Metabolic Profiling of Saponin-Rich *Ophiopogon japonicus* Roots Based on ^{1}H NMR and HPTLC Platforms[1]

Authors
Yanhui Ge[1,2*], Xiaojia Chen[3*], Dejan Gođevac[1,4], Paula C. P. Bueno[1], Luis F. Salomé Abarca[1], Young Pyo Jang[5], Mei Wang[6,7], Young Hae Choi[1,5]

Affiliations
1 Natural Products Laboratory, Institute of Biology, Leiden University, Leiden, The Netherlands
2 School of Pharmacy, Xi'an Jiaotong University, Xi'an, China
3 State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China
4 Institute of Chemistry, Technology and Metallurgy, National Institute, University of Belgrade, Belgrade, Serbia
5 College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
6 LU-European Center for Chinese Medicine and Natural Compounds, Leiden, The Netherlands
7 SU Biomedicine, Leiden, The Netherlands

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Correspondence
Dr. Young Hae Choi
Natural Products Laboratory, Institute of Biology, Leiden University
Sylviusweg 72, 2333 BE Leiden, The Netherlands
Phone: + 31 71 527 45 10, Fax: + 31 71 527 45 11
y.choi@chem.leidenuniv.nl

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ABSTRACT

Ideally, metabolomics should deal with all the metabolites that are found within cells and biological systems. The most common technologies for metabolomics include mass spectrometry, and in most cases, hyphenated to chromatographic separations (liquid chromatography- or gas chromatography-mass spectrometry) and nuclear magnetic resonance spectroscopy. However, limitations such as low sensitivity and highly congested spectra in nuclear magnetic resonance spectroscopy and relatively low signal reproducibility in mass spectrometry impede the progression of these techniques from being universal metabolomics tools. These disadvantages are more notorious in studies of certain plant secondary metabolites, such as saponins, which are difficult to analyse, but have a great biological importance in organisms. In this study, high-performance thin-layer chromatography was used as a supplementary tool for metabolomics. A method consisting of coupling ^{1}H nuclear magnetic resonance spectroscopy and high-performance thin-layer chromatography was applied to distinguish between *Ophiopogon japonicus* roots that were collected from two growth locations and were of different ages. The results allowed the root samples from the two growth locations to be clearly distinguished. The difficulties encountered in the identification of the marker compounds by ^{1}H nuclear magnetic resonance spectroscopy was overcome using high-performance thin-layer chromatography to separate and isolate the compounds. The saponins, ophiojaponin C or ophiopogonin D, were found to be marker metabolites in the root samples and proved to be greatly influenced by plant growth location, but barely by age variation. The procedure used in this study is fully described with the purpose of making a valuable contribution to the quality control of saponin-rich herbal drugs using high-performance thin-layer chromatography as a supplementary analytical tool for metabolomics research.

[1] Dedicated to Professor Dr. Cosimo Pizza 70th birthday in recognition of his outstanding contribution to natural product research.

[*] These authors contributed equally to this article.

Introduction

Recent advances in analytical technology such as NMR- and MS-based methods have allowed metabolomics researchers to start to close the gap between the whole metabolome and that which can be detected [1–3]. However, most of the signals or peaks that appear in these spectra still remain unidentified. This is partly due to the fact that plant secondary metabolites are often analogues and isomers. Many of these metabolites are present in glycosidic forms that yield numerous isomers, including stereo- and positional isomers. It can be argued that the differentiation between these metabolites is one of the most challenging aspects of compound identification, yet, it is inevitable that a solution will be found to this if a true representation of the actual metabolome of interest is to be obtained.

Among the most common glycosides in nature, saponins are typical amphiphilic glycosides with triterpenoid and/or steroid aglycones as the lipophilic moiety and sugars as the hydrophilic moiety. Saponins are widely distributed in diverse types of organisms such as plants, fungi, and marine life [4–6]. Traditionally, many saponin-rich medicinal plants have been used for diverse clinical purposes such as anti-inflammatory activity and cardiovascular diseases as well as tonics and improving metabolic balancing. For example, *Panax ginseng* [4], *Allium* species [7], *Terminalia arjuna* [8], *Clematis* species [9], *Glycyrrhiza glabra* [10], *Ilex paraguariensis* [11], *Crataegus oxyacantha* [12], and *Astragalus membranaceus* [13] have been extensively studied for their notable bioactivities. Several methods have been used for the analysis of saponins in natural products. Photocolorimetry has been employed to detect the total saponin content of natural product extracts but has great limitations, such as a high degree of false positives, the impossibility of identification of individual saponins, and data fluctuation caused by experimental conditions [14]. GC has also been applied to the analysis of saponins but requires the hydrolysis of the glycosidic link due to the structural variation largely arising from the combination of the glycosidic moiety and the aglycone, e.g., the type, position, and number of sugars [15]. Thus, GC is usually employed to classify saponins based on their aglycones. In the past, many saponin-targeted analyses have been based on HPLC coupled to diverse detectors, such as UV, refractive index, or evaporative light scattering detectors (ELSD) [16], but since the development of soft ionisation methods in MS, the LC-MS tandem has became the most popular method for natural compounds. However, in the case of complex mixtures, metabolite identification is particularly difficult due to the presence of numerous isomers and large numbers of adduct ions that interfere with the identification of molecular ions. This great technical variation means saponins are one of the most challenging groups for metabolomics analyses.

