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From traditional resource to global commodities: - A comparison of *Rhodiola* species using NMR spectroscopy - metabolomics and HPTLC

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Traditional Chinese Medicine (TCM); Nuclear magnetic resonance (NMR); High performance thin layer chromatography (HPTLC); Species (spp.)

Abstract:

The fast developing international trade of products based on traditional knowledge and their value chains has become an important aspect of the ethnopharmacological debate. The structure and diversity of value chains and their impact on the phytochemical composition of herbal medicinal products as well as the underlying government policies and regulations have been overlooked in the debate about quality problems in transnational trade. *Rhodiola* species, including *Rhodiola rosea* L. and *Rhodiola crenulata* (Hook.f. & Thomson) H.Ohba, are used as traditional herbal medicines. Faced with resource depletion and environment destruction, *R. rosea* and *R. crenulata* are becoming endangered, making them more economically valuable to collectors and middlemen, and also increasing the risk of adulteration and low quality.

Rhodiola products have been subject to adulteration and we recently assessed 39 commercial products for their composition and quality. However, the range of *Rhodiola* species potentially implicated is has not been assessed. Also, the ability of selected analytical techniques in differentiating these species is not known yet.

Using a strategy previously developed by our group, we compare the phytochemical differences among *Rhodiola* raw materials available on the market to provide a practical method for the identification of different *Rhodiola species* from Europe and Asia and the detection of potential adulterants. Nuclear magnetic resonance spectroscopy coupled with multivariate analysis software and high performance thin layer chromatography techniques were used to analyse the samples. Rosavin and rosarin were mainly present in *R. rosea* but also in *Rosea sachalinensis* Borris. 30% of the *Rhodiola* samples purchased from the Chinese market were adulterated by other *Rhodiola* spp. The utilisation of a combined platform based on ^{1}H-NMR and HPTLC methods resulted in an integrated analysis of different *Rhodiola* species. We identified adulteration at the earliest stage of the value chains, i.e. during collection as a key problem involving several species. This project also highlights the need to further study the links between producers and consumers in national and trans-national trade.

Keywords: *Rhodiola*, metabolomics, herb quality, adulteration, HPTLC, NMR

Introduction

While medicinal plants and spices have been traded for centuries on a global scale, the fast developing international trade of products now includes a large number of species which are used based on local and traditional knowledge and practice. The value chains of such products are starting to become an important topic in the ethnopharmacological debate. The structure and diversity of value chains, as well as their impact on the phytochemical composition of herbal medicinal products (HMPs) has been overlooked in quality issues in transnational trade. Different government policies and regulations governing trade in herbal medicinal products impact on such value chains.

Medicinal *Rhodiola* species, including *Rhodiola rosea* L. and *Rhodiola crenulata* (Hook.f. & Thomson) H.Ohba, have been used widely in Europe and Asia as traditional herbal medicines with numerous claims for their therapeutic effects. Faced with resource depletion and environment destruction, *R. rosea* and *R. crenulata* are becoming endangered, making them more economically valuable to collectors and middlemen, and also increasing the risk of adulteration and low quality. Poor quality and adulterated *Rhodiola rosea* products have been previously reported (Booker *et al.* 2015, Xin *et al.* 2015) and this paper investigates some aspects of the value chains that leads to the production of such products.

Adulteration of *R. rosea* products with *R. crenulata* has been previously reported but our fieldwork investigations suggested that other species may be implicated, and particularly *Rhodiola sachalinensis*, another species that appears to contain rosavins (the main marker compounds used for the identification of *R. rosea*).

The genus *Rhodiola* (Crassulaceae) comprises approximately 90 species of succulent and herbaceous perennial plants, which mainly show a circumpolar distribution across the northern hemisphere (Xia *et al.* 2005, Lu. and Lan 2013). According to the Global Biodiversity Information Facility (GBIF, 2010) (Kylin 2010), *Rhodiola* species usually grow in mountainous areas such as rock ledges, precipices, tundra, brooks and river banks (Zhu and Lou 2010).

Figure 1: Rhodiola species. A: R. rosea; B: R. crenulata; C: R. quadrifida; D: R. sachalinensis; E: R. fastigiata. Attribute: A & B: Anthony Booker; C: http://www.plantarium.ru/; D&E: Plant Photo Bank of China (PPBC) (http://www.plantphoto.cn/en)

Ethnopharmacological importance of key *Rhodiola* species:

In Europe and North America, *Sedum roseum (L.*) Scop. (commonly named under its synonym *R. rosea* L.) is the most well-known and widely used among the different species. It is also known as golden root, or artic root which reputedly demonstrates the economic importance and the geographical distribution of the plant. It has a rich history of traditional use in Russia, Europe and Asia with various uses according to the region (e.g. as shown in Table 1).

Table 1: Traditional uses of *R. rosea* in different regions

Region	**Use**	**Reference**
Russia	• Escalation of physical endurance • Remedy against fatigue and high altitude sickness • Aphrodisiac	Shikov et al., 2014; Alm, 2004
Norway	• Astringent • Cure for scurvy • Remedy against hair-loss and urinary tract disorders	Alm, 2004
Iceland and Denmark	• Alleviation of headaches	Alm, 2004
France	• Stimulant • Astringent	Panossian et al., 2010
Alaska	• Cure for sores • Remedy against tuberculosis	Alm, 2004
Mongolia	• Remedy against tuberculosis • Anticancer • Escalation of physical endurance • Treatment for lung inflammation	Brown et al., 2002; World Health Organization, 2013

In Europe, the first documented medicinal use of *R. rosea* can be traced back to Dioscorides in 77 A.D. (Brown et al., 2002). In C. v. Linne's *Materia Medica*, the root of *R. rosea* was recommended for several conditions such as headaches, ''hysteria'', hernias and discharges (C. v. Linne 1749 in Panossian et al., 2010). Throughout the years, it has appeared in many pharmacopoeias and medicinal books of different countries such as Sweden, France, Norway, Germany, Iceland, Estonia and Russia (Brown *et al.* 2002, Alm 2004, Panossian *et al.* 2010, Shikov *et al.* 2014).

In China, 73 different *Rhodiola* species have been reported, mainly in the northwest and southwest regions such as Tibet and the Sichuan province. The adaptogenic and tonic properties of the *Rhodiola* plants have been widely used in traditional Chinese and Tibetan medicine (Li and Zhang, 2008). They are generally referred to with the Pinyin name Hong Jing Tian 红景天 (red (or glorious) view of heaven) with slight alterations for each species. (Table 2).

Table 2: Examples of the similar Pin Yin names of different *Rhodiola* species in China

Scientific name	**Pin Yin name**
R. rosea L.	Qiang Wei (rose smell) Hong Jing Tian
R. sachalinensis Borris.	Gao Shan (high mountain) Hong Jing Tian
R. quadrifida (Pall.) Fisch. & C.A.Mey	Si Lie (four split) Hong Jing Tian
R. crenulata (Hook.f. & Thomson) H.Ohba	Da Hua (big flower) Hong Jing Tian
R. yunnanensis (Franch.) S.H. Fu	Yunnan (From Yunnan) Hong Jing Tian
R. kirilowii (Regel) Maxim.	Xia Ye (narrow leaf) Hong Jing Tian
R. fastigiata (Hook. f. & Thomson) S.H. Fu	Chang Bian (clustered) Hong Jing Tian

R. crenulata can be traced back to Tibetan medicine books including ''The Four Medical Tantras'' (*rgyud-bzhi* in Tibetan, *Si Bu Yi Dian* in Chinese), Yue Wang's Classical Medicinal Book (*Somaratsa* in Tibetan, Y*ue Wang Yao Zhen* in Chinese) and Jing Zhu Materia Medica (*Shel Gong Shel Phreng* in Tibetan, *Jing Zhu Ben Cao* in Chin ese (Lu. and Lan 2013)). It is used for treatment of cough, hemoptysis, pneumonia and abnormal vaginal discharge. In Traditional Chinese Medicine (TCM), it has effects of nourishing qi as well as promoting blood circulation and is mainly prescribed for qi deficiency and blood stasis (QDBS), stroke, hemiplegia and fatigue. It is commonly used in China and Tibet for treating altitude sickness.

