Determination of polyphenolic compounds in raw plant materials by 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. For this purposes mass-spectrometry and NMR spectroscopy are widely used. Both methods have their own advantages and disadvantages. But NMR spectroscopy is a non-destructive method and requires simple sample preparation. In addition, quantitative studies using NMR spectroscopy do not require authentic standard samples. We have developed various techniques for targeted metabolomic analysis of plant origin samples using NMR spectroscopy, including: determination of anthraquinone derivatives in extracts from roots and rhizomes of *Rubia tinctorum* L., determination of diosmin in *Hyssopus officinalis* L., determination glucofrangulin A in extracts from leaves of *Rhamnus frangula* L.

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.

Polyphenolic compounds play an important role in plant life. In addition, most of them have biological activity. We used ^{1}H NMR spectroscopy to develop a universal technique for target metabolomics analysis of anthraquinone derivatives of *Rubia tinctorum* L. roots

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and rhizomes, flavonoid diosmin of *Hyssopus officinalis* L. and anthraquinone derivatives of *Ramnus frangula* L. bark. All these plants are medicinal plant raw materials. Two of the three studied plants are included in the State Pharmacopoeia of the Russian Federation - *Rubia tinctorum* L. and *Hyssopus officinalis* L. [1]. The 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.

1.1 *Rubia tinctorum* L.

Rubia tinctorum L. is an herbaceous perennial plant with a woody horizontal rhizome and branched climbing shoots up to 1.5-2 meters high. *Rubia tinctorum* L. grows in Eastern Europe, the Mediterranean, Asia Minor, and Central Asia. In Russia, it is native to the south-eastern part of the country and to the Caucasus region. From prehistoric times till the end of the 19th century, when synthetic dyes were introduced, the roots and rhizomes of *Rubia tinctorum* L. have been used as a natural red dye source. At present, *Rubia tinctorum* L. is mostly utilized as a medicinal plant. It was reported to exert the nephrolithic effect, has the antidiabetic potential in type II diabetes, possesses the antimicrobial and cytotoxic activities, and significantly reduces the genotoxicity of several carcinogens [2 - 8].

1.2 *Hyssopus officinalis* L.

Hyssop (*Hyssopus officinalis* L.) is a branched subshrub that can reach heights up to 60 cm. It belongs to the Lamiaceae family. Initially, the plant was widespread in the Mediterranean and Central Asia. Now it can be found in Russia in a wild state. As a rule, hyssop is founded in the forest and steppe zones of the European part of our country, in the Caucasus and Altai. The plant prefers to grow on dry hills and rocky places.

1.3 *Rhamnus frangula* L.

Rhamnus frangula (Frangula alnus Mill. syn. *Ramnus frangula* L.) is a shrub usually up to 7 m high [9,10]. It is a light-loving species that thrives in temperate forests, mainly in slightly acidic, moist soils. *Ramnus frangula* L. has been used in folk medicine due to its extremely laxative effect of the bark or decoctions of the black berries since the Middle Ages [11].

2 Materials and methods

2.1 Plant material, solvents and standards

Crushed commercial samples: leaves of *Hyssopus officinalis* L., bark of *Ramnus frangula* L. and roots and rhizomes of *Rubia tinctorum* L. were analyzed in this research. The following solvents and reference samples were used: dimethyl sulfoxide-d6 (≥99.9%, Sigma-Aldrich, CAS number 2206-27-1), a standard samples of diosmin (CAS number 520-27-4), ruberythric acid (CAS № 152-84-1) and lucidin3-primveroside (CAS № 29706-59-0).

2.2 Sample preparation and experiments

Based on our previous work [12] optimal sample preparation was carried out for all samples. Approximately 20 mg (exact weight) of the powder were placed into a 2 mL

microcentrifuge tube, and 0,8 mL of deuterated dimethyl sulfoxide was added. The sample was sonicated for 30 min in an ultrasonic bath (Sapphire-9.5 TTC, Russia), placed into the laboratory shaker (IKA VXR S0000, Germany), mixed, and centrifuged (Eppendorf Mini Spin, Germany) for 5 min (14,000 rpm). The supernatant was collected and transferred into a 5 mm NMR tube prior to analysis.

The main experiment was carried out on a JNM ECA-600 NMR spectrometer (JEOL, Japan) with an operating frequency for protons of 600 MHz under the following conditions: relaxation delay - 40 sec., points - 32768, sweep - 22 ppm, acquisition time – 1.98 sec., scans – 16, angle – 45º.

Post-registration processing of ^{1}H NMR spectra was analyzed using Delta IV software (Japan). The phase, baseline and signal integration were adjusted manually.