A present trend of life science is to investigate organisms in a holistic manner, rather than targeting certain aspects or groups of compounds to obtain pseudo-profiles. Many medicinal plants that contain saponins as major metabolites have been investigated for potential correlations between the metabolome and other factors, such as the variety, bioactivity, and seasonal and geographical variations. For example, *A. membranaceus* roots that contain saponins as major metabolites have been chemically profiled with the expectation of identifying key chemomarkers that relate to differences in age and location of cultivation. However, when ^{1}H NMR was employed as the analytical platform, while total saponins were associated with these factors, it was difficult to identify individual saponins due to a lack in characteristic ^{1}H resonances and to regions of intense overlapping within the aliphatic range [13].

Similar issues were experienced with metabolomics studies on the *Ilex* species. Whereas numerous phenolic metabolites were identified, saponins and triterpenoids were almost impossible to identify. The correlation of metabolites with genetic (species or variety within the species) and environmental factors (geographical location and planting time) was thus attributed mainly to phenolics, such as chlorogenic acid analogues and arbutin, due to the difficulty posed by the low content of very diverse saponins in the extracts [11].

Recently, high-performance thin-layer chromatography (HPTLC) has been revisited as a supplementary tool for metabolomics to expand its application from mere chemical screening to a versatile profiling tool [17, 18]. This is due to advantages such as the availability of derivatization reagents, short run times, and the possibility of performing simultaneous, parallel analysis. Furthermore, the ease of preparative HPTLC adds to its great potential to solve the issues with metabolomics studies of saponin-rich materials. These metabolites can be visualised on HPTLC plates and then extracted from them to be subjected directly to NMR- or MS-based analysis for further chemical elucidation.

Ophiopogon japonicus (L. f) Ker-Gawl (Asparagaceae) roots, one of the most famous saponin-rich drugs in traditional Chinese medicine (TCM) were used as a model to analyse the feasibility of applying HPTLC-based methods as supplementary tools following initial general (macroscopic) metabolomics, particularly for saponin-containing drugs. The roots, both as a single plant medicine or in mixtures with other plants, have been used to treat cardiovascular and chronic inflammatory diseases for thousands of years in East Asia. A number of previous chemical studies have shown that *O. japonicus* has a high amount of steroidal saponins, polysaccharides, and homoisoflavonoidal compounds, all of which possess different therapeutic effects. This has been well reviewed by Min-Hui Chen [19].

The metabolic variation of plants is largely mediated by both genetic and environmental factors [20, 21]. Similar to many other medicinal plants, *O. japonicus* roots are currently grown in several locations across China. Previous studies have shown that the quality of *O. japonicus* roots is influenced by different factors such as species [22] and growth location [23, 24]. Among these factors, the geographical origin of *O. japonicus* has been proven to produce most of the variation in its clinical activity. For example, Lin and colleagues reported that *O. japonicus* from the Zhejiang province had a higher total homoisoflavonoid content and better antioxidant activity than that of *O. japonicus* from Sichuan province [23]. It was also demonstrated that the Zhejiang *O. japonicus* extract had a stronger antioxidant and anti-inflammatory capacity than the Sichuan *O. japonicus* extract. Interestingly, both *O. japonicus* root extracts exhibited cytotoxicity with different selectivity on cancer cell lines *in vitro* [24]. Ge also reported that *O. japonicus* from different producing areas possessed different properties

with cytotoxicity and drug-metabolising enzyme induction [25]. The fact that these results are bioactivity-based and this bioactivity is related to metabolites indicates that the regional variation largely affects the level or type of metabolites. Another factor to be investigated is age, since this has been strongly related to the quality of root-based medicinal drugs, such as ginseng roots [26]. However, in the case of *O. japonicus* roots, the effect of age on a wide range of metabolites is still unknown.

