The determination of flavan-3-ol content in the root of *Rhodiola Kirilowii*

AGNIESZKA GRYSZCZYŃSKA*, SEBASTIAN MIELCAREK, WALDEMAR BUCHWALD

Institute of Natural Fibres and Medicinal Plants
Libelta 27
60-707 Poznań, Poland

*corresponding author: phone: +4861 6659540, fax.: +4861 6659551,
e-mail: agnieszka.gryszczynska@iwnirz.pl

S u m m a r y

According to a new classification, nearly 90 species belong to the *Rhodiola* family. However, only three of them are most frequently used in alternative medicine. In addition to *R. rosea* and *R. quadrifida*, *R. Kirilowii* can also be included in this group. This species was investigated for flavan-3-ol content. Due to beneficial properties of this class of compounds (free radical scavenging and reactive oxygen forms), the researchers decided to examine 5 substances in the root and in two extracts, alcoholic and aqueous ones. Depending on the matrix analysed, the content of particular components varies. Furthermore, it is also affected by extraction time as well as a particular extraction solvent employed. An ultra performance liquid chromatograph connected to a tandem mass spectrometer (Waters) was used for the assay. The application of this analytical method allows to detect very small amounts of analytes.

Key words: *flavan-3-ols, Rhodiola Kirilowii, UPLC-MS/MS*

INTRODUCTION

Rhodiola (golden root) belongs to the *Crassulacae* family. It grows primarily in the cold climate of the Eastern Hemisphere on rocky substrate, moist slopes, and cliffs. According to the Index Kewensis (1885), the *Rhodiola* genus consists of 15 species. The following species are most frequently used in pharmacology: *R. rosea, R. quadrifida,* and *R. Kirilowii*. A more recent classification of this family includes ca. 90 species, out of which 70 grow in China at an altitude of 1000-5600 m a.s.l. [1].

Representatives of this plant family have been used in traditional eastern medicine for centuries. The medicinal properties of plants of the genus *Rhodiola* are compared to the pharmacological use of *Ginseng* and *Manyprickle Acathopanax*. The positive advantage of the use of golden root is the fact that no undesired effects are observed [2]. Not only Asian alternative medicine utilises the medicinal activity of *Rhodiola*. Extracts from this plant are used both in Europe and in the USA [3].

Due to the richness of different types of compounds, *Rhodiola* shows a protective effect on human organism. Oral application of *R. Kirilowii* extract has a protective effect on people with circulatory system disorders who live at high altitudes above sea level [4]. This preparation is also given to astronauts, pilots, and mountain climbers [5, 6]. Alternative medicine uses *Rhodiola* root to enhance the ability to prevent the oxygen deficiency in the organism, to reduce the content of glucose blood and to increase the antithrombotic activity [5]. It is also used as an antibacterial, antifungal, anti-inflammatory. In *in vitro* assays, the hepatitis C virus and *Mycobacterium tuberculosis* inhibitory activity has also been confirmed. In China this plant is traditionally used as a tonic water, antibacterial and anti-inflammatory medicine, which shows the anti-coagulation effect and reduces content of glucose in blood [7].

Flavan-3-ols, also called proanthocyanidins, show many properties beneficial for the organism. Among others, the use of supplements containing *Rhodiola* extract protects the organism against many pathogenic agents. Flavan-3-ols exhibit bioactive (antioxidative), activity, thus, they protect against the harmful effects of free radicals and reactive oxygen forms. They also show anticarcinogenic, anti-inflammatory, antiallergic, antimutagenic, and antiaging activity as well as improve the functioning of liver [3]. In the literature there are also reports on the fact that catechins prevent obesity as well as tests on animals have confirmed their hypocholesterol activity [8].

The researches concerning interactions between proanthocyanidins and organisms are very popular. Plants containing substances from this class of compounds can be attractive to insects or show deterrent properties, thereby protecting themselves against pathogenic agents and environmental stress.

