$ | proton | $\delta$ | $\Delta\delta/\Delta T^a$ | carbon | $\delta$ |
| $H_{3a}$ | 7.51 | 0.23 | $C_1$ | 127.8 | $H_{3a}$ | 7.53 | 0.03 | $C_{1''}$ | 127.9 |
| $H_{2a}$ | 6.20 | −0.17 | $C_2$ | 114.8 | $H_{2a}$ | 6.24 | −0.70 | $C_{2''}$ | 115.3 |
| $H_2$ | 7.02 | 0.11 | $C_3$ | 147.0 | $H_{2''}$ | 7.02 | −0.06 | $C_{3''}$ | 146.7 |
| $H_5$ | 6.76 | 0.40 | $C_5$ | 116.7 | $H_{5''}$ | 6.76 | 0.39 | $C_{5''}$ | 116.5 |
| $H_6$ | 6.92 | −0.01 | $C_4$ | 149.8 | $H_{6''}$ | 6.94 | −0.09 | $C_{4''}$ | 149.5 |
| | | | $C_6$ | 123.0 | | | | $C_{6''}$ | 123.0 |
| | | | $C_{1a}$ | 171.4 | | | | $C_{1a}$ | 168.7 |
| | | | | | | | | $C_1$ | 173.5 |

[a] Expressed in parts per $10^9$ (ppb) per K.

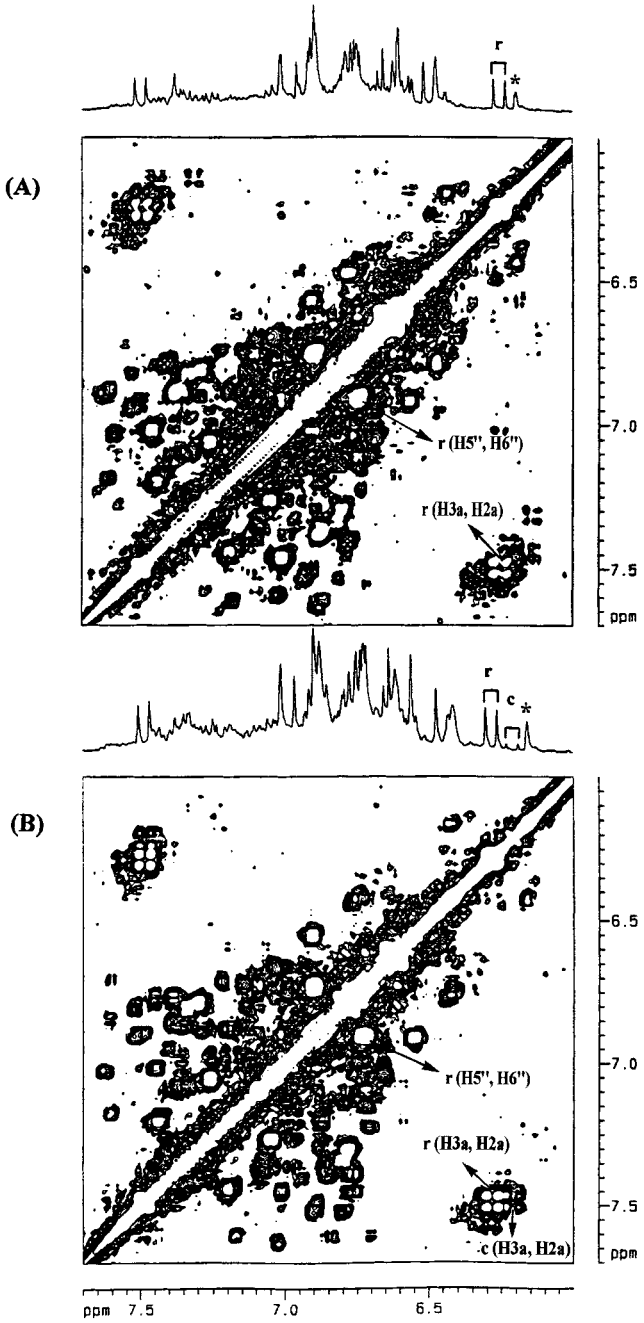



**Figure 2.** 400 MHz $^1H-^1H$ DQF-COSY (high-frequency region) spectra of a methanolic fraction of the *O. onites* species, 90 mg of material in 0.5 mL of $CD_3OD$: (A) 298 K; (B) 243 K. The arrows denote the cross-peaks of the spin system of r($H_{3a}$, $H_{2a}$) r($H_{5''}$, $H_{6''}$) and c($H_{3a}$, $H_{2a}$). The asterisk (*) denotes an unknown resonance.