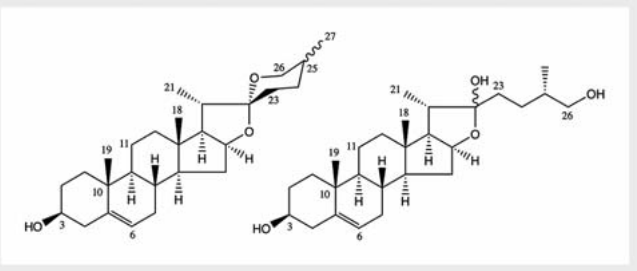
In this investigation, *O. japonicus* root samples from two locations (the Zhejiang and Sichuan provinces in China) at different ages (1–3 years old) were initially analysed by ^{1}H NMR to obtain an overall metabolic picture. Following this, specific metabolites selected from the ^{1}H NMR analysis were further analysed by HPTLC via saponin-targeted profiling. The compounds were separated and derivatised as bands, and then extracted for further analysis by MS-based techniques (UPLC-MS and GC-MS).

Results and Discussion

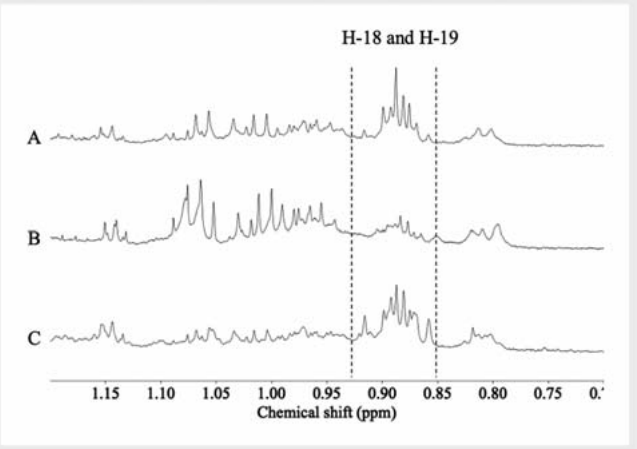
Of the wide range of metabolites found in *O. japonicus* roots, homoisoflavonoids and steroidal saponins (two basic skeletons, spirostanol and furostanol) (▶ **Fig. 1**) are known to be the two major metabolic groups that are mainly associated with their pharmacological activities. Consequently, the metabolites of those two groups are currently used as chemical markers for quality control of these plants. A UV-based method, HPLC-UV, has been adopted for the chemical analysis of homoisoflavonoids, whilst ELSD coupled to HPLC was applied for saponins [19]. Due to the low concentrations of the compounds of interest, samples of *O. japonicas* roots require lengthy pretreatment for their concentration, such as liquid-liquid extraction [27], solid-phase extraction [28], or column chromatography with porous polymers [14].

In this study, chemical profiling with ^{1}H NMR was used to obtain a metabolic overview of the roots. The most highly detected key metabolites were sugars (glucose, raffinose, stachyose, sucrose, and some oligosaccharides), amino acids (alanine, arginine, aspartic acid, asparagine, glutamic acid, glutamine, isoleucine, leucine, proline, threonine, tryptophan, tyrosine, and valine), and some organic acids such as acetic, *trans*-aconitic, formic, fumaric, and lactic acids. The homoisoflavonoids and some characteristic methyl signals of saponins within the range δ 0.70–1.25 (▶ **Fig. 2**) were detected, but at extremely low concentrations. The complexity of the chemical structures of saponins results in a variation of individual chemical shifts of their methyl signals. However, those of H_3-18 and H_3-19 at δ 0.8–1.0 are not changed significantly by structural variations. As a result, the signals may be used as distinguishing marker signals of the total saponin content within the metabolite matrix, even when they are partly overlapping with some amino acid signals such as those from leucine.

To gain further insight into the *O. japonicus* metabolome, the ^{1}H NMR data were then analysed by orthogonal partial least square (OPLS) modelling analysis or OPLS-discriminant analysis (DA). Firstly, the samples collected from two of the most famous cultivation locations in China, the Sichuan and Zhejiang provinces, were compared (▶ **Fig. 3A**). The model used two classes (Sichuan and Zhejiang) and validated the correlation by the permutation test ($Q^2 > 0.7$ and p value in CV-ANOVA < 0.01). The metabolites