PURPOSE

The purpose of the present study was to determine the flavan-3-ol content in the root of *Rhodiola Kirilowii*. An ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC-MS/MS; Waters) was used for the determination of the content of these compounds. This method leads to precise identification of compounds by using soft ionization as the detection method.

MATERIALS AND METHODS

Plant material

Rhodiola Kirilowii root was collected in October 2009 from field crops of the Institute of Natural Fibres and Medicinal Plants.

Reagents

The following reagents were used in the experiment: methanol, acetonitrile LC-MS (Fluka), diethyl ether HPLC (SIGMA), sodium sulphate, formic acid HPLC (Merck). Comparison substances: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigalocatechin gallate (ChromaDex), D-(-)-salicine (SIGMA).

Comparison solution

The stock solution concentration was c=0.1 mg/ml. An exact amount of 1.00 mg of a comparison substance was weighed out in a 10 ml volumetric flask, was dissolved in 8.0 ml of methanol and the solution was made up to the mark with the same solvent (stock solution).

Methanol solution in the amount of 1.0 ml with 1 mg/ml was transferred to a 10 ml volumetric flask. The solution was made up to the mark with water (working solution).

A number of dilutions of each analyte were prepared in order to determine the standard curve. The solution mixture was prepared by taking an adequate volume of the working solution and making up the solution to 1.0 ml. In this way, a number of dilutions was obtained in a range of 100–1000 ng/ml ($n=5$).

Preparation of *Rhodiola Kirilowii* root extracts

Preparation of 50% ethanol extract

The powdered plant material was extracted with 50% *v/v* ethanol using the percolation method at a 1:10 ratio of plant material to solvent. After evaporating the alcohol under reduced pressure at a temperature of 40–45°C and then freezing at –55°C, the percolate obtained was lyophilized.

Preparation of aqueous solution

The powdered plant material was extracted with purified water for 3 hours at 90ºC, with a ratio of plant material to solvent of 1:10. Subsequently, after gauze and cotton filtering, the extract was frozen at –55ºC and lyophilized.

The dry extracts were placed into polyethylene containers with a lid equipped with a dehydrator and stored at a temperature of 20–25ºC.

Preparation of a test sample

Plant material, dry, aqueous and hydroalcoholic extracts from *Rhodiola Kirilowii* root

In order to prepare a test sample, the method of flavan-3-ol extraction from bark, developed by P. Mammela [9] was adapted for the needs of the present study. An exact amount of ca. 0.5 g of plant material (ca. 0.5 of dried powdered (0.315)) *Rhodiola Kirilowii* root was weighed out and placed in a 20 ml volumetric flask. 15.0 ml of 80% (*v/v*) methanol was added and the solution was subjected to ultrasounds for 60 min. at a temperature of 20–25ºC. Subsequently, the solution was then made up to the mark with the same solvent and filtered on a quantitative filter paper. The filtrate was concentrated to evaporate the methanol up to a volume of about 1/5 in a rotary evaporator in vacuum. The residue was extracted with 4 ´ 16.0 ml of diethyl ether. The combined ether extracts were dried with anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator in vacuum. The dry residue was dissolved in 4.0 ml of 10% (*v/v*) methanol and transferred quantitatively to a 5 ml volumetric flask. 0.023 ml of 0.5 mg/ml D-(-)-salicine (IS) was added and the solution was made up to the mark with 10% (*v/v*) methanol. The sample was filtered through a membrane filter with a diameter of 0.20 μm.

LC-MS/MS assay

The assay was performed using an ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC-ESI MS/MS; Waters). The separation of analytes was performed on an Acquity UPLC BEH C18 column, 1.7 μm 2.1´50 mm (Waters). Mobile phase: phase A: 0.1% (*v/v*) HCOOH solution in water, phase B: 0.1% (*V/V*) HCOOH solution in acetonitrile. Mobile phase flow rate was: 0.20 ml/min. The assay was performed in gradient elution: 0.0 min. – 97% of phase A, 7.5 min. – 68% of phase A, 9.0 min. – 97% of phase A. Column temperature was 25ºC; ion source temperature: 120ºC; desolvation temperature: 350ºC. Gas flow rate: desolvation gas: 700 L/h; cone gas: 10L/h. The chemical structures of particular falvan-3-ols are shown in Fig. 4.

