Analysis of the Marker Compounds of *Rhodiola rosea* L. (Golden Root) by Reversed Phase High Performance Liquid Chromatography

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An HPLC method permitting the first simultaneous detection of 5 marker compounds (salidroside, rosarin, rosavin, rosin, rosiridin) of *R. rosea* was developed. A separation was achieved within 27 min by using C-18 column material, a phosphate buffer/acetonitrile gradient system and at a separation temperature of 60 °C. All five compounds could be detected at concentrations as low as 0.62 μg/ml and were clearly assignable in *R. rosea* plant material and commercial products. Therefore, this quantitative and qualitative applicability of the method offers efficient and reliable means for the evaluation of *R. rosea* and products thereof.

Key words *Rhodiola rosea*; HPLC; phenylpropanoid; salidroside; rosavin

The genus *Rhodiola* (Crassulaceae) consists of nearly 200 species and among them *Rhodiola rosea* L. is the best known. The plant is indigenous to the arctic regions of eastern Siberia, but it is also found in the northern parts of Europe and Alaska. It is commonly known as golden or arctic root, can reach 75 cm in height and has yellow, fragrant flowers.[1] Similar to Siberian ginseng, the root of *R. rosea* is traditionally used as a tonic in Russia, and also as an antidepressant and antiinflammatory drug.[2,3] The first *Rhodiola* products were introduced as adaptogens to the western world several years ago.[1]

Intensive research on *Rhodiola* has been performed in the former Soviet Union, resulting in the isolation of several classes of compounds. Phenylpropanoids like rosarin (**2**), rosavin (**3**) and rosin (**4**) are not only typical for *R. rosea*, but are also pharmacologically active as antioxidants and neurostimulants.[4—6] The same activities were found for the hydroxyphenethyl glucoside salidroside (**1**), whereas rosiridin (**5**), a monoterpene, has mainly stimulant properties.[7]

Although several HPLC methods for the analysis of **1** or **3** in *R. rosea* have been reported, none of these allows an individual detection of all five marker compounds in one run.[8—10] Compounds **1** and **3** are not unique for *R. rosea* and their determination is therefore not sufficient for an exact identification.[4,11] Commercial *R. rosea* extracts are usually standardized for the content of "salidrosides" and "rosavins," without specifying the composition of these groups or the methods by which the material was standardized. Thus, in our ongoing effort to develop analytical methods for the quality assurance of dietary supplements, we have developed an HPLC method for the separation of compounds **1**—**5**.

Results and Discussion

The separation of *R. rosea* marker compounds is challenging because of several reasons. As seen in Fig. 1, compounds **2**—**4** only differ in their sugar moieties and a sensitive detection of **1** and **5** is only possible at low and rather unspecific wavelengths (Fig. 2). In addition, **1** is much more polar than the other marker compounds, thus the total separation time has to be increased excessively in order to obtain an acceptable separation.

By carefully assessing column materials of different manufacturers and optimizing the separation conditions, all five marker compounds could be baseline separated in 27 min (Fig. 3 shows sample NPC-RR-1). Optimum results were obtained with a Luna C-18 column from Phenomenex. Other stationary phases like Lichrosphere 5 RP18, Aqua 5 μ C18 or Selectosil 5 C18 could not resolve **3** and **4**, an unacceptable baseline drift was observed, or **1** was merging with other compounds of similar polarity. The use of a buffer at pH 7.0 as mobile phase improved the peak symmetry of all peaks of interest. Acidic buffer systems or the addition of acid were not advantageous since compound **5** was overlapped by peak

