# Determination of chlorogenic and gallic acids by UPLC-MS/MS

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Summary

The aim of our study were qualitative and quantitative analyses of two polyphenolic acids: chlorogenic and gallic acids. These compounds were determined in two species of *Rhodiola: R. kirilowii* and *R. rosea*. After collecting plants, aqueous and hydroalcoholic extracts were prepared. In order to identify analysed polyphenolic compounds ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS, Waters) was used. Gallic acid is commonly found in the roots of these plants. Aqueous extract in both species is a rich source of gallic acid. The UPLC-MS/MS studies allow to use this analytical method for determination of polyphenolic acids accordance with the requirements of ICH. Chromatographic method developed by our team is more precise then previously published.

***Key words:*** *Rhodiola kirilowii, Rhodiola rosea, chlorogenic acid, gallic acid, UPLC-MS/MS*

## INTRODUCTION

Chlorogenic and gallic acids belong to the group of polyphenols. Compounds from this group have antioxidant activity. In the human organism they protect against reactive oxygen species (ROS) that are chemically reactive molecules participating in many biochemical processes [1]. It is assumed that foods rich in chlorogenic acid can protect against the development of civilization diseases such as cancer, cardiovascular disease, age-related and neurodegenerative diseases [2]. Chlorogenic acid has antiviral, anti-inflammatory, antioxidant activities and inhibits the hepatic glucose-6-phosphatase [3]. Gallic acid has anti-inflammatory, antimutagenic, anticancer, antioxidant activities and possesses antifungal, antiviral and antibacterial properties [4].

Use of some *Rhodiola* species in medicine is well known and documented. The advantage of the use of golden root (*Rhodiola rosea*) is the fact that no side effects are observed [5, 6]. Golden root is highly valued in traditional herbal medicine. The secondary metabolites accumulated in roots and rhizomes show the wide spectrum of pharmacological activities and adaptogenic effects. The adaptogenic effects of *Rhodiola* sp. are manifested in the prevention of tiredness, in the increase of learning and memory capacity, prevention of age related disorders and diseases, avoidance and elimination of depression as well as sexual disorders. To main bioactive compounds present in *R. kirilowii* belong: salidroside, *p*-tyrosol, daucosterol, *β*-sitosterol, lotaustralin and epigallocatechin gallate (EGCG) [7-12]. In *R. rosea* there are found active substances such as cynnamic alcohol, glucosides, phenylpropanoids [13], phenylethanoids, flavonoids (rodiolin, rodionin, tricin), monoterpenes, triterpenes and phenolic acids [14-16].

It is well known that large differences in the production of secondary metabolites can be observed in plants of the same species depending on the plant source, growth conditions, developmental stage and type of examined tissue. The regulation of biosynthetic pathways that produce most natural products from *Rhodiola* definitely requires further study. It is also known that these plants are an

extremely polymorphic but their diversity at molecular level was not determined so far [8,15,16].

Based on this information, it can be concluded that *Rhodiola* is a very promising prospective plant for the medicine, beverage and food industry. The rapidly growing acceptance is observed in herbal supplements industry in Europe and the United States. It suggests that demand for *Rhodiola rosea* and *Rhodiola kirilowii* will continue to grow at a rapid rate in future.

The aim of our study were qualitative and quantitative analyses of polyphenolic compounds such as chlorogenic acid and gallic acid in *Rhodiola rosea* and *Rhodiola kirilowii* using ultra performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS, Waters).

## MATERIAL AND METHODS

### Plant material

In the study *Rhodiola kirilowii* (Regel.) Maxim and *Rhodiola rosea* L. roots were used. The plants were collected in October 2009 from field crops of the Institute of Natural Fibres and Medicinal Plants in Poznan. Roots were dried in a room temperature (22–24ºC).

### Preparation of plant extracts

Dry roots were powdered (0.315) and two kinds of extracts were prepared: aqueous extract and 50% *(v/v)* ethanol extract.

### Preparation of aqueous extract

The powdered dry roots were extracted with purified water for 3 h at 90ºC (material to solvent ratio 1:10). After filtering, the extracts were frozen at –55ºC and then lyophilised. The dry plant extracts were stored at a temperature of 20–25ºC.