Phytochemical and pharmacological research:

Research on the phytochemistry and pharmacology of *Rhodiola* spp. was initiated in the 1960s in the Soviet Union and Scandinavia, mainly focusing on *R. rosea* (Brown et al., 2002). After the turn of the century the interest in this plant spread globally. Intensive phytochemical research led to the detection of known and novel compounds in *R. rosea* and related species (Ma et al., 2006; Yousef et al., 2006). Between 2000 and 2015 an increased number of publications stemming from Asian research groups have focused on the detection of novel compounds from *Rhodiola* species, usually in combination with their respective pharmacological assessments (Fan et al., 2001; Nakamura et al., 2007; Nakamura et al., 2008).

There are more than a few hundred pharmacological studies on medicinal *Rhodiola species* (mainly on *R. rosea)* that show a wide range of activities reflecting their diverse traditional use. They possess adaptogenic and stress-protective (neuro-cardio and hepato protective); cardioprotective; antioxidant effects as well as stimulating effects on the central nervous system including on cognitive functions such as attention, memory and learning; anti-fatigue effects; antidepressive and anxiolytic effects; endocrine activity normalising; life-span increasing effects. (Aslanyan *et al.* 2010, Sarris *et al.* 2011, Panossian *et al.* 2013). The main active compounds are reputedly phenylpropanoids (rosavin, rosarin, rosin) and phenylethanoids (salidroside and tyrosol).

Quality issues of medicinal *Rhodiola* spp.

Rhodiola roots and rhizomes are highly valuable products traded at an international level. Since the majority of *R. rosea* and *R. crenulata* raw material supplied still comes from wild-collection their intensive collection leads to scarcity. (Galambosi, 2006; (Lu. and Lan 2013)

Herbal preparations of *Rhodiola* species (mainly *R. rosea*) are extensively utilised around the globe. There is an increasing number of commercial products available on the American, Asian and European markets, either as food supplements or herbal medicines. *R. rosea* herbal monographs have been included in many Pharmacopoeias worldwide. On the other hand, *R. crenulata* is the only species used medicinally in TCM (Table 3).

Due to this rapid increase of *Rhodiola* raw material demand, other *Rhodiola* species such as *R. fastigiata, R. sahcalinensis, R. quadrifida, Rhodiola sacra* (Prain ex Hamet) S.H.Fu and

Rhodiola serrata H. Ohba have been sold on the market (Xin *et al.* 2015). Since there is not any consistent worldwide quality control programme, inadequate quality assessment of *Rhodiola* spp. is a common issue. This raises concerns about possible adulteration and misidentification issues. The lack of genuine drug material, confusion over the Chinese Pin Yin name of the drug when sourcing from China and accidental or deliberate adulteration during the manufacturing stage may contribute to low quality of final products.

The analytical techniques currently available focus on identifying *R. rosea* or *R. crenulata* through chromatographic methods. Other species of *Rhodiola* have generally not been considered. *R. sachalinensis* presents a particular problem as it may contain similar marker compounds to *R. rosea*. (and some sources suggest that it is the same species – see www.kew.org/mpns-portal)

Table 3: Generation of *Rhodiola* spp. recorded in selected pharmacopoeias and publications

Pharmacopeia / Publication	Recorded *Rhodiola* species	Medicinal use part	Herbal product
Department of Health and Ageing, Australian Government	*Rhodiola rosea*	Root (Rhizome)	Dry extract
Committee on Herbal Medicinal Products, European Medicines Agency (2012)	*Rhodiola rosea*	Rhizome et radix	Extract
United States Pharmacopeia (32th Edition)	*Rhodiola rosea*	Rhizome et radix	Dry extract, tincture
Chinese Pharmacopoeia (2010)	*Rhodiola crenulata*	Rhizome et radix	Extract
Russian Pharmacopoeia (12th Edition)	*Rhodiola rosea*	Rhizome et radix	Extract

Integrated analytical platform approach

NMR-based metabolomics

NMR-based metabolic fingerprinting has been used in numerous food and medicinal species focusing on their quality assurance as well as their pharmacology. Such comparative studies include Danggui [*Angelica sinensis* (Oliv.) Diels] and Engelwurz / European Angelica (*Angelica archangelica* L.) (Li *et al.* 2014). Metabolomic differences between different *Tussilago farfara* L. accessions (Zhi *et al.* 2012) and different *Salvia miltiorrhiza* Bunge production sites (Jiang *et al.* 2014) were also studied by NMR fingerprinting coupled with multivariate analysis. Compared to GC-MS and LC-MS, NMR has some advantages such as non-selectiveness, high reproducibility, and good stability (Simmler *et al.* 2014). At the same time, structural information on metabolites could also be obtained from NMR directly. Therefore, NMR could be regarded as an ideal choice for chemical comparison and identification of the phytochemical differences of medicinal plants.

HPTLC

Since the NMR- metabolomic approach is not a validated pharmacopoeial method, there is a need to be compared to a standard method like high performance thin layer chromatography (HPTLC). This method is widely used for the authentication and quality control of herbal substances (Reich *et al.* 2008). Compared to NMR-based metabolic fingerprinting, HPTLC could be highly effective with relatively lower price (Booker *et al.* 2014). HPTLC can also be helpful for the identification of specific compounds. Therefore, we chose these two complementary approaches in this study.

A third analysis strategy using DNA bar coding was used to help verify some of the samples (details are given in the supplementary material (S2)).

Material and methods

Sampling and preparation of plant material

42 batches of *Rhodiola* market samples (i.e. not authenticated) were collected between October 2014 and January 2015 from different suppliers including retail outlets, Internet, pharmaceutical companies in seven different locations (Beijing, Guangdong, Qinghai, Anhui, Hebei, Jilin and Hong Kong SAR) in China, Germany and Russia. These raw-material samples, were mainly labelled as *R. rosea*, *R. crenulata*, *R. sachalinensis* and *R. quadrifida.* 18 batches of authenticated plant material were provided by Agroscope institute

(Switzerland). The samples were rhizomes of *R. rosea* plants propagated from different wild Swiss populations (Mattmark, Carrasino, and Nomnon) or botanical gardens (Switzerland and Germany). In addition, authenticated *R. rosea* samples which were grown from seeds or provided to the institute by Dr. Bertalan Galambosi were also included. Lastly, in July 2015, samples of *R. crenulata* and *R. fastigiata* roots and rhizomes were collected from Garze, Sichuan, China (altitude 4,500 metres). These samples were authenticated by Professor Shuyuan Li, (Guangdong Pharmaceutical University, Guangzhou, China). Botanical reference materials (BRMs) for *R. rosea, R. crenulata* and *R. sachalinensis* were obtained from the National Institute of Food and Drug Control (NIFDC, China), Dr. William Schwabe (Germany) and Agroscope (Switzerland). BRMs for *R. quadrifida* and *R. fastigiata* were provided by Professor Alexander Shikov (Saint-Petersburg Institute of Pharmacy, Russia) and Dr. Anthony Booker (UCL School of Pharmacy- *R. fastigiata* authenticated by Professor Shuyuan Li, Guangdong Pharmaceutical University, Guangzhou, China), respectively.

