INVESTIGATION OF GLYCOSIDES IN RHODIOLA ROSEA WITH USE OF HPLC

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Contents

Abstract 3
Introduction 3
Bioactive compounds in Rhodiola Rosea 4
Methods 6
HPLC 6
2-way ANOVA 7
Tukey test 8
Method of validation 8
Results 9
Discussion 11
Data analysis 11
Production of R.Rosea 12
Method validation 12
References 13
Appendix 14
Appendix A: Sample preparation and method description 14
Appendix B: 15
Appendix C: 15
Appendix D: 15

Abstract

The goal of this paper is to locate the best possible location to produce R. Rosea. This is done via analysis of data obtained from previous research done. The data will be analyzed with a 2-way ANOVA, as this would prove whether there are significant differences in the amount of product yield vs the locations and gender of the plants. After analysis it is confirmed that location plays a major part in the percentage of bioactive compounds found within R. Rosea.

Introduction

Rhodiola Rosea is a medium-sized yellow plant, that mainly grows in cold and mountainous areas, and is therefore not found naturally within Denmark. The plant has over an extended period of time been used as a "cure all" drug by many people. While this has been commonly accepted by most health professionals, actual research behind the compounds within Rhodiola Rosea has been sorely lacking[1]. Research regarding the overall root extract from the plant is plentiful, but the plant contains a multitude of potentially relevant bioactive compounds, research into whether these compounds have specific uses within the medicine is underway.

Rhodiola Rosea is considered an adaptogen[2, 3], which means it increases the body's resistance to physical, biological, and chemical stressors that could occur within the body. Adaptogens are known to provide a multitude of benefits, including increased energy, reduction of stress, increased endurance, anti-oxidative effects, and perhaps even life-span increasing effects[1].

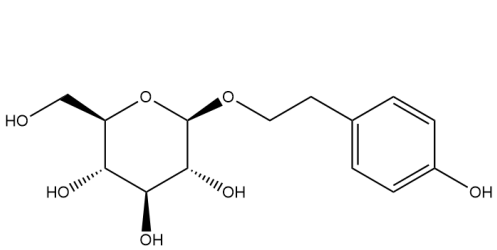
Rhodiola Rosea extracts is sold in several countries as an over-the-counter medicine and is believed to have little to no present side effects, it must be said, that few trials have been run on humans, and therefore possible side effects have yet to be found.

Bioactive compounds in Rhodiola Rosea

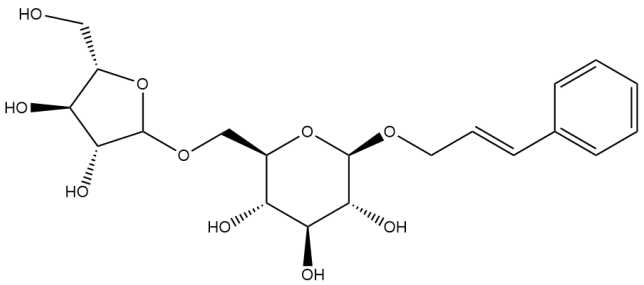
The plant Rhodiola Rosea contains a multitude of different potentially relevant compounds[4](Figure 1). Many of these compounds are fairly similar, and we will therefore be focusing mainly on Rosarin and Rosavin, as these are the compounds, we have obtained statistical data from.

Rosarin and Rosavin are both glycosides, which means they contain sugars bonded to another or multiple other functional groups. Glycosides are a common molecule within plants, and these molecules can be hydrolyzed by the body, releasing the bound functional groups into the body. This concept is well known and is used in many different forms of medicine, for example cardiac glycosides, used to treat heart diseases[5]. With this information, it is of great interest to determine which glycosides within the R. Rosea extracts that exhibit the positive changes, therefore it is necessary to separate the many different compounds and analyze them.

