# Fast screening of some flavonoids content in raw plant materials: opportunities of $^1$H NMR spectroscopy

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**Abstract.** Environmental monitoring and assessment of the prospects for extracting biologically active substances (BAS) from various types of plant biomass requires the development of simple and fast methods for measuring their content in raw materials. A new approach for measuring the content of various flavonoids groups in plant raw material using $^1$H NMR spectroscopy has been developed, which allows to characterize its resource capabilities and study the effects on their composition different environmental factors without complex sample preparation and standard samples.

## 1 Introduction

The concept of effective environmental management is not limited by the main environmental imperative - the conservation of environmental biodiversity. Its economic component is the rational consumption of natural raw materials. Trees and higher plants are important renewable resources of the biosphere, the processing methods of which should be non-waste and produce not only cellulose, construction and composite materials. Scientifically-based their use involves obtaining a wide range of food, medical and technical products. Assessment of the content of biologically active substances in biomass should be based on environmentally friendly quantitative analytical methods free from complex sample preparation, derivatization, and the need for standard samples. Taking into account the multicomponent nature of the metabolite extracts, NMR spectroscopy meets these requirements due to sufficient sensitivity, informativity and standardless quantitative measurements.

Among the extractive polyphenolic compounds of plant biomass, a special place is occupied by flavonoids, of which more than 10,000 are identified. We used $^1$H NMR spectroscopy to develop a universal technique for assessing the content of flavonoids with unique biological activity in significantly different objects: taxifolin **1** in Siberian larch wood (*Larix sibirica*), flavonolignans **2-8** in the seeds of Milk Thistle (*Silybum marianum*), group of flavonol glycosides (FG) **9** in the leaves of a Ginkgo biloba (*Ginkgo biloba*). The

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aim of the work is to study the possibility for determination of the above biologically active substances content in the studied extracts without using of standard samples for the purpose of eco-monitoring and assessment of their resource potential for drugs creation.
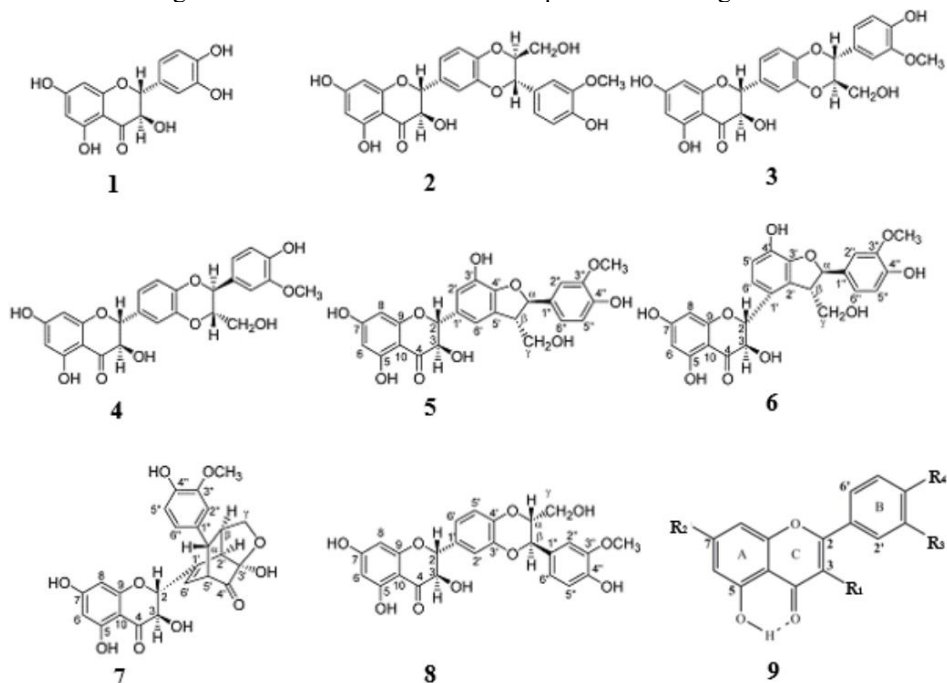


**Fig. 1.** Structural formulas of the main biologically active substances of the considered extracts: 1. Taxifolin; 2. Silybin A; 3. Silybin B; 4. Isosilybine A; 5. Isosilybine B; 6. Silicritin; 7. Isosilicritin; 8. Silidianin; 9. FG of Ginkgo biloba.

**Taxifolin.** Russia's coniferous forests contain about half of the world's timber reserves, and the largest area ($\approx$260 million ha) is occupied by larch ($\approx$37%) [1], which UNESCO identified as a symbol of Russia. A unique phenomenon of Siberian larch (*Larix sibirica*) is a high content of **1** in its wood (up to 4%), which is an order of magnitude higher than its content in any other types of plant materials. Due to its ability to inhibit lipid peroxidation, **1** is recognized as a stronger natural antioxidant.