All the collected samples were deposited in the herbarium of the UCL School of Pharmacy (London, UK). A detailed description of the investigated samples including their origins and representative symbols are provided in supplement (S1).

Crude root samples were ground to powder using a household grinder (EK1665ROFOB, Salter, UK) and sieved (0.70 mm mesh). All the powder samples were kept in 1.5 mL tubes (Eppendorf AG.) at 4 °C until use.

Solvents, reagents and reference compounds

Deuterium oxide (D_2O), methanol-d_4 (99.8% D, MeOD), dimethyl sulfoxide-d_6 (DMSO-d_6) and tetramethylsilane (TMS) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Salidroside, gallic acid, rosarin and rosavin were purchased from Sigma-Aldrich Chemicals (St Luis, USA). Tyrosol was purchased from Acros organics (New Jersey, US). Water used in this study was purified by using ULTRAPURE water system (Millipore, Germany). All other chemicals were of analytical grade.

^{1}H-NMR spectroscopy

Sample preparation

900 µL of MeOD-d_4 was added for extraction. The samples were vortexed (Rodamixer, UK) for 30 s and sonicated at an ultrasound bath (Fisher, XB22, UK) for 10 min. The solutions

were centrifuged for 10 min at 14,000 rpm (EBA21, Hettich, Faust Laborbedarf AG, Germany). 600 µL of supernatant was transferred to a 5mm diameter NMR spectroscopy tube and the samples were submitted for NMR spectroscopic analysis. The one and two dimensional ^{1}H-NMR spectra were recorded on Brucker Avance 500 MHz spectrometer (Bruker Analytic, Germany), which was equipped with a QNP (^{31}P, ^{13}C, ^{15}N and ^{1}H) 5mm cryoprobe. The acquisition parameters were: size of the spectra 64 k data points, line broadening factor = 0.16 Hz, pulse width (PW) = 30 degrees and the relaxation delay d1 = 1 s. The acquisition temperature was 298 K.

In order to assess the coherence of the results obtained, two samples from the same batch were subjected to NMR analysis on the different days of examination. To minimize the error caused by root selection during sample grinding, any samples weighed more than 500g were analysed twice.

Data analysis

The resulting spectra were manually phased and automatic baseline corrected by Topspin 3.2 (Bruker, Germany) for organic fractions. Signals between δ 5.20 - 4.40 ppm and δ 3.35 - 3.22 ppm were removed prior to statistical analysis due to the presence of methanol-d_4. The total area of peaks (δ 10.00 - 0.00 ppm) was integrated into small (0.04 ppm) buckets by bucketing (binning) function using AMIX or ACD-Labs in order to generate a number of integrated regions of the data set. The buckets obtained were then imported to Microsoft EXCEL (2013) where the samples were re-labelled and their species information was added.

Principal component analysis (PCA) was performed with SIMCA-P 13.0 (Umetrics, Umeå, Sweden) for metabolomic analysis of the generated dataset. Scaling mode of Pareto (Par) and Unit Variance (UV) were tested to optimize the analysis model with best R^2X value.

HPTLC

Sample preparation

1 ml of ethanol was added to 50mg of weighed samples for extraction. The solutions were then mixed on a rotary mixer (Rodamixer, UK) for 30 s, sonicated in an ultrasound bath (Fisher, XB22, UK) for 10 min and centrifuged for 10 min at 14,000 rpm. The supernatant was used for HPTLC analysis. The reference standard solutions of salidroside, rosarin,

rosavin, gallic acid and tyrosol were prepared at a concentration 1mg/mL in methanol. Both the reference material and the test samples were stored at 4^{o}C.

Data analysis

Samples were applied to the plates as bands 8 mm wide by using Linomat 5 semi-automatic applicator with 100 µL syringe. The space between bands was 2.0 mm and the rate of application was 90 nL· $s^{-1.}$ The number of tracks per plate was 15 and 5µl of standard and sample solution were applied.

The temperature and relative humidity were controlled to 21-24°C and 33 %, respectively. 10 mL solvent was poured into the right inlet for development and 25 mL solvent was poured into the left inlet for saturation. Plates were previously air dried for 10 s and developed in a 20 cm × 10 cm twin-trough chamber (Analtech, USA) lined with Whatman filter paper (20 cm × 10 cm) and saturated with mobile phase (Ethylacetate, methanol, water, formic acid (77:13:10:2) vapour for 20 min. The development distance was 70.0 mm from the lower edge.

The developed plates were derivatised by dipping in sulphuric acid reagent, using a CAMAG chromatogram immersion device and heated at 100°C on a plate heater for 5 min. Sulphuric acid reagent was prepared with procedure as follow: 20 mL sulfuric acid was carefully added to 180 mL ice-cold methanol and mixed. The plates were visualised using CAMAG visualizer under white light, UV 254 nm and at UV 366 nm, photographed and uploaded to HPTLC computer software (VisionCats).

Results and discussion

^{1}H-NMR and multivariate statistical analysis

By incorporating the whole region (0-10 ppm) and Par scaling a significant clustering is observed in *R. rosea* samples (Figure 2). *R. rosea* can be differentiated distinctly from the rest of the species based on their principal component variability.

Figure 2: Scores plot of five different species of Rhodiola (R. rosea, R. crenulata, R. quadrifida, R. sachalinensis, R. fastigiata), showing principle component 1 and principal component 2.

According to the spectra of the species (Figure 3), the aromatic region (6-8 ppm) is dominated by the main marker compounds (rosavin and salidroside). Hence, this region was

analysed independently using Par scaling (Figure 4). Based on the scores plot produced, *Rhodiola* species were separated more clearly compared to the scores plot of the whole region.

Figure 3: ^{1}H-NMR spectra of the reference compounds, salidroside and rosavin, together with the spectra of botanical reference material. 1: R. fastigiata, 2: R. quadrifida, 3: R. crenulata, 4: R. sachalinensis, 5: R. rosea, 6: rosavin and 7: salidroside.(From bottom to top) A: Whole region (0-10ppm); B: aromatic region (6-8ppm)

Figure 4: Scores plot of Rhodiola samples using the aromatic ^{1}H-NMR region and Pareto scaling

R. crenulata and *R. quadrifida* were also separated from the rest of the species. However, in this model they were clustered together. This means that there is no crucial metabolomic difference between them in the aromatic region. At this point it was considered important to visually inspect the spectra of the BRM's and detect any differences that they might be gone with the integration of the data. *R. crenulata* BRM has an additional quartet at 6ppm not detected in the rest of the species. This quartet can also be found in all the other *R. crenulata* samples investigated (figures not shown).

Therefore, an effective separation between *R. crenulata* and *R. quadrifida* samples can be accomplished by combining the PCA results with the detection of the additional peaks on the ^{1}H-NMR spectra only present in *R. crenulata* samples between 5-6ppm.

We also studied the group-pair comparisons in PCA model with Pareto scaling (Figure 5). The score plots showed that *Rhodiola* species separated well. (A: *R. crenulata* with *other Rhodiola species*; B: *R. rosea with other Rhodiola species;* C: *R. crenulata* with *R. rosea*).

Figure 5: Score plots of group comparison between Rhodiola species. A: R. crenulata (red) with other Rhodiola spp. (blue); B: R.rosea (green) with other Rhodiola spp. (blue); C: R. crenulata (red) with R. rosea (green).

