Analysis of Plant Complex Matrices by Use of Nuclear Magnetic Resonance Spectroscopy: St. John's Wort Extract

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The efficiency of two-dimensional homonuclear $^1H-^1H$ correlated spectroscopy and two-dimensional reverse heteronuclear shift correlation spectroscopy (i.e., heteronuclear multiple quantum correlation) in characterizing and evaluating the relative content of herbal extract constituents is demonstrated. These experiments are able to fully assign the proton and carbon resonances of all three classes of constituents present in dried commercial extract of St. John's wort, that is, flavonols, phloroglucinols, and naphthodianthrones, with particular regard to the very unstable phloroglucinols. In addition, shikimic and chlorogenic acids, sucrose, lipids, polyphenols, and traces of solvents of the extractive process (methanol) were also identified. These experiments can be considered to be a very simple and fast analytical method for determining the quality and stability of the titled commercial extract. They represent a generally applicable technique for a rapid screening and a specific measurement of other commercial phytochemicals or, in selected cases, an alternative to the classical analytical techniques such as high-performance thin-layer chromatography, high-performance liquid chromatography, capillary gas chromatography, and electrophoresis.

Keywords: *^1H NMR; ^{13}C NMR; 2D-NMR experiments; St. John's wort; commercial dried extract; quality control; stability testing*

INTRODUCTION

The analysis of complex mixtures has always been a crucial tool especially in food chemistry and more recently in the analysis of plant constituents (*1*−*3*). Quality control and stability testing are in general achieved through high-resolution chromatography techniques such as high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), capillary gas chromatography (GC), and electrophoresis using specific detectors such as ultraviolet−visible (UV−vis) spectroscopy, infrared (IR) spectroscopy, and coupled systems such as mass spectrometry (MS). Recently, the combination of chromatographic separation with nuclear magnetic resonance (NMR) spectroscopy (i.e., HPLC-NMR) has also been developed (*4*, *5*).

All of these analytical techniques are recognized as very powerful methods in the field of herbal drugs and their preparations, but it is often not easy to develop a simple and rapid chromatographic separation of the constituents because they have different solubilities, polarities, and sizes. However, the fingerprint obtained is, in general, quite complicated due to the presence of many similar constituents such as isomers, epimers, and conformers or glycosides containing different sugar chains. In addition, these techniques cannot reveal unknown plant metabolites that may also be present and may contribute to the biological activity of the phytochemicals. Finally, the chromatograms can become very complicated due to degradation signals with those of the constituents.

To provide harmonization within the European Union (EU), the Herbal Medicinal Product Working Party (HMPWP) of the European Agency for Evaluation of Medicinal Products (EMEA) established that the quality and stability of herbal drugs, their preparations, and herbal medicinal products should be determined by appropriate fingerprint chromatograms, overall methods of assay, and physical and sensory tests or other appropriate tests for complete characterization (*6*).

To find a suitable method of assay of commercial phytochemicals, one- and two-dimensional NMR experiments were considered as an analytical instrument to fully characterize them and to evaluate the stability of their constituents. These experiments represent a powerful method for the analysis of complex mixtures containing molecules with relatively similar size, as are often encountered in natural product chemistry (*7*). Furthermore, high-field strengths make it possible to obtain highly resolved NMR signals. Thus, in the past decade NMR has played an increasingly important role in the compositional study of food, particularly fruit juices (*8*−*10*).

In this study, a commercial dried extract of *Hypericum perforatum* L. (popularly called St. John's wort) was evaluated to demonstrate that NMR spectroscopy is a suitable analytical tool for herbal drug composition that does not require fractionation or isolation steps. The marketed St. John's wort extracts are standardized in their hypericin content, as required by the European Pharmacopoeia monograph (*11*), although a number of constituents with documented biological activity are also present and include other naphthodianthrones (principally pseudohypericin), flavonols (rutin, hyperoside, isoquercitrin, quercitrin, and quercetin), and phloroglucinols (hyperforin, adhyperforin, and others) (*12*). Herbal preparations based on this extract are largely marketed as dragees, drops, and capsules, especially in Europe and in the United States, due to the claimed antide-

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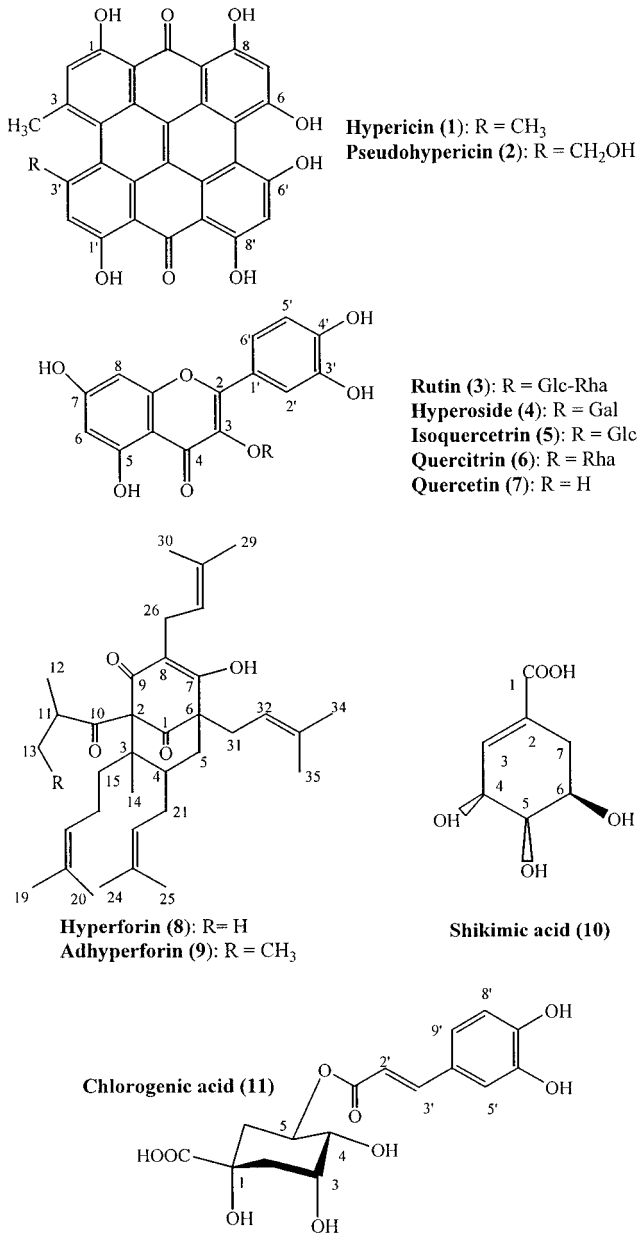

Figure 1. Chemical structures of compounds **1**−**11**.

pressant activity of polyketides (i.e., naphthodianthrones and phloroglucinols) (*13*−*15*). In addition, through one-dimensional ^{1}H NMR experiments the relative amounts and stabilities of constituents of the extract, with special regard to the very unstable phloroglucinols, were also evaluated, and the data obtained were compared with those derived by the conventional HPLC analysis.

