Rapid Extract Dereplication Using HPLC-SPE-NMR: Analysis of Isoflavonoids from *Smirnowia iranica*

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A novel hyphenated technique, HPLC-SPE-NMR, was used for accelerated identification of isoflavonoids from the roots of *Smirnowia iranica*. The extract constituents eluted from a HPLC column were automatically trapped on solid-phase extraction (SPE) cartridges, and NMR spectra were acquired with concentrated solutions after solvent change. The structures of 10 new isoflavonoids (**1**, **4**, **5**, **7**−**10**, **12**, **13**, **16**) and of seven previously described constituents (**2**, **3**, **6**, **11**, **14**, **15**, **17**) were elucidated from NMR spectra acquired in the HPLC-SPE-NMR mode. Multiple peak trapping on the same SPE cartridge increased analyte amounts and provided access to 2D NMR data. It was demonstrated that linear accumulation of material is possible in up to seven repeated trapping steps. The use of HPLC-SPE-NMR speeded up dereplication of the *S. iranica* extract considerably by providing detailed information about the constituents of a complex, essentially crude extract prior to their preparative-scale isolation or extract prefractionation, and the information obtained could be used to direct preparative isolation work. In connection with structure elucidation of isoflavonoids containing *O*-methylated 1,2,3-benzenetriol moieties as the B-ring, *O*-methylation-induced changes of chemical shifts of aromatic hydrogens were found to depend on the conformation of the resulting methoxy group, i.e., on the number of its ortho substituents. The recognized regularities will be useful in structure determination of partially *O*-methylated polyphenols based on 1D ^{1}H NMR spectra obtainable from HPLC-SPE-NMR experiments, diminishing dependence on 2D NMR data and ^{13}C NMR chemical shifts.

Isoflavonoids form a distinct subclass of flavonoids, formed by aryl migration in an original flavonoid precursor.[1−3] In contrast to the almost ubiquitous occurrence of flavonoids, isoflavonoids are found primarily in the Leguminoseae (Fabaceae) and only rarely in other families of flowering plants.[4] Isoflavonoids appear to have pronounced effects on human health. Many of them possess significant estrogenic properties, which may be associated with adverse as well as beneficial health effects of plant products containing isoflavonoids. For example, isoflavonoids of the soy plant (*Glycine max*) are thought to give protection against estrogen-dependent cancers, notably breast cancer, by antagonizing action of natural female sex hormones, and to reduce postmenopausal symptoms by mimicking action of these hormones.[5−10]

Close to a thousand natural isoflavonoids have been characterized, and those from the soy plant and licorice root (*Glycyrrhiza* species) are among the most extensively studied.[1−12] Most isoflavonoid-containing plants appear to contain a variety of related compounds differing in oxygenation, methylation, and prenylation level and modified by various secondary transformations. Identification of isoflavonoids present in a particular source thus poses a considerable analytical challenge. HPLC-UV (with photodiode array detectors), GC-MS, and HPLC-MS have been used frequently for the analysis of complex isoflavonoid mixtures. However, truly unambiguous structural characterization of isoflavonoids would normally require use of NMR spectroscopy. In this contribution, we describe the performance of HPLC-SPE-NMR experiments at 600 MHz for on-line characterization of isoflavonoids and other constituents of the roots of *Smirnowia iranica*, a leguminous plant previously shown to contain isoflavonoids.[13]

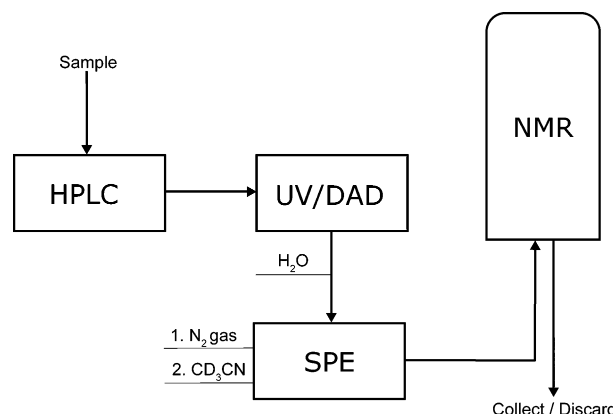

Figure 1. Principle of operation of the HPLC-SPE-NMR instrument used in this work. Separation of sample constituents is performed by reversed-phase HPLC. Detection of chromatographic peaks in a photodiode array detector results in automated analyte trapping by solid-phase extraction after dilution of the HPLC eluate with water. SPE cartridges with trapped analytes are dried and the analytes transferred to an NMR spectrometer using acetonitrile-d_3.

HPLC-SPE-NMR is a new hyphenated technique that uses solid-phase extraction (SPE) as an interface between chromatography and NMR.[14−21] Thus, analytes eluted in chromatographic bands are automatically trapped on SPE cartridges, the cartridges are dried, and the analyte is eluted from the SPE cartridge into an NMR flow-probe with a small amount of appropriate solvent (Figure 1). Start and stop signals for peak trapping are provided by an on-line UV (or optionally MS) detector. The main advantages of this technique, contrasting traditional HPLC-NMR implementations,[22] are that in principle the whole amount of analyte eluted from a HPLC column is concentrated in the NMR flow-cell and that a solvent change to a deuterated

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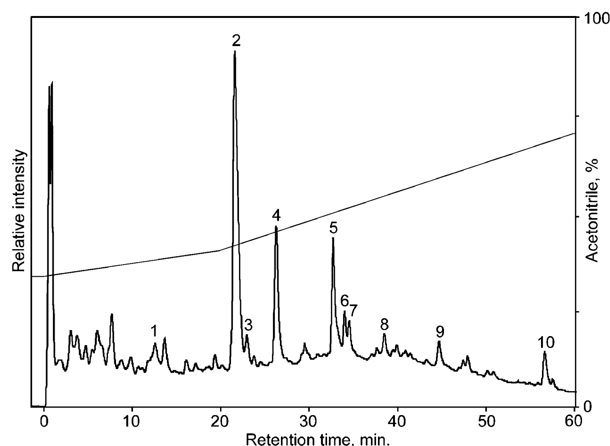

Figure 2. HPLC of the ethanolic extract of *S. iranica* roots on a C_{18} column; acetonitrile gradient profile in water is shown. The chromatogram shows average absorbance at 254 and 300 nm; peaks used for HPLC-SPE-NMR experiments are labeled 1−10.

NMR solvent takes place following HPLC separation using nondeuterated eluents. Moreover, multiple SPE trappings are possible, resulting in a substantial increase of the amount of material available for the NMR measurement. The HPLC-SPE-NMR technique used successfully in the present work for identification of *S. iranica* isoflavonoids is believed to be a generally applicable analytical platform in the field of phytoestrogens as well as many other classes of natural products.

Results and Discussion

An ethanol extract of powdered roots of *S. iranica* was initially passed through preparative-scale C_{18} SPE cartridges using a protocol designed to remove the most hydrophobic components such as fatty acids and chlorophyll. After this initial extract cleanup, a HPLC method resulting in a good separation of constituents was developed; the chromatogram of this essentially crude extract turned out to be very complex, with numerous UV-absorbing constituents (Figure 2). Use of a photodiode array detector ensured detection of all absorbing constituents. The extract was subjected to HPLC-SPE-NMR analysis using averaged absorptions at 254 and 300 nm to trigger trapping on the SPE cartridges. Ten peaks were selected for the analysis (Figure 2), covering a broad range of compound polarities (as expressed by chromatographic retention times) in order to test the scope of the HPLC-SPE-NMR technique. Since retention of the extract constituents was good on HPLC columns packed with C_{18} material, C_{18}-type SPE cartridges (2 × 10 mm) were used for peak trapping. A makeup flow of water was added postcolumn in a 1:1 ratio in order to change the partition coefficients for increased affinity of compounds to the stationary phase of the SPE cartridges.

