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Full Length Research Paper

Fast identification of flavonoids in the roots of *Sophora flavescens* by on-flow LC-NMR

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Sophora flavescens **Ait (Leguminosae) is a Chinese herbal medicine. Sophorae radix, the dried roots of *S. flavescens*, has been used for various diseases including atherosclerosis and arrhythmias. For the direct identification of the compounds present in Sophorae radix, the hyphenated LC-NMR technique has been applied. WET solvent suppression and scout scan techniques were used to suppress solvent peaks in on-flow LC-NMR experiment. Six prenylflavonoids were identified successfully as kushenol U (1), kurarinone (2), sophoraflavanone G (3), leachianone A (4), kuraridin (5) and kushenol A (6).**

Key words: LC-hyphenated, on-flow LC-NMR, prenyl-flavonoid, *Sophora flavescens*.

INTRODUCTION

In the natural product analysis, the hyphenated techniques of HPLC with nuclear magnetic resonance (NMR) (LC-NMR) is increasingly used for phytochemical analysis (Andrade et al., 2002; Kang et al., 2008; Tode et al., 2009). This technique combines the separation power of HPLC with the structural information provided by NMR (Figure 1). A number of advantages over the traditional off-line techniques are present in this hyphenated technique: (1) no need for conventional separation procedures, (2) easy to identify the closely related compounds, (3) an unique insight into the composition of mixtures at an early analytical stage. However, LC-NMR contains several limitations: (1) requirement of conventional isolation procedures for a novel natural product due to data loss by the solvent suppression, (2) need for ^{13}C NMR data by off-line NMR spectroscopy to characterize the structures of new compounds, (3) detection of major peak due to the low sensitivity (Wolfender et al., 2001). Two modes, on-flow and stop-flow, are available in the LC-NMR. In general, on-flow mode is used to screen for the presence of particular groups of compounds or to gain a general overview in the mixture while stop-flow mode is chosen to acquire more exact information including two-dimensional NMR spectra such as COSY, HMBC and HSQC (Keifer et al, 2000; Elipe et al., 2010).

In this study, on-flow LC-NMR techniques were applied to the direct identification of prenylflavonoids in the Sophorae radix. Sophorae radix, the dried root of *Sophora flavescens* Ait (Leguminosae) is a well known traditional Chinese medicines widely spread in China, Japan and Korea. It has been used frequently as the traditional medicines such as antipyretic, diuretic, anthelmintic and stomachic and for the treatment of diarrhea, gastrointestinal hemorrhage and eczema. A variety of flavonoid compounds including flavanone, flavanol, valvanonol, chalcone, isoflavone, isoflavanonone and pterocarpans have been reported in this plant (Zhang et al., 2007). Among them, prenylflavonoids are major components of Sophora radix. A wide range of biological functions has been reported for prenylated flavonoids, e.g. anti-tumor, anti-bacterial, anti-virus, anti-oxidant and anti-nitric oxide production (Yazaki et al., 2009). Even a single prenylated compound may show multiple effects. For instance, kurarinone exhibits estrogenic, anti-glycosidase, anti-tyrosinase and anti-lipoxygenase acitivities, indicating possible application on preventing osteoporosis and diabetes, skin whitening,

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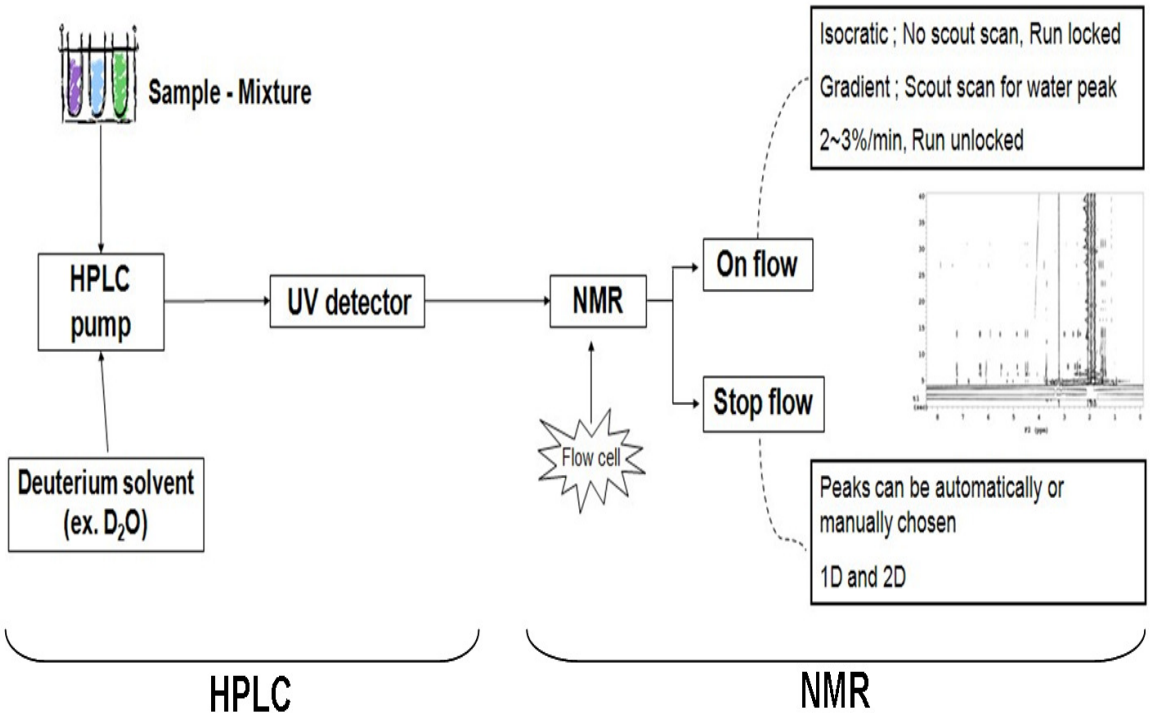