The main differences were between δ 7.5 - 7.3 ppm (PC1) and δ 7.0 - 6.8 ppm (PC2). The chemical shift of the main variable metabolites were mainly rosavin, rosarin and cinnamyl alcohol derivatives.

The metabolites detected were elucidated by the analyses of the ^{1}H-NMR spectra as well as the comparison with the reference compounds, together with the in-house NMR chemical

shift database (Mudge *et al.* 2013, Luo *et al.* 2015). The summary of the assignment of ^{1}H-NMR spectral peaks obtained from the *R. rosea*, *R. crenulata* and *R. sachalinensis* BRM extracts are provided in supplement (S3).

HPTLC analysis

The band position and visibility of the standards rosavin, rosarin and salidroside (Figure 7) appear with characteristic colours and increasing retention factors (Rfs) 0.19, 0.26 and 0.31 respectively Fig.7, track H). Under UV light 254nm (track D), salidroside is not visible. Under 366nm, after derivitisation with sulphuric acid, rosavin and rosarin appear as pale pink bands and salidroside as a green one (track H).

Gallic acid shows good visibility under UV 254 nm (track A), while it is not easily detected under UV 366 nm at a dark blue back-round (track E). Tyrosol is visible in 254 nm but less clear in 366 nm.

Figure 6: Left: HPTLC results of standard compounds under UV 254 nm (rosavin Rf = 0.19, rosarin Rf = 0.26, gallic acid Rf = 0.58); Right: HPTLC results of standard compounds under UV 366 nm, after derivatisation with sulphuric acid (rosavin Rf =0.19, rosarin Rf = 0.26, salidroside Rf = 0.31, gallic acid Rf = 0.58, tyrosol Rf = 0.76)

The raw plant material obtained from the market was also studied by our HPTLC method (list of samples in Supplement S4). Under UV 254 nm (Fig. 8), there were two obvious bands among these samples (Rf= 0.27 and 0.48). However, due to lack of reference standards, their identity remains unknown. Further studies need to be conducted using NMR and TLC-MS. The majority of the samples investigated contained concentration of tyrosol similar to the standard raw material used (R24, R30 and R31). Samples R1-R6 contained lower levels of this compound possibly due to their longer storage time. Therefore, tyrosol could be considered as a marker to study duration of *Rhodiola* storage. It was also found that only five samples (R9, R25, R58, R59 and R24) contained high levels of rosavin, which turned out to be *R. rosea*. Moreover, this result can also be verified by the NMR results (Figure 5). However, it is not evident whether there is adulteration of *R. sachalinensis* in *R. rosea* since their metabolites are similar.

Under UV 366nm after derivatisation eight samples (R1, R5, R6, R15, R27, R32, R61 and R64) had low content in salidroside (Rf = 0.31). These samples could have been kept for rather long time after collection and the salidroside content could have decreased due to lack of good storage environment.

Figure 7: HPTLC results for all Rhodiola market samples, mobile phase (Ethylacetate, methanol, water, formic acid (77:13:10:2)

Combining the results of HPTLC and ^{1}H-NMR multivariate statistical analysis, we also analysed the adulteration rate among all the market samples (supplement, S4).

30% of the *Rhodiola* samples collected from the market were not *R. rosea* or *R. crenulata*. Some *R. rosea* samples were also being sold as *R. crenulata.* 47.7 % of raw materials samples were not labelled properly and their species information were not clearly illustrated to customers. This highlights the lack of proper local government policies and good quality control strategies.

According to our study, different *Rhodiola* species (including *R. rosea* and *R. crenulata)* can be found in the Chinese market. However, they are neither sold separately nor well identified. Therefore, there is a high potential of adulteration and substitution among these species.

Qualitative and quantitative analysis of mixtures

Since in the value chain mixing of batches and, therefore, potentially also of species also is of major concern, the possibility of qualitatively and quantitatively detecting plant mixtures was also investigated. The additional species chosen for this study was *R. crenulata* which is considered to be the most common adulterant of *R. rosea*. The selected BRMs were weighed individually in different proportions and then added together in an Eppendorf reaction tube. The rest of the sample preparation was identical to the methodology for the ^{1}H-NMR

spectroscopy. The samples were renamed as seen in Table 4. After the acquisition of the spectra, they were baseline and phase corrected and zeroed to the TMS peak in Topspin 3.2.

Table 4 ^{1}H-NMR-based detection of plant mixtures by

Sample name	Mg of *R. rosea* BRM	Mg of *R. crenulata* BRM
RR100	100	00
RR80RC20	80	20
RR60RC40	60	40
RR40RC60	40	60
RR20RC80	20	80
RC100	00	100

In all samples, the salidroside peak intensity remains almost the same since this constituent is present in both species. The peaks of rosavin are gradually decreasing with the addition of *R. crenulata*, whereas the characteristic quartet at 6ppm due to the presence of an unknown compound is increasing with the addition of *R. crenulata* and it is not detected in *R. rosea* at all (Figure 9).

Figure 8: ^{1}H-NMR spectra of the whole region (left) and the aromatic region (right) of the R. rosea and R. crenulata mixtures

The acquired spectra were bucketed using Amix and only focused on this region (6ppm). When the whole quartet was integrated into a single bucket, the observed increase of its intensity was not adequately represented. Therefore, the bucket size used changed to 0.02ppm and only incorporated the first peak of the quartet (6.0028-6.0048ppm). The buckets obtained from Amix when then transferred into Excel, where the relationship between the bucket value and the percentage of *R. crenulata* in the mixture was expressed graphically as a calibration curve. The bucket value of the respective peak is increasing in a linear mode (Figure 10).

Figure 9: Calibration curve showing the bucket value of the peak versus the percentage of R. crenulata within a mixture of R. crenulata and R. rosea

Similar results can also be obtained with HPTLC analysis. The HPTLC fingerprints produced consist of the over-spotted BRM extracts in different volumes as seen in Table 5. The final volume applied was 5 μL.

Table 5: Sample preparation for the detection of plant mixtures by HPTLC

R. rosea 100%	RR100	*R. rosea BRM*	5μL
R. rosea 80% and *R. crenulata* 20%	RR80	*R. rosea BRM*	4μL
		R. crenulata BRM	1μL
R. rosea 60% and *R. crenulata 40%*	RR60	*R. rosea BRM*	3μL
		R. crenulata BRM	2μL
R. rosea 40% and *R. crenulata 60%*	RR40	*R. rosea BRM*	2μL
		R. crenulata BRM	3μL
R. rosea 20% and *R. crenulata 80%*	RR20	*R. rosea BRM*	1μL
		R. crenulata BRM	4μL
R. crenulata 100%	RC100	*R. crenulata BRM*	5μL

As seen in Figure 11, when the loading volume of the *R. rosea* decreases, the representative markers of this species (rosavin and rosarin) decrease as well. However, the band for salidroside, (since it occurs in both species) remains almost the same.

Figure 10: HPTLC fingerprints of all R. rosea and R. crenulata mixtures under UV 254nm (tracks 1-6), white light and SAR (tracks 7-12) and UV 366nm and SAR (tracks 13-18).

By gradually increasing the *R. crenulata* proportion, several bands gradually appear above salidroside that could potentially be used as markers for the qualitative and semi-quantitative HPTLC analysis of mixtures of these two *Rhodiola* species. Further work needs to be carried out to determine the identity and species-specificity of these compounds.

Conclusions

This study provided a method for distinguishing five different species of *Rhodiola* and suggests possible methods for quantifying different species within mixtures. The metabolomic and phytochemical differences between these different species has been demonstrated through NMR spectroscopy and HPTLC analysis. Species represented with only a small number of samples will need further investigation in order to accurately define their chemical characteristics.