(Table 1). Furthermore, the concentration of one of the two acids in the mixture is significantly larger than that of the other. Consequently, the 2D NMR off-diagonal cross-peaks of the acid in lower concentration level are hidden under the much stronger cross-peaks of the acid at higher level. Figure 2A shows the high-frequency region of the 2D $^1H-^1H$ DQF-COSY spectrum of the methanolic extract. The strongest cross-peaks ($H_{3a}$, $H_{2a}$) and ($H_{5''}$, $H_{6''}$) are diagnostic of the $H_{3a}$, $H_{2a}$, $H_{2''}$, $H_{5''}$, and $H_{6''}$ protons of the caffeic acid moiety. Moreover, the existence of very strong cross-peaks at (5.12, 3.11), (5.12, 2.97), and (2.97, 3.11) ppm indicates a coupling network that can be attributed to rosmarinic acid. This also indicates that the acid that exists in high concentration is rosmarinic acid, as shown also by TLC on silica gel plates. The $H_{3a}$, $H_2$, $H_5$, and $H_6$ protons of caffeic acid are not identified due to overlapping. The $H_{2a}$ proton of caffeic acid, which is strongly overlapped in the 1D spectrum with an unknown strong peak at 6.2 ppm (denoted by an asterisk), could be resolved only partially in the 2D spectrum. However, performing 2D DQF-COSY NMR spectra at low temperatures can solve this resolution problem.

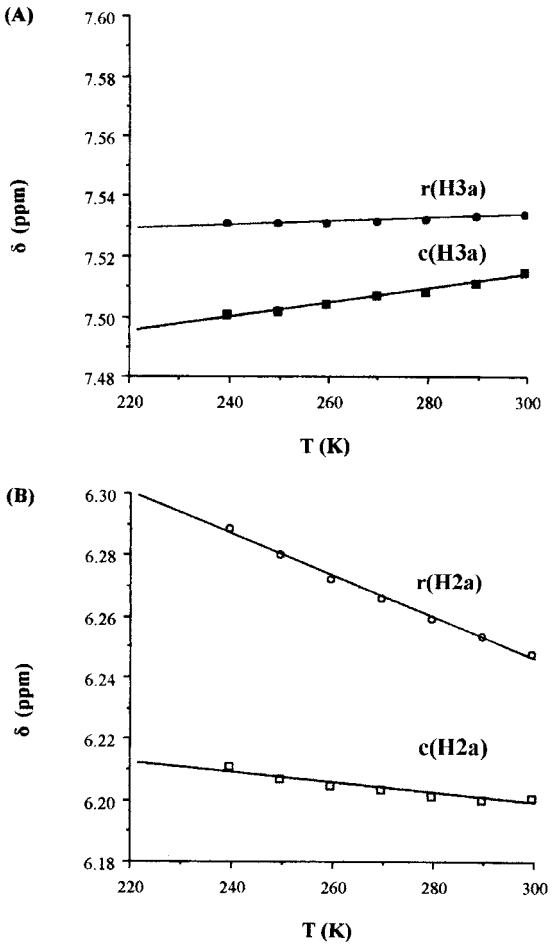



**Figure 3.** Temperature dependencies of the proton chemical shifts of (A) c($H_{3a}$) and r($H_{3a}$) and (B) c($H_{2a}$) and r($H_{2a}$) in $CD_3$-OD, concentration 20 mM, on a Brüker AMX-400 instrument.

The temperature dependence of proton chemical shifts is a technique commonly used to investigate solute–solvent accessibility and to improve resolution of strongly overlapped signals, particularly in the field of peptides and proteins (Deslaurier and Smith, 1980). Hydrogen-bonded amide (peptide) protons generally have larger chemical shifts compared to those of non-hydrogen-bonded species (Stevens et al., 1980). It is postulated that raising the temperature breaks intramolecular hydrogen bonds less readily than those involving the solvent. Additional factors such as conformational changes and temperature effects on the population of vibrational levels may be invoked (Muller and Reiter, 1964). To the best of our knowledge, variable-temperature proton chemical shifts have not been utilized as a means to reduce spectral degeneracy analysis of plant extracts.