Figure 1. Work scheme of LC-NMR.

anti-inflammatory, respectively. The prenyl moiety plays a crucial role in these divergent biological activities (Row et al., 2006). In this study, six prenylflavonoids could be identified as kushenol U (1), kurarinone (2), sophoraflavanone G (3), leachianone A (4), kuraridin (5) and kushenol A (6) by applying on-flow LC-NMR technique.

MATERIALS AND METHODS

Plant material

The dried roots of *S. flavescens* were purchased from Deok-hyun herb shop, Gyundong Market, Seoul (native to Yeongcheon, Kyeongbuk). The dried roots of *S. flavescens* were grinded with electric mixer before extraction.

Extraction

The dried sample (10 g) was extracted twice with 50 mL ethanol (99.9%) in an ultrasonic bath for an hour and then, the extraction solution was combined and filtered as described in Gülçin (2005) and Gülçin et al. (2007). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C.

Structural identification by LC-NMR spectroscopy

The extract (50 mg) was dissolved in 1 mL of methanol and filtered in a 0.45 µm membrane filter. Twenty microliter of this solution was directly injected into the Varian LC–NMR system (Varian, Palo Alto, CA, USA). A YMC hydrosphere C_{18} reverse phase column (4.6 × 150 mm, 3 µm particle size) was used for the separation of each compound in the extract with the solvent gradient condition as following; from 45 ~ 65% acetonitrile in D_2O for 60 min at a flow-rate of 0.8 mL/min. Separations were monitored by absorbance detection at 320 nm. On-line LC-NMR experiments were conducted on a Varian 500 MHz spectrometer equipped with LC–NMR cold probe with a 60 µL flow-cell (active volume). Varian WET solvent suppression and "scout" scan were used to suppress the acetonitrile and the residual water peaks. Free induction decay (FID) were collected with 16 K data points, a spectral width of 12000 Hz, a 3-µs 90° pulse, a 2 s acquisition time and a 1 s pulse delay. A total of 4 transients were acquired to obtain the data. Prior to fourier transformation, an exponential apodization function was applied to the free induction decay corresponding to a line broadening of 1 Hz. The structures of each compound were compared with the literature data. The isolated kuraridin was dissolved in methanol-d_4, DMSO-d_6, acetonitrile-d_3 and acetonitrile-d_3 + 1% D_2O, respectively and subjected to NMR to assess the solvent effect in the chalcone skeleton.

RESULTS AND DISCUSSION

In real-life application LC-NMR, the most important factor is an optimized HPLC separation. Because sensitivity is still the crucial point of this hyphenated technique, it is extremely important to develop a chromatographic separation where the quantity of the available separated compound is concentrated in the smallest available elution volume. Figure 2a shows the HPLC chromatogram of extract of Sophorae radix. Many peaks were separated successfully in the gradient solvent system of acetonitrile and D_2O in the HPLC system. Among these