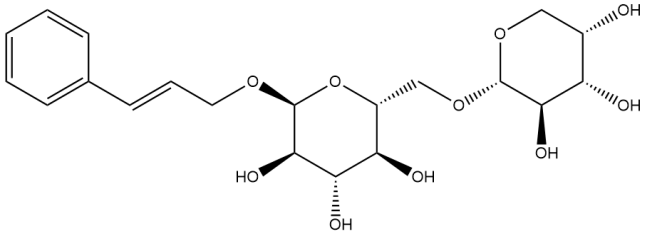
Figure 1 |Compounds of interest within R.Rosea



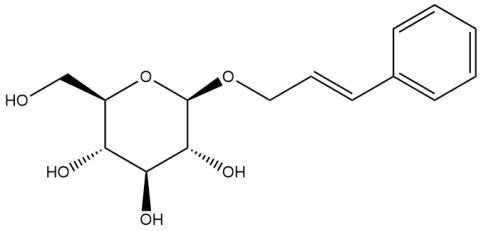
A) Salidroside



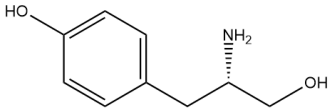
B) Rosarin



C) Rosavin



D) Rosin



E) Tyrosol

The compounds Rosavin and Rosarin make up very little of the total extract amount, accounting for only 1.1% (Rosarin) and 2.14% (Rosavin) of the total dry weight of the R.Rosea extract[4]. This is very important, since these concentrations fluctuate depending on various conditions, such as place of origin and the gender of the plant, this is further discussed in the results. Since these are already in such low concentration, minor differences in yield could radically change the effect of the supplement.

Many of the compounds within R. Rosea are believed to react with the central nervous system, in order to reduce the negative effects of stress on the body, the intricate mechanisms of each bioactive compound are not presently known[6].

Methods

To analyze the glycoside in Rhodiola Rosea typically involves the analytical method of chromatographic techniques such as the high-performance liquid chromatography HPLC, or gas chromatography GC. These analytical methods allow separation and quantification, but also identification of the glycoside that are present in the plant in this case rosavin and Rosarin. However according to literature HPLC seem to be the best analytical method even though many of the methods haven't been validated.[7]

HPLC

In this analysis HPLC is used to better understand the best source for Rosarin and Rosavin. HPLC stands for High-Performance Liquid Chromatography and works by running a solvent containing a sample through a column with filled with solid adsorbent material, at high pressure. This then separates the different compounds in the sample since they interact differently with the column, at the end of the column there is a detector that then detects the compounds as they reach it. This then leads to the chromatogram that shows peaks at different times.

There are advantages and limitations to using HPLC (**Table 1)** but is has been chosen to be best fit for the analysis.

Table 1 | Advantages and limitations of HPLC. [8]

Advantages • Applicable to diverse analyte types • Precise and highly reproducible quantitative analysis • HPLC coupled with mass spectrometry (HPLC/MS) • High separation power with sensitive detection
Perceived limitations • Lack of an ideal universal detector • Less separation efficiency than capillary gas chromatography (GC) • Still arduous for regulatory or quality control (QC) testing

By chosen HPLC separation technique or any other technique for that matter, there are some parameters to consider to best separate the analyte and some of these are. 1. The choice of column with are crucial for the separation of the glycosides rosavin and rosarin. Here the revers phase liquid chromatography is commonly used because water can be uses as a solvent, and because the glycoside of interest is relative polar which reduces the retention time. Furthermore, the length of the column, diameter and particle size also have effects on the separation and time. 2. The mobile phase, since Rhodiola Rosea consist over 140 compounds[1] with verity of polarity gradient elution is consisted for the HPLC for reduce the retention time[9]. 3. Flow rate witch have an effect on the peaks shape and analyze time and so on. However, to figure out if the choice of some of these parameters is acceptable, calculation of retention factor and the selectivity factor can be done, but since the operation is done under gradient eluent the concept on retention factor makes it much more complex.[9]

The samples were analyzed by HPLC on a Phenomenx Luna C18 150 x 4.5 mm, 5 µm column by a gradient, with a flowrate of 0.8 mL/min. The two eluents used are MillQ with 0.1% formic acid and acetonitrile with 0.1% formic acid.