### Preparation of 50% *(v/v)* ethanol extract

The powdered dry roots were extracted with 50% *(v/v)* ethanol using the percolation method at plant material to solvent ratio 1:10. After the evaporation of the alcohol in reduced pressure at a temperature of 40–45ºC the extracts were frozen at –55ºC and than lyophilised. Dry plant extracts were stored at a temperature of 20–25ºC.

## Standard solutions

To preparation calibrate curves of chlorogenic acid and gallic acid, standards from ChromaDex and acetylsalicylic acid as an internal standard (Sigma Aldrich) were implemented. All substances were dissolved in methanol and different concentration of stock solutions were prepared. Calibration curves were prepared at 5 different levels of concentration in the range of 100–1000 ng/ml.

## Sample preparation

Roots of *Rhodiola*

Nearly 2.5 g of dried powdered root from *Rhodiola kirilowii* or 1.5 g of *R. rosea* was weighed out and placed in a 100 ml round-bottom flask. To 20 ml of 70% (*v/v*) methanol 0.5 ml of methanolic solution of acetylsalicylic acid (IS) was added. That sample was heated under a reflux condenser in the boiling point of the solvent for 45 min. After cooling, the sample was filtrated and extraction was repeated two more times. All extracts were connected to evaporate the methanol up to dry in a rotary evaporator in vacuum. A dry residue was dissolved in 3 ml of 70 % (*v/v*) methanol. Sample was transferred quantitatively to 5 ml volumetric flask. Subsequently, the solution was made up to the mark with the 70% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 $\mu$m.

Extracts from *Rhodiola*

Nearly 0.1 g of dried extract from *Rhodiola kirilowii* or *Rhodiola rosea* was weighed out and placed in a 100 ml round-bottom flask. To 20 ml of 70% (*v/v*) methanol 0.5 ml methanolic solution of acetylsalicylic acid (IS) was added. That sample was heated under a reflux condenser in the boiling point of the solvent for 45 min. After cooling, the sample was filtrated and extraction was repeated two more times. All extracts were connected to evaporate the methanol up to dry in a rotary evaporator in vacuum. A dry residue was dissolved in 3 ml of 70 % (*v/v*) methanol. Sample was transferred quantitatively to 5 ml volumetric flask. Subsequently, the solution was made up to the mark with the 70% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 $\mu$m.

## LC-MS/MS assay

Concentration of chlorogenic and gallic acids in the *Rhodiola* species were determined by ultra performance liquid chromatography connected with a tandem mass spectrometry (UPLC-ESI MS/MS; Waters). The separation of polyphenolic acids were performed on the Acquity UPLC BEH C18 column, 1.7 $\mu$m, 2.1 · 50 mm

(Waters). Mobile phase: phase A: methanol, phase B: water. Mobile phase flow rate was 0.35 ml/min. The assay was performed in isocratic elution: 95% of phase A. Column temperature was 24ºC; ion source temperature: 100ºC; desolvation temperature: 300ºC. Gas flow rate: desolvation gas: 700 l/h; cone gas: 10 l/h. Polyphenolic acids and acetylsalicylic acid were analyzed in the negative-ions source. Fragmentation of chlorogenic acid m/z 359→191 Da, gallic acid m/z 169→125 Da and acetylsalicylic acid (IS) m/z 179→137 Da was used to determinate concentration.

## Validation

Validation of this extraction method was conducted in accordance with the requirements of ICH. The calibrations curves were prepared on the 5 different concentrations of substances. Retention time, peak area and regression coefficient were checked to accept recurrence and linearity of calibration curves. The calibration curves were prepared in the range 100-1000 ng/ml. After that, the precision of extraction was done for 6 samples. An accurancy of this method was conducted for 3 different level. Percentage of recovery ranged between 95.8 and 99.2%.

## Statistical method

For the polyphenolic acids and internal standard, the regression analysis was performed at 5 concentration levels. Concentration of compounds was carried out for all samples in 6 repeats. The average and relative standard deviations (RSD) for those results were determined.

## RESULTS

Table 1 shows basic data of precision and fragmentation of analysed compounds. Precision and linearity were evaluated by using regression analysis for compared substances (tab. 2). The recovery of analytical method was analysed for *R. kirilowii* roots using the enrichment method by adding to the sample dry hydroalcoholic extract of *R. rosea* with the determined level of chlorogenic and gallic acid. Detection of every analysed compound was conducted in negative ions source. The signals visible in the chromatogram came from the parent ion fragmentation $[M-H]^-$.