MATERIALS AND METHODS

Solvents and Reagents. Dimethyl-d_6 sulfoxide (99.8%) was purchased from Euriso-top (Gif-Sur-Yvette, France). Acetonitrile and methanol were of HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Plant Material. A commercial sample of the extract of *H. perforatum* L. (lyophilized extract, lot 8J2152) was kindly provided by Aboca S.p.A. (Sansepolcro, Arezzo, Italy).

Thermal and Photostability Testing. A sample (350 mg) of extract was put in a glass vial and placed for 7 h in a Solarbox 1500 instrument equipped with a xenon lamp (1500 W) emitting radiation in the range of 310−700 nm (Angelantoni Industry S.p.A., Massa Martana, Perugia, Italy), as suggested by ICH guidelines for photostability testing of new drug substances and medicinal products (*16*). The sample was exposed, providing an overall illumination of 176 klx and an integrated energy of 75.8 W/m^2. After exposure, the samples were covered with aluminum foil and analyzed both by HPLC and by NMR.

Another sample of extract (350 mg) was put in a glass vial, covered with aluminum foil, and exposed to stress temperature conditions at 50 °C in an oven for 10 days. Samples were also analyzed by HPLC and NMR.

NMR Spectroscopy. The ^{1}H, ^{1}H−^{1}H COSY, selective TOCSY, and HMQC spectra were recorded at 300 K on a Bruker Avance-600 spectrometer operating at 600.13 MHz (14.1 T) using a 5-mm inverse probe equipped with a z-shielded gradient. Data processing was achieved with an SGI/02 computer using XWin-NMR software version 2.6. Samples (50 mg/0.8 mL) were dissolved in deuterated dimethyl sulfoxide, and the solvent signal was used for spectral calibration (^{1}H 2.49 ppm). Proton spectra were run using the standard pulse sequence program "zg" for recording 1D experiments. The time domain size was 32K, number of scans 4, spectral width 7440 Hz, Fid resolution 0.23 Hz, acquisition time 2.2 s, and relaxation delay 1 s. Processing parameters were 64K number of points and 3 Hz, FFT, line broadening.

The ^{1}H−^{1}H COSY experiments were acquired using gradient pulses for selection, type COSY magnitude 90°. Acquisition parameters were as follows: time domain size, 2K; number of experiments, 512; number of scans, 2; dummy scans, 16; spectral width, 7440 Hz in both dimensions. Fid resolution was 14.5 Hz in F2 and 29.1 Hz in F1, acquisition time was 138 ms, relaxation delay was 1 s, number of points was 1024 or 512, and filter function applied was squared sine in both dimensions, with magnitude calculation of phase along columns. No phase correction was applied along rows.

The HMQC experiments were acquired via heteronuclear zero and double-quantum coherence, phase sensitive using TPPI with decoupling during acquisition, peak type selection using gradient pulses with coherence selection step after t_1. Acquisition parameters were as follows: time domain size, 2K; number of experiments, 256; number of scans, 16; dummy scans, 16; spectral width, 7440 Hz (^{1}H) or 37730 Hz (^{13}C). Fid resolution was 3.633 Hz in F2 and 147.400 Hz in F1, acquisition time 138 ms, F1 (^{13}C) at 110 ppm, and relaxation delay 1 s. Processing parameters were as follows: number of points 1024 and 512; filter function applied, 60°, squared sine. The total experiment time was 1 h and 20 min.

Selective TOCSY experiments were performed using the eburp 1/25 selective pulse shape program (PW = 200, TRIMPWR = 54) and acquired as 16K datapoints (SW = 4400 Hz, AQ = 2.00 s). Off-line data processing was performed using the manufacturer's NMR data processing software (Bruker WinNMR packages).

The ^{13}C NMR experiments were recorded overnight at 300 K on a Bruker AC-200 spectrometer operating at 50.1 MHz. The same solvents and spectral calibrations (^{13}C: 39.9 ppm) of ^{1}H NMR experiments were used.

HPLC Analysis. The dried extract (2.50 mg/mL) and samples submitted to thermal and photostability testing were dissolved in a mixture of HPLC grade solvents (methanol/acetonitrile/water, acidified to pH 2 with formic acid, 3:1:1). These were sonicated and filtered through a cartridge-type sample filtration unit with a poly(tetrafluoroethylene) (PTFE) membrane (d = 13 mm, porosity = 0.45 μm, Lida Manufacturing Corp.) before HPLC analysis.

The HPLC system consisted of an HP 1090L instrument with a diode array detector controlled by an HP 9000 workstation (Hewlett-Packard, Palo Alto, CA). The column was a Protein C4, 5 μm, 250 mm, 0.5 mm i.d., 300 Å (Vydac Separation Group, Hesperia, CA) maintained at 26 °C. The method previously reported (*12*) was employed; injected volume was 25 μL of solution, and chromatograms were monitored at 230, 254, 270, 350, and 590 nm and evaluated at 270 nm.