After drying with a flow of pressurized nitrogen gas, the SPE cartridges were eluted with acetonitrile-d_3 for NMR data acquisition with a 30 μL (active volume) NMR flow-probe. 1D ^{1}H NMR spectra were acquired using 1D NOESY pulse sequence with presaturation of residual solvent signals (water and acetonitrile-d_3) during mixing time and relaxation delay. The resulting 1D NMR spectra allowed full or partial structure elucidation of 17 extract components (compounds **1**−**17**) as described below. In ambiguous cases, 2D NMR spectra were recorded (see below). HPLC-MS data were acquired separately to obtain masses of the extract components. Positive- as well as negative-ion detection mode was used because of variations of ionizability of individual extract constituents, and the MS results were used to assist structure elucidation by HPLC-SPE-NMR. HPLC-MS proved to be helpful in confirming the presence of exchangeable hydroxy groups, the resonances of which were fully or partly suppressed by saturation transfer from the irradiated water resonance.

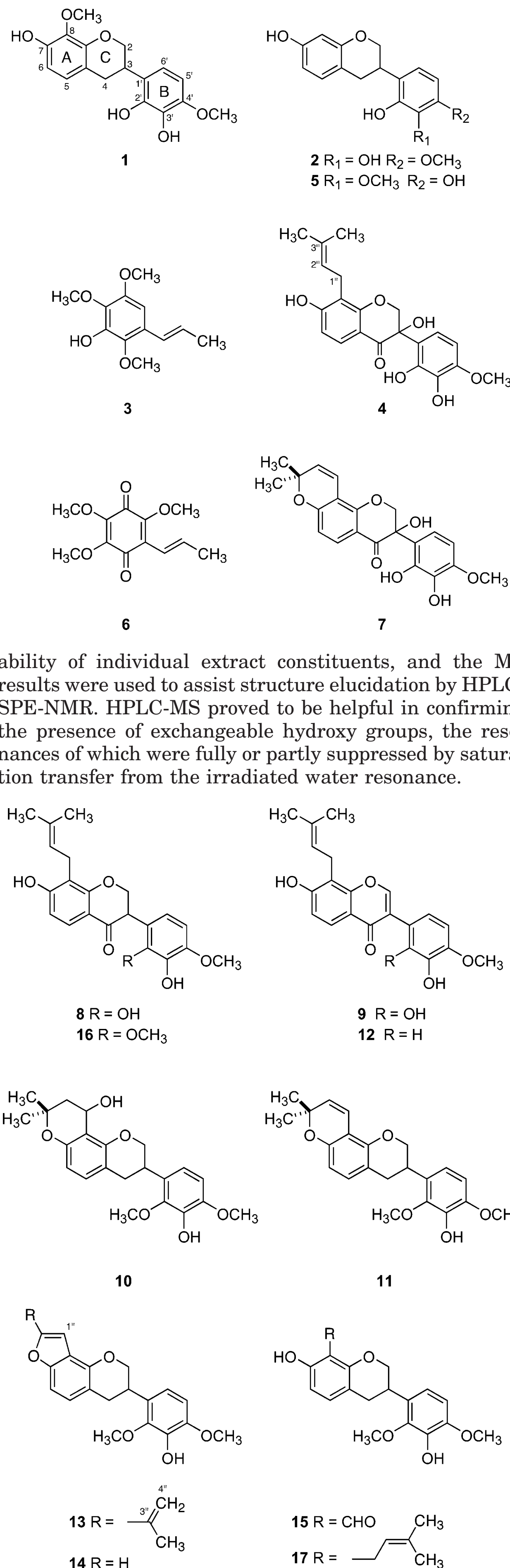

Table 1. 600 MHz ^{1}H NMR Spectroscopic Data Obtained in the HPLC-SPE-NMR Mode from a Crude Extract of *Smirnowia iranica* Roots[a,b]

position	**1**	**2**	**4**	**5**	**7**	**8**
H-2	4.33 ddd (10.6, 3.7, 2.0)	4.23 ddd (10.6, 3.7, 2.0)	4.27 d (11.8)	4.24 ddd (10.2, 3.4, 1.9)	4.30 d (11.8)	4.55 dd (11.2, 5.3)
	4.03 t (10.6)	3.98 t-like(10.6)	4.88 d (11.8)	3.98 dd (10.2, 9.5)	4.93 d (11.8)	4.65 dd (11.2, 10.3)
H-3	3.42 m	3.42 m		3.42 m		4.09 dd (10.3, 5.3)
H-4	2.84 m	2.84 m		2.84 ddd (15.5, 5.0, 1.9)		
	2.96 ddd (16.0, 10.5, 1.0)	2.93 ddd (16.0, 10.5, 1.0)		2.94 ddd (15.5, 10.7, 1.0)		
H-5	6.67 br d (8.4)	6.91 br d (8.2)	7.63 d (8.8)	6.91 br d (8.2)	7.70 d (8.8)	7.63 d (8.6)
H-6	6.39 d (8.4)	6.33 dd (8.2, 2.6)	6.59 d (8.8)	6.34 dd (8.2, 2.5)	6.51 d (8.8)	6.56 d (8.6)
H-5′	6.50 d (8.6)	6.49 d (8.6)	6.49 d (8.8)	6.49 d (8.6)	6.50 d (8.8)	6.49 d (8.6)
H-6′	6.61 d (8.6)	6.60 d (8.6)	6.88 d (8.8)	6.79 d (8.6)	6.87 d (8.8)	6.60 d (8.6)
H-1″			3.28 br d (7.0)		6.60 d (10.1)	3.29 br d (7.1)
H-2″			5.15 tsp (7.0, 1.0)		5.72 d (10.1)	5.18 tsp (7.1, 1.4)
CH_3-3″			1.75 br s		1.44 s	1.75 br s
			1.66 br s		1.43 s	1.67 br s
OCH_3	3.78 s	3.81 s	3.81 s	3.80 s	3.82 s	3.81 s
	3.81 s					
other		H-8: 6.24 d (2.6)		H-8: 6.24 d (2.5)		

position	**9**	**10**	**12**	**13**[c]	**15**	**16**
H-2	8.27 s	4.27 ddd (10.3, 3.6, 2.4)	8.08 s	4.34 ddd (10.6, 3.7, 2.0)	4.37 ddd (10.6, 3.7, 2.2)	4.50 ddd (11.6, 5.7)
		3.97 t (10.3)		4.11 t (10.6)	4.12 t (10.6)	4.53 t (11.6)
H-3		3.42 m		3.54 m	3.50 m	4.12 dd (11.6, 5.7)
H-4		2.83 m		2.97 ddd (15.6, 5.0, 2.0)	2.85 ddd (15.6, 5.0, 2.2)	
		2.92 ddd (15.7, 11.0, 1.0)		3.05 ddd (15.6, 10.7, 1.0)	2.93 ddd (15.6, 10.7, 1.0)	
H-5	8.00 d (8.6)	6.82 br d (8.3)	7.88 d (8.6)	7.00 br s	7.28 br d (8.6)	7.64 d (8.6)
H-6	7.05 d (8.6)	6.29 d (8.3)	6.95 d (8.6)	7.00 br s	6.43 dd (8.6, 0.6)	6.57 d (8.6)
H-5′	6.63 d (8.6)	6.65 d (8.6)	6.96 d (8.3)	6.65 d (8.6)	6.65 d (8.6)	6.59 d (8.4)
H-6′	6.77 d (8.6)	6.72 d (8.6)	7.01 dd (8.3, 2.2)	6.71 d (8.6)	6.72 d (8.6)	6.68 d (8.4)
H-1″	3.56 br d (7.2)	3.72 t (6.6)	3.50 d (7.2)	6.58 (s)	CHO: 10.33 d (0.6)	3.30 br d (7.3)
H-2″	5.25 tsp (7.2, 1.4)	2.46 dd (17.0, 6.6)	5.22 tsp (7.2, 1.4)			5.19 tsp, (7.0, 1.0)
		2.81 m				
CH_3-3″	1.81 br s	1.27 s	1.81 br s	1.57 br s		1.75 br s
	1.67 br s	1.21 s	1.67 br s			1.67 br s
OCH_3	3.86 s	3.81 s	3.86 s	3.83 s (two)	3.83 s (two)	3.72 s
		3.83 s				3.84 s
other			H-2′: 7.06 d (2.2)		7-OH: 11.73 s	

[a] In CD_3CN; chemical shift values are standardized to the residual CD_2HCN signal set to δ 1.94. [b] Multiplicity of signals is given as follows: s, singlet; d, doublet; t, triplet; sp, septet; m, multiplet; br, broadened signal due to long-range coupling; coupling constants (apparent splittings) are given in parentheses as numerical values in Hz. [c] Signals of the olefinic hydrogens (H-4″) not observed due to low amount of material and poor S/N ratio; all resonances of **13** were observed in a spectrum recorded with isolated sample in chloroform-*d* (see Experimental Section).