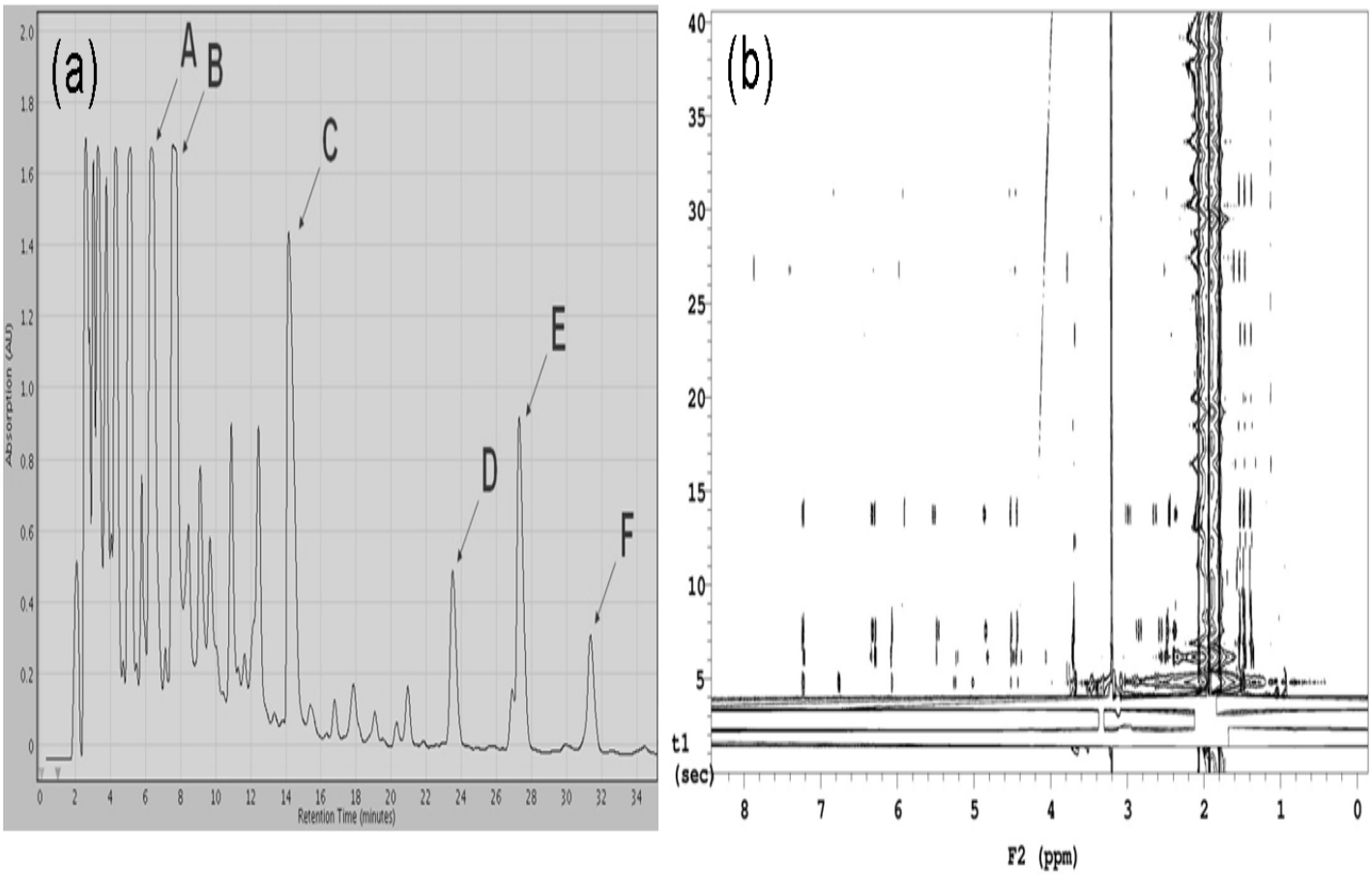


Figure 2. HPLC chromatogram (a) and on-flow LC-NMR chromatogram (b) of the extract of Sophorae radix (the dried roots of *S. flavescens*). HPLC chromatogram was recorded from gradient reverse-phase LC-NMR with detection at 320 nm. The NMR spectra data was collected from major six peaks (A to E). Horizontal and vertical represent the ^{1}H NMR chemical shifts in ppm and retention time, respectively.

peaks, NMR data could be collected from the major six peaks (A – F). The solvent suppression technique is necessary in order to achieve a reduction of the NMR signal entering the receiver for observing small analyte signals in the presence of much larger signals from the mobile phase. Solvent signal suppression is efficiently performed by using three techniques: presaturation, soft-pulse multiple irradiation and WET presaturation. Among them, the WET technique was chosen in this study. The WET technique uses a series of variable tip-angle solvent-selective radio frequency (RF) pulses, where each selective RF pulse is followed by a dephasing field gradient pulse (Smallcombe et al., 1995). In an acetonitrile:water solvent gradient system, the relative positions of the acetonitrile and water resonances change according to the mobile-phase composition (Keifer, 2010). The scout scan technique allows us to automatically track the frequencies of the largest NMR resonances. The scout scan information is then used to drive the WET suppression.