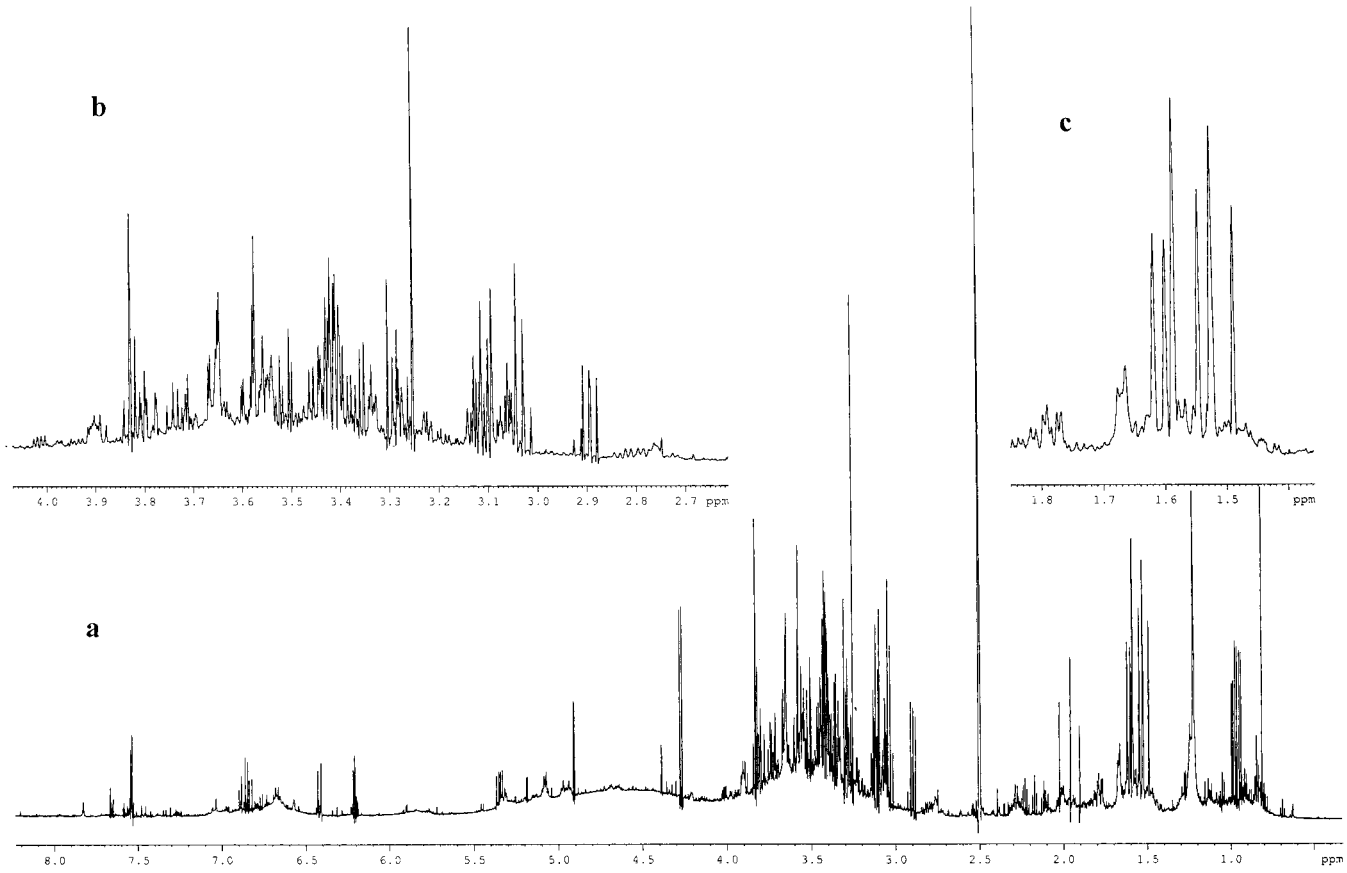

Figure 2. (a) Full resolution-enhanced 600 MHz ^{1}H NMR spectrum of St. John's wort commercial extract in dimethyl sulfoxide. (b and c) Horizontally expanded regions (2.6−4.1 and 1.35−1.85 ppm) of the spectrum to clarify the splitting pattern of individual resonances.

RESULTS AND DISCUSSION

St. John's wort extract contains more than 10 principal and characteristic constituents, both aglycons and glycosides (Figure 1). They are represented by flavonols, mainly 3-glycosides of quercetin, that is, rutin (**3**), hyperoside (**4**), isoquercitrin (**5**), quercitrin (**6**), and quercetin (**7**) itself; naphthodianthrones, represented by hypericin (**1**) and pseudohypericin (**2**), considered as total hypericins; and two principal, nonaromatic, structurally related phloroglucinols, that is, hyperforin (**8**) and adhyperforin (**9**), considered as total hyperforins. In addition, the presence of other constituents such as shikimic acid (**10**), chlorogenic acid (**11**), sucrose, and lipids was also confirmed in the extract by NMR analysis.

In an initial investigation, the ^{13}C NMR spectrum of the sample (data not shown) was recorded overnight. However, it showed resonances of only the main constituents (flavonoids and hyperforins) and the signals were strongly overlapped, so only limited information was provided about the overall chemical composition of St. John's wort extract.

^{1}H NMR, with its greater sensitivity, may provide better detection of the constituents, and sufficient line-narrowing can be achieved (Figure 2). Thus, some characteristic resonances of the proton spectrum were analyzed according to the data (chemical shifts and coupling constants) found in the literature (*17*−*24*) and by comparison with chemical shifts observed for single-constituent solutions. Spectral assignments of the constituents were carried out on the basis of 1D-NMR and primarily by means of 2D-NMR spectra (Figures 3 and 4) and are presented in Table 1.

The ^{1}H NMR spectrum was characterized by four main regions: a low-field region between 6.0 and 9.0 ppm with signals principally due to aromatic protons of quercetin derivatives and also olefinic protons of naphthodianthrones and chlorogenic acid; a mid-low-field region between 5.5 and 4.5 ppm with signals due to anomeric protons of sugar units and olefinic protons of hyperforins; a mid-field region between 4.5 and 3.0 ppm with signals due principally to the sugar protons of the glycosides; and a high-field region between 2.7 and 0.7 ppm with signals due to aliphatic protons of sugar residues (Me-6 of rhamnose), aromatic methyls of naphthodianthrones, and aliphatic protons of phloroglucinols.

*Quercetin Derivatives (**3**−**7**).* Some aglycon resonances were easily attributed due to their chemical shifts, together with their splitting and couplings shown by cross-peaks in the COSY experiments. In particular, the signals at 6.21 and 6.41 ppm were attributed to protons linked to C-6 and protons linked to C-8 of quercetin and its derivatives. Thus, these chemical shifts are not affected by glycosylation at C-3, so both aglycon and glycosides gave identical resonances (*22*). These data were confirmed through HMQC experiments (Figure 5) by the connectivities with the carbon resonances at 97.6 and 93.8 ppm, respectively. The other aromatic signals of flavonol ring B appeared between 6.6 and 7.8 ppm, and the assignments were made by analysis of the HMQC experiments (Figure 5) after comparison with the data reported in the literature (*17*, *22*). In general,