The material eluted as peak 1 (t_R = 12.8 min) was a mixture of two coeluting components, as shown by the presence of two aliphatic $-CH_2-CH-CH_2-$ spin systems, characteristic of isoflavans. According to the negative-mode ESIMS data, there was a difference of 30 amu between the two components (*m/z* 317 and 287, $[M - H]^-$), corresponding to a methoxy group. The component present in slight excess (compound **1**) contained two methoxy groups, whereas the minor component (compound **2**) had one methoxy group. Compound **1** exhibited two AB spin systems of the two aromatic rings, whereas compound **2** showed one AB spin system and a coupling pattern corresponding to a 1,2,4-trisubstituted benzene. Since one AB doublet in each compound was broadened by long-range coupling, these doublets could be assigned to H-5 and the long-range coupling to $J_{4,5}$. This automatically assigned all hydrogen resonances of the A-ring in both compounds. Thus, compounds **1** and **2** have two and one substituent in the A-ring, respectively, and both have three oxygen substituents in the B-ring. As the methoxy groups were the only substituents apparent in the NMR spectrum, the remaining substituents must be hydroxy groups. Although the exact positions of the methoxy groups could not be determined from a superficial analysis of 1D NMR data, the structures of **1** and **2** were inferred from consideration of *O*-methylation-induced chemical shift changes and confirmed by 2D experiments as detailed below. The ^{1}H NMR data of **1** and **2** are shown in Table 1. Compound **2** was previously synthesized by hydrogenation of the corresponding pterocarpan[23] and later isolated from several *Sophora* species and named arizonicanol A;[24] the reported ^{1}H chemical shifts (in acetone-d_6) of the latter are closely similar to those found in the present work (Table 1). Compound **1** has not been reported previously, although its methyl ethers have been characterized.[25,26]

The ^{1}H NMR spectrum obtained with peak 2 (t_R = 21.8 min), the major peak in the chromatogram (Figure 2), also revealed the presence of two coeluting compounds in a ratio of 4:3. One of these was identified as **3** (*m/z* 225, $[MH]^+$) by comparison of ^{1}H NMR spectrum of a previously isolated sample[13] in acetonitrile-d_3 (see Supporting Information). The minor component of peak 2 was identified as **4**, a novel representative of the relatively rare 3-hydroxyisoflavanones. Thus, its spectrum showed two coupled geminal hydrogens at δ 4.27 and 4.88, an ortho-coupled pair of resonances at δ 7.63 and 6.59, another ortho-coupled pair at δ 6.88 and 6.49, signals characteristic of a prenyl group, and a single methoxy substituent (Table 1). Signals at δ

4.27 and δ 4.88 were assigned to the diastereotopic methylene hydrogens at C-2; chemical shifts and splitting patterns of these hydrogens prove the presence of a hydroxy group at C-3.[27−29] No further signals attributable to the C-ring were observed in the ^{1}H NMR spectrum. Compared to isoflavans such as **1** and **2**, the resonance of H-5 (coupled to the signal at δ 6.59) was shifted downfield to δ 7.63, which is a characteristic effect of the carbonyl group at C-4. The signals at δ 6.88 and 6.49 are due to two ortho-coupled hydrogens in the B-ring, leaving three positions available for oxygen substituents. Since only one methoxy and one prenyl group were observed, three of the total of five substituted positions must be hydroxylated. This was confirmed by MS data (*m/z* 385, [M − H]$^-$). The exact substitution pattern of **4** was elucidated as discussed below.

The ^{1}H NMR spectrum obtained from the minor peak 3 (t_R = 23.2 min) showed signals corresponding to only one compound, designated as **5**. Its ^{1}H NMR spectrum showed the presence of a single methoxy group and was almost identical to that of the isoflavan **2**, except that the B-ring doublet at δ 6.60 in the latter was shifted to δ 6.79. Thus, compound **5** (*m/z* 287, [M − H]$^-$) is a positional isomer of **2**. Detailed discussion of structure **5** is deferred to the discussion of 2D NMR spectra (see below). This is the first report on the isoflavan **5** as a natural product, although its triacetate was previously obtained by hydrogenation of the corresponding isoflav-3-ene derivative and characterized by MS.[30]

^{1}H NMR analysis of peak 4 (t_R = 26.5 min) revealed the presence of two compounds in equal amounts. One of the two constituents was identified as compound **6** (*m/z* 239, [MH]$^+$) by comparison with a reference spectrum of an authentic sample[13] (see Suporting Information). The second compound (**7**) was a variant of the 3-hydroxyisoflavanone **4** found in peak 2, with a modified prenyl side chain. Thus, the spectrum exhibited an AB pattern of olefinic hydrogens at δ 6.60 and 5.72 (J_{AB} = 10.1 Hz) and two methyl group signals at δ 1.43 and 1.44. These features demonstrated the presence of a 2,2-dimethyl-2*H*-pyran ring formed by cyclization of the 8-prenyl group present in **4** with the hydroxy group at C-7. The remaining signals observed in the ^{1}H NMR spectrum of **7** were practically identical to those of **4**, in agreement with the molecular formula $C_{21}H_{20}O_7$ confirmed by negative-mode ESIMS (*m/z* 383, [M − H]$^-$). Because of the identity of its B-ring resonances with those of **4** (Table 1), the new 3-hydroxyisoflavanone could be formulated as **7** (see below).

The ^{1}H NMR spectrum obtained from the material eluted as peak 5 (t_R = 33.0 min) showed the presence of three compounds, an isoflavanone, an isoflavone, and an isoflavan in the ratio of 6:3:2. Structures of these compounds were established as **8**, **9**, and **10**, respectively, on the basis of the following interpretation. The major constituent (**8**) showed two sets of ortho-coupled hydrogens and an ABX system characteristic of the C-ring in isoflavanones, four signals of a prenyl group, and a single methoxy substituent (Table 1). As for compounds **4** and **7**, the aromatic signals of **8** at δ 7.63 and 6.55 were assigned to the A-ring on the basis of the large shift value of H-5, and the other pair of AB doublets to the B-ring. On the basis of ESIMS data (*m/z* 371, [MH]$^+$), the isoflavanone has three hydroxy groups. The chemical shifts of H-5′ and H-6′ are compatible only with the presence of a methoxy substituent at C-4′ as discussed below, and thus the major constituent of peak 5 could be formulated as a novel isoflavanone **8**.

The second most abundant component eluted with peak 5 exhibited a characteristic singlet of H-2 of an isoflavone (δ 8.27), which replaced the ABX pattern observed for **8**. The remaining structural features were similar to those observed for the latter; the introduction of double bond in the C-ring of **9** was also reflected in a shift of H-5 from δ 7.63 to δ 8.00 due to conjugation effects (Table 1). Thus, the compound was formulated as the novel isoflavone **9** (see below), compatible with ESIMS (*m/z* = 367, [M − H]$^-$).

The minor compound found in peak 5 was identified as the new isoflavan **10**. Most of its ^{1}H NMR signals were very similar to those of the previously isolated[13] isoflavan **11** (see Supporting Information for reference data recorded with an authentic sample in acetonitrile-d_3), except that the AB pattern of the olefinic hydrogens of the 2*H*-pyran moiety was replaced by an ABX pattern (Table 1), indicating hydration of the double bond. Since the chemical shifts of H-5′ and H-6′ were practically identical to those of **11**, the two compounds have the same substitution pattern of the B-ring. ESIMS data (*m/z* = 385 and 387, respectively [M − H]$^-$ and [MH]$^+$) corroborated the proposed structure of **10**, but the configuration at C-1″ was not determined.

The material eluted as peak 6 (t_R = 34.2 min) was immediately determined to be the new isoflavone **12** (*m/z* 351 and 353, respectively [M − H]$^-$ and [MH]$^+$), showing a high degree of similarity with compound **9**. Thus, according to ^{1}H NMR data (Table 1), C-2′ was not oxygenated. The B-ring structure was determined on the basis of 2D NMR correlations described later.