Figure 3 shows the temperature dependence of the chemical shifts of $H_{3a}$ and $H_{2a}$ protons of caffeic (c) and rosmarinic acid (r), and Table 1 indicates the chemical shifts, $\delta$ (ppm), and the values of chemical shift differ-

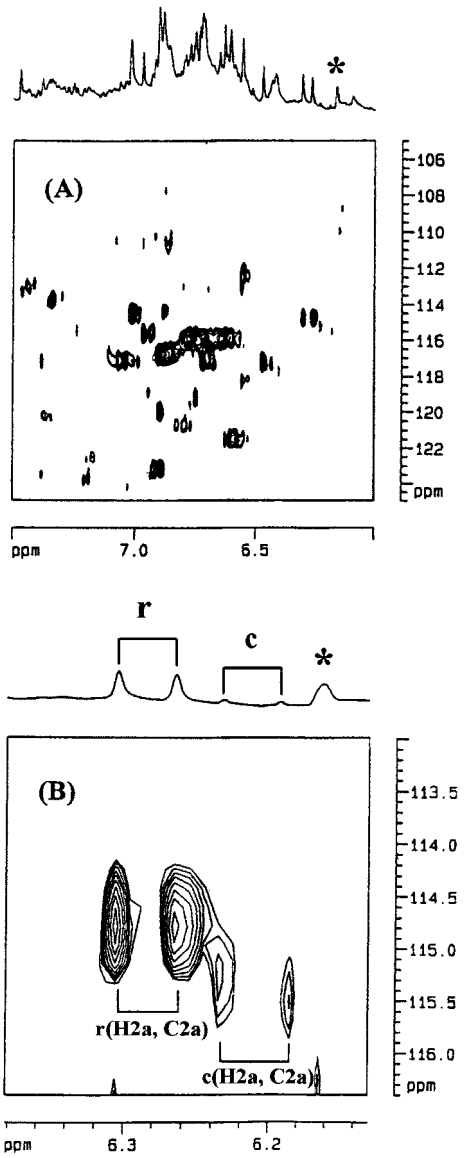
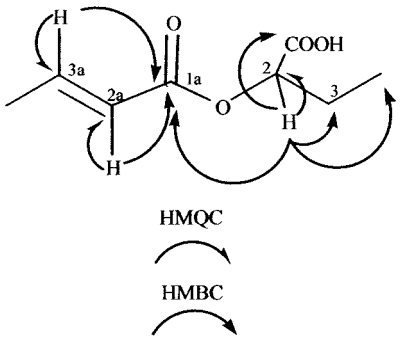



**Figure 4.** (A) High-frequency region of a 2D $^1H-^{13}C$ HMQC gradient-enhanced NMR spectrum at 243 K of the methanolic extract of the *O. onites* of Figure 2. (B) Selected region of the spectrum of Figure 5A, at lower contour level, which denotes the cross-peaks of the spin system of r($H_{2a}$, $C_{2a}$) and c($H_{2a}$, $C_{2a}$). The asterisk (∗) denotes an unknown resonance.

ence, $\Delta\delta$, in relation to variation in temperature ($\Delta T$) of caffeic and rosmarinic acid. It is evident that $\Delta\delta/\Delta T$ for r($H_{2a}$) ($\sim -0.70$ ppb per K) is significantly larger than that of the c($H_{2a}$) proton ($\sim -0.17$ ppb per K). This allows the differentiation of the respective cross-peaks at low temperatures. Figure 2B shows the high-frequency region of the 2D COSY spectrum recorded at 243 K. The cross-peak connectivity of the $H_{3a}$ and $H_{2a}$ protons of caffeic acid can be assigned unequivocally despite the fact that the concentration of rosmarinic acid in the extract is significantly larger than that of caffeic acid. The c($H_{2a}$) proton is clearly resolved even in the 1D spectrum, and it is not overlapped with the unknown strong peak at 6.2 ppm [denoted by an asterisk (∗), Figure 2B].

From the above it is concluded that the temperature dependence of the proton chemical shifts may be the basis for an effective methodology to reduce spectral degeneracy in mixture analysis. From a practical point of view this can be better exploited in the 2D $^1H-^1H$ DQF-COSY experiment in which cross-peaks coincide only when both pairs of coupled resonances have identical chemical shifts (Sanders and Hunder, 1993).