**Flavonolignans.** *Silybum marianum* is an annual/biennial plant of the Asteraceae family, now growing and cultivated worldwide. It has been used for centuries in Mediterranean medicine mainly to treat liver, kidney, spleen, and gallbladder diseases. Silymarin is a well-established *Silybum marianum* seeds standardized dry extract containing mainly flavolignans (more than 65%) as well as polymeric and oxidized polyphenolic compounds. The main silymarin flavonolignas are **2-8** and taxifolin (Figure 1), where the flavonoid part (taxifolin) of the molecule is fused with a lignan. Content of the amount of **2-8** in raw materials is usually more 2% [2].

**Flavonol glycosides.** Preparations based on extract from the leaves of *Ginkgo biloba* are the most popular plant nootropics in the world. Its medicinal properties were known more than 2,000 years ago and are used now for treatment central nervous system diseases, such as dementia and Alzheimer's disease.

The extract from ginkgo leaves contains a wide variety of FG (more 70 are classified and more 30 are isolated) [3]. FG of ginkgo has from one to three carbohydrate residues and their acylated derivatives which aglycones are predominantly quercetin, kaempferol

and isoramnetin, content of which is minimal. The content of **9** is more than 0.5% in raw materials.

## 2 Materials and Methods

### 2.1 Plant material, solvents and standards

Finely ground absolutely dry samples of *Larix sibirica* wood (LLC "Scientific and Production Association "Wood Chemistry", Irkutsk, Russia), *Silybum marianum* seeds (CJSC "Pharmacenter VILAR", Moscow, Russia) and *Ginkgo biloba* leaves (CJSC "Evalar", Biysk, Russia), as well as commercial samples of extracts from this raw material.

A dimethylsulfoxide-$d_6$ (DMSO-$d_6$) solvent (≥99.9%, Sigma-Aldrich, CAS Number 2206-27-1) was used. Taxifolin (USP Reference Standard, CAS Number 480-18-2), silybin (USP Reference Standard, CAS Number 22888-70-6) and diosmin (USP Reference Standard, CAS Number 520-27-4) standards have also been used.

### 2.2 Sample preparation and experiments

50,0±0,5 mg of the raw material powder were placed in eppendorf and 1 ml of DMSO-$d_6$, containing 0,1% of diosmin standard, was added. Eppendorfs were shaken for 10 minutes on Vortex, 10 minutes were sonicated and placed into a centrifuge (14,000 rpm) for 5 minutes. The supernatant was transferred to a standard 5 mm diameter NMR tube and the $^1$H NMR spectrum was recorded.

The NMR spectra were acquired at 298 K with a JEOL JNM ECA 600 NMR spectrometer (JEOL, Japan) with an operating frequency of 600 MHz for protons under the following conditions: flip angle - 45°, acquisition time – 3.5 s, the relaxation delay - 4 s, spectra width - 18 ppm, spectrum offset - 7 ppm, free induction decay (FID) data points - 32 kbyte, the number of scans - 64, the time for each spectrum was about 8 minutes. Data acquisition and processing were performed with Delta IV. The phase and baseline correction were done automatically, the integration was done manually. RSD is less 2%.

## 3 Results and Discussion

Earlier during the screening of flavonoids content in dietary supplements, we found [5] that $^1$H NMR spectra in a wide range of concentrations (1-50 mg / ml) contain narrow proton signals of a hydroxyl group in position 5 (5-OH) in the range from 11.8 to 13.5 ppm. The protons of 5-OH form a strong intramolecular hydrogen bond with the oxygen of the neighboring carbonyl group, so these nuclei are deshielded and their signals appear in DMSO-$d_6$ in the above range depending on the presence of a multiple bond in position 2-3 and the nature of the substituents. For example, for flavones - 12.80-13.50 ppm, flavonols - 12.40-12.65 ppm, flavonones - 12.0-12.20 ppm, flavononols - 11.80-11.96 ppm. [4,5]. Using of the 5-OH groups proton signals, it is possible to identify the FG in the extracts of plant origin and also to determine their content, because signals of aromatic and aliphatic protons do not appear in this region of the spectrum. As follows from Figure 1, all flavonoids **1–9** are characterized by the presence of 5-OH.

Biologically active substances of plant materials are usually extracted solvents with low boiling point (water, methanol, ethanol, acetone, ethyl acetate), which are subsequently removed. The presence of a solvent is possible for quantitative estimates of the **1–9** content from $^1$H NMR spectra, if 5-OH proton signals with a chemical shift >11 ppm are used. DMSO is an environmentally friendly non-toxic organic solvent with the highest dissolving ability for flavonoids. The high basicity of DMSO contributes to the narrowing of the

signals of 5-OH protons in the spectrum due to the slowdown of proton exchange. Diosmin was chosen as an internal standard for quantitative measurements of the **1–9** contents, the 5-OH protons $T_1$ relaxation time of which is less than or equal to the analyte protons $T_1$. The 5-OH protons $T_1$ of diosmin and **1-9** does not exceed 2 seconds in DMSO-$d_6$, which significantly reduces spectrum acquiring time. The 5-OH proton signal of diosmin is quite narrow, located in the same spectral region (12.95 ppm) with the 5-OH proton signals of analytes (Figure 2).