The ^{1}H NMR spectrum of the compound eluted as peak 7 (t_R = 35.0 min) was identified as **13** (*m/z* 367, [MH]$^+$). Its ^{1}H NMR spectrum was reminiscent of that of the previously isolated **14**,[13] but the signal of H-2″ was missing and replaced by signals of a 2-propene group (Table 1). Because of a low amount of material present in peak 7, the isolated **13** was used for full characterization by 2D NMR experiments (see below).

The 1D ^{1}H NMR spectrum obtained with peak 8 (t_R = 38.8 min) showed a mixture of two compounds in a ratio of 9:10. These were identified as isoflavan **15** and isoflavanone **16** (which was present in slight excess). The isoflavan (*m/z* 331, [MH]$^+$) exhibited a characteristic spin systems of the C-ring, two pairs of ortho-coupled aromatic ring hydrogens, two methoxy group singlets, a low-field singlet at δ 11.73, and a doublet at δ 10.33 (J = 0.6 Hz) coupled to the A-ring resonance at δ 6.43 (Table 1). The second AB doublet of the A-ring (H-5) exhibited long-range coupling to H-4, in a manner similar to that for **1**, **2**, and **5**. The low-field signals demonstrated the presence of an aldehyde group at C-8 with an intramolecular hydrogen bond to a hydroxy group at C-7; the aldehyde hydrogen is coupled to H-6 along a five-bond zigzag path. The positions of the methoxy groups in the B-ring followed from the identity of chemical shifts of H-5′ and H-6′ with those observed for **11** and **14** in the same solvent (see Supporting Information). The ortho-hydroxy aldehyde **15** was subsequently isolated on a preparative scale as described below. This compound was recently isolated from *Sphaerophysa salsula* and named sphaerosin S3.[31]

The major component eluted as peak 8 was identified as a novel isoflavanone **16**. Its spectrum was similar to that of **8**, but two methoxy group resonances were observed, in agreement with ESIMS data (*m/z* 385 and 383; respectively [MH]$^+$ and [M − H]$^-$). The additional methoxy group in **16** was placed on the basis of consideration of *O*-methylation-induced shifts, as discussed later.

Peak 9 (t_R = 44.7 min) was also composed of two compounds, present in a ratio of 10:1. The minor and the major component were identified as **14** and **17**, respectively;

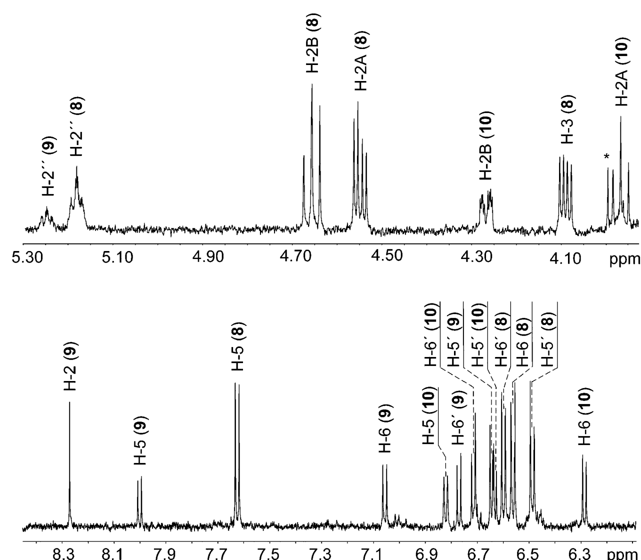

Figure 3. Diagnostic signals in the 1D ^{1}H NMR spectrum obtained in HPLC-SPE-NMR mode with peak 5 (containing compounds **8**, **9**, and **10**) after three trappings on the same SPE cartridge (C_{18}); * denotes an impurity.

both isoflavans were earlier isolated from *S. iranica*.[13] The identity of the compounds was confirmed by comparison of their HPLC-SPE-NMR data (Table 1) with ^{1}H NMR spectra of authentic samples[13] dissolved in acetonitrile-d_3 (see Supporting Information). Negative-mode ESIMS data were in agreement with the structures **14** and **17** (*m/z* respectively 325 and 369, $[M - H]^-$). The compound eluted with the last peak selected for the analysis, peak 10 (t_R = 56.3 min), was identified as **11** (glyasperin H)[13] by the same method. Positive as well as negative mode ESIMS data confirmed the molecular mass of **11** to be 368.

The 1D NMR data obtained with peaks 1−10 confirmed the usefulness of the HPLC-SPE-NMR experiments for analysis of isoflavonoids of *S. iranica*. Although the HPLC peaks in many cases contained two or more coeluting components, the 1D NMR data were sufficient for assignment of the constituent to a particular isoflavonoid subclass and for determination of the types of substituents present. Such information will normally be sufficient for extract dereplication, i.e., determination of whether a particular component should be isolated and purified, for example for pharmacological evaluation. An example of a 1D NMR spectrum obtained in HPLC-SPE-NMR mode, illustrating identification of coeluting constituents, is shown in Figure 3.

Full structural analysis of isoflavonoids, including rigorous determination of all substituent positions, will usually require 2D NMR experiments, for which larger amounts of material are preferred. Since the HPLC-SPE-NMR technique in principle offers a unique possibility of increasing the amount of analyte by repeated injections and repeated trappings on the same SPE cartridge,[14,15,19−21] the performance of this approach was tested. Peak 10 was selected as a test case for the multiple trapping experiments, because glyasperin H (**11**) is a representative compound, present in the extract in quite small amounts (Figure 2).

The results obtained clearly demonstrated the advantage of multiple trappings. Thus, up to seven repeated trappings of peak 10 resulted in a linear increase of the signal-to-noise in the NMR spectra (Figures 4 and S1, Supporting Information). Besides improving quality of the 1D spectra, the multiple trappings option enables acquisition of both

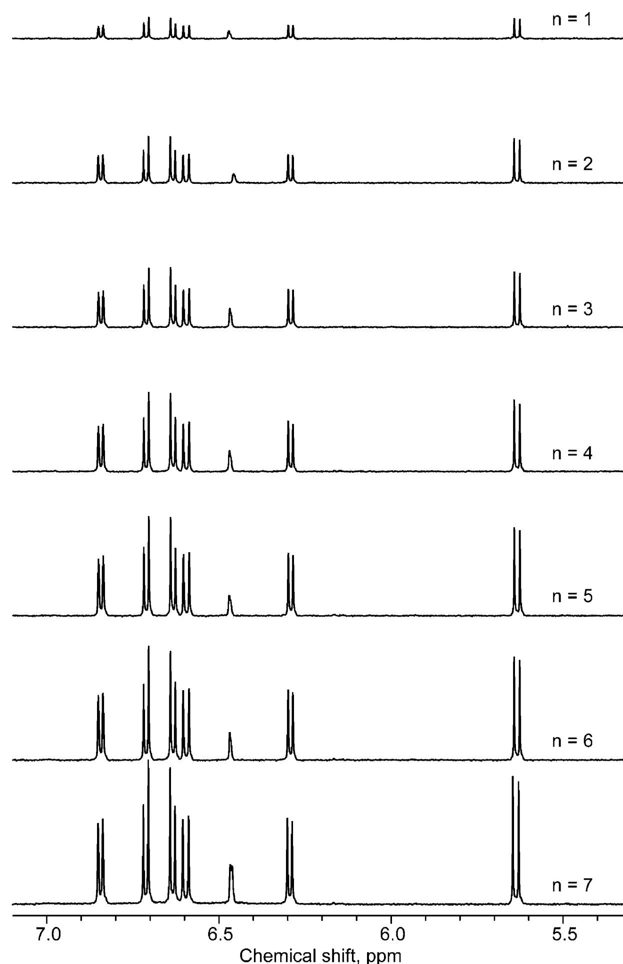

Figure 4. 1D spectra obtained in HPLC-SPE-NMR mode with peak 10 (compound **11**) after repeated analyte trappings on the same SPE cartridge; n = number of injections.

