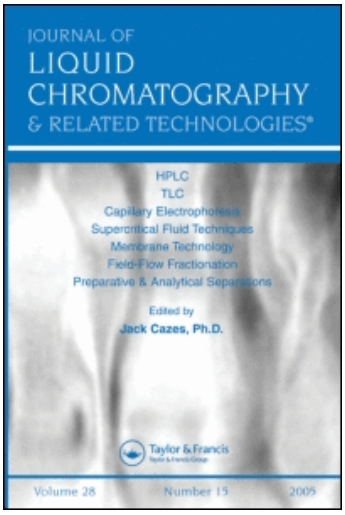

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Abstract: A rapid and sensitive liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS) assay for the determination of five pharmacologically active compounds (PAC) extracted from the traditional Chinese medicine, *Rhodiola*, namely salidroside, tyrosol, rhodionin, gallic acid, and ethyl gallate has been developed. In this method, PAC could be baseline separated and detected with DAD at 275 nm. The validation of the method, including sensitivity, linearity, repeatability, and recovery, was examined. The linear calibration curves were acquired with correlation coefficient >0.999 and the limits of detection LOD (at a signal-to-noise ratio = 3:1) were between 0.058 and 1.500 μmol/L. It was found, that the amounts of PAC varied with different species of *Rhodiola*. The established method is rapid and reproducible for the separation of five natural pharmacologically active compounds from extracts of *Rhodiola* with satisfactory results.

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Keywords: High performance liquid chromatography, Atmospheric pressure chemical ionization mass spectrometry, Pharmacologically active compounds, *Rhodiola*

INTRODUCTION

Within the pharmaceutical industry, the identification of novel active compounds through the use of powerful tools can be considered to be one of the most important discoveries for natural drugs. Samples isolated for these compounds often are initially generated as complex mixtures consisting of multiple components; powerful analytical tools are required for both the separation and characterization of the compounds in these mixtures in a useful manner. Specifically, the intrinsic complexity of natural product extracts represents both a source of extremely high compound diversity and a significant separation/deconvolution challenge.

For the drug discovery efforts currently taking place within the pharmaceutical industry, natural product extracts have been found to provide a valuable source of molecular diversity, which is complementary to that provided by traditional synthetic organic methods or combinatorial chemistry. However, there exists a need for analytical tools that can facilitate the separation and characterization of components from these sources in a rapid manner. Chromatography has served as an effective technique for the separation of many of the components in complex mixtures. Although numerous publications were reported for the analysis of effective components from different traditional Chinese medicines (TCM),[1–6] further composition analysis from TCM plants can be considered to be one of the most important procedures in order to discover natural drugs. Various methods such as paper chromatography and thin-layer chromatography can been used, but these methods are nonspecific and nonsensitive. Gas chromatography (GC) offers high sensitivity for a wide range of compounds. However, it generally requires a derivatization step before chromatography. Traditionally, reversed-phase liquid chromatography has served as an effective technique for the separation of many of the components in complex mixtures that are under evaluation for drug discovery. Reversed-phase column packing has been successfully utilized in conjunction with volatile mobile phases to provide rapid high-throughput characterization of samples via liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS).

Rhodiola is a traditional Chinese medicine belonging to the family of Crassulaceae that is mainly distributed in northern atmospheres with about 90 species recorded in the world and above 70 species found in China.[7] The extracts of *Rhodiola* exhibit different efficacy from clinical examinations, such as exciting the central nervous system, anti-virus, anti-lacking of oxygen, anti-cold, and anti-tatigue.[8–12] In this study, five pharmacologically active compounds (PAC), namely salidroside, tyrosol, rhodionin, gallic acid, and ethyl gallate isolated from several species of *Rhodiola*, were determined

conveniently by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). The suitability of the developed method for the rapid and sensitive analysis of PAC from actual samples is satisfying.