Figure 2b represents the on-flow LC-NMR chromatogram of ethanol extract present in the roots of *S. flavescens* separated using a C_{18} column. In the chromatogram, the horizontal axis represents ^{1}H NMR chemical shifts in ppm and the vertical axis shows retention time. ^{1}H NMR spectra of 6 major peaks were collected as shown in Figure 3. The solvent peaks were suppressed successfully around δ 1.8 ppm for acetonitrile and δ 4~5 ppm for water. By the analysis of ^{1}H NMR spectrum of on-flow LC-NMR and comparison with the literature data, we could determine the chemical struc-tures of six prenylflavonoids as kushenol U (1), kurarinone (2), sophoraflavanone G (3), leachianone A (4), kuraridin (5) and kushenol A (6) as shown in Figure 4 (Iinuma et al., 1990; Yamahara et al., 1990; Woo et al., 1998; Kuroyanagi et al., 1999; Kim et al., 2006; Quan et al., 2008). The ^{1}H NMR data extracted from on-flow LC-NMR were summarized and presented in Table 1.

Among six major peaks investigated in this study, 5 compounds had the prenylated flavanone skeleton composed of three ring structure (ring A to C) as shown in (Figure 4). The ^{1}H NMR spectrum of peak A (RT 6.3 min) revealed 2 AB system with the proton signals at δ 7.23 (2H, *d*, 8 Hz, H-2′, 6′) and δ 6.76 (2H, *d*, 8 Hz, H-3′,

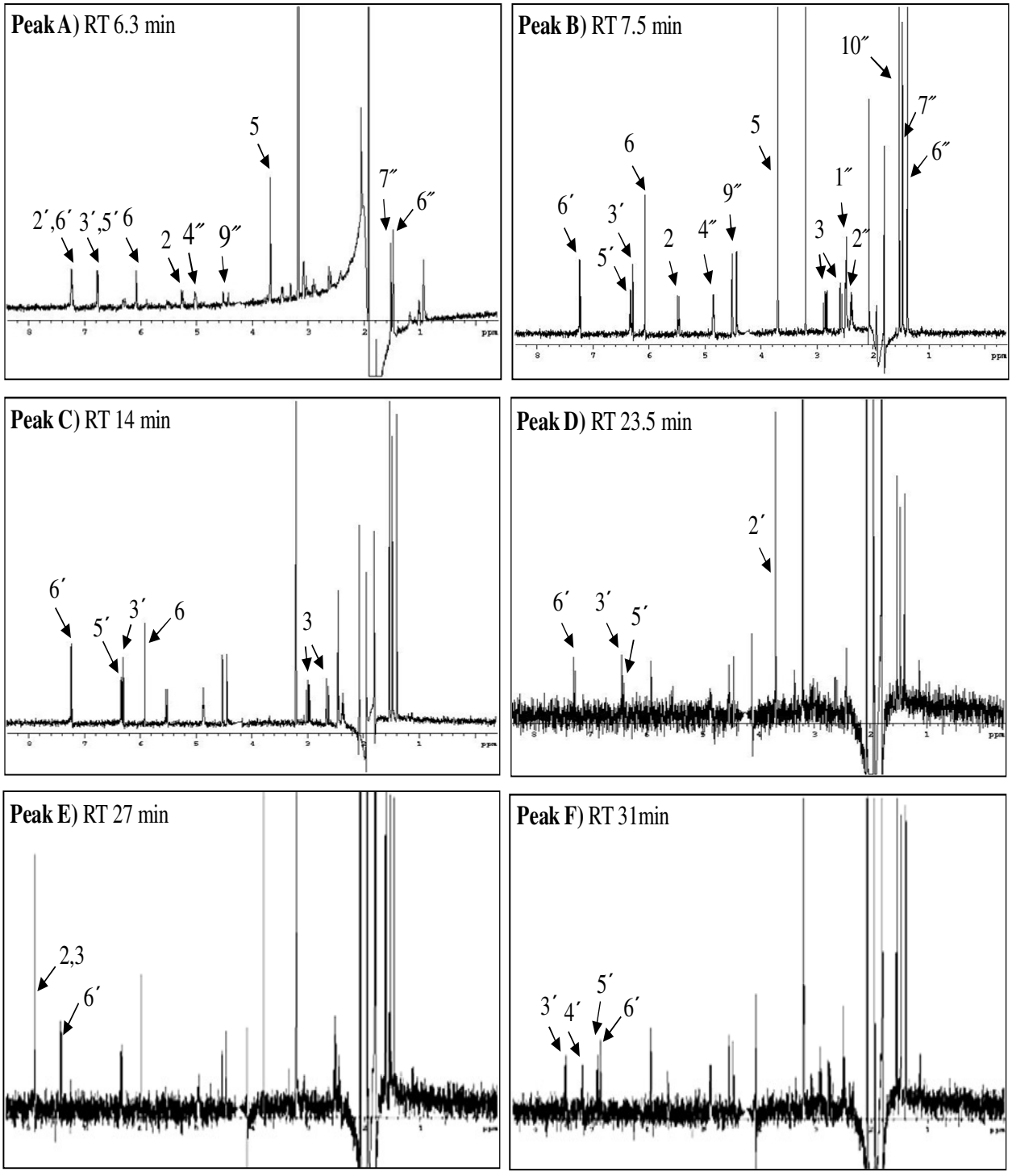
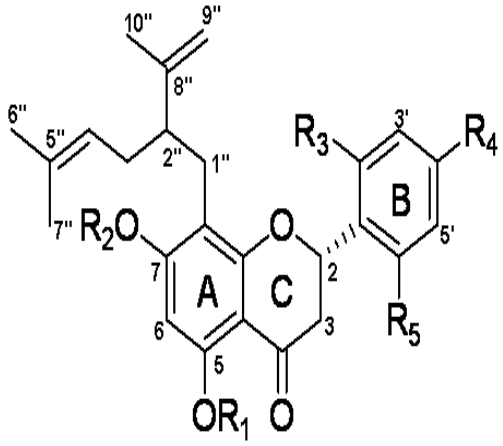
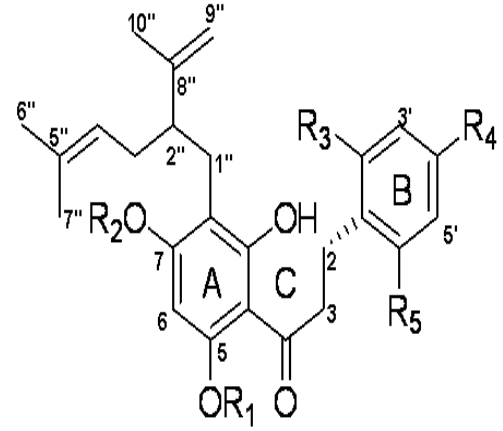