2-way ANOVA

2-way analysis of variance (2-way ANOVA) is a statistical technique used to determine whether there are significant differences between two or more groups based on two independent variables. In a 2-way ANOVA, there are two independent variables or factors, and the goal is to determine whether there is a significant interaction between the two factors, as well as the main effects of each factor. In this case 2-way ANOVA is used to determine the difference between location, gender and %yield of relevant compounds.

The null hypothesis for this 2-way ANOVA is that there is no significant interaction between the two factors. The alternative hypothesis is that there is a significant difference between the groups. A chosen level of significance is chosen, in this case 0.05, this is used to determine whether the null hypothesis is rejected or accepted. The sum of squares of the individual samples are calculated together with the sum of the “intersection”. These values are used to calculate F and P-values, the P-value will used together with the level of significance to reject or accept the null hypothesis [[10].

Tukey test

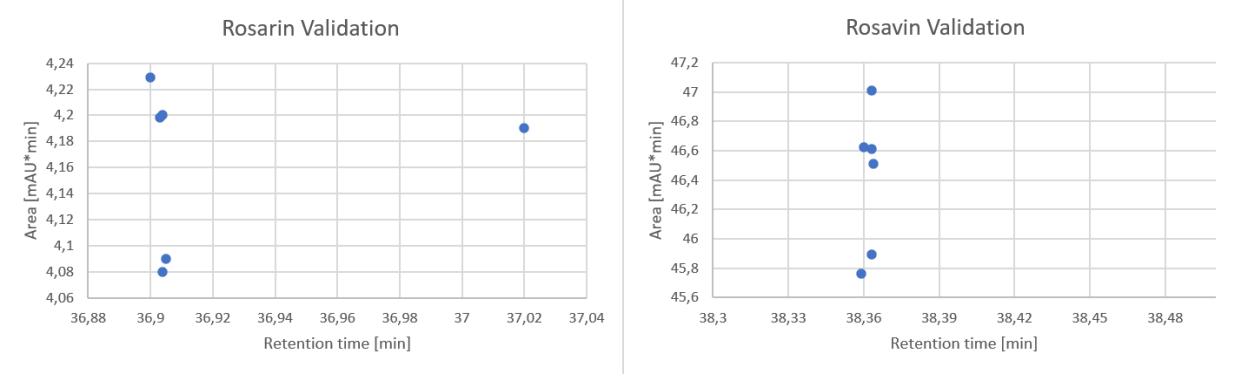
Tukey's test is usually used as a post-hoc test, after doing an ANOVA test. Tukey's test determines the minimum significant difference between 2 mean groups, therefore it is used after first performing an ANOVA test.

Tukey's test works by calculating the between the means of 2 groups, it then compares this value to a critical value based on the samples size and the number of groups being analyzed. If the calculated range is larger than the critical value, then the 2 groups will be considered to have significantly different means[11].

Method of validation

To identify which peaks of the HPLC are the active compounds, Rosavin and Rosarin samples are run on their own to find their retention time and area. This is done with an external standard for both samples at a concentration of 0.1, to ensure precision this was done 6 times for each (**Figure 2**). Doing the validation only one outlier was found for Rosarin, with a deviation of 0.117 min.