**Scheme 1**




**Two-Dimensional $^1H-^{13}C$ Heteronuclear Experiments.** Further improvement in resolution and assignment information can be achieved by the use of two-dimensional $^1H-^{13}C$ heteronuclear experiments (Sanders and Hunder, 1993; Griesinger et al., 1996; Günther, 1996). The sensitive $^1H$ nucleus is used for signal detection, and this method was introduced as reverse or inverse shift correlation. Practically, 2D $^1H-^{13}C$ heteronuclear multiple-quantum coherence (HMQC) experiments allow the observation of protons that are directly connected to carbons via one bond. Two advantages, which result from gradient selection of coherence, are the elimination of the need for phase cycling and a significant reduction of coherent artifacts (Hurd and John, 1991; Norwood, 1994; Günther, 1996). Figure 4 shows the $^1H-^{13}C$ HMQC gradient-enhanced spectrum at 243 K of a methanolic extract of *O. onites*. The distinction between the cross-peaks of r($H_{2a}$, $C_{2a}$) and c($H_{2a}$, $C_{2a}$) is now more obvious than in the 2D DQF-COSY spectrum recorded at the same temperature.

The $^1H-^{13}C$ heteronuclear multiple-bond correlation (HMBC) gradient NMR experiment is particularly useful in that it connects protons to carbons via two or three bond couplings, and therefore it can be used to connect proton spin systems that are interrupted by nonprotonated carbon atoms. However, the literature related to mixture analysis is very poor. As far as we know, only Lin and Shapiro (1997) have utilized a combination of HMBC with TOCSY to assign the structures of various components from a mixture of six, structurally similar, esters. The combination of the observed $^1H-^{13}C$ HMQC and HMBC correlations allows the connection of the proton and carbon-13 spins across the molecule, which means that we can examine the ester group of rosmarinic acid as shown in Scheme 1.

Figure 5 shows the $^1H-^{13}C$ HMBC spectrum of rosmarinic acid. The $H_{3a}$ and $H_{2a}$ protons are coupled with the ester carbon $C_{1a}$, which resonates at 168.7 ppm (Figure 5B). This ester carbon indicates also a cross-peak with the $H_2$ proton, which resonates at 5.12 ppm. The $H_2$ proton indicates three further cross-peaks: with $C_1$ at 173.5 ppm, with $C_3$ at 38.0 ppm, and with $C_{1'}$ at 129.2 ppm (Figure 5A). In contrast, the carboxyl carbon $C_{1a}$ of caffeic acid, which resonates at 171.0 ppm, indicates cross-peaks only with the $H_{3a}$ and $H_{2a}$ protons (spectrum not shown). This significant difference in the $^{13}C$ shieldings and the number of cross-peaks of the −COOR and −COOH carbons (Breitmaier and Voelter, 1989) offers the solution to the problem of the unequivocal assignment of caffeic and rosmarinic acid in the