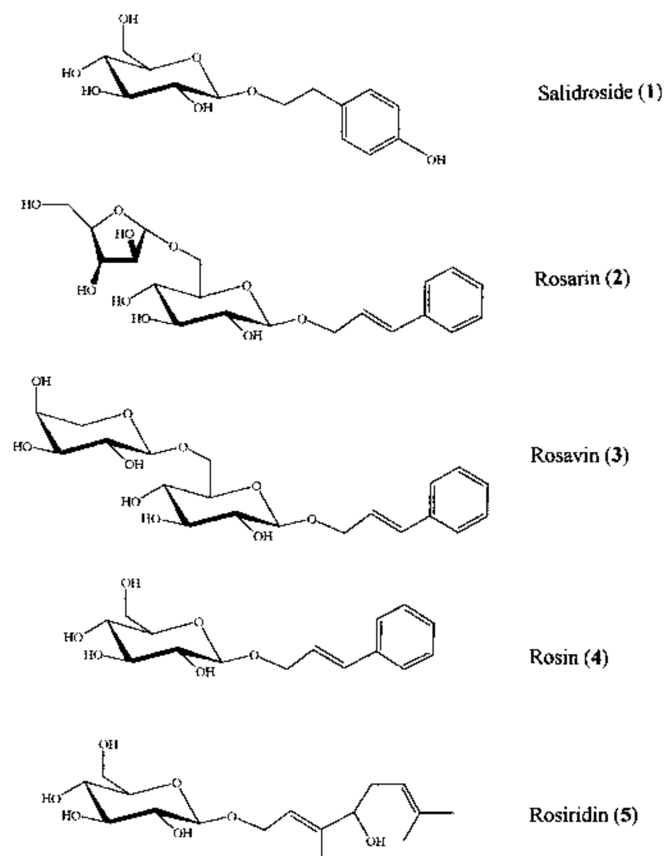

Fig. 1. Structures of the *R. rosea* Marker Compounds **1**—**5**

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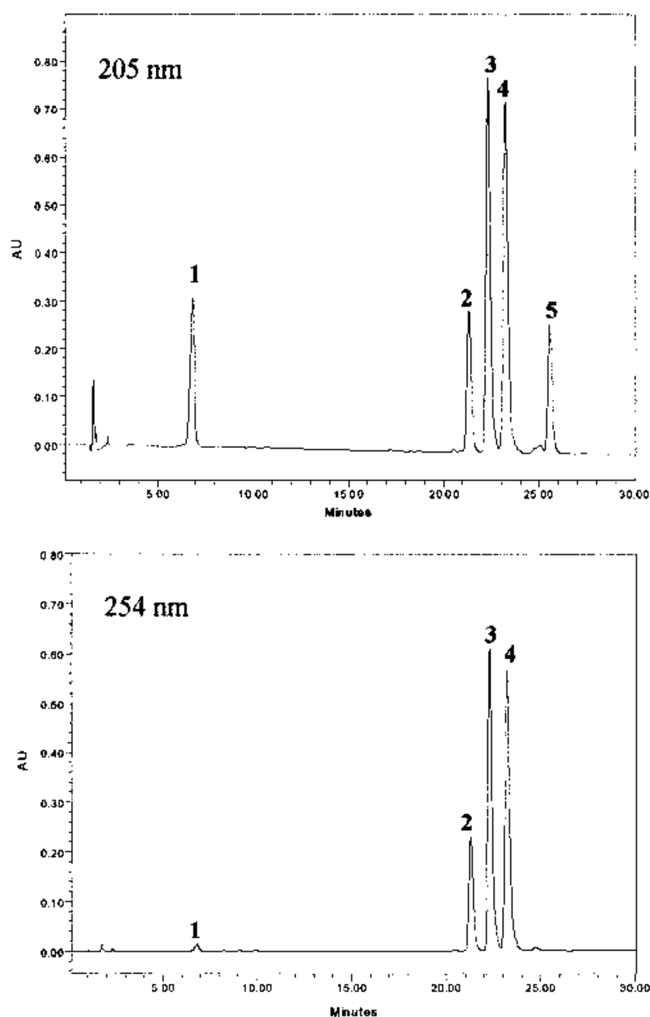

Fig. 2. Chromatogram of a Standard Mixture Separated by RP-HPLC

Column: Luna C18, 5 μm particle size, 150×4.6 mm; mobile phase: 25 mM phosphate buffer (pH 7.0)/acetonitrile, from 95/5 to 80/20 in 30 min; flow rate: 1.00 ml/min; detection: 205 and 254 nm; injected sample volume: 10 μl; temperature: 60 °C. Assignment of peaks according to Fig. 1.

a (unidentified compound) under these conditions. A higher separation temperature of 60 °C resulted in a significantly reduced separation time without any decrease of the peak resolution.