^{1}H−^{1}H and inverse ^{1}H−^{13}C 2D NMR correlation spectra within reasonable time. The ability to acquire good-quality 2D NMR data in the HPLC-SPE-NMR mode is illustrated for peaks 2 and 10 in Figures 5 and 6. Thus, each peak was trapped six times in the usual way, before elution and performing 2D NMR experiments (see Experimental Section for details). Significantly, the HPLC-SPE-NMR mode heteronuclear correlation experiments provided ^{13}C NMR chemical shifts, important for structure elucidation of natural products.

The HSQC and HMBC spectra of the compounds present in peak 2 allowed unambiguous identification of the position of the methoxy group in compound **4**. It is well established that ^{13}C NMR chemical shifts of methoxy groups attached to an aromatic ring and flanked by two ortho substituents lie in a narrow chemical shift range around δ 60, whereas ortho-monosubstituted methoxy groups appear around δ 56.[32−35] The two types of methoxy groups have the $O-CH_3$ bond perpendicular to or in the plane of the benzene ring, respectively, resulting in differences in oxygen lone-pair delocalization.[36,37] The single methoxy group present in **4**, with ^{13}C NMR chemical shift δ 56.8 obtained from HSQC and HMBC experiments recorded in the HPLC-SPE-NMR mode (Figure 6), has

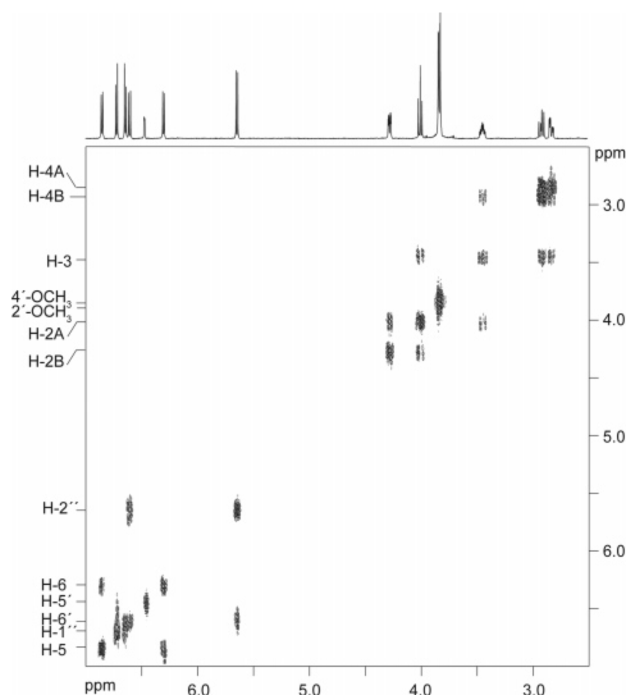

Figure 5. COSY spectrum obtained in HPLC-SPE-NMR mode after six trappings (C_{18} cartridge) with peak 10 containing compound **11** (total acquisition time 6 h 10 min).

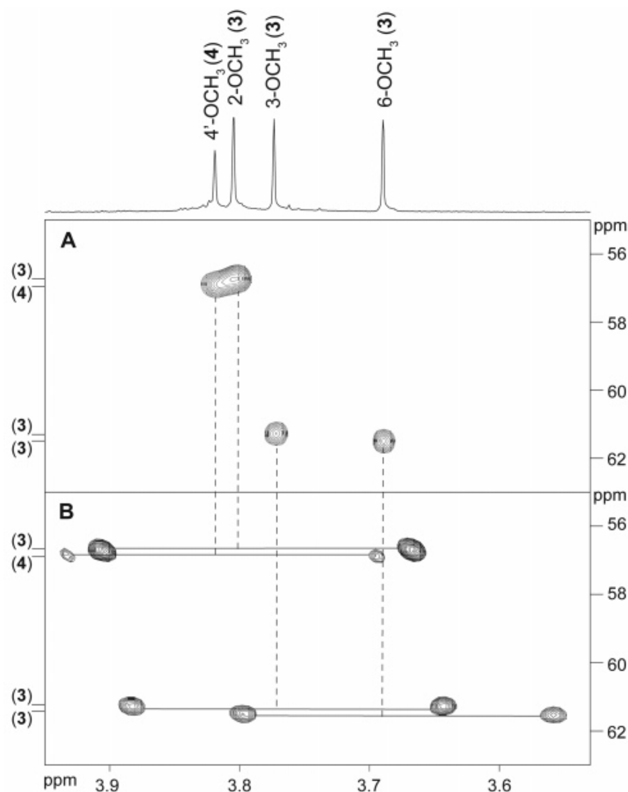

Figure 6. Fragments of heteronuclear correlations obtained with peak 2 (mixture of compounds **3** and **4**) showing ^{13}C chemical shifts of methoxy groups. (A) HSQC spectrum (total acquisition time 9 h 40 min). (B) One-bond correlations in HMBC spectrum (total acquisition time 15 h 7 min). The spectra were obtained in HPLC-SPE-NMR mode after six trappings.

therefore only one ortho substituent. That the methoxy group is attached to C-4′ and not to C-7 was confirmed by the HMBC spectrum. The spectrum showed a correlation between the methoxy group protons and a carbon signal at δ 149.4, which correlated to H-6′; the signal at δ 149.4 must therefore belong to C-4′. Since the chemical shifts of H-5′ and H-6′ in **7** are identical to those of **4**, the substitution pattern in the B-ring of these two compounds must be the same, as already mentioned. These experiments show that fully unambiguous structural assignments (apart from determination of chirality) of previously unknown natural products can be achieved using HPLC-SPE-NMR.

In connection with elucidation of methoxy group positions in polyoxygenated B-rings of isoflavonoids such as those described in this work, it is also of interest to discuss the possible utility of 1D ^1H NMR data in the absence of 2D heteronuclear correlation spectra, i.e., to consider chemical shift changes of aromatic hydrogens caused by *O*-methylation of phenol groups. Conversion of phenol to anisole causes only small changes of chemical shifts of H_{ortho} and H_{meta} of +0.08 and +0.04 ppm, respectively, and has a negligible effect on H_{para} (in chloroform-*d*).[38] However, analysis of assigned ^1H NMR chemical shifts of methyl ethers of 1,2,3-benzenetriol and 4-methyl-1,2,3-benzenetriol available in the literature (considering spectra recorded in the same solvents) shows more complex relationships. For example, conversion of 2-methoxy-1,3-benzenediol[39,40] to 1,2,3-trimethoxybenzene[41−43] causes chemical shift changes of aromatic hydrogens paralleling closely those observed for conversion of phenol to anisole (in chloroform-*d*). However, conversion of 2,6-dimethoxyphenol[44,45] to 1,2,3-trimethoxybenzene[41−43] causes a shift of about +0.20 ppm at the position para to the *O*-methylation site, with a negligible change of H_{meta} (in chloroform-*d*). *O*-Methylation of 3-methoxy-4-methyl-1,2-benzenediol[46] to 2,6-dimethoxy-3-methylphenol[46] causes only small chemical shift changes of both aromatic hydrogens, whereas conversion of 3-methoxy-6-methyl-1,2-benzenediol[46] to the latter causes a shift of H_{para} of +0.21 ppm (in methanol-d_4). The same shift of H_{para} of +0.21 ppm is observed with chloroform-*d* as solvent on going from 2,3-dimethoxy-6-methylphenol[47] to 1,2,3-trimethoxy-4-methylbenzene.[46] Also, large chemical shift changes of both aromatic hydrogens are observed upon conversion of 3-methoxy-6-methyl-1,2-benzenediol[47,48] to 1,2,3-trimethoxy-4-methylbenzene[46] (0.19−0.23 ppm, in chloroform-*d*). It appears clearly that when an ortho-disubstituted phenol group is methylated, the shift of H_{para} is much larger than in the case of a hydroxy group with only one (or no) ortho substituent. This effect is in fact similar to the effect previously observed for ^{13}C NMR chemical shifts of ring carbons in methoxyphenols[34] and is presumably due to differences in conformational effects of ortho substituents between hydroxy and methoxy groups, manifested as differences in lone electron pair delocalization. In flavonoids, the effects of *O*-methylation on chemical shifts of B-ring hydrogens may be further complicated by changes of conformer populations for rotation about the C-3−C-1′ bond and by hydrogen-bonding effects. However, a conclusion that we derive from the literature data cited above is that chemical shift changes of aromatic hydrogens caused by *O*-methylation of flavonoids may be a useful structure elucidation tool; *O*-methylation-induced shifts observed for ortho-disubstituted phenols are large for H_{para} and are thus fundamentally different from those in phenol itself, where the shifts are small and diminish from the ortho via the meta to the para position.[38]