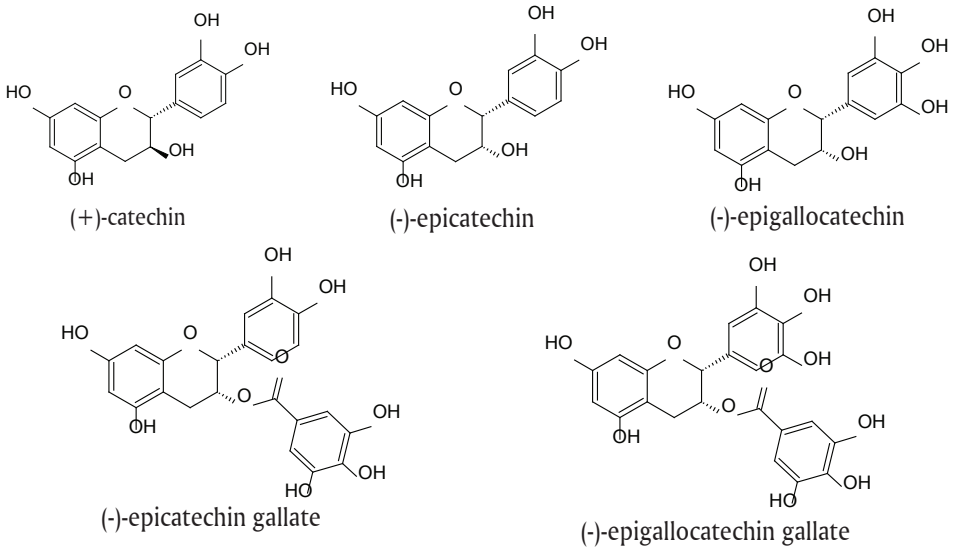

Figure 4.
Structures of flavan-3-ols

RESULTS AND DISCUSSION

Calibration curves for flavan-3-ols

Calibration curves were made for all the analytes. In order to make them, standard solutions were prepared with concentrations of 100–1000 ng/ml. In this way, the following curve parameters were determined: (+)-catechin y=2.502x-141.463 (r^2=0.991), (-)-epicatechin y=2.729x-165.783 (r^2=0.991), (-)-epigallocatechin y=6.083x-652.836 (r^2=0.995), (-)-gallate epicatechin y=0.505x-35.074 (r^2=0.996), (-)-gallate epigallocatechin y=4.421x-451.039 (r^2=0.993), using D-(-)-salicine (IS) y=0.184x-4.155 (r^2=0.991).

System suitability test

Flavan-3-ol content in *Rhodiola Kirilowii* root

The flavan-3-ol extraction method was subjected to optimization which was designed to determine the content of: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-gallate epicatechin, (-)-gallate epigallocatechin, using D-(-)-salicine (IS) in particular plant materials. The following plant materials were investigated: *Rhodiola Kirilowii* root, hydroalcoholic and aqueous extracts. The analytical meth-

od employed was evaluated for precision, linearity and accuracy. Precision and linearity were evaluated by using regression analysis for each of the comparison substances used (tab. 2.) The accuracy was analysed using the enrichment method by adding to the sample dry hydroalcoholic extract with the determined level of flavan-3-ols (Tab. 3.). The content of these components was determined by means of two methods: HPLC DAD and UPLC MS/MS.

Table 1.

Characteristic parameters of flavan-3-ol detection

compound	retention time [min]	RSD_{time} (n=15) [%]	RSD_{area} (n=5) [%]	fragmentation m/z
(+)-catechin	3.53	0.15	6.33	289→109
(-)-epicatechin	4.19	0.20	8.04	289→109
(-)-epigallocatechin	3.28	0.11	5.41	305→125
(-)- epicatechin gallate	5.49	0.06	4.86	441→169
(-)-epigallocatechin gallate	4.27	0.08	2.91	457→169
D-(-)-salicine (IS)	2.72	0.31	1.33	285→123

Table 2.