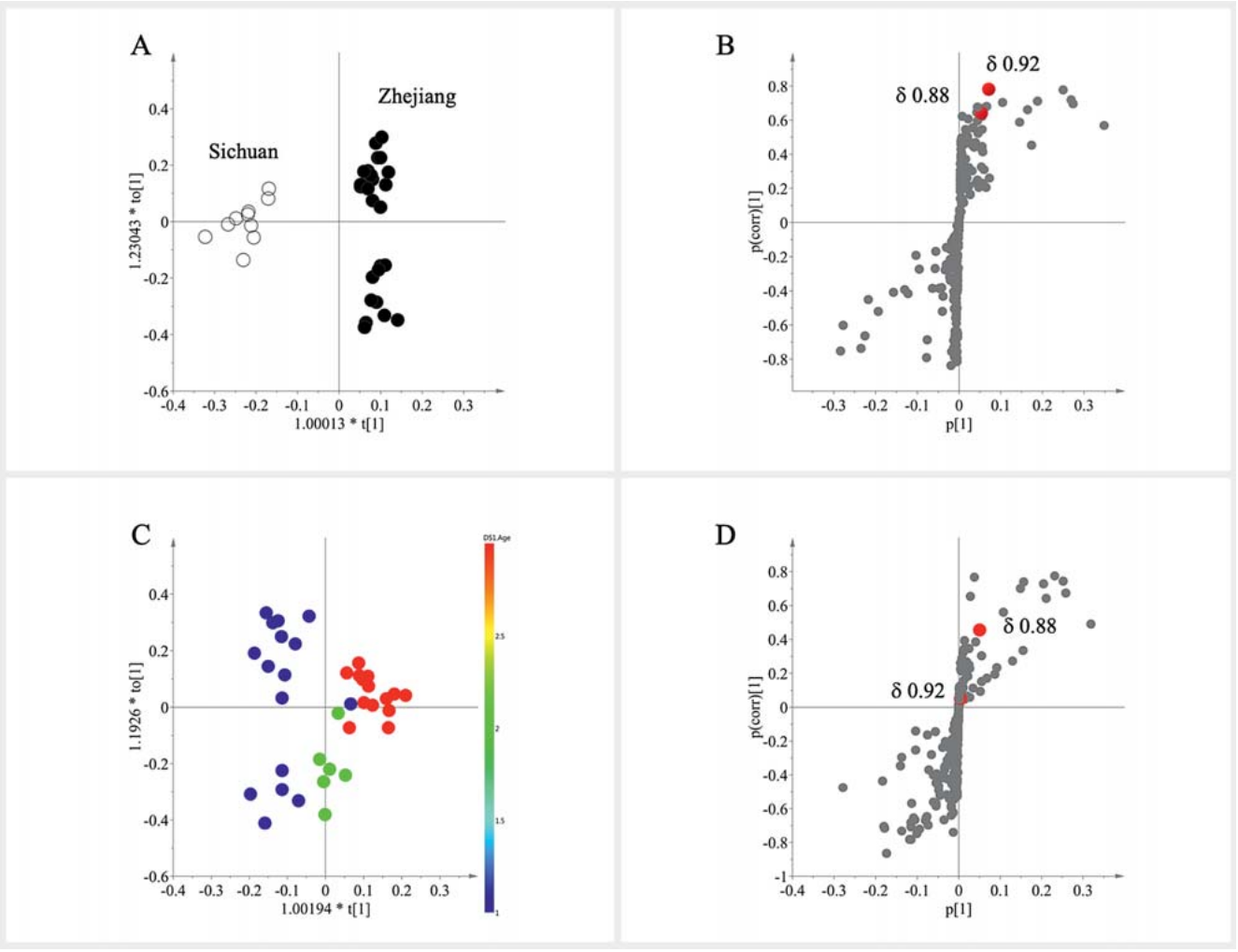
▶ **Fig. 1** Basic chemical structures of spirostanol and furostanol of *O. japonicus* roots.



▶ **Fig. 2** Typical ^{1}H NMR spectra (600 MHz) of *O. japonicus* roots collected from Sichuan, China (**A**: 1-year-old) and Zhejiang, China (**B**: 1-year-old; **C**: 3-year-old) in KH_2PO_4 buffer in D_2O-CH_3OH-d_4 (1 : 1, v/v) within the range δ 0.70–1.20.

responsible for the separation were found to be sugars. Interestingly, two methyl signals at H-18 and H-19 of the steroidal saponins as indicated by the S-plot in ▶ **Fig. 3B** were found to be more abundant in the Zhejiang samples.

Although it is evident that the *O. japonicus* roots collected from two different locations were clearly distinguishable, the age of the plants was also a potential factor to consider. The Sichuan samples used in this study were all 1 year old, while the Zhejiang samples ranged from ages 1–3 years old. Unsurprisingly, the highest variation was observed in these samples. Thus, the separation between the two samples could also be influenced by differences in the age of the roots. In order to address this issue more specifically, the ^{1}H NMR spectra from the samples were analysed by OPLS with the age variable as the Y-data set to evaluate whether there is a true effect of age on the metabolome. As shown in ▶ **Fig. 3C**, the effect of age was found to be of equal significance to that of the location ($Q^2 > 0.7$ and p value in CV-ANOVA < 0.01) and some sugars appeared to be associated only with the older samples. The contribution of saponins to the separation of age was not as straightforward, because as seen in S-plot of ▶ **Fig. 3D**, the characteristic signals of the saponins, H-18 and H-19, were not as clearly distinguished as in the case of collection places.