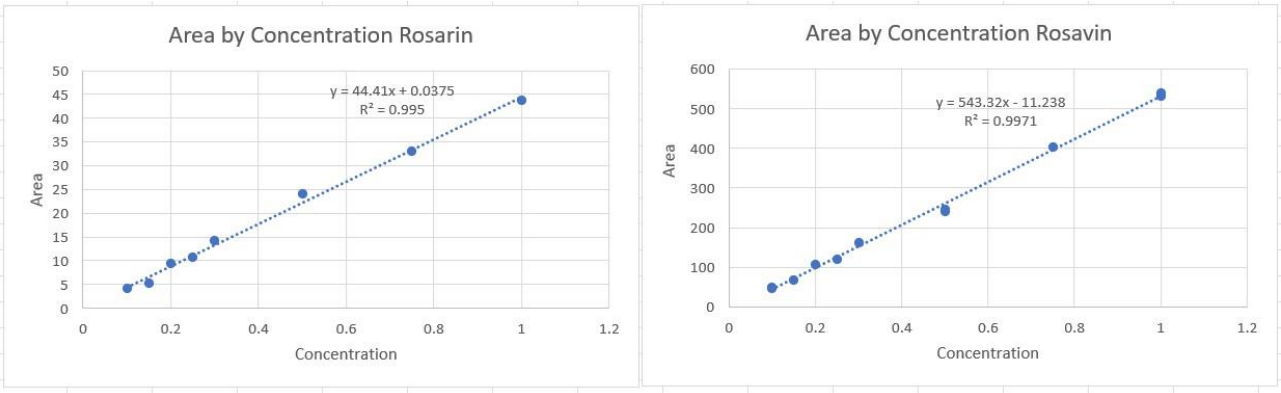
Figure 2 | Method validation for Rosarin and Rosavin at concentration of 0.1.



Results

After running the samples on HPLC the chromatograms for each sample were used and based on the external standards done previously the peaks for the various compounds of interest were extracted and the data was compiled in spreadsheet, values below threshold of detection (0.0891 mAU*min for Rosarin and 0.1021 mAU*min) for Rosavin were set to 0. Following this, standard curves were created for both Rosarin and Rosavin to convert from the peak area to a concentration value.

Figure 3 | Linear Regression



As seen in (Figure 3 | Linear Regression) the resulting curves fit to the regression line with an R^2 value of 0.995 for Rosarin and 0.997 for Rosavin both indicating a high correlation between the values. Using these curves all area values found in the dataset were converted into concentrations and 2-way ANOVA tests were performed to determine if there were significant differences in concentration across the different points of origin and between plants of different sex. The results shown in the table below indicate that for Rosavin the threshold for significant difference (PR < 0.05) is met only for origin. In the following table for Rosarin the threshold is met for both the origin and the intersection but again significance is not met for sex.

Rosavin ANOVA by Sex and Origin					
	df	sum_sq	mean_sq	F	PR(>F)
C(sex)	1	0.333445	0.333445	1.040945	0.310016
C(origin)	4	9.086232	2.271558	7.091326	0.000044
C(sex):C(origin)	4	3.087381	0.771845	2.409538	0.054051
Residual	102	32.67357	0.320329	NaN	NaN

Rosarin ANOVA by Sex and Origin					
	df	sum_sq	mean_sq	F	PR(>F)
C(sex)	1	22.1551	22.1551	2.19385	1.42E-01
C(origin)	4	1686.12	421.53	41.74093	1.09E-20
C(sex):C(origin)	4	319.801	79.95047	7.91689	1.34E-05
Residual	102	1030.07	10.09872	NaN	NaN

Figure 4 |Rosarin concentration (left) Rosavin concentration (right)