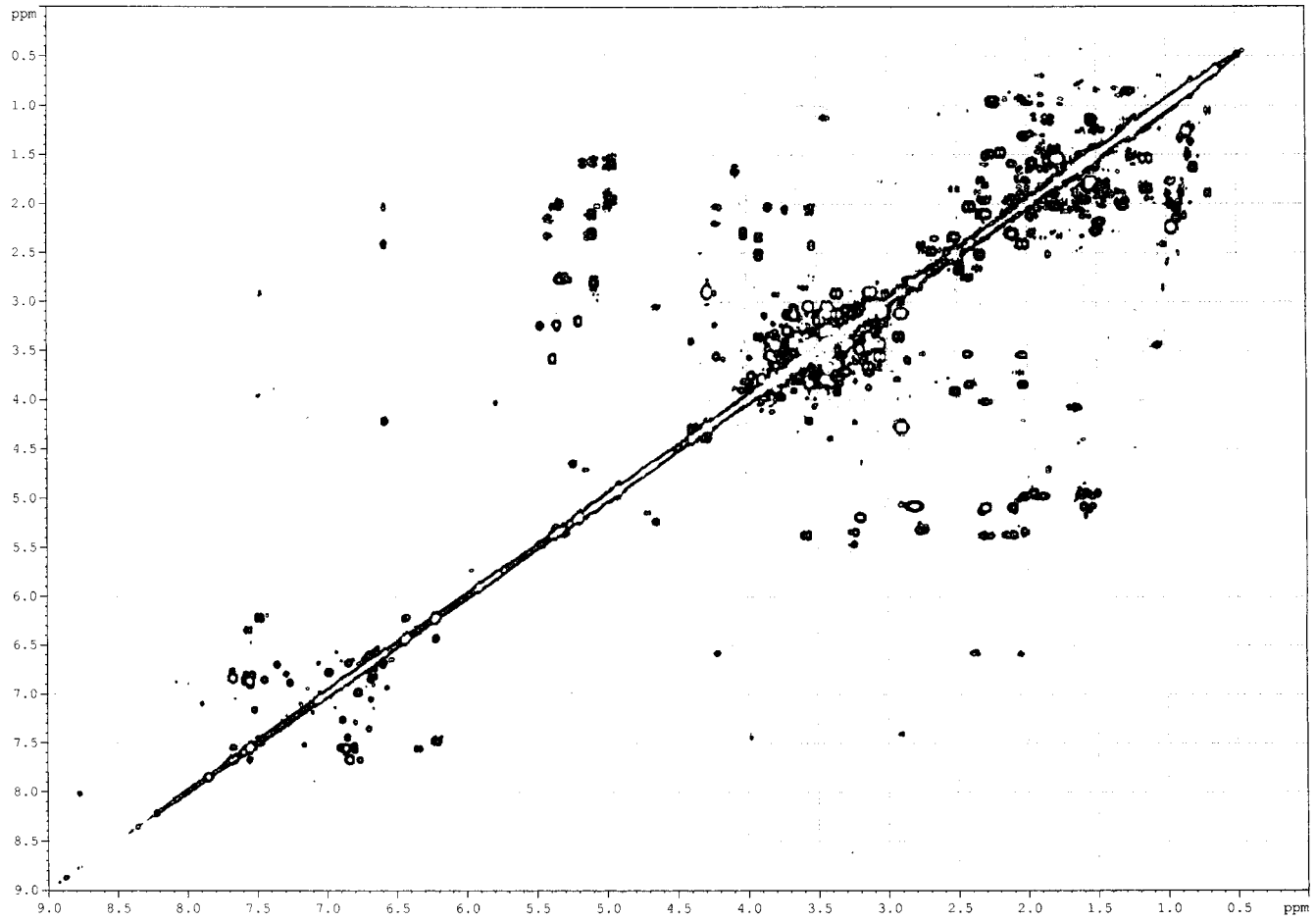

Figure 3. $^{1}H-^{1}H$ COSY spectrum of St. John's wort commercial extract.

glycosylation of C-3 of flavonols affects only the resonances of the 2′ proton and carbon, whereas the signals of the 5′ and 6′ protons and carbons are not affected. On the basis of these data, the proton resonance at 6.65 ppm, which showed a correlation at 115.5 ppm in the HMQC spectrum, was attributed to the 5′ proton and carbon of both aglycon and glycosides. Two signals due to H-6′ were found at 7.53 and 7.64 ppm, which showed a correlation with the signals at 121.8 and 122.2 attributable to aglycon and glycosides, respectively. On the other hand, the H-2′ resonance at 7.55 ppm, which correlated in the HMQC spectrum with the signal at 116.2 ppm, was attributable to the glycosides, whereas the resonances at 7.65 and 115.3 ppm corresponded to the aglycon quercetin.

*Hypericins (**1**−**2**).* The aromatic protons of hypericins were identified by their chemical shift, splitting, and cross-peaks in the COSY (Figure 3) and HMQC (Figure 5) experiments and confirmed by comparison with data reported in the literature (*18*). Protons linked to the 3 and 3′ carbons of hypericin were represented by a broad singlet at 7.40 ppm and correlated in the COSY experiments with the methyl at 2.85 (aromatic methyl at C-4, carbon resonance at 22.3 ppm), whereas signals of protons 7 and 7′ were at 6.51 as a singlet. The first aromatic resonance in the HMQC experiments showed a correlation with the carbon resonance at 118.8 ppm, whereas the other showed a cross-peak with the signal at 105.3 ppm. These data confirmed the presence of the hypericin (**1**) moiety. The presence of pseudohypericin (**2**) was shown by the cross-peaks in the COSY between a broad singlet at 7.48 ppm and oxymethylene protons at 3.98 ppm, the assignment of which was confirmed by HMQC experiments (^{13}C resonances at 116.2 and 65.8 ppm, respectively).

*Shikimic Acid (**10**).* NMR signals due to shikimic acid were easily seen by the characteristic $^{1}H-^{1}H$ COSY connectivities between H-3 (6.58 ppm) and H-4 (4.21 ppm) and long-range connectivities between H-3 and H-7a and H-7b (2.40 and 2.04 ppm), as shown in Figure 6 (*3*). In the same manner, H-5 and H-6 resonance signals (3.28 and 3.57 ppm) were also found by TOCSY experiments. The presence of shikimic acid was confirmed by the characteristic HMQC connectivities of H-3 with the ^{13}C resonance at 139.7 ppm, of H-4 with the ^{13}C resonance at 65.8 ppm, and of H-7a and H-7b with the ^{13}C resonance at 34.0 ppm, whereas the other signals were overlapped and not unambiguously assigned (*10*).

*Chlorogenic Acid (**11**).* Chlorogenic acid, previously reported by some authors (*12*), was also shown with the aid of chemical shifts and coupling constants. The characteristic signals were the protons at 6.38 and 7.60 ppm with large coupling constants (16 Hz) due to the trans configuration in the proton spectra that showed connectivities in the HMQC (Figure 5) with the ^{13}C resonances at 115.2 and 148.0 ppm, respectively. The proton signals of the aromatic ring were overlapped with those of ring B of quercetin derivatives (H-5′) between 6.6 and 7.1 ppm, and their assignment was done by HMQC (Figure 5). Thus, the proton resonance at 7.05 ppm and the carbon resonance at 114.8 ppm with which it was correlated were attributed to H-5′ and C-5′, respectively. The signal at 6.85 ppm and its cross-peaks