Method recovery (n=3)

sample	recovery [%]				
	(+)–catechin	(-)-epicatechin	(-)-epigallo-catechin	(-)-gallate epicatechin	(-)-gallate epigallocatechin
20%	90.33	92.71	96.83	96.28	97.37
40%	85.45	95.79	96.44	99.86	101.13
80%	91.73	84.43	85.39	106.00	100.17

Table 3.

Method precision (n=6)

sample	(+)-catechin		(-)-epicatechin		(-)-epigallo-catechin		(-)-gallate epicatechin		(-)-gallate epigallocatechin	
	content [%]	RSD	content [%]	RSD	content [%]	RSD	content [%]	RSD	content [%]	RSD
root	0.000097	2.06	0.000288	2.08	0.019584	7.53	0.005294	4.55	0.135435	2.00
50% ethanol extract	0.000377	1.86	0.001651	1.51	0.097357	3.81	0.004045	3.51	0.266494	2.72
aqueous extract	0.000565	2.12	0.000858	3.38	0.031558	5.81	0,005138	2.80	0.304449	3.78

The ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC-MS/MS) allows to perform the a quantitative assay based

on a specific qualitative assay. The qualitative assay is designed to select proper fragmentation parameters, characteristic for given comparison substances. This process is possible thanks to the presence of two quadruples; the first of them is sensitive to the parent ion, while the other one is sensitive to the fragmentation ion. An MRM chromatogram is an image of this process. Figure 1 shows the MRM chromatogram of the *Rhodiola Kirilowii* root assay. The compounds were assayed using soft ionization in the negative ion source (ESI-). The signals visible in the chromatogram come from the parent ion fragmentation $[M-H]^-$. The fragmentation of particular analytes is presented in table 1 and in figure 5. The selection of the most specific fragmentation allows the identification of a small content of an analyte. The content of particular components is presented in table 3.