Owing to the high tannin content of the plant a steady increase of the column backpressure was observed while analyzing *Rhodiola* samples. From our previous experience with a similar problem we attempted to remove the tannins with polyamide.[12] This procedure was not successful, since both, the tannins and also some compounds of interest were removed. Therefore the column was washed with 0.1% phosphoric acid in methanol after each injection. This limited the buffer concentration to 25 mM, hence preventing the problem of salt precipitation in the mobile phase.

Methods for the assessment of product quality of pharmaceuticals require the determination of certain analytical parameters (accuracy, linearity, limit of detection, precision and peak purity) in order to establish their validity. The accuracy of our method was confirmed by performing a recovery experiment, where one sample (NPC-RR-1) was spiked with known amounts of the standard compounds. The recovery rates obtained were all between 97.49% (for **4**) and 100.88% (for **3**). Table 1 combines the calibration data for **1**—**5** and indicates the linearity of the detector signal in the concentration range tested (15.6 to 500.0 μg/ml). The limit of detection was between 0.16 (for **3** and **4**) and 0.62 μg/ml (for **5**). An indicator for precision is the relative standard deviation (δ). All samples were injected in triplicate, and as Table 2 shows, a maximal δ-value of 3.15% was obtained. Peak purity was confirmed by studying the photodiodearray (PDA) spectra of the peaks of interest; no indication of any impurity was found.

Prior to the analysis of different *R. rosea* samples, the efficiency of our extraction method was verified. Sample NPC-RR-3 was extracted under optimized conditions and each extraction step analyzed separately. After the third extraction a minimum of 95.2% of each compound was extracted; thus a threefold extraction of the samples was considered to be exhaustive.

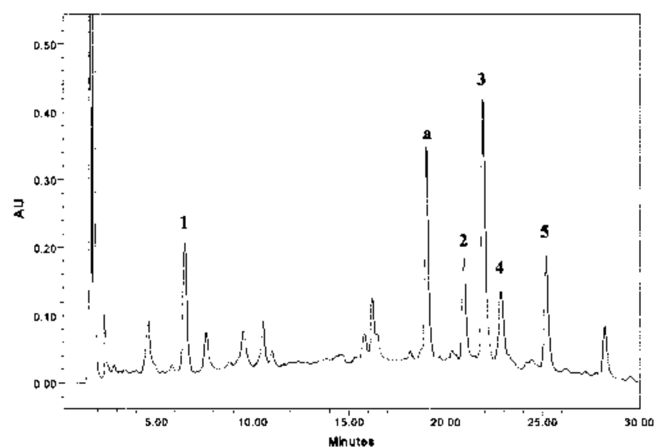

Fig. 3. Separation of a Methanolic *R. rosea* Root Extract (Sample NPC-RR-1) Separated under Optimized Conditions

Peak assignment according to Fig. 1, conditions same as Fig. 2, detection at 205 nm.

Table 1. Calibration Data for Compounds **1**—**5**, Including Correlation Coefficient (R^2), Regression Equation[a)] and Limit of Detection (LOD)

Compounds	R^2	Regression equation	LOD (μg/ml)
1	0.9999	$y=1.57\times10^4X$	0.56
2	0.9999	$y=2.84\times10^4X$	0.19
3	0.9999	$y=3.52\times10^4X$	0.16
4	0.9998	$y=3.89\times10^4X$	0.16
5	0.9999	$y=1.29\times10^4X$	0.62

a) *y* Reflects the peak area, *X* the amount of compound in μg/ml.

Table 2. Analysis of Different *R. rosea* Samples (Values in g/100g) and the Suggested Daily Dose of the Phenylpropanoids **2**—**4** in mg

Compounds	NPC-RR-1	NPC-RR-2	NPC-RR-3	NPC-RR-4	NPC-RR-5
1	0.27 (1.60)	0.04 (0.63)	0.09 (0.41)	0.06 (0.41)	0.22 (0.45)
2	0.11 (0.02)	0.02 (2.38)	0.02 (2.45)	0.02 (0.96)	0.07 (3.15)
3	0.35 (1.33)	0.10 (1.54)	0.07 (0.06)	0.06 (0.96)	0.22 (0.52)
4	0.08 (1.23)	0.03 (1.95)	0.03 (1.19)	0.02 (0.88)	0.07 (0.96)
5	0.38 (0.23)	0.16 (1.51)	0.11 (0.37)	0.07 (0.15)	0.29 (0.79)
2—**4**/d	—	—	0.78	2.94	6.87

Relative standard deviations are given in parentheses ($n=3$).