EXPERIMENTAL

Instrumentation

Experiments were performed using a LC/MSD-Trap-SL liquid chromatography/mass spectrometry (1100 Series LC/MSD Trap, a complete LC/MS/MS). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1315A). Ion source type, APCI (Positive/Negative model); nebulizer pressure 60 psi; dry gas temperature, 450°C dry gas flow, 5.0 L/min. APCI Vap temperature 350; Corona Current (nA) 4000 (pos); Capillary voltage 3500V. An Eclipse XDB-C8 column (150 × 4.6 mm 5 μm) was used. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C. The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an APCI (ESI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. A twenty-minute gradient elution (A was 20:80 acetonitrile/water(v/v); B was 60:40 acetonitrile/water(v/v); and C was acetonitrile), was selected for the separation of five pharmacologically active compounds. The samples were detected with DAD at 275 nm. The mobile phase was filtered through a 0.2 μm nylon membrane filter (Alltech, Deerfiled, IL).

Chemicals

HPLC grades of methanol and acetonitrile were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used in this study were also of analytical grade unless otherwise stated. The five standard compounds (structures and molar masses shown in Figure 1): salidroside, tyrosol, rhodionin, and gallic acid were isolated from *Rhodiola crenulata* and ethyl gallate from *Rhodiola Coccinea*, and the purity of each of the five standard compounds was more than 99%. Their structures were confirmed by comparing their melting points, ^{1}H-NMR, IR, UV, and MS data with those given in the literature.[13,14]

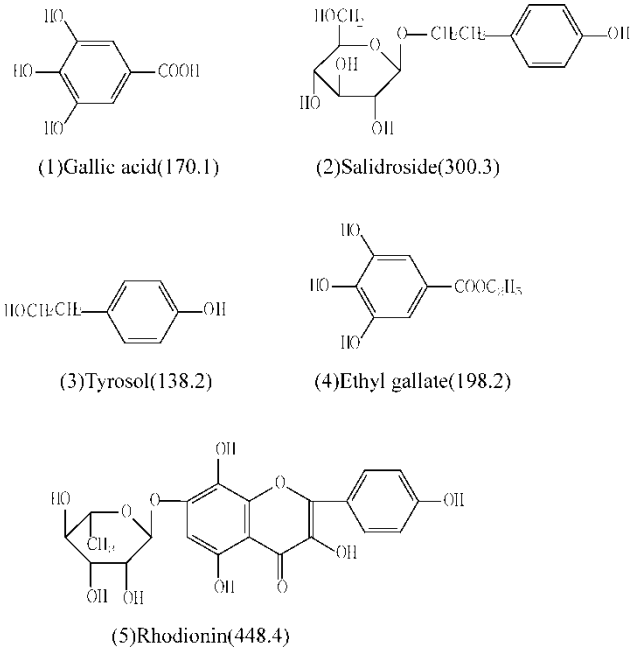

Figure 1. Structure of the natural product test standards.

Preparation of Standard and Sample Solutions

The standard solutions of five polar components (1.0×10^{-3} mol/L) were prepared by dissolving corresponding amounts of pure compounds in 10 mL of 50% acetonitrile. The corresponding low concentration of solution (1.0×10^{-5} mol/L) was obtained by diluting the stock solution with acetonitrile. When not in use, all reagent solutions were stored at 4°C in a refrigerator or at −20°C in a refrigerator until analysis.

Five species of *Rhodiola*, *Rhodiola himalensis* (Baqing, County, Tibet, 4300ATH), *Rhodiola kirilowii* (Changdu, County, 4620 ATH), *Rhodiola Coccinea* (Changdu, County, 4590 ATH), *Rhodiola sexifolia* (Linzhi, District, 3130 ATH), and *Rhodiola chrysanthemifolia* (Baqing, County, 4300 ATH), were collected from different areas in Qinghai-Tibet Plateau and washed successively with 20 mL each of water and deionized water. The washed *Rhodiola* were dried under a stream of nitrogen and broken to powdered samples. To a 10 mL round bottom flask, a 1.0 g powdered *Rhodiola* and 10 mL 80% methanol was added. The contents of the flask were allowed to incubate at room temperature for 24 h, then immersed in a sonicator water bath and the sample was sonicated in 5 min intervals for 30 min. The contents were then centrifuged at a speed of 4000 rpm for 15 min. The supernatant was collected and stored at 4°C in a refrigerator until analysis.