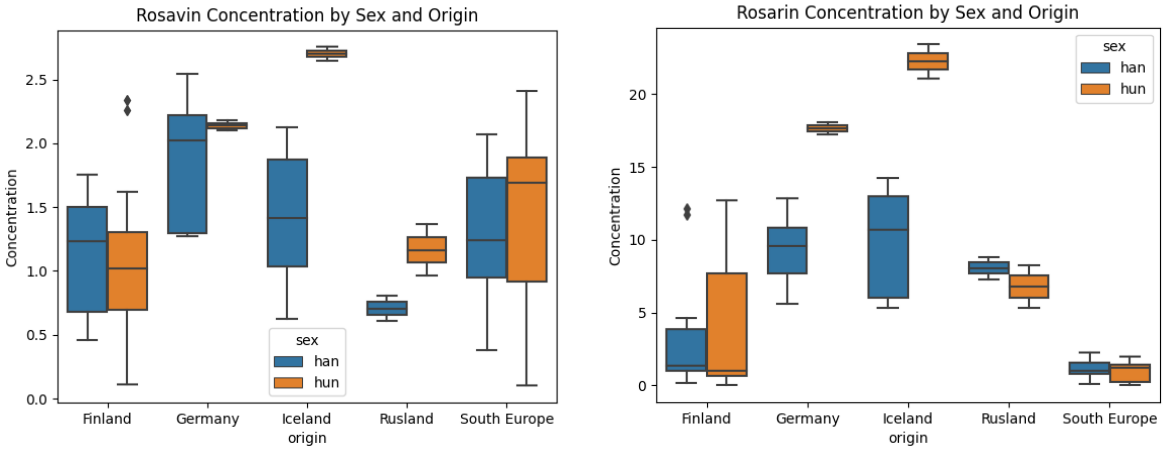
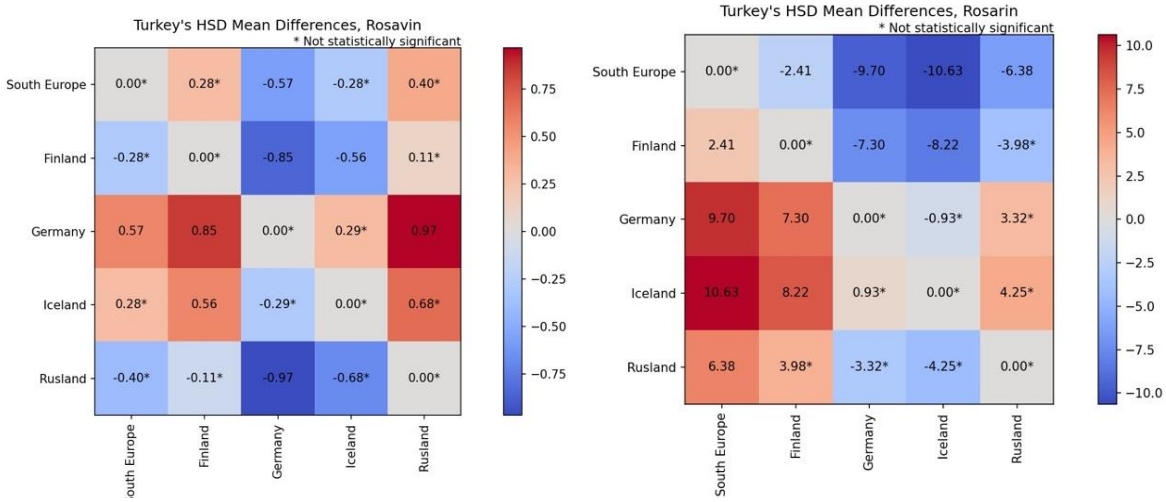


Figure 5 | Tukey test rosavin (left) Rosarin (right)



Looking at the graph of the data in (Figure 4 |Rosarin concentration (left) Rosavin concentration (right)) we can see the variation in the dataset and following this a post hoc analysis was conducted using Tukey's HSD test to quantify the differences between the points of origination. The results are summarized in (Figure 5 | Tukey test rosavin (left) Rosarin (right)); note that Russia has only 4 data points associated with it which likely contributes to the lack of significance, more testing would need to be done to truly determine any reliable information about the bioactive chemical concentration from Rhodiola Rosea originating in Russia. Low sample size is also likely the cause of the lack of significant differences with Iceland and Germany as well, this due to the barrier for rejecting the null hypothesis being higher when there are less data points.