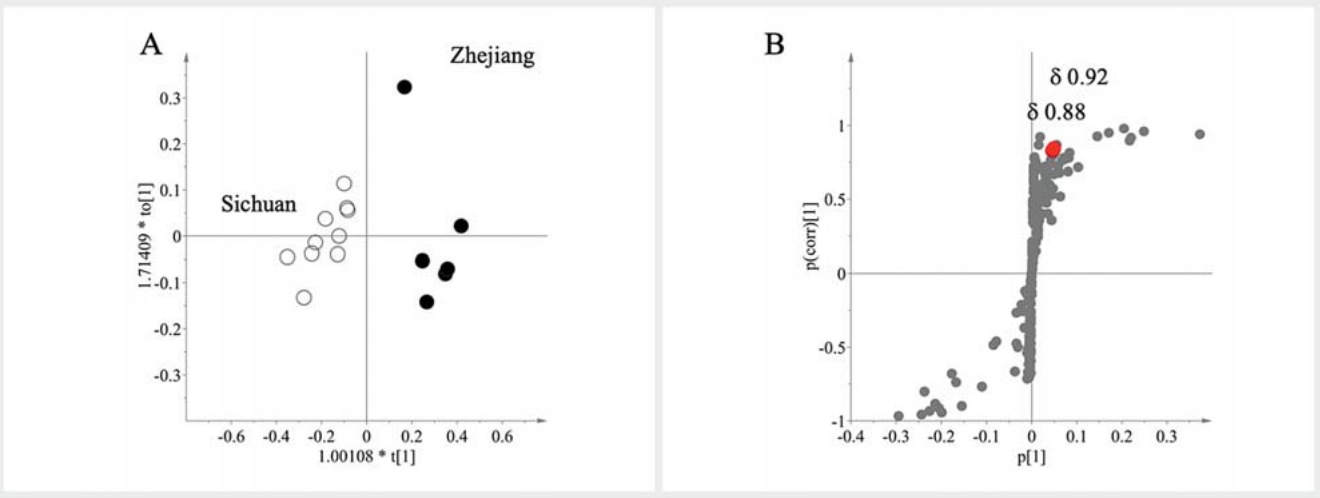


▶ **Fig. 3** OPLS-DA based on ^{1}H NMR data using the collection location (Sichuan and Zhejiang in China) of *O. japonicus* roots as two classes (**A**: score plot and **B**: S-plot). OPLS modelling analysis using the age of *O. japonicus* roots as Y-variables (**C**: score plot and **D**: S-plot).

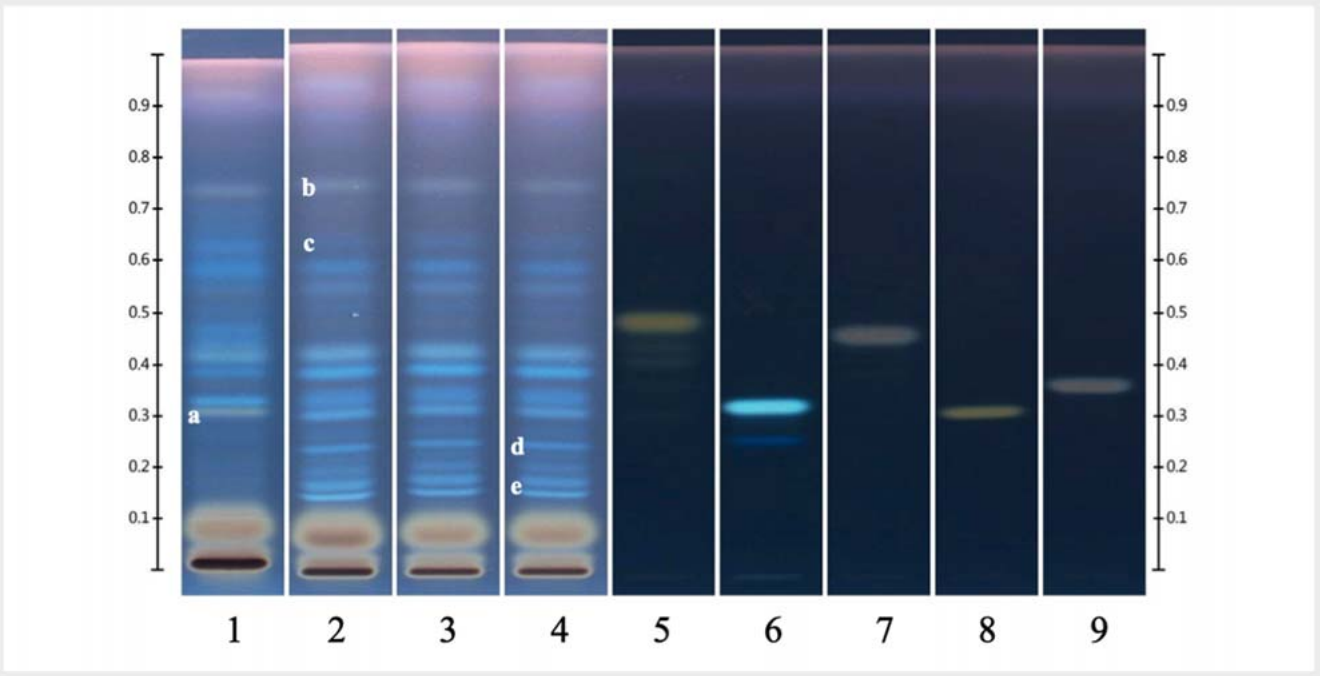
As a next step, the samples of the same age but from the two different locations were compared after removing older samples of the Zhejiang collection. As can be observed in ▶ **Fig. 4A**, a very clear separation between Sichuan and Zhejiang samples was obtained, with some sugars and saponins as potential chemomarkers (▶ **Fig. 4B**).