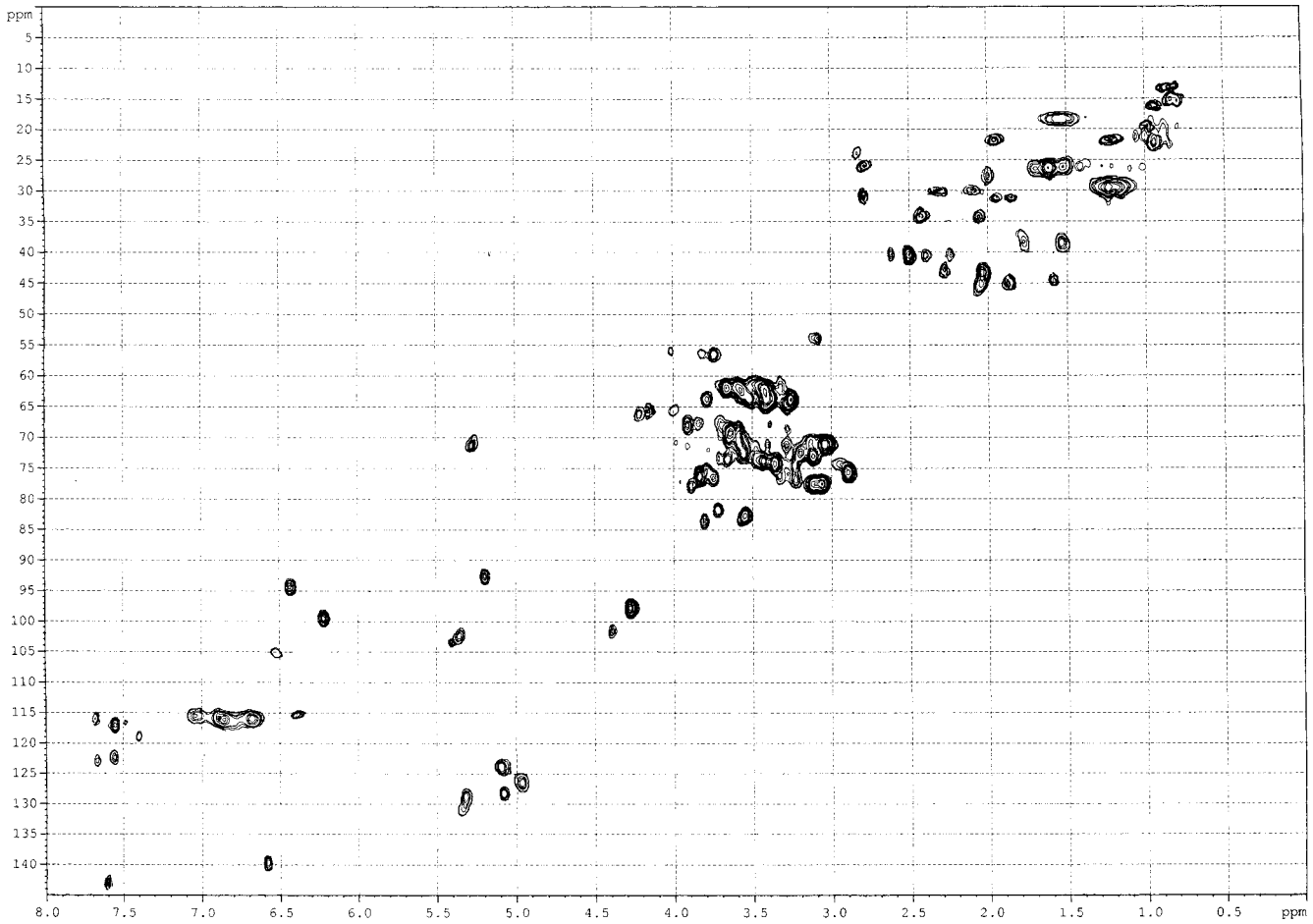

Figure 4. $^{1}H-^{13}C$ HMQC spectrum of St. John's wort commercial extract.

Table 1. Resonance Assignments with Chemical Shift of Constituents Identified in 600 MHz ^{1}H and $^{1}H-^{13}C$ NMR Spectra of St. John's Wort Extract

molecule	^{1}H shift (δ)	assignment	observed	^{13}C shift (δ)
chlorogenic acid	7.05, 6.85, and 6.89	H-5′, H-9′, and H-8′	1D, HMQC	114.8, 115.1, and 115.5
chlorogenic acid	6.38 and 7.60	H-2′ and H-3′	1D, COSY, HMQC	115.2 and 148.0
chlorogenic acid	5.32 and 4.19	H-5 and H-3	1D, COSY, HMQC	71.2 and 66.3
chlorogenic acid	3.57	H-4	1D, COSY, TOCSY	
chlorogenic acid	2.07 and 2.25; 2.11 and 2.32	H-2eq and H-2ax; H6ax and H-6eq	1D, COSY, HMQC	43.5, 37.1
hyperforins	4.95, 5.05, 5.10, 5.21	H-22, H-27, H-17, and H-31	1D, COSY, HMQC	126.2, 127.4, 123.1, and 128.2
hyperforins	2.80, 1.49, and 1.47	CH_2-26, $C\overline{H}_3$-29, and $C\overline{H}_3$-30	1D, COSY, HMQC	23.2, 24.5, 18.0[a]
hyperforins	1.88 and 1.98, 1.58, and 1.57	CH_2-21, $C\overline{H}_3$-24, and $C\overline{H}_3$-25	1D, COSY, HMQC	32.0, 17.8,[a] 25.9
hyperforins	2.78, 1.96, and 2.01	CH_2-31, $C\overline{H}_3$-34, and $C\overline{H}_3$-35	1D, COSY, HMQC	25.3, 21.4, 26.8
hyperforins	2.29 and 2.09, 1.58, and 1.60	CH_2-16, $C\overline{H}_3$-19, and $C\overline{H}_3$-20	1D, COSY, HMQC	30.1, 18.2, 26.0
hyperforins	1.79 and 1.55	CH_2-15	1D, COSY, HMQC, TOCSY	38.6
hypericins	7.40 and 2.85	H-3 and H-3′, CH_3-Ar	1D, COSY, HMQC	118.8 and 22.3
hypericins	7.48 and 3.98	H-3′ and $CH_2O\overline{H}$-Ar pseudohypericin	1D, COSY, HMQC	116.2 and 65.8
hypericins	6.51	H-7 and H-$\overline{7}$′	1D, HMQC	105.3
lipids	0.85	$C\overline{H}_3(CH_2)_n-$	1D, COSY	
lipids	1.22, 1.24, and 1.28	$-\overline{(CH_2)}_n-$	1D, HMQC	29.4, 29.1, and 29.2
methanol	3.09		1D, HMQC	54.0
quercetin derivatives	7.55 and 7.65	H-2′ glycosides and H-2′ aglycon	1D, HMQC	116.2 and 115.3
quercetin derivatives	7.53 and 7.64	H-6′	1D, HMQC	121.8 and 122.2
quercetin derivatives	6.65	H-5′	1D, HMQC	115.5
quercetin derivatives	6.21 and 6.41	H-6, H-8	1D, COSY, HMQC	97.6 and 93.8
quercetin derivatives	5.38 and 5.44	anomeric protons of sugar linked to C-3	1D, HMQC	101.3 and 101.8
quercetin derivatives	4.39	anomeric proton of terminal rhamnose	1D, HMQC	100.7
shikimic acid	2.40 and 2.04	H-7a and H-7b	1D, COSY, HMQC	34.0
shikimic acid	3.28 and 3.57	H-5 and H-6	1D, TOCSY	
shikimic acid	6.58 and 4.21	H-3 and H-4	1D, COSY, HMQC	139.7 and 65.8
sucrose	5.18 and 4.26	H-1 fructose and H-1 GLC, respectively	1D, HMQC	91.8 and 95.6

[a] Interchangeable.