Discussion

Data analysis

Looking at the results we can draw several conclusions with relatively high certainty. First, based off the ANOVA test we can confirm that location has significant effects on the quantity of bioactive chemicals in Rhodiola Rosea. Specifically, we can see in Turkey's test that Iceland has the highest concentration of Rosarin followed so closely by Germany that the difference between the two is not statistically significant. For Rosavin the post hoc analysis indicates Germany has the highest concentration followed by Iceland although there is not enough data for each to conclusively say Germany has a statistically significant difference over Iceland although transitively it does seem that way.

Another important thing to note is that depending on application more research is a worthwhile consideration, in our data we could not conclude that sex makes a significant difference in bioactive compounds but for Rosarin the interaction of sex and origin is significant which indicates that depending on the region there may be significant bioactive compound variation between plants of different sex like can be seen with Iceland. The same may be true for Rosavin but at least for our data the interaction is not significant.

Production of R.Rosea

The differences in Rosarin and Rosavin could have a major impact in the production of commercially available R. Rosea extract, since these are considered important bioactive compounds, and as previously discussed, might have a significant impact on the effectiveness of the supplements. This would make way for optimization of production, and if the factors responsible for the increased amounts of these bioactive compounds are uncovered, it would open up the possibility of effective production within artificial conditions such as greenhouses.

Method validation

Method validation was done with an external standard at only on concentration, this could have been improved. If external standards still were decided to be best fit, it would be beneficial to run them at different concentrations to see if they act differently, and it would also have made it possible to see at which concentration they are detectable at. A method that might have been better is an internal standard, this would make it possible to see exactly where in the samples the two compounds are. Also, it considers how the compounds react with the solution and will yield higher accuracy and precision. Doing the validation this way will also ensure a higher degree of robustness due to the effects the other compounds might have on Rosavin and Rosarin.

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Appendix

Appendix A: Sample preparation and method description

Extraction of Rhodiola rosea

1. Samples were chopped with a knife and approx. 1g is transferred to a 50 mL centrifuge tube.
2. 5 mL of MeOH is added and samples were homogenized aprox. for 1 min with an Ultra turax.
3. Samples were extracted overnight in a shaker
4. After that the samples were centrifuged for 5 min at 5000 rpm.
5. The supernatant was transferred a glass vial
6. 3 mL MeOH is added to samples and there are one again extracted overnight
7. Samples are the centrifuged and the supernatant is the transferred to the same glass vial as the first 5 mL
8. Samples are the evaporated in a speedvac and then resuspended in 1 mL of water:MeOH 1:1 (note: for a few a different volume was used)
9. Finally, they are filtered through a 0.22µ syringe filter into a HPLC vial.

HPLC analysis

Sample were analyzed by HPLC on a Phenomenx Luna C18 150 x 4.5 mm, 5 µm column by a gradient.

Flowrate: 0.8 mL/min.

Eluent A: MillQ + 0.1% Formic acid. Eluent B: Acetonitrile + 0.1% Formic acid

Time	%B
0	5
70	26
73	100
85	100
87	5

97 5

Data were recorded on PDA at the following wavelengths: 200,205,221,252,280,300,360 nm.

Appendix B:

UV-Vis4: Found under resources in instrumental analytisk kemi og statestik on sdu.itslearning.com

https://syddanskuni.sharepoint.com/sites/GroupKE530-3BioactivecompoundsinRhodiolarosea/Delte%20dokumenter/General/Group%208/UV-Vis4.pdf

Appendix C:

RoseaHPLCF22: found under resources in instrumental analytisk kemi og statestik on sdu.itslearning.com

https://syddanskuni.sharepoint.com/sites/GroupKE530-3BioactivecompoundsinRhodiolarosea/Delte%20dokumenter/General/Group%208/RoseaHPLCF22.pdf

Appendix D:

RosenRodDataKPTE4F22: Found under resources in instrumental analytisk kemi og statestik on sdu.itslearning.com

https://syddanskuni.sharepoint.com/sites/GroupKE530-3BioactivecompoundsinRhodiolarosea/Delte%20dokumenter/General/Group%208/RosenRodDataKPTE4F22.xlsx