The information obtained from the ^{1}H NMR-based metabolic profiling confirmed that the metabolic levels of *O. japonicus* roots were strongly influenced by their geographical origin and age. However, the investigation into the individual metabolites that are key indicators for the distinction between the influencing factors was highly difficult due to low concentrations and signal congestion, particularly for saponins. Thus, a supplementary technique based on an HPTLC method targeting these compounds was applied to the same set of samples.

In recent years, HPTLC has been proposed as a supplementary tool for metabolomics with applications to ginseng, ginkgo, and pine resins [17, 18]. Its application for the study of the saponin-rich *O. japonicus* seemed promising as a way of separating these compounds for better identification by ^{1}H NMR, for example. Thus, the 36 samples of the roots were subjected to HPTLC, and a typical chromatogram obtained with *O. japonicus* roots collected from different geographical locations and ages is shown in ▶ **Fig. 5**. The intensity of all of the R_f values of the HPTLC chromatograms was measured by a visualiser under UV 366 nm after treatment with anisaldehyde-H_2SO_4. Each R_f value was normalised to that of the corresponding R_f value of the QC samples, made from a combined pool of all 36 roots of *O. japonicus* to reduce technical variations. Multivariate data analysis from ^{1}H NMR data was further confirmed with the principal component analysis and OPLS-DA models based on this normalised HPTLC data. As shown in ▶ **Fig. 6A**, clear separations were achieved between the samples of different collection locations and ages. In the case of geographical variation, HPTLC analysis targeting the saponins indicated that the samples collected from the two provinces were clearly distinguished by main clusters, which was consistent with the ^{1}H NMR data. For the identification of the contributing metabolites, the analysis of the loading plot showed that most of HPTLC



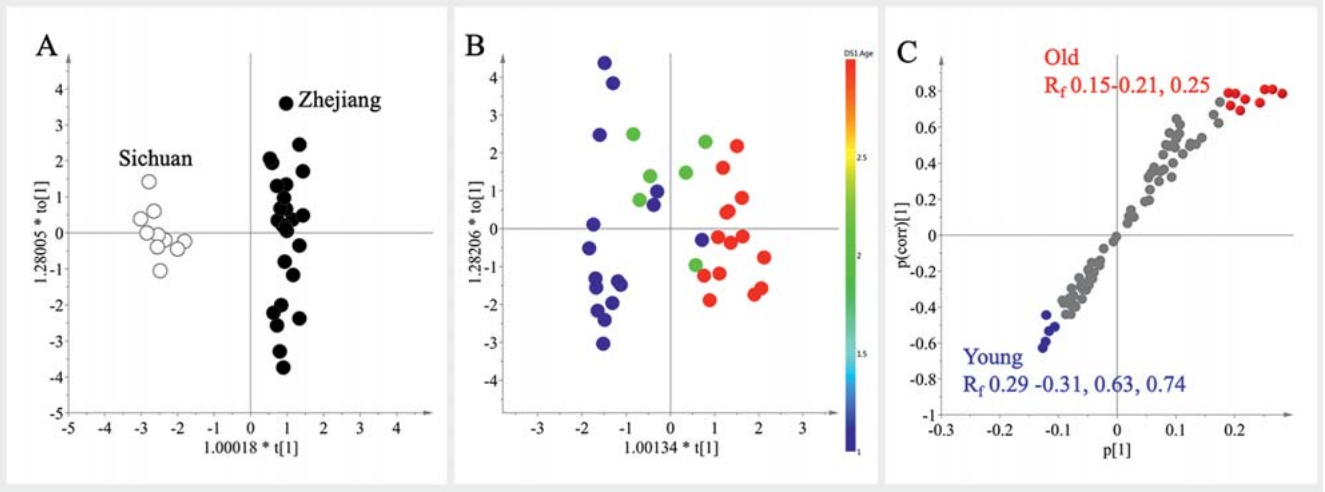
▶ **Fig. 4** OPLS-DA based on ^{1}H NMR data using the collection location (Sichuan and Zhejiang in China) of 1-year-old *O. japonicus* roots as two classes (**A**: score plot and **B**: S-plot).