Figure 3. ^{1}H NMR spectra of each peak from on-flow LC-NMR (500 MHz in acetonitril/D_2O).

n5′) in ring B of the flavanone skeleton. Peaks at δ 5.25 (1H, *m*, H-2), δ 2.90 (1H, *m*, H-3a) and δ 2.61 (1H, *br d*, 17 Hz, H-3b) represent the protons of ring C. The proton signals at δ 5.01 (1H, *m*, H-4″), δ 4.52 (1H, *s*, H-9a″), δ 4.42 (1H, *s*, H-9b″), δ 2.41 (2H, *m*, H-1″), δ 2.24 (1H, *m*, H-2″), δ 1.53 (3H, *s*, H-10″), δ 1.51 (3H, *s*, H-7″), and δ 1.48 (3 H, *s*, H-6″) were assigned as lavanduyl functional group. The peak at δ 6.07 (1H, *s*) represented the signal of H-6 in ring A and the peak at δ 3.67 was assigned to OCH_3 at C-5. Therefore, peak **A** could be identified as



compound	R_1	R_2	R_3	R_4	R_5
1	CH_3	H	H	OH	H
2	CH_3	H	OH	OH	H
3	H	H	OH	OH	H
4	H	H	OCH_3	OH	H
6	H	H	OH	H	H



compound	R_1	R_2	R_3	R_4	R_5
5	CH_3	H	OH	OH	H

Figure 4. Chemical structures of prenylflavonoid compounds from the extract of sophorae radix identified by LC-NMR analysis.

kushenol U (1) by the comparison of NMR data with the reported literature data. Peak B (RT 7.5 min) demonstrated similar ^{1}H NMR spectrum of 1. The proton signals at δ 6.28 (1H, *d*, 1.5 Hz, H-3′), δ 6.32 (1H, *dd*, 8, 1.5 Hz, H-5′) and δ 7.23 (1H, *d*, 1.5 Hz, H-6′) represented the ABX system of ring B. Thus, peak B was confirmed

Table 1. ^{1}H NMR spectral data of compounds 1 - 6 from on-flow LC-NMR.