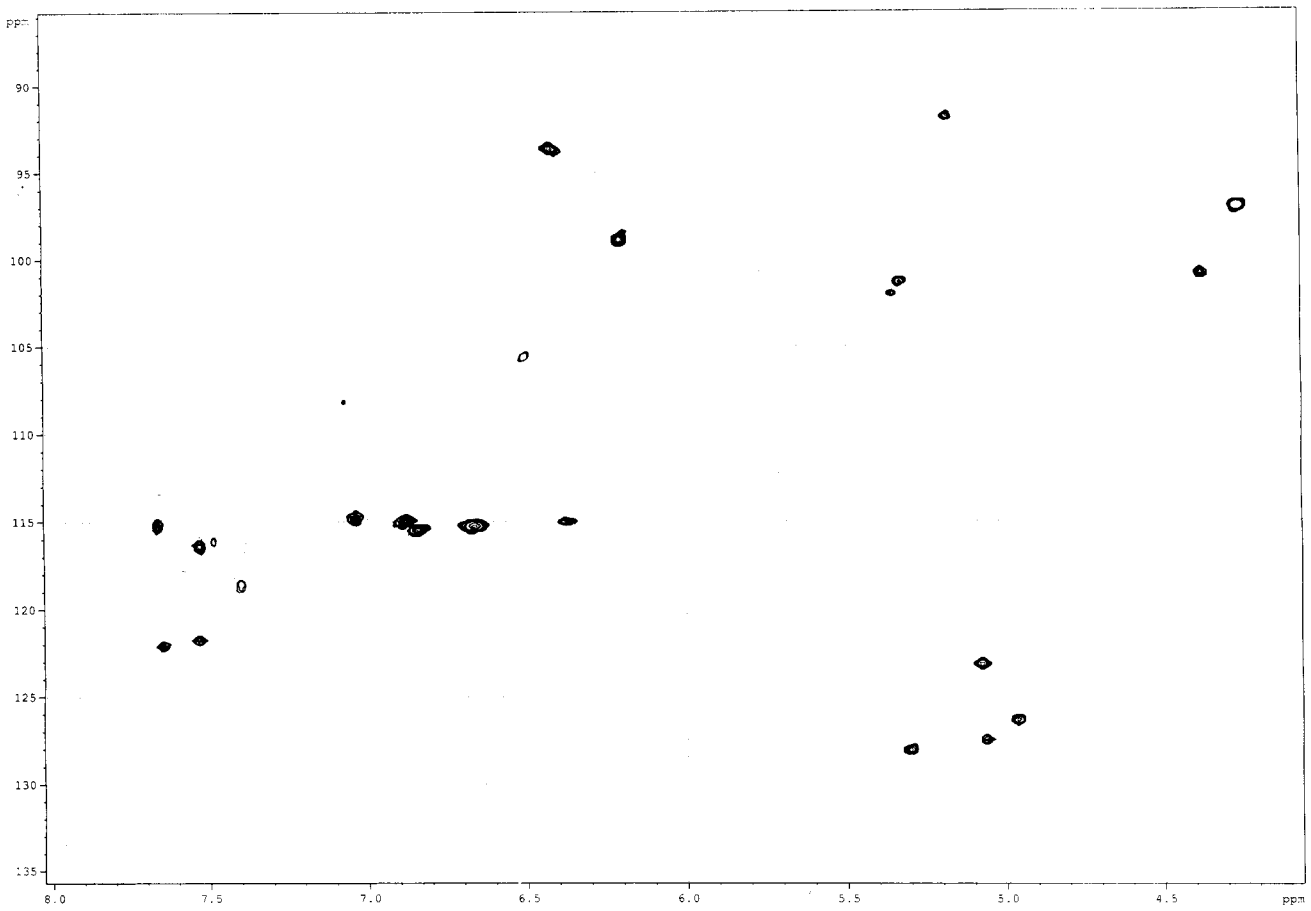

Figure 5. Expanded region of the $^{1}H-^{13}C$ HMQC experiment of the resonances of aromatic, olefinic, and anomeric signals of constituents **1**−**11**.

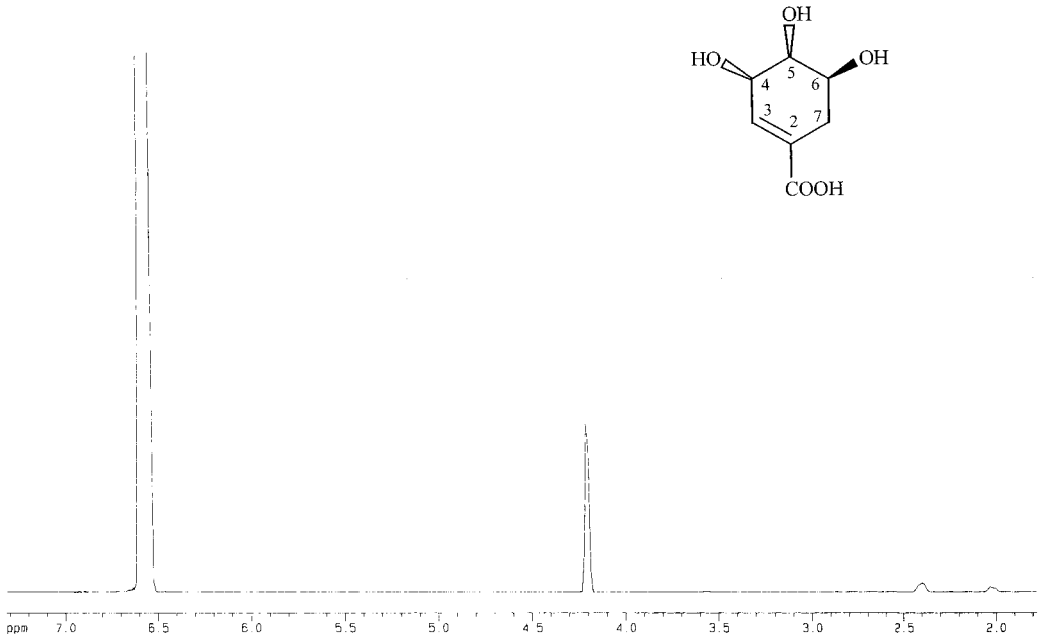

Figure 6. Resonances of coupled protons H-3 and H-4 and H7a and H-7b of shikimic acid extracted by means of the $^{1}H-^{1}H$ COSY experiment of St. John's wort commercial extract.

in the HMQC with the resonance at 115.5 ppm were attributed to H-8′ and C-8′, respectively, and, finally, the resonance at 6.89 ppm showed a correlation with the signal at 115.1 ppm and these were attributed to H-9′ and C-9′, respectively. The presence of the quinic acid unit of chlorogenic acid was evident from COSY and TOCSY experiments. Thus, in the TOCSY spectra a set of characteristic correlated peaks was apparent at 5.21, 4.14, 3.57, 2.07, 2.11, 2.25, and 2.32 ppm, and these signals were attributed by COSY connectivities to H-5, H-3, H-4, H-2eq, H6ax, H-2ax, and H-6eq, respectively (*25*). In addition, the chlorogenic acid molecule was confirmed by some characteristic HMQC connectivities of H-5 with the ^{13}C resonance at 71.2 ppm,