RESULTS AND DISCUSSION

The objective of this study was to develop a simple and sensitive method that can be used to analyze PAC from natural plants, such as different species of *Rhodiola*, by high performance chromatography with atmospheric pressure chemical ionization detection (APCI source indentification). The main challenge of the present work was to test the feasibility of the method in a variety of conditions.

HPLC Separation

For the simultaneous separation of FPAC (five pharmacologically active compounds), an Eclipse XDB-C8 column in conjunction with a gradient elution was used, the linear gradient was as follows: Eluent A was 20:80 acetonitrile/water(v/v); B was 60:40 acetonitrile/water(v/v); and C was acetonitrile. During conditioning of the column and prior to injection, the mobile phase composition was 70% A and 30% B. The percentage of mobile phase was changed as follows after injection: 100% (A) from 0 to 5 min, 0–50% (B) from 5 to 10 min (C = 0); 50–0% (B) and 0–100% (C) from 10 to 15 min (A = 0); 100% (C) from 15 to 20 min (A = 0, B = 0). Under these conditions, FPAC was separated within 12 min (Figure 2). The resolution was not significantly affected by the pH of the mobile phase. It would be suited for the separation of FPAC components with good resolution, over a wide range of pH's from 4.0 to 9.0. The separation of FPAC could also be achieved by isocratic elution with 20% acetonitrile (20 : 80 acetonitrile/water (v/v)) as mobile phase, however, it took about 30 min for the complete separation of FPAC. At the same time, the elution of ethyl gallate and gallic acid obviously increased in retention value and exhibited serious tailed peaks. After further experiments, it was found that if the pH value of mobile phase A was adjusted to 4.0–9.0, a complete baseline resolution for all PAC components in conjunction with a gradient elution could be achieved within the shortest time.

LC/APCI/MS

Prior to use, the instrument was checked to meet the sensitivity defined by the manufacturer. The HP1100 LC/MSD-Trap-SL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy, specification, and sensitivity were achieved over the entire mass range. APCI source and instrument parameters were optimized by infusing the FPAC components that were isolated from an HPLC column with DAD detection, and into the post column on-line mass spectrometer. The ionization and fragmentation of the isolated