On the basis of these conclusions, the structures of isoflavonoids detected in the above HPLC-SPE-NMR experiments can be discussed further. Since compounds **1** and **2** have practically identical chemical shifts of the B-ring hydrogens (Table 1), compound **1** must have only one methoxy group in the B-ring, occupying the same position

as in compound **2** (if both methoxy groups of **2** were placed in the B-ring, one of them would be ortho-disubstituted and would cause a substantial difference in chemical shift of H_{para} between the two compounds). Comparison of the chemical shifts of H-5′ and H-6′ in **1** and **2** with those of the dimethoxylated isoflavans **11**, **14**, and **17** (the structures of which were determined by 2D experiments[13]) strongly suggests that the single methoxy group present in the B-ring of **1** and **2** is attached to C-4′. If this is correct, the second methoxy group in **11**, **14**, and **17** caused a shift of H_{para} (H-5′) by +0.13−0.17 ppm relative to **1** and **2** (Table 1 and Supporting Information), close to the value expected on the basis of the above discussion; if the methoxy group in **1** and **2** was attached to C-2′ or C-3′, the chemical shifts of H-5′ or H-6′ would have to be considerably larger than observed (Table 1). To verify this conclusion, a HSQC spectrum was recorded with peak 1 in the HPLC-SPE-NMR mode (six SPE trappings). The spectrum showed ^{13}C NMR resonances of the methoxy groups in **1** at δ 56.7 and 60.1 and in **2** at δ 56.8, which provided a definitive proof that the methoxy group in the B-ring of both compounds is attached to C-4′ (i.e., has a single ortho-substituent), in agreement with the conclusions based on consideration of the *O*-methylation shifts. At the same time, the ^{13}C NMR chemical shifts of the methoxy groups in **1** proved that the second methoxy group is attached to C-8.

The isoflavan **5** has one methoxy group at a position different from that in **2**, i.e., either C-2′ or C-3′. The high chemical shift of H-6′ and the low chemical shift of H-5′ (Table 1) are only compatible with the presence of the methoxy group at C-3′. Accordingly, a HSQC spectrum obtained with peak 3 (six SPE trappings) showed the methoxy group carbon at δ 61.1. While this ^{13}C NMR chemical shift is compatible with methoxylation at either C-2′ or C-3′, the chemical shifts of C-5′ (δ 104.8) and C-6′ (δ 122.6), obtained from the HSQC spectrum, are only compatible with the methoxylation of C-3′.[34] Thus, the chemical shift values of C-5′ and C-6′ in **2** are, respectively, δ 104.2 and 118.1 (from the HPLC-SPE-NMR mode HSQC spectrum). Estimation from the latter values of changes expected by moving the methoxy group from C-4′ to C-3′ using the rules developed by Fujita et al.[34] gives values very similar to those actually observed for **5**, whereas markedly different chemical shifts of C-5′ and C-6′ (δ about 109 and 118) are expected if the methoxy group is moved from C-4′ to C-3′. Once again, the well-established rules[32−37] for ^{13}C NMR chemical shifts confirmed the prediction based on the ^{1}H NMR chemical shift rules proposed in the present work.

The HMBC spectrum of **4** (Figure 6), in addition to providing information about the site of the methoxy group, confirmed the position of the prenyl group at C-8 (correlation between H-1″ and C-8 at δ 114.5 and between the latter and H-6). The abnormally high chemical shift of H-6′ in **4** and **7** (as compared to the isoflavans discussed above) can be attributed to the substituents in the C-ring, cf., for example, the difference $\Delta\delta$ 0.29 ppm between chemical shifts of H-6′ in the 3-hydroxyisoflavonone secundifloran and the corresponding isoflavan having identical substituents in the rings A and B,[24,27] which is equal to the differences observed in the present case (Table 1).

The chemical shifts of H-5′ and H-6′ in **8** are the same as in **1** and **2** (Table 1), and a different (than proposed) position of the methoxy group in **8** is incompatible with the *O*-methylation shifts discussed above. However, these considerations do not take into account a possible effect of the carbonyl group at C-4, and an independent proof of the structure **8** was desirable. Since we selected compound **8** for preparative-scale isolation (see Experimental Section), a full set of 2D NMR spectra (COSY, NOESY, HSQC, and HMBC) could be recorded with purified material. These data provided unequivocal proof of structure **8** [NOE between OCH_3 and H-5′; ^{13}C NMR chemical shift of the methoxy group δ 56.1 (in chloroform-*d*)]. This confirmed once again the predictions based on consideration of chemical shifts of H-5′ and H-6′ and in addition showed that the effect of the carbonyl group on these chemical shifts is small.[24,26,29,49] The additional methoxy group in compound **16** must be placed at C-2′ on the basis of a shift of H-5′ by +0.10 ppm. In fact, the changes in chemical shifts of H-5′ and H-6′ between **8** and **16** were very similar to the corresponding changes observed between **1** or **2** and **11**, **14**, or **17** (Table 1 and Supporting Information), in which the *O*-methylation sites were proven by 2D experiments.[13]

The structures of **9** and **12** were similarly confirmed by 2D NMR spectra obtained with isolated material (see Experimental Section). Preparative purification and 2D NMR spectra of isolated compound **15** also confirmed the B-ring methoxylation pattern inferred from the chemical shifts of H-5′ and H-6′.

Thus, 2D NMR spectra of constituents of *S. iranica*, obtained either in HPLC-SPE-NMR mode or after preparative isolation, fully confirmed all structural assignments made from 1D ^{1}H NMR spectra obtained from HPLC-SPE-NMR experiments with a crude extract of the plant material. While the major purpose of the 2D NMR experiments performed in the present work was to test the predictive power of the ^{1}H NMR spectra for assignments of methoxy group location, the usefulness of HPLC-SPE-NMR resides in the possibility of making either partial or full and rigorous structure elucidation (except for chirality) from analytical-scale HPLC for targeted isolation purposes. Thus, information provided by HPLC-SPE-NMR will enable fully qualified decisions about which compounds present in a crude extract should be isolated on a preparative scale, for example to assess pharmacological activity. Since structure determination of natural products after classical isolation and purification is very laborious and often results in reisolation of trivial or otherwise unwanted constituents, the possibility of focusing purification efforts on selected compounds is a major advantage. Although earlier HPLC-NMR implementations can in principle be used in the same way,[50,51] HPLC-SPE-NMR circumvents the problem of peak broadening in HPLC columns caused by stopped-flow methods, a difficulty that increases with the number of peaks in the chromatogram. Moreover, the analyte concentration and accumulation, together with solvent change to a deuterated solvent inherent to HPLC-SPE-NMR, is an advantage compared to peak storage in capillary loops.[52] The fact that the NMR spectra are acquired in the HPLC-SPE-NMR experiments in a pure solvent, rather than in a mixed and continuously changing solvent resulting from gradient HPLC elution, enables chemical shift comparisons with reference compounds and facilitates NMR data interpretation. In the present work, HPLC-MS was applied in parallel with HPLC-SPE-NMR to provide information about molecular masses of the analytes. Because of pronounced differences in ionizability of extract constituents, inherent in the electrospray ionization technique, detection of positive as well as negative ions was necessary.