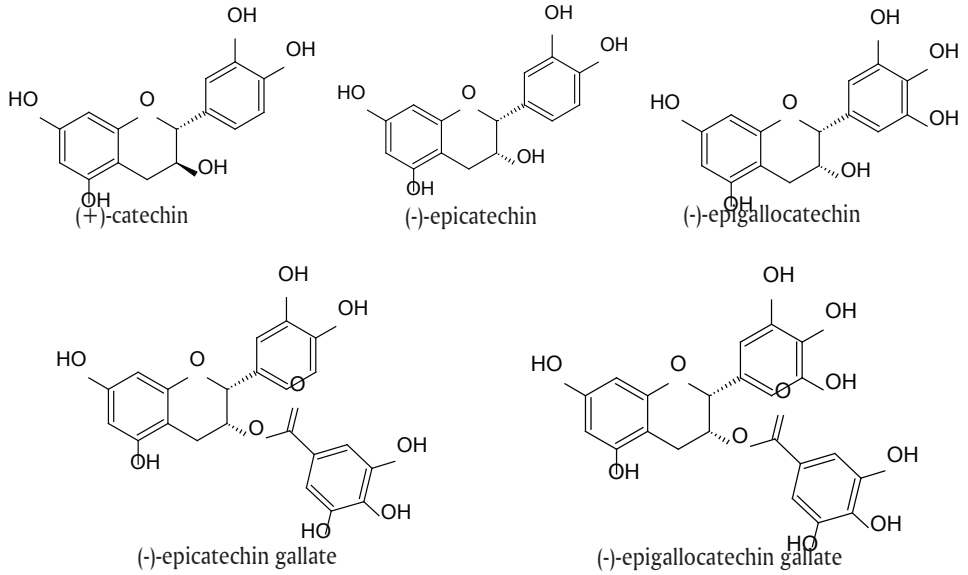

Figure 5.
Fragmentation of particular flavan-3-ols

When analysing the obtained results it can be said that the content of particular components depends on the matrix analysis employed. The compounds, i.e. (-)-epicatechin and (-)-epigallocatechin, are found at highest concentrations in the ethanol extract, while (+)-catechin and (-)-gallate epigallocatechin in the aqueous extract. The plant material is the largest carrier of (-)-gallate epicatechin. The protracted procedure of extract preparation is probably responsible for such a situation. The preparation of an extract from plant material lasts one hour, while of the other extracts – more than a dozen hours. This may significantly affect the transfer of substances to the extraction solvent. When looking at figures 1, 2, 3, which showing the chromatograms of the extracts and of *Rhodiola Kirilowii* root, certain

significant differences can be noticed. The MRM chromatogram of the aqueous extract (Fig. 3.) has additional signals in the channels 289→109, 305→125 and 457→169. In the (+)-catechin and (-)-epicetechin decay channel, two additional signals can be distinguished with t_r=4.26 and t_r=5.46. The (-)-epigallocatechin channel has an additional peak with t_r=2.37, whereas the (-)-epigallocatechin gallate channel – a peak with t_r=4.54. M. In their paper related to different catechin and epicatechin isomers, Kofink et al. [10] report that depending on temperature and pH these substances can change their spatial configuration as a result of the epimerization reaction. The study conducted by M. Kofink and his coworkers also proves that the epimerization reaction does not occur under the influence of the transmission of oxygen, light, and UV rays through the (-)-epicatechine solution. Hence, external factors are not the cause of additional peaks [M. Kofink 2007]. Therefore, due to the similarity in the decomposition of the compound and in tension parameters, it can be presumed that these can be the same substances, but being different spatial isomers. Due to the lack of other isomer standards, it is difficult to confirm the spatial structure of the additional peaks.

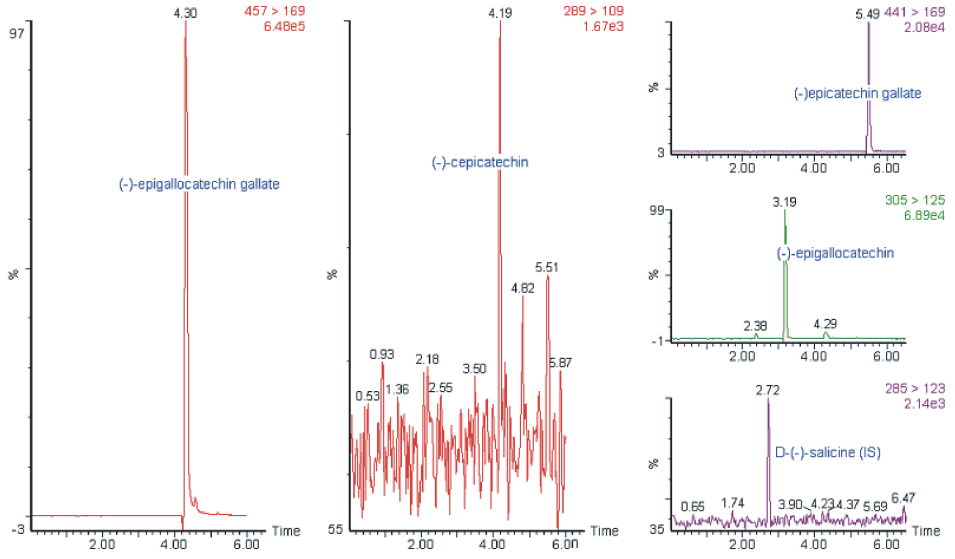

Figure 1.
The MRM chromatogram showing fragmentation of flavan-3-ols from *Rhodiola Kirilowii* root

This research project was financed by the Ministry of Science and Higher Education under grant no. N N405 306136.