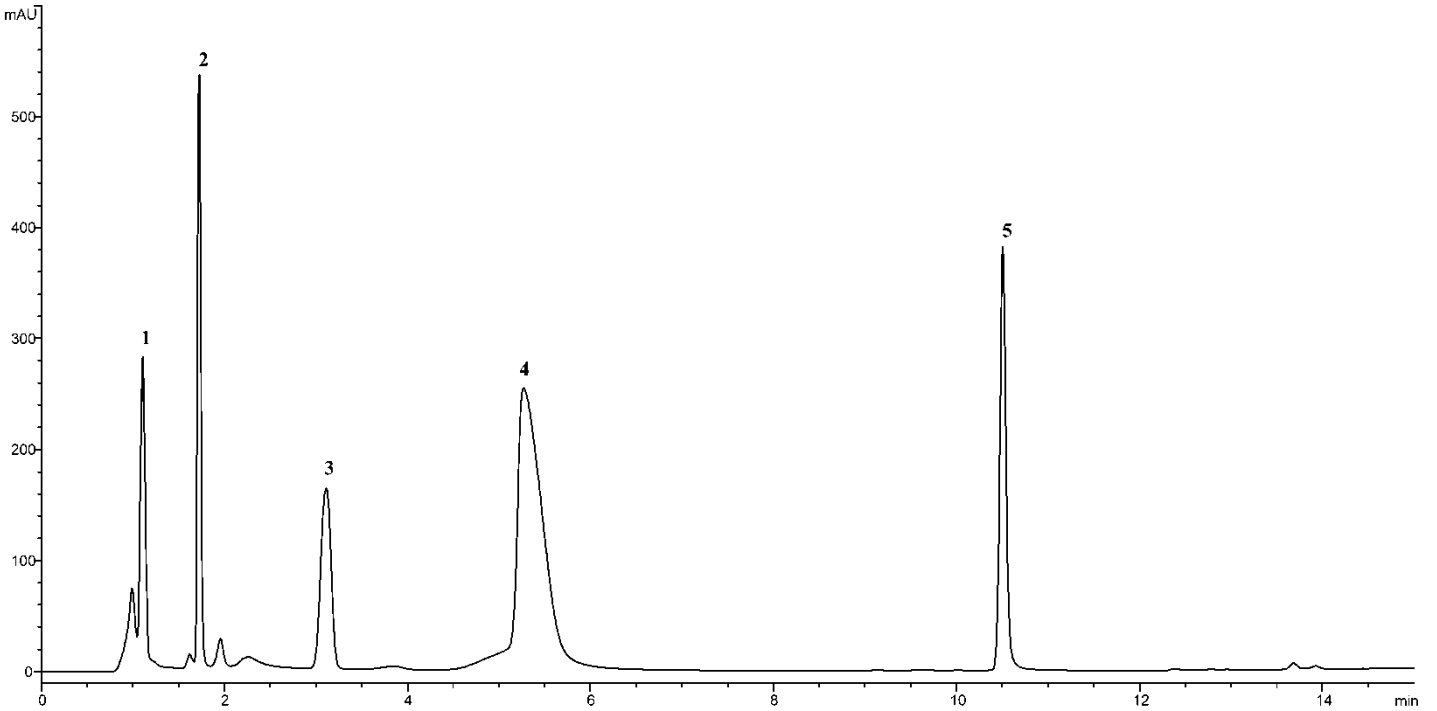

Figure 2. Typical chromatogram for FPAC components. Column temperature is set at 30°C; Column 150 × 4.6 mm Eclipse XDB-C8 (5 μm); flow rate = 1.0 mL min^{-1}; Gradient conditions described as in experimental parts. Peaks: 1. gallic acid; 2. salidroside; 3. tyrosol; 4. ethyl gallate; 5. rhodionin.

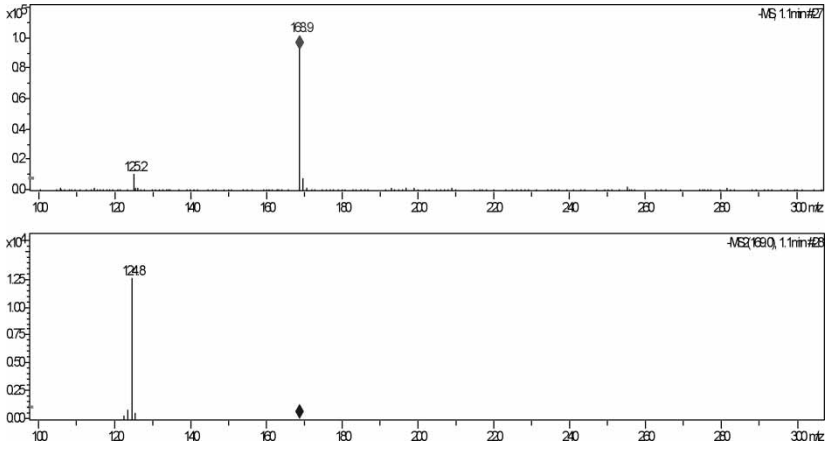

Figure 3. The profile of ion mass spectra and scanning of the isolated representative gallic acid. Typical MS chromatogram of gallic acid from full scanning range from 100 to 900 amu under APCI negative-ion mode; gallic acid was isolated from an Eclipse XDB-C8 column using DAD detection, and into the on-line mass spectrometer (top: molecular ion MS; bottom: MS/MS).

FPAC components was studied by mass spectrometry with atmospheric pressure chemical ionization detection at APCI negative-ion mode. As expected, every FPAC component produced an intense molecular ion peak at m/z $[M\text{-}H]^+$ (see Figure 3). The selected reaction monitoring was based on the m/z $[M\text{-}H]^+(168.9) \rightarrow 124.8$ transition for gallic acid; $[M\text{-}H]^+(299) \rightarrow 178.8$ transition for salidroside; $[M\text{-}H]^+(137) \rightarrow 118.9$ transition for tyrosol; $[M\text{-}H]^+(197) \rightarrow 168.9$ transition for ethyl gallate transition; $[M\text{-}H]^+(447) \rightarrow 300.9$ transition for rhodionin. Although other endogenous compounds were present in the samples, there was no detectable signal from the blank sample under the negative-ion mode detection.