▶ **Fig. 5** Typical HPTLC chromatogram of *O. japonicus* roots in this study developed on silica gel HPTLC plates (20 × 10 cm, F254) using a mixture of ethyl acetate, formic acid, and acetic acid-water (100 : 11 : 11 : 27, v/v/v/v) as the mobile phase. Plates were sprayed with anisaldehyde-H_2SO_4 and viewed at UV 366 nm. *O. japonicus* roots from Sichuan, China (**1**: 1-year-old); from Zhejiang, China (**2**: 1-year-old; **3**: 2-year-old; **4**: 3-year-old); **5**: Ophiopogonin A; **6**: Ophiojaponin C; **7**: Prosapogenin A; **8**: Ophiopogonin D; **9**: Ophiopogonin D′. **a**: R_f 0.29–0.31, **b**: R_f 0.74, **c**: R_f 0.63, **d**: R_f 0.25, **e**: R_f 0.15–0.21.

bands, including all the saponins tested in the study, were higher in the Zhejiang samples except, interestingly, the band with R_f 0.29–0.31 (**a** in ▶ **Fig. 5**), which increased in the samples collected in Sichuan province, as shown in the HPTLC chromatogram in ▶ **Fig. 5**. The band was identified as ophiopogonin D by comparison with a reference compound.

Geographical location is not, per se, a single factor, but includes numerous other features such as temperature, soil conditions, altitude, and/or rainfall. In the past, the variation produced by these factors focussed on phenolic constituents. Lin and colleagues reported that *O. japonicus* roots from Zhejiang province might have a higher level of homoisoflavonoids than those from



▶ **Fig. 6** Score plot of OPLS-DA based on HPTLC data using the collection location (Sichuan and Zhejiang in China) of *O. japonicus* roots as two classes (**A**). OPLS modelling analysis using the age of *O. japonicus* roots as Y-variables (**B**: score plot and **C**: S-plot).

Sichuan, but found no significant difference between the total phenolic contents and types [23]. However, the variation in saponin contents of roots from different collection locations has not been investigated.

A strong correlation between *O. japonicus* root age and metabolic levels was detected both within the HPTLC data and ^{1}H NMR analysis as validated by a high Q^2 value in the permutation test (> 0.5) and a low p value in CV-ANOVA (< 0.01) (▶ **Fig. 6B**).

Similar to ^{1}H NMR analysis, the S-plot was employed to detect the marker compounds for the age factor. The R_f 0.29–0.31, 0.64, and 0.74 bands were found to be higher in the young roots whilst the R_f 0.15–0.21 and 0.25 bands were strongly correlated with the old roots (▶ **Fig. 6C**). Among these, the R_f 0.29–0.31 bands were identified as ophiojaponin C or Ophiopogonin D.

The age of some roots has been reported to be correlated with the saponin level both quantitatively and qualitatively. For example, the total content of saponins and senegenin of *Polygala tenuifolia* exhibited a sustained decreasing trend with increasing root age according to the study conducted by Teng et al. [29]. Ma et al. [30] found no significant change in the total saponin levels in *Astragali* roots up to 4 years old, whilst Peng et al. [31] found that 5-year-old roots of *A. membranaceus* plants contained the highest saponin levels as compared to 1-, 2-, 8-, and 10-year-old roots.

The quality of metabolomics studies can be evaluated by both the amount of identified metabolites and the intensity of the response of analytical signals. Currently available analytical methods for metabolomics are progressively improving both in their accuracy and sensitivity. However, admittedly, there is still a huge gap between the real metabolome and the detected metabolome, mainly due to the large number of the metabolites that remain unknown. In this sense, HPTLC can been considered a promising supplementary tool for metabolomics. Its application to saponin-rich *O. japonicus* roots allowed for the evaluation of its potential and feasibility as such a tool. Our results revealed interesting and significant differences related to the environmental factors which can influence the metabolite abundances. A clear distinction was detected between samples from the two main provinces Zhejiang and Sichuan, since main clusters could be observed both in ^{1}H NMR data and HPTLC data, and it was evident that more saponins were found in the younger roots from the Zhejiang province. Considering that saponins are one of the most important metabolites for the therapeutic efficacy of many medicinal plants, the use of HPLTC provided a satisfactory solution to the challenge posed by their identification within a complex mixture. This places this technique in a strong position as a supplementary analytical tool for metabolomics based on R_f values and intensity of the chemical markers.