In conclusion, the HPLC-SPE-NMR technique applied to an essentially crude extract of *S. iranica* roots resulted

in structure elucidation of 17 constituents present in 10 chromatographic peaks, selected for the above-described proof-of-the-concept experiment. This approach is believed to be generally applicable to phytoestrogens and other relatively nonpolar natural products. The structure elucidation has been achieved much faster than that in the earlier investigation[13] employing traditional extract fractionation schemes. Good quality NMR data allowed firm structural conclusions to be drawn even in the case of coeluting constituents, and the feasibility of routine acquisition of 2D spectra, including heteronuclear correlation experiments that provide ^{13}C NMR chemical shifts, was demonstrated in multiple-trapping mode even for minor chromatographic peaks. Although NMR spectra give no information about chirality, the knowledge of absolute configuration is not a necessary prerequisite for decisions about which compounds identified in a crude extract should be isolated on a preparative scale; hyphenation of HPLC with circular dichroism[53] can be employed if required (in the present work, optical rotation was determined only for isolated, pure compounds, see Experimental Section). Because HPLC-SPE-NMR can provide much useful structural information about constituents of complex extracts in a short time, we envisage a growing importance of this particular HPLC-NMR technique as an extract dereplication tool in natural products research.

Experimental Section

General Experimental Procedures. The HPLC-SPE-NMR instrument consisted of a Bruker LC22 quaternary solvent delivery pump with Degasys Populaire degasser, and an Agilent 1100 autosampler, a Bruker DAD UV detector, a Knauer K100 Wellchrom pump for postcolumn water delivery, a Spark Prospekt 2 solid-phase extraction device, and a Bruker Avance 600 spectrometer (hydrogen frequency 600.13 MHz) equipped with a 30 μL inverse ^{1}H{^{13}C} flow-probe operating at 25 °C. All analytical-scale HPLC separations were performed on a 150 × 4.6 mm i.d., 3 μm particle size, Phenomenex Luna C_{18}(2) column with a precolumn. Chromatography, peak trapping, and analyte transfer from the SPE unit to the NMR spectrometer were controlled with HyStar ver. 2.3 software, and NMR experiments were conducted with Xwin-nmr ver. 3.1 software (Bruker BioSpin). NMR spectra of compounds isolated on a preparative scale were obtained with either a Bruker Avance 600 spectrometer equipped with a 5 mm inverse ^{1}H{^{13}C} probe or a Bruker Avance 400 spectrometer equipped with a 5 mm normal configuration ^{13}C{^{1}H} probe (hydrogen frequency 600.13 and 400.13 MHz, respectively). HPLC-MS experiments were performed on an Agilent 1100 Series LC/MSD trap instrument. Preparative HPLC separations were performed on a chromatograph consisting of a Gynkotek P 580 pump, Rheodyne 7725 manual injector, Shimadzu SPD-10AV spectrophotometric detector operating at 254 or 270 nm, and a recorder, using a 25 cm × 2 cm i.d., 5 μm particle size, Phenomenex Luna C_{18}(2) column eluted isocratically with water−acetonitrile (1:1). Optical rotations were measured using a Perkin-Elmer 241 polarimeter.

Plant Material. Roots of *Smirnowia iranica* H. Sabeti were collected in August 1996 at the altitude of 900−950 m in the Kashan District, Isfahan, Iran, and stored in a cold and well-ventilated place. A voucher specimen (number 11054) was deposited in the herbarium of the Isfahan Research Centre of Natural Resources and Animal Science, Isfahan, Iran. This is the same collection of the plant material as used in the previous work.[13]

Sample Preparation. Dried and powdered roots of *S. iranica* (14 g) were extracted with 0.5 L of EtOH for 24 h at room temperature. The mixture was sonicated for 10 min, the extract was filtered and evaporated to dryness, and the residue (0.8 g) was redissolved in 15 mL of CH_3CN-H_2O (1:1) and loaded on a Varian Bond Elut C_{18} SPE cartridge (5 g, conditioned with CH_3CN and equilibrated with H_2O). The cartridge was eluted with 10 mL of CH_3CN-H_2O (1:1) and then with 2 × 10 mL of CH_3CN-H_2O (95:5), and the combined extracts were evaporated to give 650 mg of prepurified crude extract.

HPLC-SPE-NMR Experiments. A solution of prepurified crude extract (19 mg/mL) was prepared in CH_3CN-H_2O (1:1). The solvent system consisted of CH_3CN in H_2O (30% at 0 min rising linearly to 40% at 20 min and 70% at 60 min) with a flow rate of 0.8 mL/min. The chromatography was monitored at 254 and 300 nm, using the averaged absorption at these two wavelengths to define absorbance thresholds to trigger SPE trapping. The HPLC eluate was diluted with H_2O (0.8 mL/min) prior to trapping on 10 × 2 mm i.d. C_{18} HD SPE cartridges (Spark Holland), conditioned with 500 μL of CH_3-CN at 6 mL/min and equilibrated with 500 μL of H_2O at 1 mL/min. The cartridges were dried with pressurized nitrogen gas for 30 min each, and the analytes eluted with CD_3CN (99.8 atom %, Cambridge Isotope Laboratories) into the flow-probe. The total amount of CD_3CN used for elution and transfer was 282 μL at a transfer rate of 240 μL/min. 1D NMR spectra were typically obtained after one or three trappings, and 2D NMR spectra after six trappings.

NMR Experiments. 1D ^{1}H NMR spectra obtained in HPLC-SPE-NMR mode were recorded using 1D NOESY pulse sequence for dual presaturation of solvent resonances (HOD and CD_2HCN) during the mixing time (100 ms) and relaxation delay (2.4 s). For most samples, 256 scans were used, acquiring 64 K data points with a spectral width of 20 ppm. Phase-sensitive ^{1}H−^{1}H COSY spectra were recorded using a gradient-based pulse sequence with WET suppression of residual solvents signals, with 10 ppm spectral width, 2048 × 128 data points (processed with forward linear prediction to 2048 × 512), and 2.4 s relaxation delay. Sensitivity-enhanced ^{1}H−^{13}C HSQC spectra were acquired with the following parameters: spectral width 10 ppm for ^{1}H and 160 ppm for ^{13}C, 1028 × 128 data points (linear prediction to 512), and 2 s relaxation delay. ^{1}H−^{13}C HMBC spectra (without low-pass filter) were optimized for $J_{C,H}$ = 7.7 Hz and acquired using the following parameters: spectral width 10 ppm for ^{1}H and 200 ppm for ^{13}C, 1028 × 256 data points (linear prediction to 512), 1.5 s relaxation delay. No solvent suppression was included in the HSQC and HMBC experiments. The NMR spectra acquired in HPLC-SPE-NMR mode were calibrated internally using solvent signal (δ 1.94 for residual ^{1}H signal, δ 1.39 for ^{13}C signal). Spectra of isolated compounds were acquired at 25 °C in 5 mm tubes in CD_3CN or $CDCl_3$, using TMS as internal reference (at 600 MHz for ^{1}H-detected experiments and at 100 MHz for ^{13}C and DEPT135 spectra). COSY spectra were acquired in phase-sensitive mode; HSQC and HMBC (optimized for $J_{C,H}$ = 7.7 Hz) were acquired with gradient-selection; NOESY spectra were obtained with a mixing time of 600 ms.

HPLC-MS Experiments. These experiments were performed with the prepurified crude extract using the same column and identical chromatographic method as that used in the HPLC-SPE-NMR experiments. ESIMS were acquired in positive- as well as negative-ion mode, with the following instrument settings: drying gas temperature 350 °C, nebulizer pressure 50 psi, drying gas 10 L/min, spray voltage 3000 V, trap drive 40 (arbitrary units).

Preparative Isolation. Isolation of selected constituents of *S. iranica* roots was achieved with fractions originating from the previous work.[13] Thus, various fractions were tested for the presence of required constituents by HPLC and 600 MHz ^{1}H NMR, and the selected fractions resolved fully or partly by preparative reversed-phase HPLC (see General Experimental Procedures). This yielded 335 mg of **8**, 28 mg of a 1:1 mixture of **10** and **12**, traces of **13**, and 12 mg of **15**.