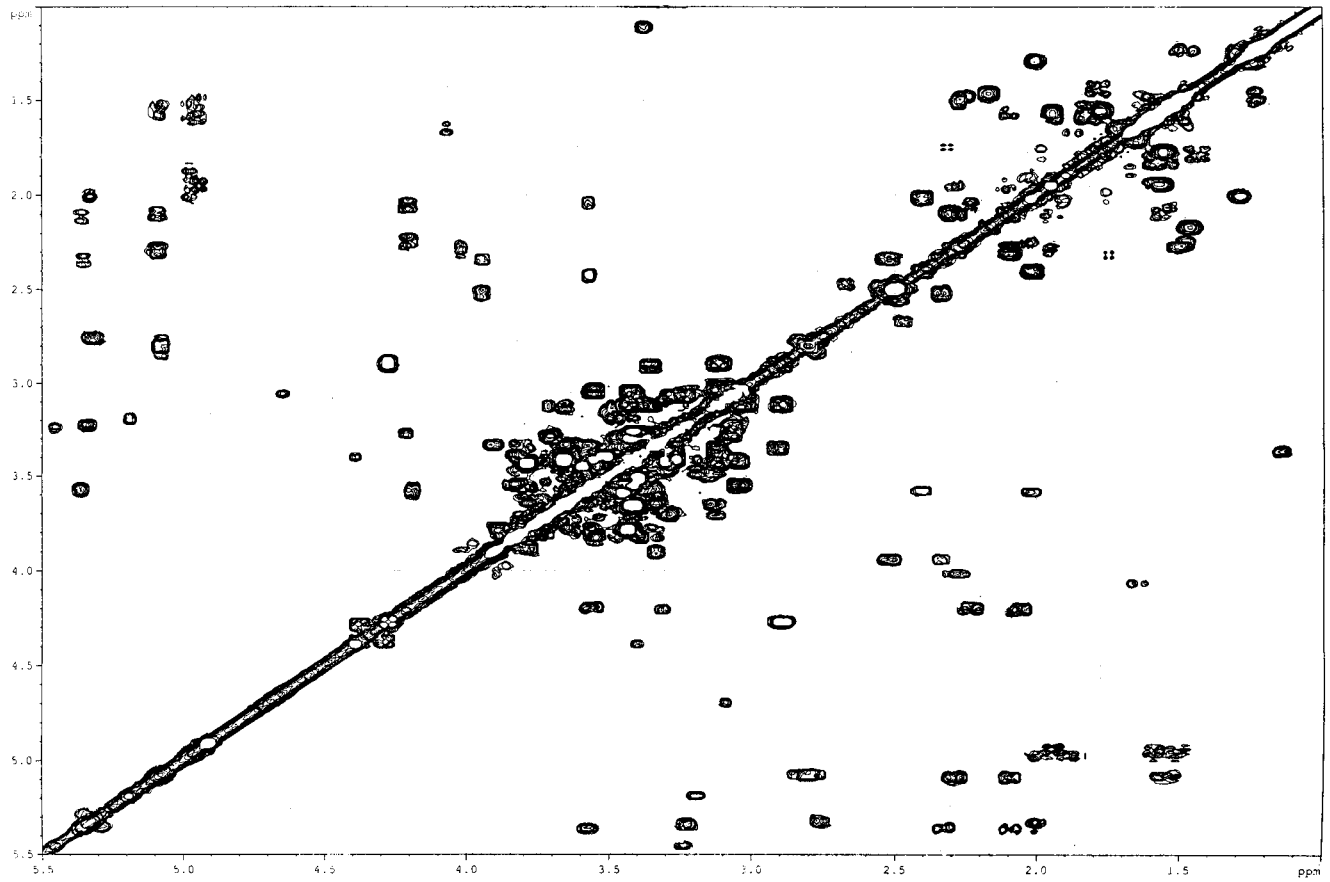

Figure 7. Subspectrum (1.0−5.5 ppm) of the $^{1}H-^{1}H$ COSY experiment of St. John's wort commercial extract to clarify the coupling pattern of individual resonances.

of H-3 with the ^{13}C resonance at 66.3 ppm, of H-2 ax and H-2eq with the ^{13}C resonance at 43.5 ppm, and of H-6eq and H-6ax with the ^{13}C resonance at 37.1 ppm (*10*).

Sugar Moieties. Three principal anomeric proton and carbon signals of the sugar units linked at 3-OH of quercetin were evident. The resonances at 5.38 and 5.44 ppm, which correlated with the signals at 101.3 and 101.8 ppm, respectively, were attributed to anomeric protons and carbons of glucose of rutin (**3**) and galactose of hyperoside (**4**), respectively. The anomeric signals of the terminal rhamnose of rutin (**3**) were at 4.39 and 100.7 ppm. The anomeric signals of the quercitrin (**6**) and isoquercitrin (**5**) were probably hidden by the anomeric signals of the main constituents rutin (**3**) and hyperoside (**4**). Furthermore, characteristic ^{1}H and ^{13}C resonances of sucrose were present at 5.18 and 4.26 and 91.8 and 96.5 ppm, respectively (*10*). As expected, the signals of the sugar region of the spectra were strongly overlapped and not significant for the fingerprint of the extract.

*Hyperforins (**8** and **9**).* The signals of hyperforins were seen as a set of correlated peaks in the COSY (Figure 7) and HMQC (Figures 5 and 8) spectra. Thus, these constituents are characterized by the presence of a quinoid structure containing aliphatic methine and methylene protons with resonances from 1 to 2.7 ppm with isoprenyl side chains, that is, vinyl (4.8−5.3 ppm), allylic methyl (1.4−1.8 ppm), and allylic methylene (1.6−3.1 ppm) protons (*19*−*21, 24*). The identification of signals corresponding to the isoprene side chains was the crucial point of this investigation. Vinylic protons of the isoprenyl chains were not easily identifiable in the proton spectrum due to overlap with other proton signals in the zone between 4.8 and 5.3 ppm, a typical range of the anomeric protons of sugars also. However, in the COSY experiments it was very easy to distinguish these vinylic protons of the hyperforins from the anomeric signals. Thus, vinylic protons showed cross-peaks in the aliphatic region with the signals of vinylic methyls and methylenes of the isoprenyl chains of hyperforins. These resonances were distinct from those of anomeric signals that showed connectivities only with signals in the sugar region (3.0−4.5 ppm), as shown in Figure 7 and reported in Table 1.

These features were confirmed by the HMQC experiments in which the chemical shifts of anomeric carbons (not above 106 ppm) were very different from those of vinyl carbons of the isoprenylic chains (120−130 ppm) as shown in Figure 8. Using similar HMQC experiments, all of the aliphatic signals of hyperforins were also identified, and it was demonstrated that among the hyperforin signals, those of the characteristic region between 1.4 and 1.8 ppm were due to the methyl and methylene protons of the isoprenyl chains of the phloroglucinol derivatives (four moieties).

Other Compounds. The presence of methanol (^{1}H NMR signal at 3.09 ppm and ^{13}C NMR signal at 54.0 ppm), probably due to the extraction process of the sample, was also shown. In addition, signals due to fats (see Table 1) and polyphenols (numerous signals between 8.0 and 8.5 ppm) were also present.

As a second step of our investigation, the stability of St. John's wort extract was evaluated to assess whether the phytocomplex is stable and the proportional content of metabolites remains constant, as suggested by the