Validation of the new methodology in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ) were carried out for individual solutions of **1** or **2+3** at concentrations from 0.01 to 40 mg/ml. The linear correlation for **1** is described by the equation: y=0.9447x+0.2973, n=7, r=0.9986. LOD (S/N=3) was found to be 0.01 mg/ml for **1** (0.02 for **2+3**) and LOQ (S/N=10) was 0.03 mg/ml for **1** (0.05 mg/ml for **2+3**). The obtained results give the possibility of using the methodology for another plant materials containing flavonoids in smaller quantities than in the biomass of *Larix sibirica* wood or *Silybum marianum* seeds.

To assess the variations in the resource potential of *Larix sibirica* as a source **1**, its content in samples from different parts of wood (root, trunk) of one living tree of mature age (100-120 years) in the vicinity of Irkutsk city was monitored during the year. It was found that the content of **1** in the root biomass is about 50% of the sum of extractive substances in DMSO-d6 and varies from 0.8% in spring to 3.4% in autumn. The trunk biomass always contains **1** 2-3 times less, which is consistent with the results [1]. Comparison of the DMSO-d6 effectiveness as extractant **1** with the commonly used solvents (methanol/water, acetone, ethyl acetate) showed the completeness of extraction. The results of the $^1$H NMR and HPLC-UV methods [6] are consistent, although the latter is needs more time, requires standard samples and has errors 4-5 times higher.
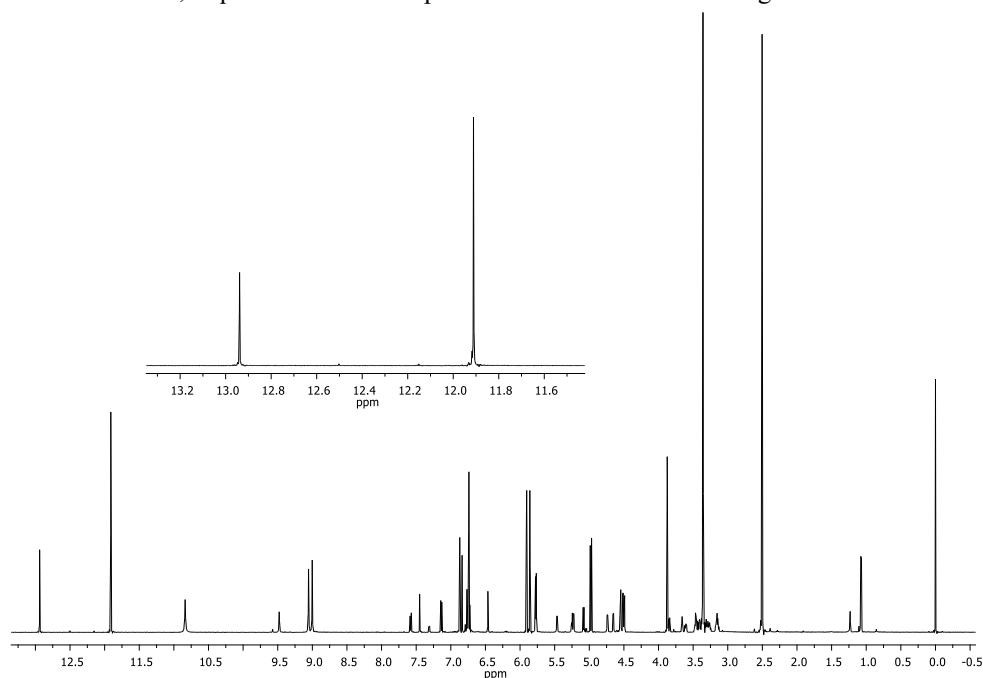