There is a need to study the links between producers and consumers especially when in trans-national trade and re-enforce the hypothesis that poor quality and adulterated products can be products of poorly governed value chains, particularly at the early stages of supply. Moreover, it can be argued that through the establishment of well-controlled and well managed value chains it is possible to better prevent accidental or deliberate contamination and adulteration from occurring

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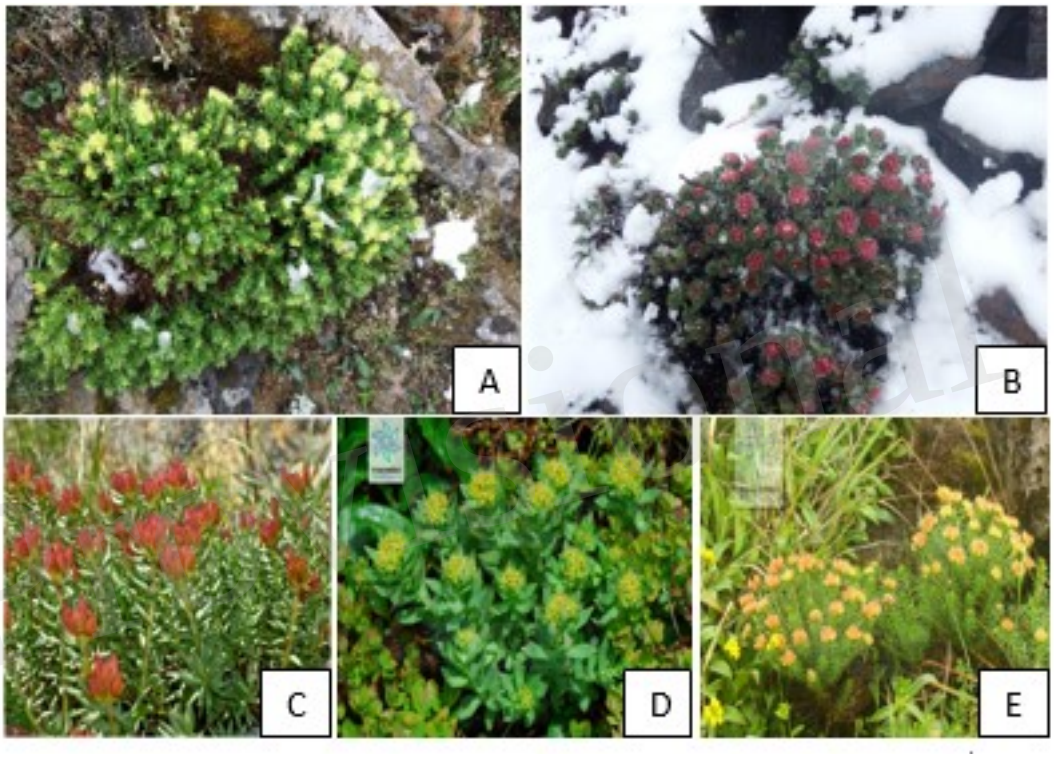
Figure 01.JPEG



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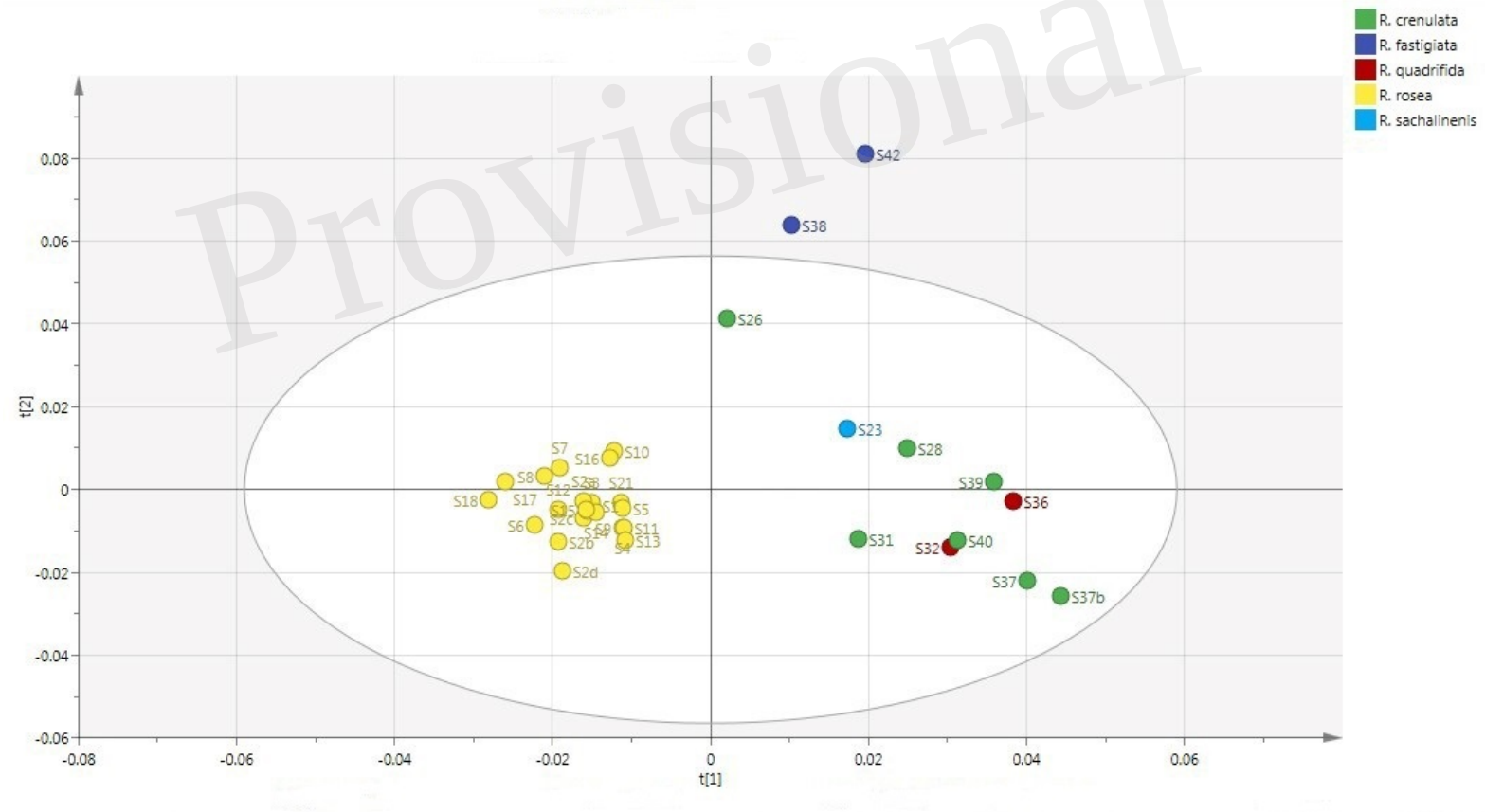