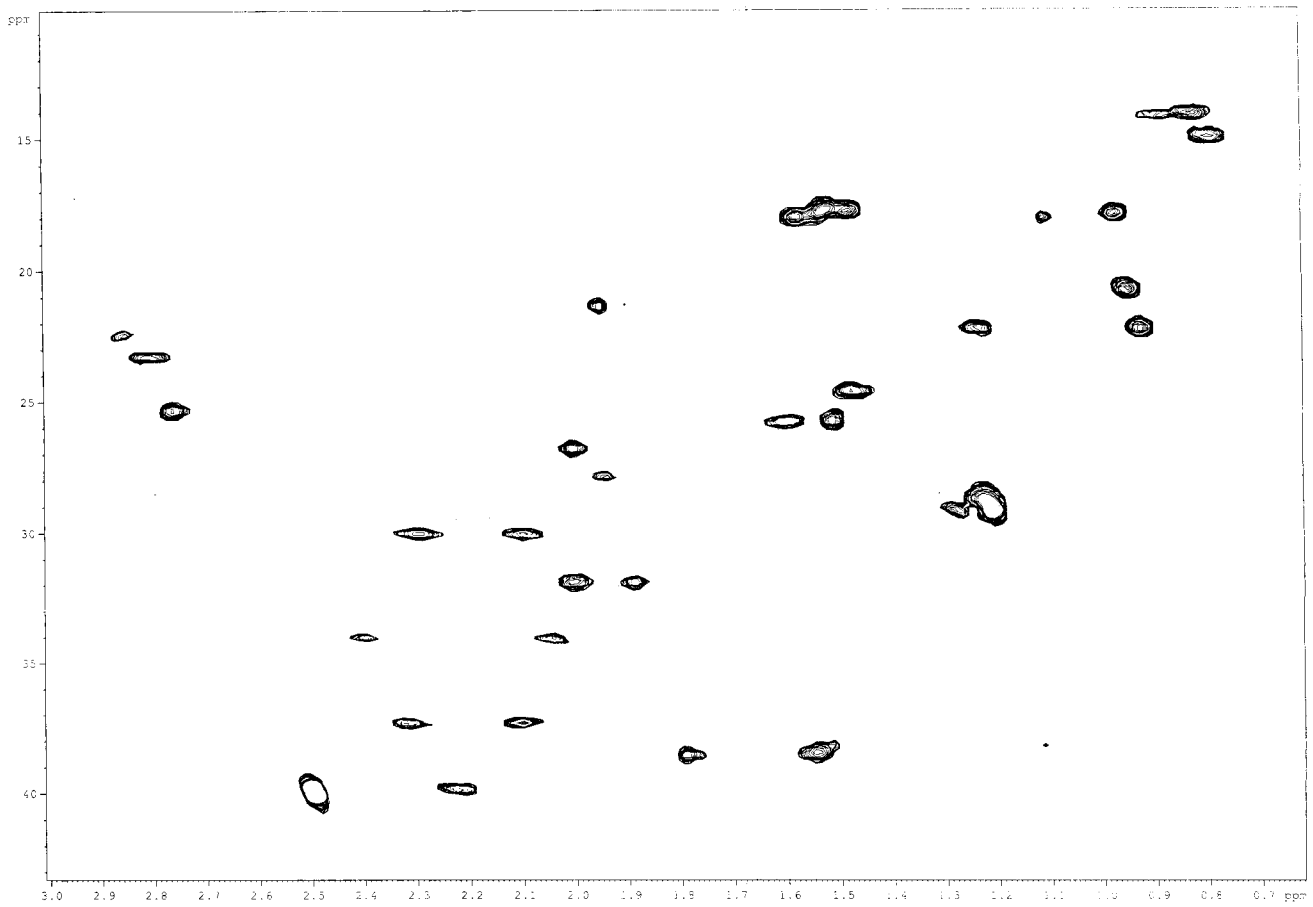

Figure 8. Expanded region of the $^{1}H-^{13}C$ HMQC experiment of the resonances of aliphatic resonances of constituents **1**−**11**.

HMPWG directives (Figure 9). Previously, many studies have pointed out that hyperforins are unstable toward light and temperature (*19*−*21*, *23*, *26*, *27*).

On the basis of the assignments made, from analysis of the spectra obtained with the degraded samples, it was quite easy to document the decreased intensity of vinyl, methyl, and methylene proton signals of the isoprenylic chains of hyperforins, due to oxidative cleavage of the isoprenyl side chains, whereas signals of the other molecules were not significantly modified. Due to the difficulty in using an intensity reference peak, only qualitative and semiquantitative considerations were made, on the basis of relative intensity changes.

To evaluate the quantitative degradation of the hyperforins, the proportional content of the signals between 1.4 and 1.8 ppm, corresponding to the vinyl methyls and methylene of the isoprenyl chains, and the signals of the H-6 of the flavonol skeleton were compared (Figure 8). This aromatic proton was chosen because the high stability of flavonols is well-known; thus, its area remained constant during the stress experiments, and, furthermore, its signal was sufficiently isolated to evaluate the proportional content of hyperforins. To verify the validity of all these findings, HPLC analysis was also carried out to confirm the NMR results, using a method reported in the literature (*12*) and modified for our analytical needs (*28*). The sample submitted to photostability testing according to ICH guidelines showed a residual 53.0% (standard deviation of ±1.08) content of hyperforins by integrating the proton spectra and a value of 52.2% (standard deviation of ±0.04) by HPLC analysis. The sample submitted to thermal stability showed a residual 94.4% (standard deviation of ±1.12) content of hyperforins by integrating the proton spectra and a value of 96.3% (standard deviation of ±0.06) by HPLC analysis. The results obtained by the two different analytical methods are comparable.

In this investigation high-resolution NMR spectroscopy is proposed as an alternative, nonchromatographic method for the quality and stability assessment of a commercial extract of an herbal drug to obtain a complete analysis of the extract. Many functional groups can be easily and conclusively identified by their characteristic ^{1}H and/or ^{13}C chemical shifts, determined by detailed analysis of 2D experiments, and compared with the literature data. From these NMR results, especially 2D experiments, the technique can be considered to be a valid alternative method to obtain a fingerprint for the assurance of quality and stability and, therefore, safety and efficacy of crude extracts of herbal drugs.

A critical comparison with the routine classical analytical methods shows that NMR experiments can be readily performed without the necessity of pretreatment; are not time-consuming (all experiments can be performed in <2 h); are nondestructive and do not consume large amounts of reagent; are versatile, not depending on the nature of the extract, providing a fingerprint of the extract; and are inexpensive because specific columns and international standards are not required. The method may have lower precision than other methods but is sufficient for pharmaceutical applications, especially to evaluate stability in normal and accelerated conditions.

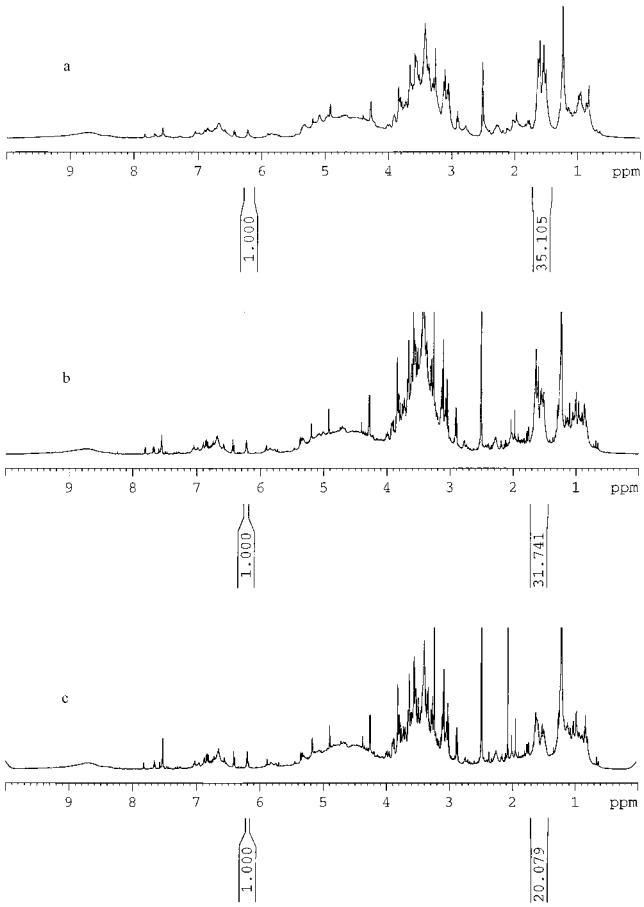

Figure 9. ^{1}H NMR spectra of St. John's wort commercial extract (a) and extract submitted to thermal (b) and photostability (c) testing.

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