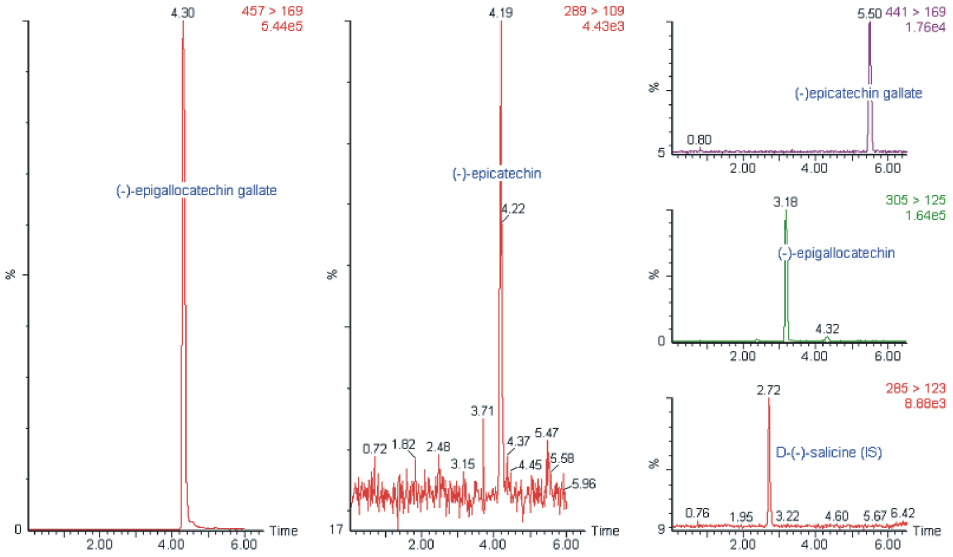

Figure 2.
The MRM chromatogram showing fragmentation of flavan-3-ols from *Rhodiola Kirilowii* hydroalcoholic extract

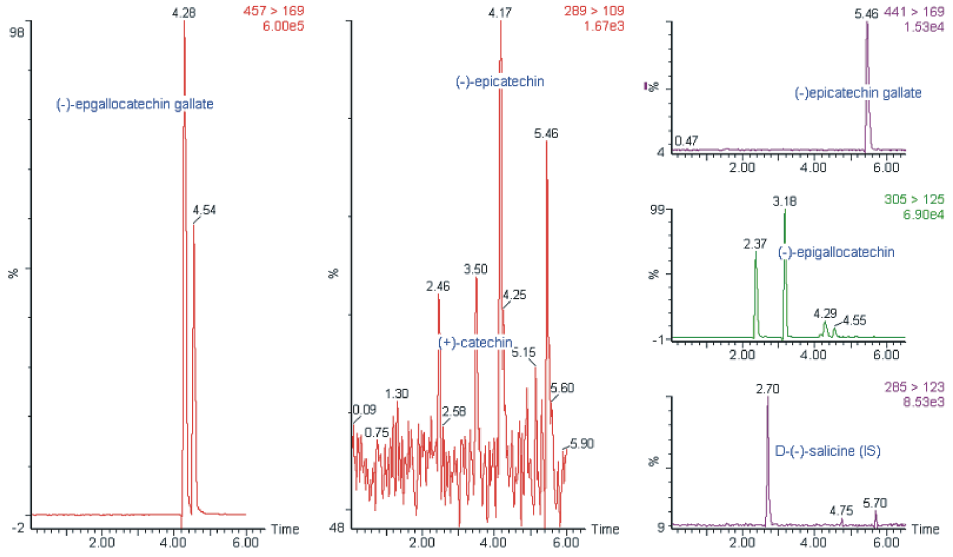

Figure 3.
The MRM chromatogram showing fragmentation of flavan-3-ols from *Rhodiola Kirilowii* aqueous extract