Figure 1 presents the MRM chromatogram showing fragmentation of chlorogenic and gallic acids in *Rhodiola kirilowii* and figure 2 showing *Rhodiola rosea* roots.

**Table 1.**

Characteristic parameters of chlorogenic and gallic acids detection

| Compound | Retention time [min] | $RSD_t$ ($n$=15) [%] | $RSD_p$ ($n$=5) [%] | Fragmentation m/z [Da] |
|---|---|---|---|---|
| Chlorogenic acid | 0.41 | 0.63 | 4.22 | 353→191 |
| Gallic acid | 0.41 | 0.00 | 2.22 | 169→125 |
| Acethylsalicilic acid (IS) | 0.34 | 0.00 | 7.82 | 179→137 |

**Table 2.**

Method of recovery (n=3)

| Sample | Chlorogenic acid | Gallic acid |
|---|---|---|
| 20% | 97.4±5.2 | 99.1±5.9 |
| 40% | 95.8±6.1 | 99.2±4.5 |
| 80% | 98.1±3.6 | 98.6±5.1 |

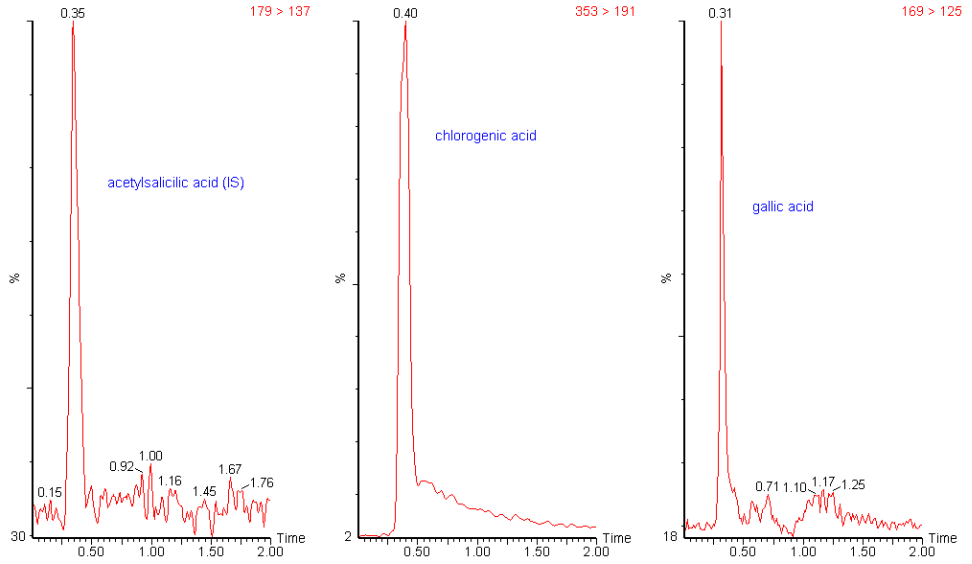


Figure 1.

The MRM chromatogram showing fragmentation of chlorogenic and gallic acids from *Rhodiola kirilowii* roots.

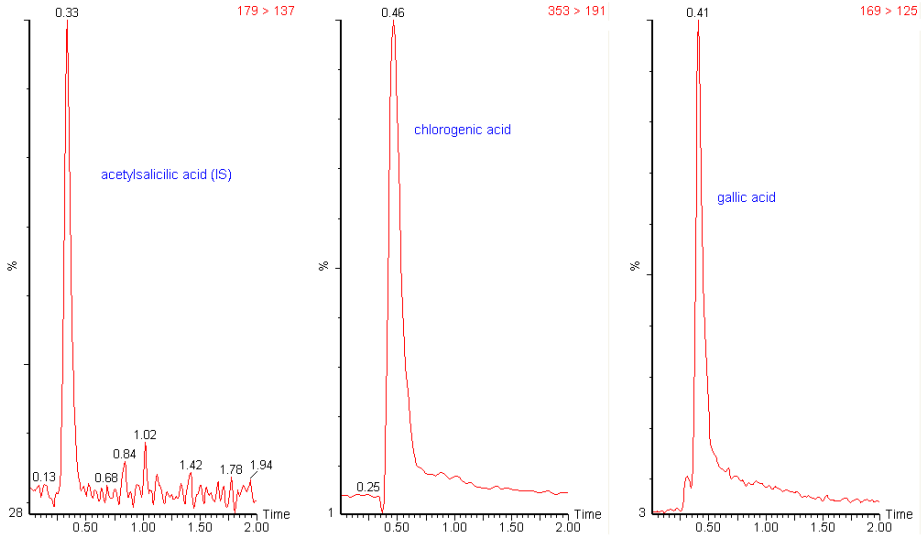


Figure 2.