Ultraviolet Absorption of FPAC

The ultraviolet absorption of FPAC was investigated in an acetonitrile solution. The absorption wavelength of FPAC was obtained with the on-line scanning range of 190 to 400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 215 and 275 nm for gallic acid; 198, 225, and 275 nm for salidroside; 214 and 260 nm for tyrosol; 198, 224, and 275 nm for ethyl gallate; 195, 224, and 275 nm for rhodionin, respectively. Absorption exhibited higher intensity in low wavelength, however, serious interferences from background electrolyte solution were also observed. The ultraviolet detection was, therefore, selected at 275 nm.

Linearity and Repeatability

Method repeatability was examined by preparing and measuring a standard solution, and carrying out six successive injections of the standard solution within one day. The relative standard deviations (RSDs) of retention times and peak areas are listed in Table 1. The linear calibration curve was constructed using the regression of the peak area versus concentration of the calibration standards. The results are listed in Table 2.

Analysis of Samples

Extracted methanol solutions from five species of *Rhodiola* plants, including salidroside, tyrosol, rhodionin, gallic acid, and ethyl gallate, were analyzed for the determination of their contents by using the proposed method. The representative chromatogram of *Rhodiola* sexifolia is shown in Figure 4. The results obtained are presented in Table 3. In order to examine the reliability of the method, the recoveries of five standards were investigated. The recovery was determined by addition of known amounts of four standards (tyrosol, rhodionin, gallic acid, and ethyl gallate) into the methanol extract of *Rhodiola sexifolia*, and salidroside into the methanol extracts of *Rhodiola chrysanthemifolia*, under the same conditions stated above. The recoveries of these five compounds were found to be in the range of 96.7–103.5% (see Table 4).

As can be seen, gallic acid widely existed in each kind of *Rhodiola*, and corresponding contents are higher than those of tyrosol, rhodionin, and ethyl gallate. Gallic acid has been found to be pharmacologically active as an antioxidant, antimutagenic, and anticarcinogenic agent in clinical examination.[15,16] The different efficiencies exhibited in clinical examination may be attributed to the gallic acid content existing in various kinds of *Rhodiola*. Saildroside, which have been found in the four species of *Rhodiola* (*Rhodiola chrysanthemifolia*, *Rhodiola kirilowii*, *Rhodiola himalensi* and *Rhodiola Coccinea*). Three species of *Rhodiola* (*Rhodiola sexifolia* and *Rhodiola kirilowii*) contained tyrosol. Ethyl gallate only existed in *Rhodiola sexifolia*. Rhodionin existed in two species of *Rhodiola* (*Rhodiola sexifolia*

Table 1. Repeatability for retention time and peak area (n = 6)

Analytes	Retention time RSD (%)	Peak area RSD (%)
Gallic acid	0.087	0.636
Salidroside	0.048	0.499
Tyrosol	0.067	0.284
Ethyl gallate	0.088	0.645
Rhodionin	0.092	0.853

Table 2. The linear equation and the related parameters

Analytes	Regression equation	Concentration (mmol/L)	Correlation coefficient	Detection limits[a] (μmol/L)	MS $(M\text{-}H)^+$
Gallic acid	$Y = 6.307 \times 10^6x + 41.509$	0.004–1.0	0.9998	0.058	168.9
Salidroside	$Y = 2.462 \times 10^6x + 29.283$	0.004–1.0	0.9998	0.067	299.0
Tyrosol	$Y = 2.455 \times 10^6x + 33.713$	0.008–1.0	0.9998	0.143	137.0
Ethyl gallate	$Y = 6.452 \times 10^6x - 730.879$	0.030–2.0	0.9996	1.323	197.0
Rhodionin	$Y = 7.997 \times 10^5x - 64.387$	0.030–2.0	0.9994	1.500	447.0

[a] $S/N = 3$.