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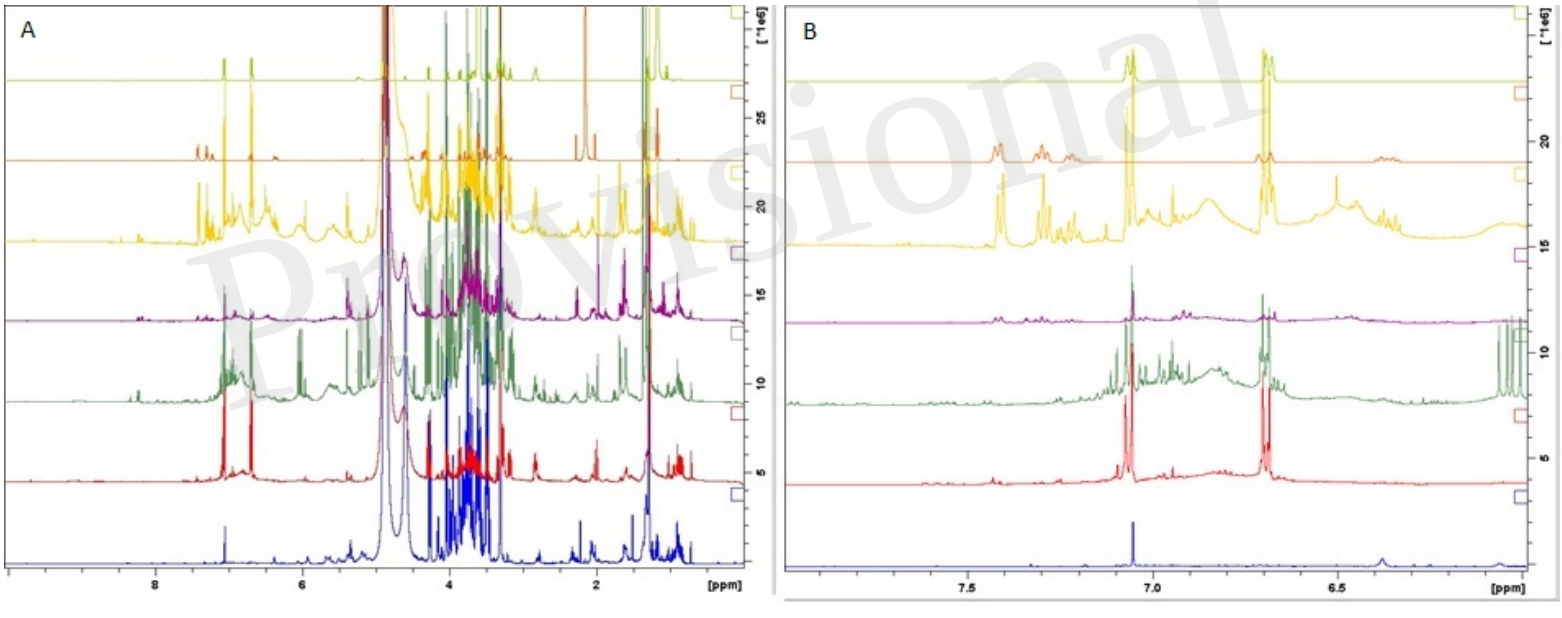

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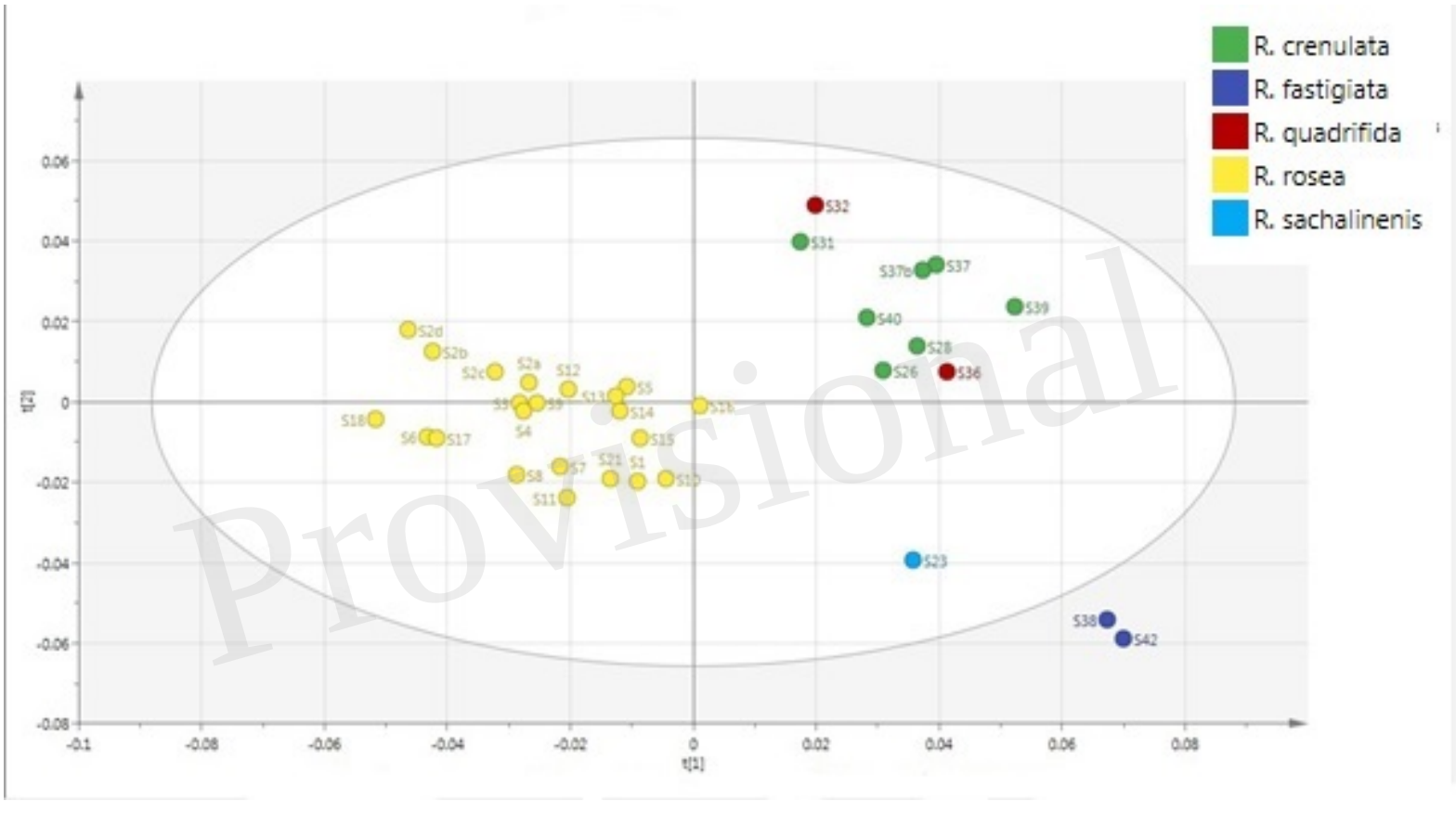