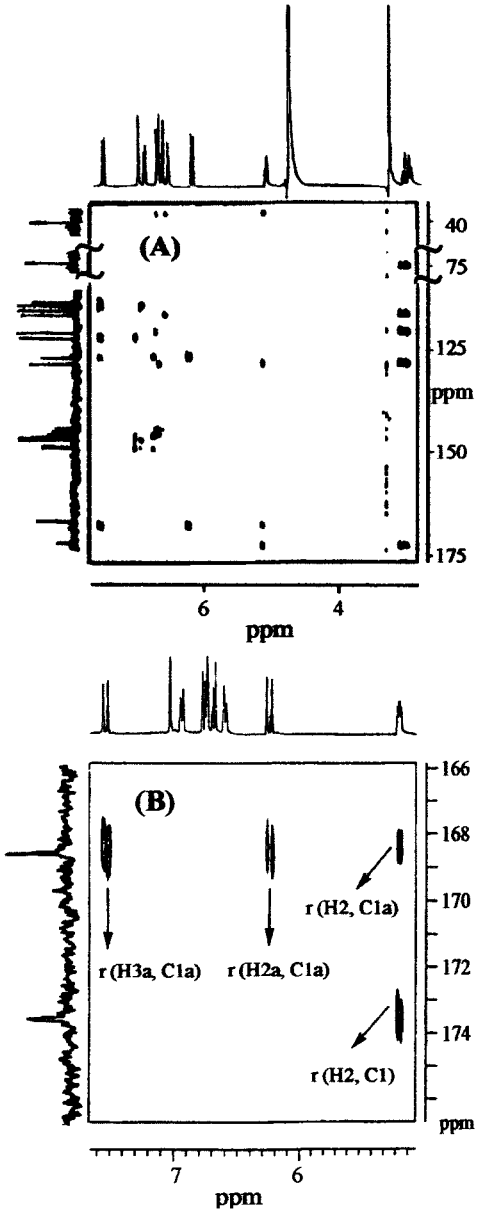



**Figure 5.** (A) 2D $^1H-^{13}C$ HMBC gradient-enhanced NMR spectrum at 298 K of rosmarinic acid, in $CD_3OD$, concentration 20 mM, on a Brüker AMX-400 instrument. The $^1J_{CH}$ filter was set to 3 ms, and the delay for the evolution of the long-range coupling was set to 90 ms. (B) Selected region of the spectrum of Figure 5A.

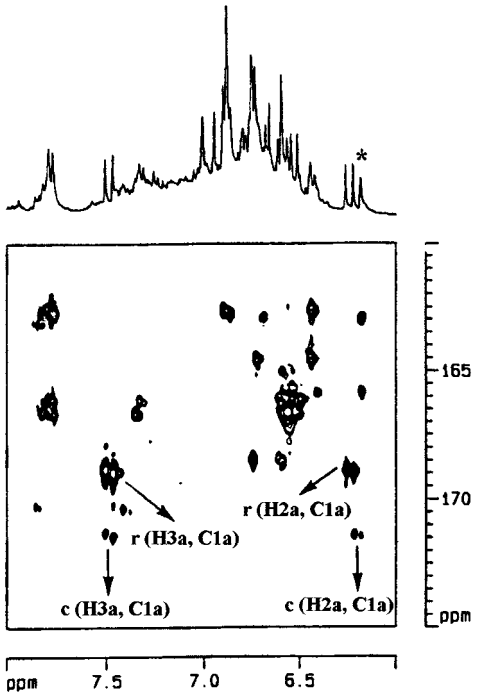



**Figure 6.** Selected region of the 2D $^1H-^{13}C$ HMBC gradient-cnhanced NMR spectrum at 298 K of the methanolic extract of *O. onites* of Figure 2. The $^1J_{CH}$ filter was set to 3 ms, and the delay for the evolution of the long-range coupling was set to 90 ms. The arrows denote the cross-peaks of the spin system of r($H_{3a}$, $C_{1a}$), r($H_{2a}$, $C_{1a}$), c($H_{3a}$, $C_{1a}$), and c($H_{2a}$, $C_{1a}$).

methanolic and ethanolic extracts. As shown in Figure 6, the high resolution of the 2D $^1H-^{13}C$ HMBC spectrum allowed us to determine unambiguously the individual cross-peaks of the spin system of r($H_{3a}$, $C_{1a}$), r($H_{2a}$, $C_{1a}$), c($H_{3a}$,$C_{1a}$), and c($H_{2a}$,$C_{1a}$), even at room temperature.

It is evident that the $^1H-^{13}C$ HMBC gradient NMR experiment provides significant resolution and structural information compared to the $^1H-^{13}C$ HMQC. The principal limitation of the technique is imposed by the low natural abundance of $^{13}C$ and long mixing times, which limit the obtainable signal-to-noise ratio. Furthermore, the HMBC method is more difficult to obtain on a routine basis. The sensitivity limitation can be overcome by using higher magnetic fields or better probe designs offering more sensitivity, such as microprobes (Subramanian and Webb, 1998).

**Comparison of NMR and HPLC Quantitative Results.** Quantification of rosmarinic acid was achieved by a straightforward integration of the 1D NMR spectra of the plant extracts, compared to that of $d_4$-TMSP of known concentration (0.2 mM), which was used as an internal standard. Quantification of caffeic acid was achieved with respect to the rosmarinic resonance in the low-temperature 2D $^1H-^1H$ DQF-COSY spectra. The concentrations of rosmarinic and caffeic acid in the methanolic extract of *O. onites* were found to be 18120 mg/kg of the extract and 780 mg/kg, respectively. These results are in agreement, to a certain extent, with the integration data of the respective cross-peaks derived from the $^1H-^{13}C$ HMQC and $^1H-^{13}C$ HMBC spectra. Integration data, however, of the $^1H-^{13}C$ HMBC experiment are less reliable due to the significant variation and conformational dependence of the $^2J_{^{13}C-^1H}$ and $^3J_{^{13}C-^1H}$ couplings (Breitmaier and Voelter, 1989; Günther, 1996).