3 Result and discussion

At least one signal of each biologically active substance should not be overlapped with the others to develop a method for identifying and quantifying individual biologically active substances in complex mixtures. Identification of organic substances by ^{1}H NMR spectroscopy is obtained either by comparing the spectrum of the analyzed compound with the spectrum of a standard sample, or by matching the spectral characteristics of the test sample with the data described in the literature. For this purpose, we recorded the individual ^{1}H NMR spectra of diosmin, ruberythric acid and lucidin3-primveroside reference samples as well as the ^{1}H NMR spectra of *Hyssopus officinalis* L and *Rubia tinctorum* L. extract obtained in the laboratory via direct extraction with DMSO-d_6. For the determination of the target analyte in the of *Ramnus frangula* L., we chose the other option, since we did not have the required standard samples of glucofrangulin A.

3.1 *Rubia tinctorum* L.

Comparing the ^{1}H NMR spectra of a *Rubia tinctorum* L. extract and standard samples of ruberythric acid and lucidin-3-primveroside, we can observe the next signals: two doublet proton signals at 7.62 ppm and 7.74 ppm (J = 8.5 Hz each) in the ^{1}H NMR spectrum of the standard sample of ruberythric acid and 7.62 ppm (J = 8.5 Hz) in the ^{1}H NMR spectrum of the *Rubia tinctorum* L. extract, which do not overlap with other aromatic proton signals. The coupling constants of these doublet signals also coincide. The obtained data imply that these signals can be used for the identification and quantification of ruberythric acid and lucidin-3-primveroside in the extract.

Quantitative determination was carried out by comparing the integral intensity of the target biologically active substance with the signal of residual protons of DMSO-d_6. The values obtained as a result of integration were entered into an Excel table for further calculations. Three parallel measurements were carried out for the studied *Rubia tinctorum* L. roots and rhizomes extract. Thus, as a result of calculations, the content of ruberythric acid and lucidin-3-primveroside in the studied sample: 1.67±0.02 % and 2.18±0.04 %, accordingly. The developed method was validated.

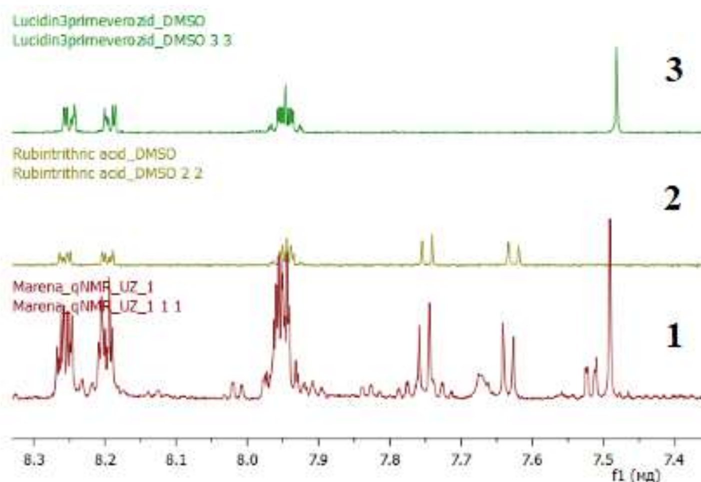

Fig. 1. Stack of ^{1}H NMR spectra in DMSO-d_6: 1) extract of roots and rhizomes of *Rubia tinctorum* L., 2) ruberythric acid standard, 3) lucidin-3-primeveroside standard.

3.2 *Hyssopus officinalis* L.

Comparing the ^{1}H NMR spectra of a *Hyssopus officinalis* L. extract and standard sample of diosmin, we can observe the next signals which do not overlap with the signals: δ 12.93 (s, 1H, OH-5), 9.46 (s, 1H, OH-3′), 7.57 (dd, J = 8.5, 2.2 Hz, 1H, H-6′), 7.44 (d, J = 2.2 Hz, 1H, H-2′), 7.13 (d, J = 8.7 Hz, 1H, H-5′), 6.82 (s, 1H, H-3), 6.76 (d, J = 2.1 Hz, 1H, H-8), 6.46 (d, J = 2.1 Hz, 1H, H-6). The coupling constants of these signals also coincide. This leads to the conclusion that presented signals can be used to identify diosmin in the extract of *Hyssopus officinalis* L. by ^{1}H NMR spectroscopy. To estimate the diosmin content in the extract of *Hyssopus officinalis* L. by ^{1}H NMR spectroscopy, we used a doublet signal at 7.44 ppm.

Quantitative determination was carried out by comparing the integral intensity of the target biologically active substance with the signal of residual protons of DMSO-d_6. The values obtained as a result of integration were entered into an Excel table for further calculations. Three parallel measurements were carried out for the studied *Hyssopus officinalis* L. leaves extract. Thus, as a result of calculations, the content of diosmin in the studied sample is 0,75±0,04%%. The developed method was validated.