H	1	2	3	4	5	6
2	5.25 (1H, m)	5.47 (1H, dd, 13, 2Hz)	5.52 (1H, dd, 13, 3Hz)	5.53 (1H, dd, 13, 3Hz)	7.87 (1H, s)	5.62 (1H, br d, 13Hz)
3	2.90 (1H, m) 2.61 (1H, br d, 17Hz)	2.85 (1H, dd, 16.5, 13Hz) 2.56 (1H, dd, 16.5, 2Hz)	2.99 (1H, dd, 17, 13Hz) 2.64 (1H, dd, 17, 3Hz)	2.97 (1H, m) 2.61(1H, m)	7.87 (1H, s)	2.91 (1H, dd, 17, 13Hz) 2.74 (1H, dd, 17, 13Hz)
6	6.07 (1H, s)	6.07 (1H, s)	5.91 (1H, s)	5.90 (1H, s)	5.98 (1H, s)	5.93 (1H, s)
2′	7.23 (1H, d, 8Hz)	-	-	-	-	-
3′	6.76 (1H, d, 8Hz)	6.28 (1H, d, 1.5Hz)	6.30 (1H, d, 2Hz)	6.42 (1H, m)	6.31 (1H, d, 2Hz)	7.46 (1H, dd, 7, 1.5Hz)
4′	-	-	-	-	-	7.16 (1H, m)
5′	6.76 (1H, d, 8Hz)	6.32 (1H, dd, 8, 1.5Hz)	6.33 (1H, dd, 18.5, 2Hz)	6.40 (1H, dd, 9, 2Hz)	6.34 (1H, dd, 8, 2Hz)	6.89
6′	7.23 (1H, d, 8Hz)	7.23 (1H, d, 8Hz)	7.23 (1H, d, 8.5Hz)	7.27 (1H, d, 9Hz)	7.4 (1H, br d, 8Hz)	6.84 (1H, br d, 8.5Hz)
1″	2.41 (2H, m)	2.47 (2H, m)	2.45 (2H, br d, 7Hz)	2.42 (2H, m)	2.52 (2H, m)	2.49 (2H, m)
2″	2.24 (1H, m)	2.38 (1H, m)	2.36 (1H, m)	2.35 (1H, m)	2.43 (1h, m)	2.34 (1H, m)
4″	5.01 (1H, m)	4.85 (1H, m)	4.49 (1H, m)	4.85 (1H, m)	4.94 (1H, m)	4.87 (1H, m)
6″	1.48 (3H, s)	1.38 (3H, s)	1.39 (3H, s)	1.38 (3H, s)	1.46 (3H, s)	1.38 (3H, s)
7″	1.51 (3H, s)	1.47 (3H, s)	1.48 (3H, s)	1.46 (3H, s)	1.54 (3H, s)	1.47 (3H, s)
9″	4.52 (1H, m) 4.42 (1H, m)	4.51 (1H, br s) 4.43 (1H, br s)	4.52 (1H, m) 4.44 (1H, d, 1.5Hz)	4.52 (1H, m) 4.43 (1H, m)	4.53 (1H, m) 4.46 (1H, m)	4.53 (1H, m) 4.46 (1H, br d, 2Hz)
10″	1.53 (3H, s)	1.53 (3H, s)	1.53 (3H, s)	1.52 (3H, s)	1.61 (3H, s)	1.54 (3H, s)
OCH_3	3.67 (3H, s)	3.69 (3H, s)	-	3.68 (3H, s)	3.78 (3H, s)	-

as kurarinone (2). The ^{1}H NMR spectrum of peak C (RT 14 min) was identical with the ^{1}H NMR spectrum of 2 excepting no presence of methoxyl group at C-5. Therefore, peak C was identified as sophoraflavanone G (3) (Iinuma et al., 1990). The ^{1}H NMR spectrum of peak D (RT 23.5 min) showed similar ^{1}H NMR spectrum to 2. The only difference was that the chemical shift of H-3′ was downfield-shifted from δ 6.28 (1H, *d*, 1.5 Hz, H-3′) to δ 6.42 (1H, *m*, H-3′) indicating the attachment of methoxyl group at C-2′ of 2. Thus, peak D was characterized as the proton signals at δ 6.31 (1H, *d*, 2 Hz, H-3′), δ 6.34 (1H, *d*, 8, 2 Hz, H-5′) and δ 7.40 (1H, *br d*, 8 Hz, H-6′) corresponding to ABX system of ring B. The down-field proton signal at δ 7.37 (2H, *s*, H-2, H-3) suggested the chalcone skeleton. Two protons at C-2 and C-3, however, were resonated as one singlet of 2H. In order to investigate the solvent effect in the proton signals of these carbons, the isolated compound was dissolved in various deuterated solvent such as methanol-d_4, DMSO-d_6, acetonitrile-d_3 and acetonitrile-d_3 +1% D_2O (Keifer, 2010) and their ^{1}H NMR spectra were collected separately with off-line NMR (Figure 5). Two doublet proton signals of H-2 and H-3 were produced when the compound was dissolved in methanol-d_4 or DMSO-d_6. However, one singlet peak of 2H was detected when 1% D_2O was added to methanol-d_4 or acetonitrile-d_3 or only acetonitrile-d_3 was used as the solvent. Therefore, we could conclude that due to the acetonitrile solvent effect on the chalcone skeleton in LC-NMR flow tube, H-2 and H-3 resonated as one singlet of 2H at δ 7.37 instead of two doublets. The peak at δ 3.78 (OCH_3, *s*, H-5) was assigned as methoxyl group. Thus, peak E was leachianone A (4). The ^{1}H NMR spectrum of peak F (RT 31 min) displayed similar ^{1}H NMR spectrum to **3**. The signals at δ 5.62 (1H, *br d*, 13 Hz, H-2), δ 2.91 (1H, *dd*, 17, 13 Hz, H-3a) and δ 2.74 (1H, *dd*, 17, 2.5 Hz, H-3b) represented the protons of ring C in the flavanone skeleton. Peak at δ 7.46 (1H, *dd*, 7, 1.5 Hz, H-3′), δ 7.16 (1H, *m*, H-2′), δ 6.89 (1H, *m*, H-5′) and δ 6.84 (1H, *br d*, 8.5 Hz, H-6′) were assigned as the protons of ring B. Peak F was identified to be kushenol A (6).