**Fig. 2.** $^1$H NMR Spectrum of *Larix sibirica* wood extract in DMSO-d6.

$^1$H NMR spectrum of flavonolignans of *Silybum marianum* seeds contain overlapping signals of 5-OH protons of components **2-8** in the range 11.9-12.0 ppm (Figure 3A), which

is free of signals from other components. Their area corresponds to their total content and allows to calculate it using the known molecular weight of **2-8**, which is equal to 482.4 g/mol. Proposed method allows to determine the suitability of using biomass, extracted from various parts of *Silybum marianum* at different periods and geographical locations of the raw materials. The quality of *Silybum marianum* also depends on the content of its oxidation products, which contain the $C_2$-$C_3$ double bond. These components have the 5-OH protons signals in the region of 12.1 - 12.5 ppm (Figure 3A). The proposed method for resource estimation of the most effective biologically active flavonolignans content in *Silybum marianum* significantly exceeds in simplicity, laboriousness, and accuracy the known method, based on HPLC-UV and recommended for quantitative determination of flavonolignans both in extracts and pharmaceutical preparations based on them [6].
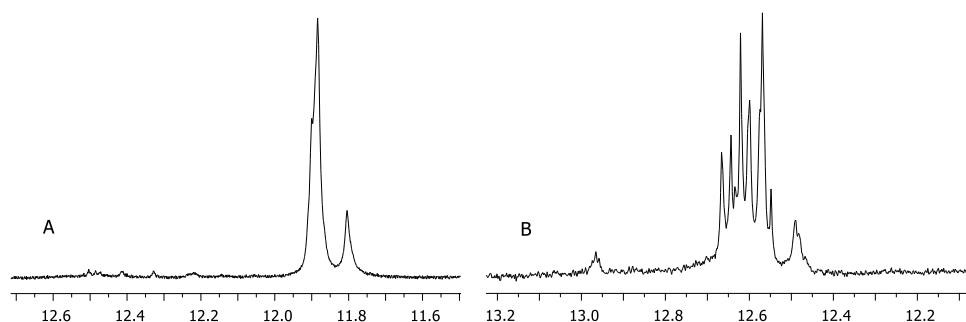


**Fig. 3.** Enlarged regions of ^{1}H NMR Spectra of A - *Silybum marianum* seeds extract and B - *Ginkgo biloba* leaves extract in DMSO-$d_6$.

According to [6], the FG content in the leaves of *Ginkgo biloba* is carried out by HPLC-UV after acid hydrolysis of the sample and recalculation of the flavonoid aglycones content to the mass of FG. The stage of sample preparation of our method does not include hydrolysis and, therefore, allows to characterize the native FG in the leaves of *Ginkgo biloba*, so their quantitative determination is direct. Figure 3B illustrates the ^{1}H NMR spectrum region of *Ginkgo biloba* extract, containing 5-OH proton signals in the 12.50-12.65 ppm region, which proves the presence of **9**. According to this Figure, analyzed sample contains at least ten dominant FG. The spectrum also has small signals at 12.47, which indicates the presence of a small amount of aglycons. The *Ginkgo biloba* extract contains flavonoids with different sizes of glycosidic part (1-3 carbohydrate residues), and, accordingly, molecular weight. The average ratio of the molecular weight of FG and their aglycones is close to 2.5 [3], and the value of the average molecular weight is about 759 g/mol. Quantitative determination of FG in different *Ginkgo biloba* samples (I-VI) was carried out by comparing the integrals of their 5-OH proton signals with the 5-OH proton signal of diosmin. The NMR results were compared with the results obtained on HPLC-UV to determine the accuracy. The results of the FG content determining by ^{1}H NMR and by HPLC in the analyzed different *Ginkgo biloba* samples are presented in Table 1.

**Table 1.** Results of FG determination using ^{1}H NMR spectroscopy and HPLC-UV (n=5, P=0,95)

| Sample | NMR | HPLC-UV |
|---|---|---|
|  | C, mg/ml | C, mg/ml |
| I | 0.19±0.02 | 0.18±0.02 |
| II | 0.32±0.01 | 0.33±0.04 |
| III | 0.19±0.02 | 0.18±0.05 |
| IV | 0.19±0.04 | 0.19±0.02 |
| V | 0.41±0.04 | 0.35±0.04 |
| VI | 0.22±0.02 | 0.18±0.02 |

The reproducibility of the NMR measurement results is less 10%. The limit of detection (LOD) of the method with a signal to noise ratio (S/N) = 3 is 0.04 mg/ml, and the limit of quantitation (LOQ) (at S/N = 10) is 0.1 mg/ml. The developed ^{1}H NMR method for the FG determination in different *Ginkgo biloba* raw material is sensitive, linear and reproducible.

## 4 Conclusion

A new approach for the rapid measurement of taxifolin in *Larix sibirica* wood, flavonolignans in *Silybum marianum* seeds, flavonol glycosides in *Ginkgo biloba* leaves by ^{1}H NMR spectroscopy is proposed. NMR spectroscopy has showed significant advantages, such as no need for standard samples and simple sample preparation, over other methods of analysis. According to the obtained results, it was determined that the use of deshielded 5-OH protons signals of flavonoids is convenient for their identification and quantification in plant materials. Validation characteristics indicate the possibility of applying of the proposed methodology for assessing plant resource potential. This approach was also tested on other plant materials containing flavonoids, such as a roots of *Scutellaria baicalensis* and leaves of *Vitex agnus-castus*, which expands its applicability.

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