4′-Methoxy-8-(3-methyl-2-butenyl)-7,2′,3′-trihydroxy-isoflavanone (8): $[\alpha]_D^{25}$ 0° (*c* 0.57, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.76 and 1.82 (each 3H, s, CH_3-3″), 3.41 (2H, d, J = 7.3 Hz, H-1″), 3.84 (3H, s, OCH_3-4′), 4.10 (1H, dd, J = 7.6 and 5.1 Hz, H-3), 4.69 (1H, dd, J = 11.4 and 5.1 Hz, H-2A),

4.83 (1H, dd, J = 11.4 and 7.6 Hz, H-2B), 5.25 (1H, tsp, J = 7.3 and 1.4 Hz, H-2″), 5.56 (1H, s, OH-3′), 6.21 (1H, s, OH-7), 6.46 (1H, d, J = 8.7 Hz, H-5′), 6.49 (1H, d, J = 8.3 Hz, H-6), 6.79 (1H, d, J = 8.7 Hz, H-6′), 7.08 (1H, s, OH-2′), 7.76 (1H, d J = 8.3 Hz, H-5); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.0 and 25.8 (CH_3-3″), 22.0 (C-1″), 46.4 (C-3), 56.4 (OCH_3-4′), 70.4 (C-2), 103.7 (C-5′), 110.9 (C-6), 114.2 (C-10), 114.3 (C-8), 115.9 (C-1′), 118.5 (C-6′), 121.0 (C-2″), 127.5 (C-5), 134.0 (C-3′), 135.4 (C-3″), 142.8 (C-2′), 146.9 (C-4′), 161.1 (C-9), 161.7 (C-7), 192.9 (C-4).

8,8-Dimethyl-3-(3-hydroxy-2,4-dimethoxyphenyl)-3,4:9,10-tetrahydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-10-ol (10): ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.32 and 1.36 (each 3H, s, CH_3-3″), 2.69 (1H, dd, J = 17.7 and 5.3 Hz, H-2″A), 2.85 (1H, ddd, J = 15.5, 5.5 and 2.3 Hz, H-4A), 2.91 (1H, dd, J = 17.7 and 5.3 Hz, H-2″B), 2.95 (1H, ddd, J = 15.5, 11.0 and 1.0 Hz, H-4B), 3.53 (1H, m, H-3), 3.81 (1H, t, J = 5.3 Hz, H-1″), 3.89 (3H, s, OCH_3-4′), 3.90 (3H, s, OCH_3-2′), 3.97 (1H, t, J = 10.4 Hz, H-2A), 4.34 (1H, ddd, J = 10.4, 3.6 and 2.3 Hz, H-2B), 6.42 (1H, d, J = 8.3 Hz, H-6), 6.60 (1H, d, J = 8.6 Hz, H-6′), 6.65 (1H, d, J = 8.6 Hz, H-5′), 6.86 (1H, d, J = 8.3 Hz, H-5); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.0 and 24.5 (CH_3-3″), 26.6 (C-2″), 31.7 (C-4), 31.8 (C-3), 56.0 (OCH_3-2′), 56.3 (OCH_3-4′), 69.6 (C-1″), 70.6 (C-2), 74.6 (C-3″), 106.5 (C-5′), 107.2 (C-8), 109.1 (C-6), 113.7 (C-10), 117.0 (C-6′), 127.6 (C-1′), 128.0 (C-5), 138.7 (C-3′), 145.6 (C-2′), 146.7 (C-4′), 151.9 (C-7), 152.7 (C-9).

7,3′-Dihydroxy-4′-methoxy-8-(3-methyl-2-butenyl)isoflavone (12): ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.76 and 1.86 (each 3H, s, CH_3-3″), 3.61 (2H, d, J = 7.1 Hz, H-1″), 3.91 (3H, s, OCH_3-4′), 5.28 (1H, tsp, J = 7.1 and 1.4 Hz, H-2″), 6.90 (1H, d, J = 8.7 Hz, H-6), 6.91 (1H, d, J = 8.3 Hz, H-5′), 7.11 (1H, dd, J = 8.3 and 2.1 Hz, H-6′), 7.12 (1H, d, J = 2.1 Hz, H-2′), 7.97 (1H, s, H-2), 8.06 (1H, d, J = 8.7 Hz, H-5); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.0 and 25.8 (CH_3-3″), 22.2 (C-1″), 56.1 (OCH_3-4′), 110.9 (C-5′), 114.5 (C-8), 114.8 (C-6), 115.3 (C-2′), 118.5 (C-10), 120.6 (C-2″), 121.3 (C-6′), 124.3 (C-3), 125.3 (C-1′), 125.4 (C-5), 135.4 (C-3″), 145.4 (C-4′), 146.7 (C-3′), 152.3 (C-2), 155.4 (C-9), 158.9 (C-7), 176.3 (C-4).

3-[3,4-Dihydro-8-(2-propenyl)-2*H*-furo[2,3-*h*]-1-benzopyran-3-yl]-2,6-dimethoxyphenol (13): ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.11 (3H, s, CH_3-3″), 2.99 (1H, ddd, J = 15.6, 5.2 and 1.9 Hz, H-4A), 3.07 (1H, ddd, J = 15.6, 11.0 and 0.6 Hz, H-4B), 3.63 (1H, m, H-3), 3.88 (3H, s, OCH_3-4′), 3.91 (3H, s, OCH_3-2′), 4.10 (1H, t, J = 10.3 Hz, H-2A), 4.42 (1H, ddd, J = 10.3, 3.5 and 1.9 Hz, H-2B), 5.13 (1H, quintet, J = 1.5 Hz, H-4″*E*), 5.73 (1H, m, H-4″*Z*), 6.70 (1H, m, H-1″), 6.63 (1H, d, J = 8.6 Hz, H-6′), 6.65 (1H, d, J = 8.6 Hz, H-5′), 6.97 (1H, d, J = 8.4 Hz, H-5), 7.00 (1H, dd, J = 8.4 and 0.8 Hz, H-6); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.3 (CH_3-3″), 31.6 (C-4), 31.8 (C-3), 56.3 (OCH_3-4′), 61.0 (OCH_3-2′), 70.6 (C-2), 100.1 (C-1″), 103.6 (C-6), 106.6 (C-5′), 112.4 (C-4″), 114.8 (C-10), 117.1 (C-6′), 118.6 (C-8), 126.1 (C-5), 127,4 (C-1′), 132.9 (C-3″), 138.7 (C-3′), 145.3 (C-2′), 146.7 (C-4′), 147.5 (C-9), 154.7 (C-7), 155.9 (C-2″).

3,4-Dihydro-7-hydroxy-3-(3-hydroxy-2,4-dimethoxyphenyl)-2*H*-1-benzopyrancarbaldehyde [7,3′-dihydroxy-2′,4′-dimethoxy-8-formylisoflavan, sphaerosin S3] (15): $[\alpha]_D^{25}$ +10.7° (c 0.3, $CHCl_3$), lit.[31] +30.6° (c 0.035, CH_3OH); ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.84 (1H, ddd, J = 15.7, 5.4 and 2.1 Hz, H-4A), 2.92 (1H, ddd, J = 15.7, 10.4 and 1.0 Hz, H-4B), 3.56 (1H, m, H-3), 3.89 (3H, s, OCH_3-4′), 3.93 (3H, s, OCH_3-2′), 4.06 (1H, t, J = 10.4 Hz, H-2A), 4.41 (1H, ddd, J = 10.4, 3.6 and 2.1 Hz, H-2B), 5.61 (1H, s, OH-3′), 6.46 (1H, dd, J = 8.3 and 0.6 Hz, H-6), 6.60 (1H, d, J = 8.6 Hz, H-6′), 6.65 (1H, d, J = 8.6 Hz, H-5′), 7.19 (1H, d, J = 8.3 Hz, H-5), 10.33 (1H, d, J = 0.6 Hz, CHO), 11.79 (1H, s, OH-7); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.8 (C-4), 31.5 (C-3), 56.3 (OCH_3-4′), 61.0 (OCH_3-2′), 70.9 (C-2), 106.4 (C-5), 109.0 (C-6), 110.4 (C-8), 112.6 (C-10), 116.9 (C-6′), 126.6 (C-1′), 138.8 (C-3′), 138.9 (C-5), 145.3 (C-2′), 147.0 (C-4′), 157.0 (C-9), 161.9 (C-7), 194.3 (C-1″).

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Supporting Information Available: 2D NMR connectivities (NOESY and HMBC) for **8**, **10**, **12**, **13**, and **15**, ^{1}H NMR data for compounds **3**, **6**, **11**, **14**, and **17** in acetonitrile-d_3, and Figure S1, showing the linear increase of signal-to-noise ratios in ^{1}H NMR spectra after repeated SPE trapping of **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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