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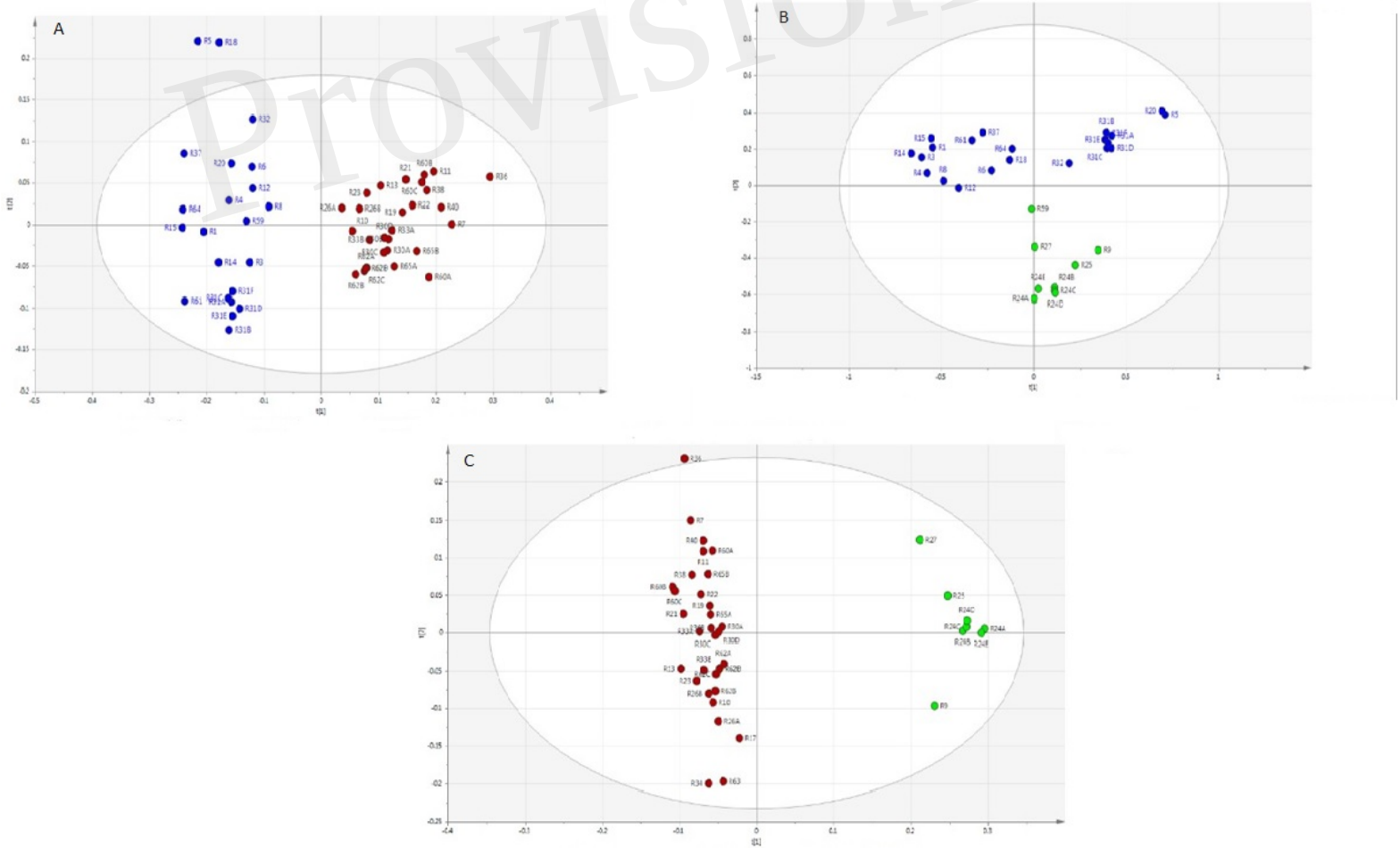

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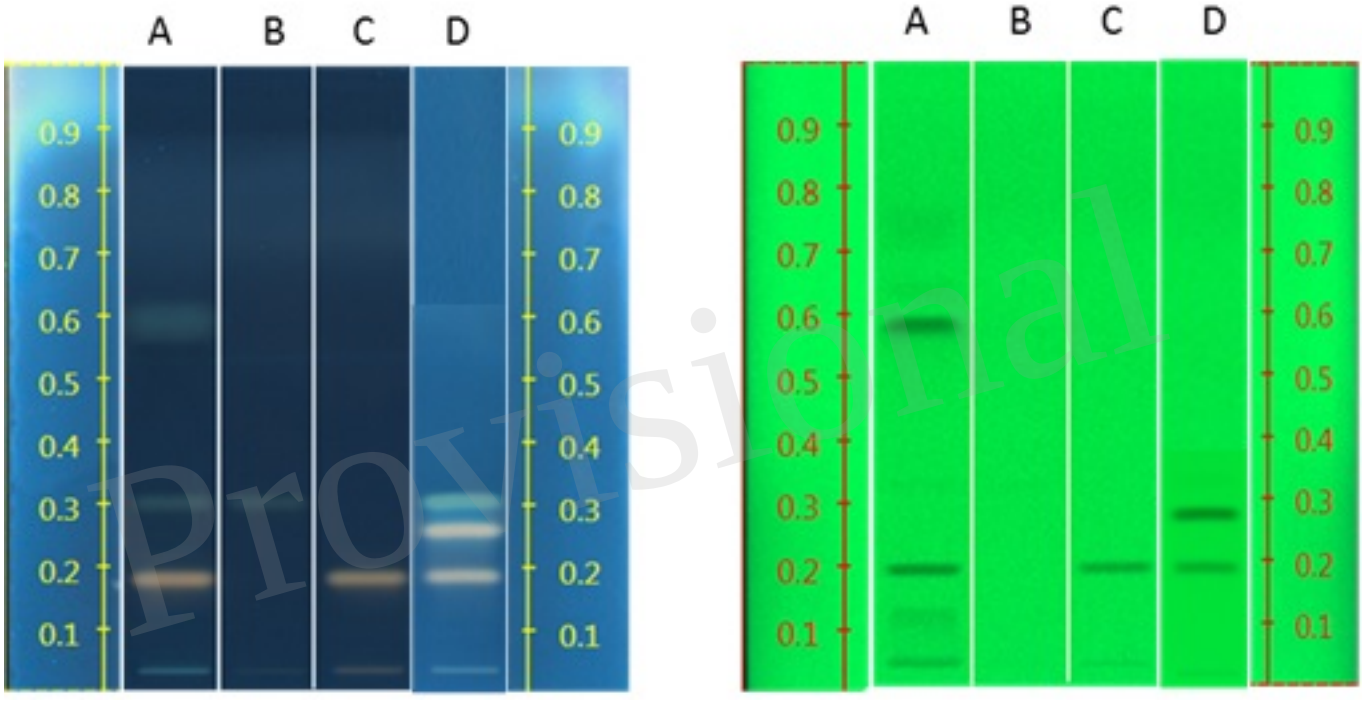

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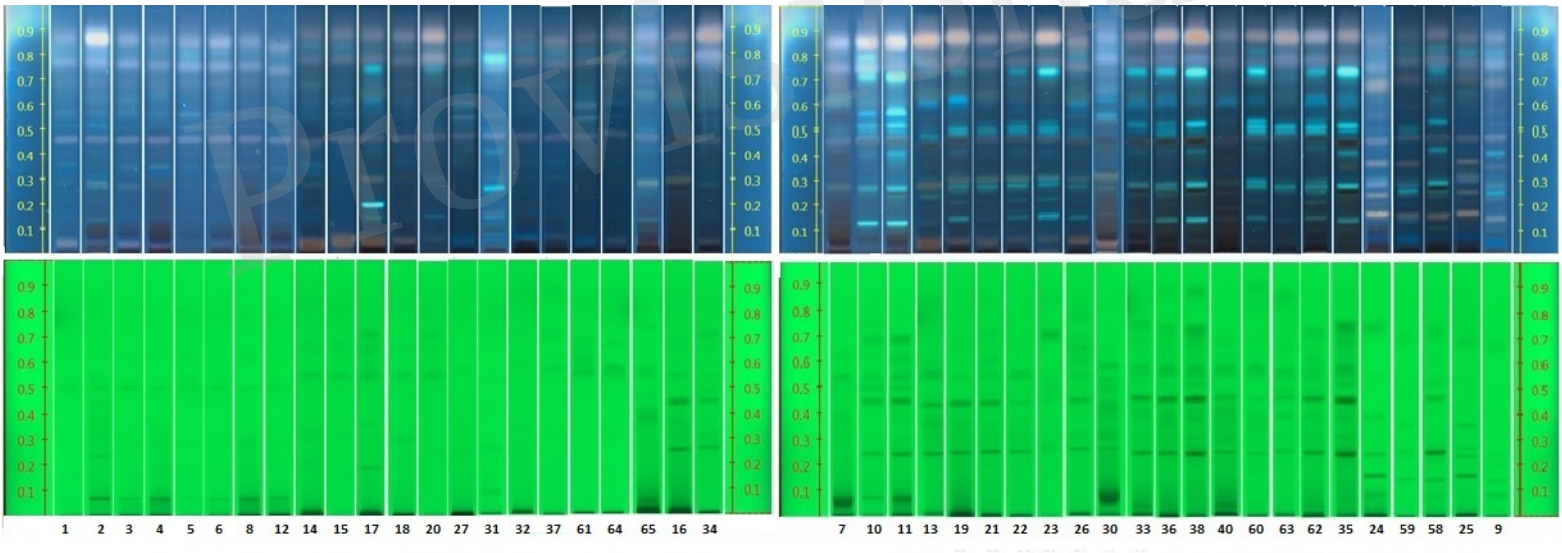