The MRM chromatogram showing fragmentation of chlorogenic and gallic acids from *Rhodiola rosea* roots

## DISCUSSION

This analytical method allows to determine the content of chlorogenic and gallic acids in the roots and extracts (tab. 3). In *Rhodiola kirilowii* roots, gallic acid is more common than chlorogenic acid. The same situation is observed in both extracts. The aqueous extract consists up to 25.590 mg/100 g of dry powdered material of gallic acid and several times less chlorogenic acid (0.219 mg/100 g of dry powdered material).

**Table 3.**

Content of chlorogenic and gallic acids in *Rhodiola kirilowii* and *Rhodiola rosea* roots and extracts

| Sample | Chlorogenic acid | | Gallic acid | |
|---|---|---|---|---|
| | Content [mg/100 g of dry powdered material] | RSD [%]* | Content [mg/100 g of dry powdered material] | RSD [%]* |
| *Rhodiola kirilowii* root | 0.051 | 3.33 | 3.852 | 1.22 |
| 50% ethanol extract | 0.121 | 4.32 | 14.257 | 2.55 |
| aqueous extract | **0.219** | 5.35 | **25.490** | 3.11 |
| *Rhodiola rosea* root | 0.095 | 5.41 | 31.574 | 4.12 |
| 50% ethanol extract | 0.173 | 3.91 | 56.199 | 2.97 |
| aqueous extract | **0.284** | 4.92 | **74.144** | 3.62 |

* – RSD – relative standard deviation (n=6)

In *Rhodiola rosea* roots and extracts, the same situation is observed in *Rhodiola kirilowii*. The aqueous extract from *R. rosea* is the richest source of gallic acid among tested products. In our study, the value of this compound is 74.144 mg/100 g of dry powdered material of this compound. Comparing two species of *Rhodiola,* the contents of both gallic acid and chlorogenic acid is the highest in the aqueous extract from *R. rosea*.

A similar analyses using a different method was performed by Lee et al. and Ohsugi et al. They also identified antioxidant compounds such as *p*-tyrosol, organic acids (gallic acid, caffeic acid and chlorogenic acid) and flavonoids in *Rhodiola rosea* and related species for alcohol and aqueous extracts pointing to a varied composition of the tested compounds depending on the prepared extracts [17,18].

In general, the presence of analysed active compounds in the roots of *Rhodiola rosea* and *Rhodiola kirilowii* is very promising due to the antioxidant properties and the possibility of protection against various civilization diseases. Thus, further study and appropriate method for determining gallic acid and chlorogenic acid are needed, taking into account the variability of chemical composition dependent on the harvest time, place of origin and kind of extracts.

## CONCLUSION

In summary, all the validation tests undertaken with use of an analytical method for polyphenolic acids were confirmed so that the ultra performance liquid chromatography – tandem mass spectrometry (UPLC-ESI MS/MS) method can be successfully used for the determination of the compounds of this group.

## ACKNOWLEDGEMENT

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## OZNACZANIE ZAWARTOŚCI KWASU CHLOROGENOWEGO I GALUSOWEGO ZA POMOCĄ UPLC-MS/MS

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Streszczenie

W Instytucie Włókien Naturalnych i Roślin Zielarskich podjęto badania mające na celu opracowanie metody detekcji kwasu chlorogenowego oraz galusowego za pomocą ultrasprawnej chromatografii cieczowej sprzężonej z tandemowym spektrometrem mas (UPLC-MS/MS, Waters). Badaniom poddano dwa gatunki różnica*: Rhodiola kirilowii* oraz *R. rosea*. Rośliny zostały wyhodowane w uprawie gruntowej w Instytucie. Przeprowadzona walidacja metody pozwoliła na jej wykorzystanie w ocenie zawartości kwasu chlorogenowego oraz galusowego w badanych roślinach, ponieważ zawartość analizowanych związków zależna jest zarówno od gatunku jak i warunków uprawy.

***Słowa kluczowe:*** *Rhodiola kirilowii, Rhodiola rosea, kwas chlorogenowy, kwas galusowy, UPLC-MS/MS*