The ^{1}H NMR spectrum of peak E (RT 27 min) showed characterized as kuraridin (5).

In conclusion, we showed LC-NMR is a useful tool in natural products research, which gives rapid and accurate information on the chemical structure. But the low sensitivity of flow NMR still remains as main obstacle, even though the introduction of cold probes and solvent

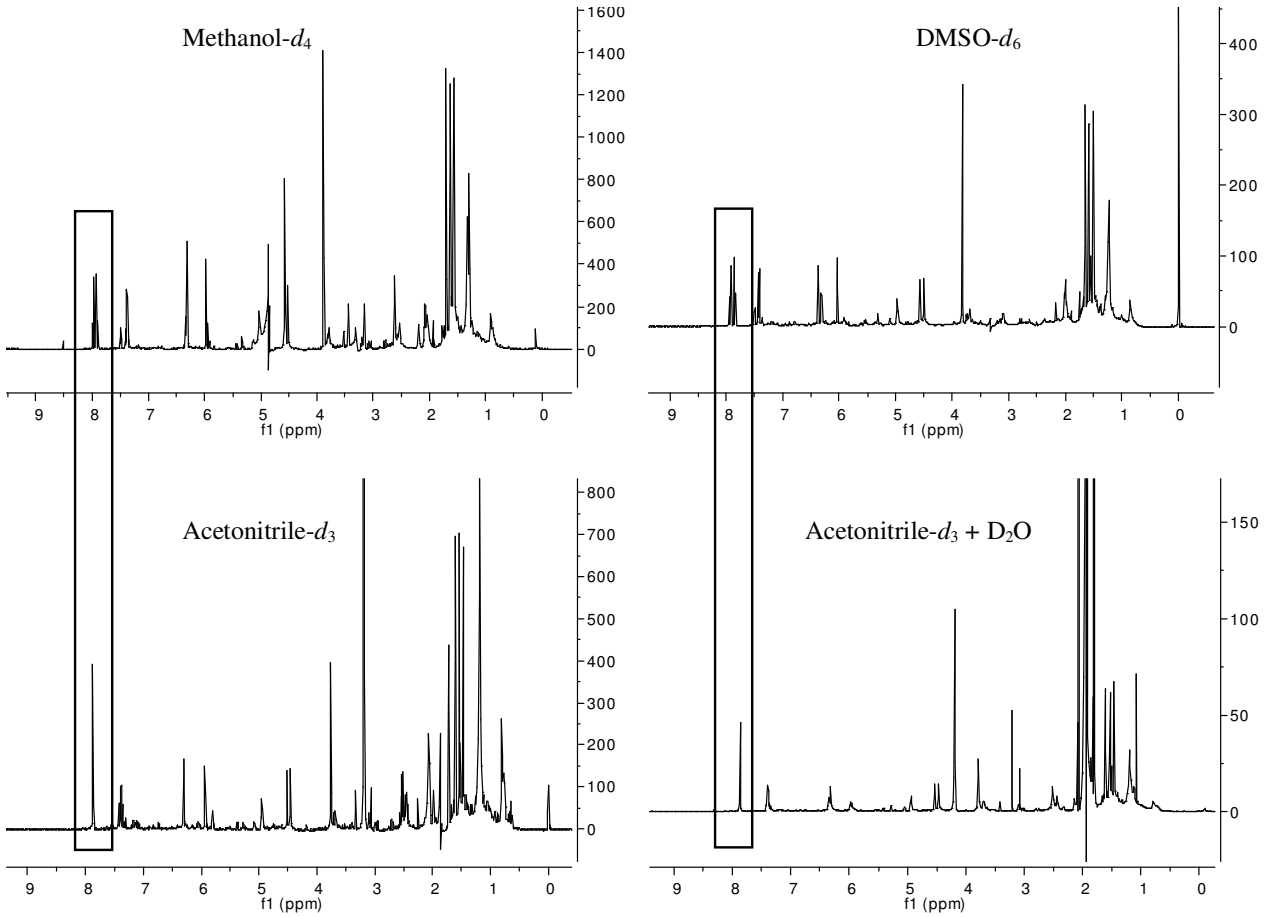


Figure 5. The solvent effect in the resonance of two protons (H-2 and H-3) in the chalcone skeleton. The isolated kuraridin was dissolved in each solvent and used for the off-line NMR analysis. The box indicates the signals of H-2 and H-3.

suppression techniques.