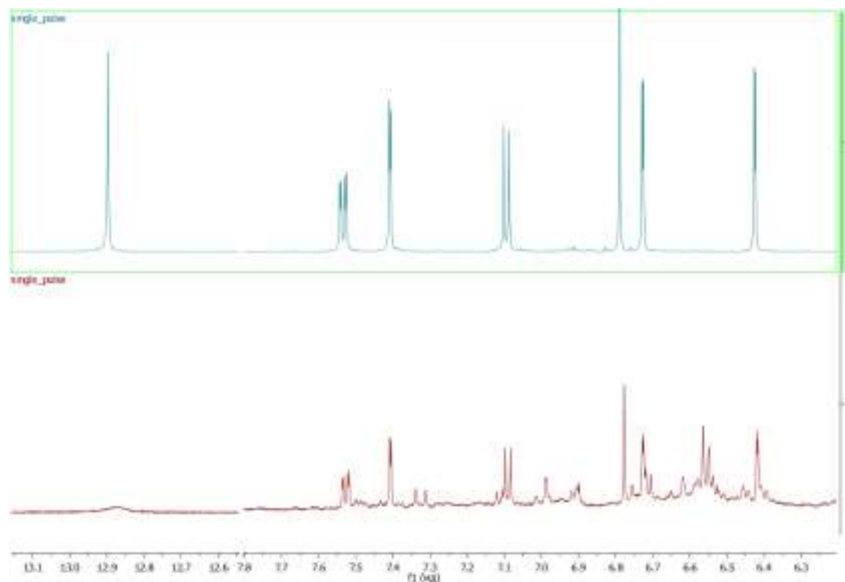

Fig. 1. Stack of ^{1}H NMR spectra in DMSO-d_6: 1) leaf extract of Hyssopus officinalis L., 2) standard sample of diosmin.

3.3 *Rhamnus frangula* L.

The ^{1}H NMR spectra of anthracene derivatives of biologically active substances of *Ramnus frangula* L. bark extract are described in [13]. Spectral data from this source were used for identification and further quantification of glucofrangulin A. To identify glucofrangulin A, signals from its protons at positions H-7, H-5 and OH-1 were used, appearing at 7.28, 7.50 and 13.00 ppm, consequently. Signal at 7.28 ppm selected for the quantitative determination of glucofrangulin A in the extract of *Ramnus frangula* L.

Quantitative determination was carried out by comparing the integral intensity of the target biologically active substance with the signal of residual protons of DMSO-d_6. The values obtained as a result of integration were entered into an Excel table for further calculations. Three parallel measurements were carried out for the studied *Ramnus frangula* L. bark extract. Thus, as a result of calculations, the content of glucofrangulin A in the studied sample is 2.09±0.07 %.

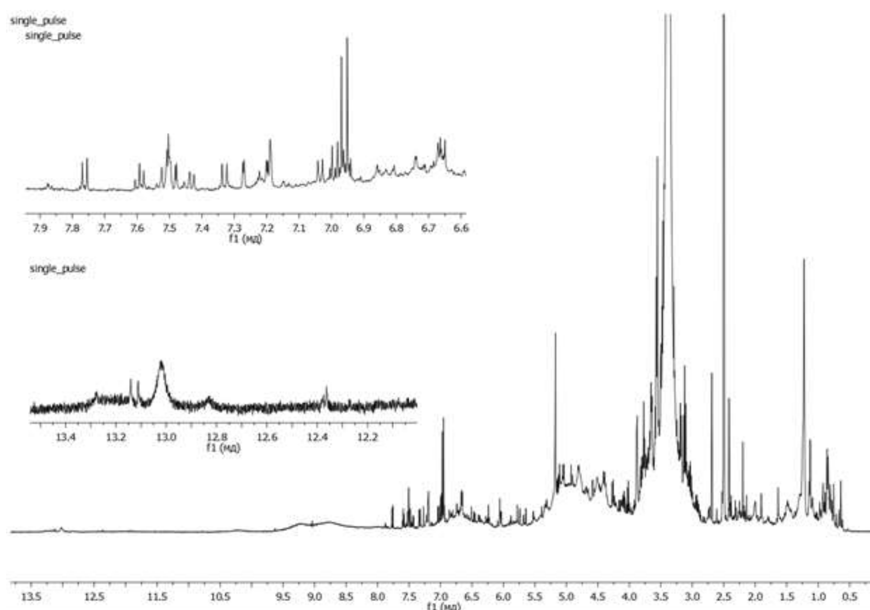

Fig. 1. ^{1}H NMR spectrum of *Ramnus frangula* L. in DMSO-d_6 also shows increased spectrum fragments of 6.6 - 7.9 ppm and 12.2 - 13.4 ppm.

3 Conclusions

A new approaches for the rapid determination of anthraquinone derivatives of *Rubia tinctorum* L. roots and rhizomes, flavonoid diosmin of *Hyssopus officinalis* L. and anthraquinone derivative of *Ramnus frangula* L. bark by ^{1}H NMR spectroscopy is proposed. NMR spectroscopy has showed significant advantages, such us no need for standard samples and simple sample preparation, over other methods of analysis. Validation characteristics indicate the possibility of applying of the proposed methodology for assessing plant resource potential.

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