Five different *Rhodiola* samples were analyzed and all five standard compounds could be readily assigned (Fig. 3). Compounds **2** and **4** always were minor constituents but present in comparable ratios; compound **5** was most dominant in all specimens. The market products NPC-RR-3 to NPC-RR-5 showed considerable variations in their content of the marker compounds (Table 2). Although all of them contained *R. rosea* extracts, the amounts found were lower than in the pure plant material (NPC-RR-1). This can be explained by the fact that the products also contained excipients or other plant material in varying percentages. The differences in these products can be illustrated even better if the manufacturers suggestions for the daily intake are implemented. If only the total phenylpropenyl glycosides **2**—**4** are compared (they are pharmacologically active and *R. rosea* typical compounds), variations from 0.78 to 6.87 mg/d were observed.

In conclusion, the developed method permitted an accurate and reliable analysis of *R. rosea* marker compounds in plant material as well as in market products. Since all requirements for a validated method are fulfilled it should be a useful analytical tool not only for scientific purposes but also for commercial applications.

Experimental

Materials Standard compounds **1**—**5** were isolated in our laboratories from a *Rhodiola rosea* root extract (Lot 70628887), purchased from Nutratech (208 Passaic Avenue, Fairfield, NJ, U.S.A.). Identity and purity of the compounds were confirmed by chromatographic (TLC, HPLC) methods and comparisons with published spectral data (IR, 1D- and 2D-NMR, HRESIMS).[13] Additional plant material (NPC-RR-1 and NPC-RR-2) was obtained from NutraSource (San Carlos, CA, U.S.A.). *Rhodiola* market products (NPC-RR-3 to NPC-RR-5) were bought in a supermarket in Oxford/MS. Voucher specimens of all samples are deposited at the NCNPR.

All solvents (methanol, acetonitrile, and water) were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.); phosphoric acid and anhydrous sodium phosphate (monobasic and dibasic) were bought from Sigma (St. Louis, MO, U.S.A.).

Sample Preparation 1.00 g of the finely powdered root or product was extracted three times with 3 ml of methanol by sonication for 10 min. After centrifugation at 3000 rpm for 10 min, the supernatants were combined in a 10 ml volumetric flask and adjusted to the final volume with methanol. Prior to use, all samples were filtered through a 0.45 μm Acrodisc syringe filter from Gelman (Ann Arbor, MI, U.S.A.). Every sample solution was injected in triplicate; relative standard deviations were below 3.15% for all experiments.

For recovery experiments, 250 mg of sample NPC-RR-1 was spiked with 1000 μl of the stock standard solution. The sample was extracted by the above procedure, and recovery rates were 99.04% (**1**), 98.7% (**2**), 100.88% (**3**), 97.49% (**4**) and 98.28% (**5**).

Calibration 2.50 mg of each standard compound was dissolved in 5.00 ml of methanol (stock solution); further calibration levels were prepared by diluting the stock solution with methanol. The range of concentrations injected varied from 15.6 to 500.0 μg/ml. All calibration levels were injected in triplicate (δ always less than 0.78%). The calibration data obtained are shown in Table 1 and indicate linearity of the detector response in the range mentioned above.

Analytical Methods HPLC analysis was performed on a Waters Alliance 2690 HPLC system, equipped with a 996 photodiode array detector (Waters, Milford, MA, U.S.A.). For all separations a Luna C18 column (150×4.6 mm, 5 μm particle size) from Phenomenex (Torrance, CA, U.S.A.) was used. The mobile phase consisted of 25 mM phosphate buffer (A), adjusted to pH 7.0 and acetonitrile (B), applied in the following gradient elution: from 95A/5B in 30 min to 80A/20B. Each run was followed by a 5 min wash with 0.1% phosphoric acid in methanol and an equilibration period for 10 min. The flow rate was adjusted to 1.0 ml/min, the detection wavelength set to 205 nm and 10 μl of sample was injected. All separations were performed at a temperature of 60 °C. Peaks were assigned by spiking the samples with standard compounds, and comparison of the UV-spectra and retention times.

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