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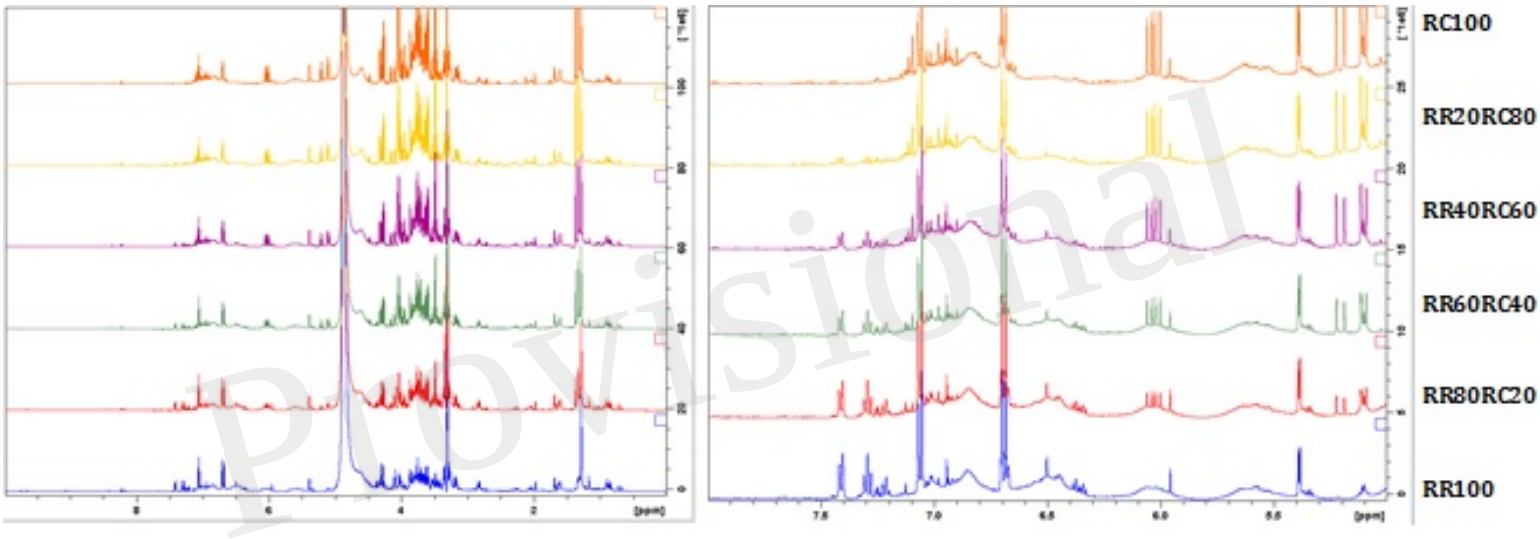

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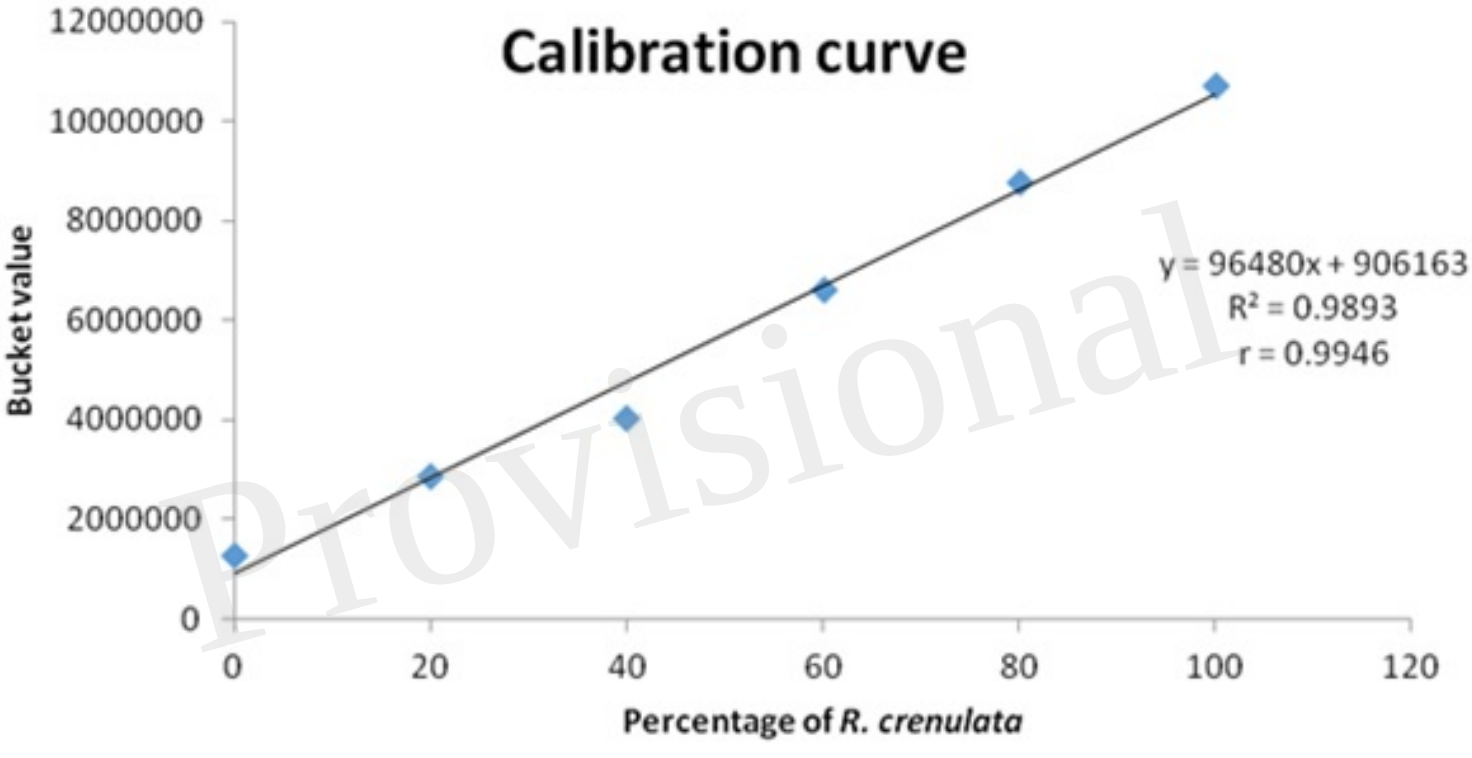

Figure 15.JPEG