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REFERENCES

Andrade FD, Santos LC, Datchler M, Albert K, Vilegas W (2002). Use of on-line liquid chromatography-nuclear magnetic resonance spectroscopy for the rapid investigation of flavonoids from *Sorocea bomplandii*. J. Chromatogr. A 953:287-291.

Elipe MVS (2010). LC/NMR and LC/MS/NMR. In: Cazes J (eds) Encyclopedia of chromatography, Taylor & Francis, New York, DOI:10.1081/E-ECHR3-120045283.

Gülçin İ (2005). The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int. J. Food Sci. Nutr., 56:491-499.

Gülçin I, Elmastas M, Aboul-Enein HY (2007). Determination of antioxidant and radical scavenging activity of basil (*Ocimum basilicum*) assayed by different methodologies. Phytother. Res., 21: 354-361.

Iinuma M, Tanaka T, Mizuno M, Shirataki Y, Yokoe I, Komatsu M, Lang FA (1990). Two flavanones in *Sophora leachiano* and some related structures. Phytochemistry 29:2667-2669.

Kang SW, Kim MC, Kim CY, Jung SH, Um BH (2008). The rapid identification of isoflavonoids from *Belamcanda chinensis* by LC-NMR and LC-MS. Chem. Pharm. Bull. 56:1452-1454.

Keifer PA, Smallcombe SH, Williams EH, Salomon KE, Mendez G, Belletire JL, Moore CD (2000). Direct-injection NMR (DI-NMR): a flow NMR technique for the analysis of combinatorial chemistry libraries. J. Comb. Chem. 2:151-171.

Keifer PA (2010). Chemical-shift referencing and resulution stability in methanol:water gradient LC-NMR. J. Magn. Reson. DOI:10.1016/j.jmr.2010.04.009.

Kim JH, Ryu YB, Kang NS, Lee BW, Heo JS, Jeong IY, Park KH (2006). Glycosidase inhibitory flavonoids from *Sophora flavescens*. Biol. Pharm. Bull. 29:302-305.

Kuroyanagi M, Arakawa T, Hirayama Y, Hayashi T (1999). Antibacterial and antiandrogen flavonoids from *Sophora flavescens*. J. Nat. Prod. 62:1595-1599.

Quan W, Lee HJ, Kim CY, Noh CW, Um BH, Oak MH, Kim KM (2008). Anti-allergic prenylated flavonoids from the roots of *Sophora flavescens*. Planta Med. 74:168-170.

Row EC, Brown SA, Stachulski AV, Lennard MS (2006). Synthesis of 8-geranyloxypsoralen analogues and their evaluation as inhibitors of

CYP3A4. Bioorg. Med. Chem., 14:3865–3871.
Smallcombe SH, Patt SL, Keifer PA (1995). WET Solvent Suppression and Its Applications to LC-NMR and High-Resolution NMR Spectroscopy. J. Magn. Reson., 117:295-303.
Tode C, Maoka T, Sugiura M (2009). Application of LC-NMR to analysis of carotenoids in food. J. Sep. Sci., 32:3659-3663.
Wolfender JM, Ndjoko K, Hostettmann K (2001). The potential of LC-NMR in phytochemical analysis. Phytochem. Anal., 12:2-22.
Woo ER, Kwak JH, Kim HJ, Park H (1998). A new prenylated flavonol from the roots of *Sophora flavescens*. J. Nat. Prod., 61:1552-1554.
Yamahara J, Kobayashi G, Iwamoto M, Chisaka T, Fujimura H, Takaishi Y, Yoshida M, Tomimatsu T, Tamai Y (1990). Vasodilatory active principles of *Sophora flavescens* root. J. Ethnopharmacol., 29:79-85.
Yazake Y, Sasaki K, Tsurumaru Y (2009). Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. Phytochemistry, 70:1739-1745.
Zhang L, Xu L, Xiao SS, Liao QF, Li Q, Liang J, Chen XH, Bi KS (2007). Characterization of flavonoids in the extract of *Sophora flavescens* Ait. by high-performance liquid chromatography coupled with diode-array detector and electrospray ionization mass spectrometry. J. Pharmaceut. Biomed., 44:1019-1028.