REFERENCES

1. Wolski T, Baj T, Ludwiczuk A, Głowniak K, Czarnecka G. Rhodiola genus taxonomy chemical composition, activity and use also phyochemical analysis of roots of two species of Rhodiola: Rhodiola rosea L. and Rhodiola quadrifida (Pall.) Fish et Mey, Postępy Fitoterapii 2008; 11(1):2-14.
2. Cui S, Hu X, Chen X, Hu Z. Determination of p-thyrosol and salidroside in three samples of Rhodiola crenulata and one of Rhodiola Kirilowii by capillary zon electrophoresis. Anal Bioanal Chem 2003; 377:370-374
3. Yousef GG, Grace MH, Cheng DM, Belolipov IV, Raskin I, Lila MA. Comparative phytochemical characterization of three Rhodiola species. Phytochemistry 2006; 67:2380-2391
4. Zych M, Furmanowa M, Krajewska-Patan A, Łowicka A, Dreger M, Mendlewska S. Micropropagation of Rhodiola Kirilowii plants using encapsulated axillary buds and callus. Acta Biologica Cracoviensia Series Botanica 2005; 47(2):83-87
5. Wójcik R, Siwicki AK, Skopińska-Różewska E, Buchwald W, Furmanowa M. The in vitro influence of Rhodiola Kirilowii extracts on blood granulocytes potential killing activity (PKA) in pigs. Centr Eur J Immunol 2009; 34(3):158-161
6. Zuo G, Li Z, Chen L, Xu X. Activity of compounds from Chinese herbal medicine Rhodiola Kirilowii (Regel) Maxim against HCV NS3 serine protease. Antiviral Research 2007; 76:86-92.
7. Skopińska-Różewska E, Bychawska M, Białas-Chromiec B, Sommer E, Furmanowa M, Mrozikiewicz PM, Buchwald W, Mielcarek S, Siwicki AK. The in vivo effect of Rhodiola Kirilowii extracts on blood granulocytes metabolic activity in mice. Centr Eur J Immunol 2010; 35(1):20-24.
8. Murakami I, Nakamura T, Ishibashi Y, Shibuya R, Ayano E, Morita-Murase Y, Nagata Y, Kanazawa H. Simultaneous determination of catechins and procyanidins in bottled tea drinks by LC/MS. Chromatography 2006; 27(1):27-33.
9. Mammela P. Phenolics in selected European hardwood species by liquid chromatography-electrospray ionization mass spectrometry. Analyst 2001; 126:1535-1538.
10. Kofink M, Papagiannopoulos M, Galensa R. (-)-Catechin in cacao and chocolate: occurrence and analysis of an atypical flavan-3-ol enantiomer. Molecules 2007; 12:1274-1288.

OZNACZANIE ZAWARTOŚCI FLAWAN-3-OLI W KORZENIU *RHODIOLA KIRILOWII*

AGNIESZKA GRYSZCZYŃSKA*, SEBASTIAN MIELCAREK, WALDEMAR BUCHWALD

Instytut Włókien Naturalnych i Roślin Zielarskich
ul. Libelta 27
60-707 Poznań

*autor, do którego należy kierować korespondencję: tel.: +4861 6659540,
faks: +4861 6659551, e-mail: agnieszka.gryszczynska@iwnirz.pl

Streszczenie

Zgodnie z nową klasyfikacją do rodziny *Rhodiola* należy ok. 90 gatunków. Jednak gatunkami najczęściej wykorzystywanymi w medycynie niekonwencjonalnej są jedynie trzy z nich. Oprócz *R. rosea* i *R. quadrifida* do tej grupy można zaliczyć *R. Kirilowii*. Gatunek ten został poddany badaniom na zawartość flawan-3-oli. Ze względu na bardzo korzystne właściwości tej klasy związków (zmiatania wolnych rodników i reaktywnych form tlenu), postanowiono zbadać zawartość 5 substancji w korzeniu i dwóch wyciągach: wodnym i alkoholowym. Zawartość poszczególnych składników różni się w zależności od analizowanej matrycy. Wpływ na to ma czas ekstrakcji, jak również zastosowanie konkretnego ekstrahentu. Do analizy wykorzystano ultrasprawny chromatograf cieczowy sprzężony z tandemowym spektrometrem mas (Waters). Zastosowanie tej metody analitycznej pozwala na wykrycie bardzo małych ilości analizowanych substancji.

Słowa kluczowe: *flawan-3-ole, Rhodiola Kirilowii, UPLC-MS/MS*