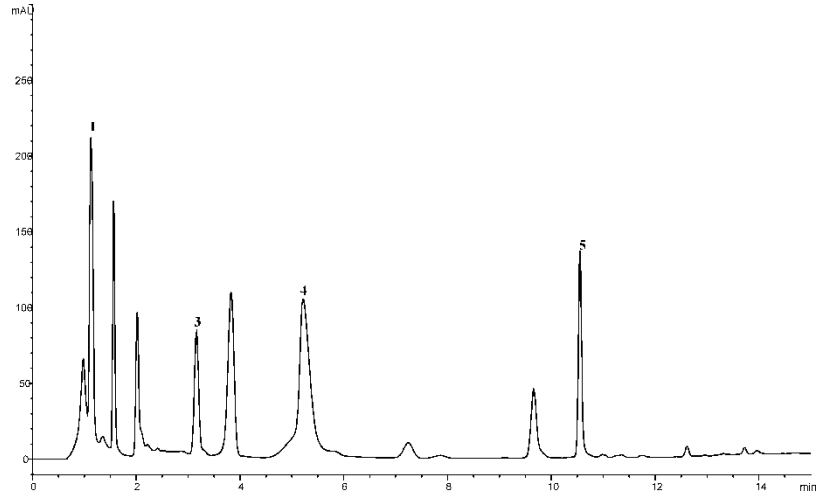

Figure 4. Chromatogram of extracts of *Rhodiola* plants using methanol as solvent. Column temperature is set at 30°C; DAD at 275 nm; Column reversed-phase Eclipse XDB-C8 (150 × 4.6 mm, 5 μm); flow rate = 1.0 mL/ min. Peaks as in Figure 2.

Table 3. The contents of individual salidroside, tyrosol, rhodionin, gallic acid and ethyl gallate in five species of *Rhodiola* (mg/g)

Analytes	*Rhodiola sexifolia*	*Rhodiola chrysanthemifolia*	*Rhodiola kirilowii*	*Rhodiola himalensi*	*Rhodiola Coccinea*
Gallic acid	1.810	0.250	4.590	2.480	1.240
Salidroside	ND[a]	2.252	4.863	4.110	3.730
Tyrosol	0.551	ND	0.120	ND	ND
Ethyl gallate	0.208	ND	ND	ND	ND
Rhodionin	0.517	0.261	ND	ND	ND

[a]No detection.

Table 4. Determination of recovery for this method (n = 5)

Compound	Added amount (ug/mL)	Found amount (ug/mL)	Recovery (%)	RSD (%)
Gallic acid	10	10.12	101.2	1.7
Salidroside	10	10.35	103.5	2.5
Tyrosol	10	9.82	98.2	1.5
Ethyl gallate	10	9.88	98.8	2.0
Rhodionin	10	9.67	96.7	2.2

and *Rhodiola chrysanthemifolia*), and the corresponding content of *Rhodiola* sexifolia is higher than that of *Rhodiola chrysanthemifolia*.

The amount of active compounds varies according to the material, probably due to two possible factors. First, ecological factors, e.g., soil type, climate, and cultivation, might affect the synthesis and turnover of secondary compounds.[17] Second, their ability to synthesize the five compounds in these populations might be subjected to genetic controls. Genetic differentiation generally has stronger effects on the contents of secondary compounds than ecological factors,[18] and the mutation of a single gene might affect the production of certain compounds.[19]

CONCLUSION

In the present study, we designed a new environmentally sensitive method that is capable of detecting FPAC with superior properties compared to the currently employed other methods, including rapid, convenient sample extraction, and excellent sensitivity. HPLC coupled with APCI negative-ion MS can provide a useful method for the detection and identification of FPAC in biologically important mixtures. Current studies are in progress to explore the applications to FPAC component analysis in various biological samples and tissues. This will necessitate further development of suitable extraction procedures and sample preconcentration techniques for the determination of FPAC from other kinds of *Rhodiola* plants.

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