Table 2 shows the concentration level of caffeic and rosmarinic acids in the plant extracts investigated with NMR and HPLC (Figure 7). It is obvious that oregano species have high levels of rosmarinic and caffeic acid, but the relative concentrations of these two phenolic acids vary widely among the subspecies. The ratio of rosmarinic to caffeic acid was found to be 64 for *Sat. hortensis*, 23 for *O. onites*, and 48 for *O. vulgare*. In the extract of *Sal. triloba* and *Sat. thymbra* we were unable to detect caffeic acid. This indicates that its concentration is below the detection limits of the 2D $^1H-^1H$ DQF-COSY experiment on our instrument (<20 $\mu$M). From Table 2 it is evident that the quantitative results using this methodology are in good agreement with values obtained with HPLC procedures for two of the extracts. For example, the contents of ethanolic extracts of *O. vulgare* and *Sat. hortensis* of rosmarinic acid were 12711 ± 1500 and 21373 ± 2584 milligrams per xilogram, respectively, whereas by HPLC the contents of rosmar-

**Table 2. Quantification (Milligrams per Kilogram) of Caffeic and Rosmarinic Acid in the Plant Extracts of the Lamiaceae Family by the Use of NMR and HPLC**

| | *O. vulgare* | | *Sat. hortensis* | | *O. onites* | *Sal. triloba* | *Sat. thymbra* |
|---|---|---|---|---|---|---|---|
| | NMR[a] | HPLC[b] | NMR[a] | HPLC[b] | NMR[a] | NMR[a] | NMR[a] |
| caffeic acid | 187 ± 25 | 140 ± 30 | 393 ± 40 | 248 ± 50 | 780 ± 69 | | |
| rosmarinic acid | 9039 ± 740 | 12711 ± 150 | 25166 ± 2150 | 21373 ± 2584 | 18120 ± 1520 | 41441 ± 3565 | 27400 ± 2259 |

[a] Values are means ± SD calculated for five measurements. [b] Values are means ± SD calculated for three measurements.

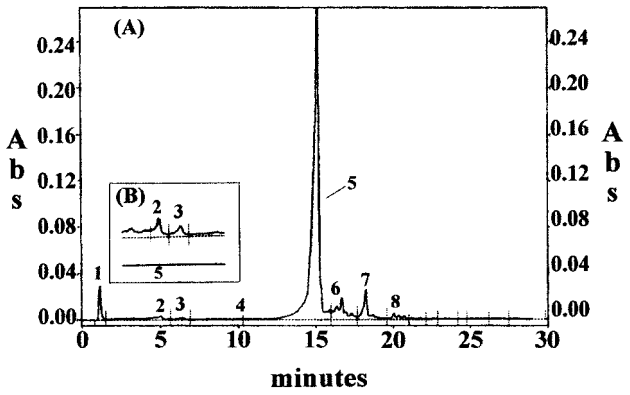



**Figure 7.** (A) HPLC/DAD chromatogram (326 nm) of the ethanolic extract of *Sat. hortensis.* Peaks 5 and 2 indicate rosmarinic and caffeic acid, respectively. (B) Selected region of the chromatogram after vertical expansion.

nic were found to be 9039 ± 740 and 25166 ± 2150 milligrams per xilogram.

In conclusion, the combination of variable-temperature two-dimensional $^1H-^1H$ COSY, $^1H-^{13}C$ HMQC, and $^1H-^{13}C$ HMBC gradient-enhanced NMR spectroscopy may be a useful analytical tool for the elucidation of the structure and quantification of complex phenol mixtures obtained from plants. The results are in good agreement with reverse-phase HPLC measurements. By the use of the above techniques structural information can be more rapidly obtained in comparison to classical analysis whereby the individual phenolic acids have to be isolated and purified prior to identification by NMR spectroscopy. The high information content of the $^1H-^{13}C$ HMBC NMR spectra may outweigh the rather unfavorable detection limits (~0.2 mM). The characterization of new structures directly from the extract is also possible. Further research is needed to extend the technique to other important classes of natural antioxidants such as flavonols. Such research is now in progress in our laboratories.

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