The confirmation of the chemical structures of compounds in these bands can be resolved with further spectrometric analysis, like MS or NMR. Thus, the hyphenation of HPTLC as an efficient automatic sample preparation technology with NMR and MS (LC- or GC-MS) has proved to be highly useful for metabolomic studies [18, 20]. However, some of the limitations related to structural features of saponins do persist, since they tend to bind strongly to the silica sorbent and show low sensitivity in LC- MS analysis requiring hydrolysis for GC-MS. Thus, integrating NMR and MS through HPTLC in the near future will result in a great leap forward for metabolomics.

Materials and Methods

Chemicals and materials

All solvents and reagents used in extraction and analyses were of analytical grade. Formic acid, methanol, ethyl acetate, and acetic acid were purchased from Sigma. The reference compounds ophiopogonin A, ophiopogonin C, prosapogenin A, ophiopogonin D, and ophiopogonin D were obtained from Shanghai Yuanye Bio-Technology Co. Ltd. The purity of all reference compounds was > 98 %. The glass plates for HPTLC (silica gel 60 F254, 20 × 10 cm) were purchased from Merck Millipore.

Plant material

Thirty-six *O. japonicus* root samples were collected from several locations in the Sichuan and Zhejiang provinces in China, the detailed information of which, including voucher specimen numbers, is described in **Table 1S**, Supporting Information. The plants were identified by one of the co-authors, Xiaojia Chen, and deposited at the Natural Products Laboratory, Institute of Biology Leiden, Leiden University, Leiden, The Netherlands.

^{1}H NMR analysis

Plant samples were prepared for ^{1}H NMR analysis using a modified version of our previous protocol [3]. Thirty milligrams of each dried and powdered sample were vortexed with KH_2PO_4 buffer (pH 6.0) in a mixture of D_2O containing 5.8 mM of trimethyl silyl propionic acid sodium salt (w/w) (TMSP) and CH_3OH-d_4 (1 : 1), ultrasonicated for 20 min at 42 kHz (Ultrasonicator 5510E-MT, Branson), and then centrifuged at 26 451 *g* at room temperature for 5 min (Heraeus Pico 17 Centrifuge, Thermo Scientific). An aliquot of 250 µL of the supernatant was transferred to a 3-mm NMR tube.

Proton nuclear magnetic resonance spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker) operating at a proton Larmor frequency of 600.13 MHz with CH_3OH-d_4 used as the internal lock. The experimental parameters were as follows: 64 scans requiring 5 min and 13 s acquisition time, 0.16 Hz/point, pulse width (PW) = 30° (11.3 µs) and relaxation delay (RD) = 1.5 s. Free induction decays were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased, baseline corrected, and calibrated toTMSP at 0.00 ppm using TOPSPIN 3.2 software (Bruker BioSpin GmbH).

High-performance thin-layer chromatography analysis

For HPTLC analysis, 30 mg of the samples (as used for the ^{1}H NMR analysis) were extracted with 1 mL of methanol in an ultrasonic bath for 20 min and then centrifuged at 26 451 *g* for 15 min. The supernatants were collected directly into 1.5 mL sample vials.

Sample constituents were separated by HPTLC using 20 × 10 cm HPTLC silica gel 60 F254 plates (Merck). A volume of 15 µL sample solution and saponin standards (250 µg/mL in MeOH) was applied onto the plates using an automated TLC autosampler (ATS-4) (CAMAG). A pooled mixture of all of the samples was also applied in all plates on the first three lanes as quality control samples.

Samples were applied with a 25-µL syringe with a bandwidth of 11.4 mm (15 bands on each plate) at 10 mm distance from the bottom and 20 mm distance from the left and right border of the plate. The mobile phase consisted of a mixture of ethyl acetate, formic acid-acetic acid, and water (100 : 11 : 11 : 27; v/v/v/v). The chamber saturation time was 25 min, and solvent migration distance was 90 mm for all analysis. Plate images were recorded using a TLC visualiser (CAMAG) before and after derivatisation with p anisaldehyde under 366 nm UV light after heating for 3 min at 105 °C on a TLC plate heater (CAMAG). All instruments and procedures were controlled by Vision Cats 2.5 software (CAMAG).

Data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII files. Spectral intensities were scaled to the internal standard and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.0–10.0 using AMIX software (Bruker). The regions δ 4.7–5.0 and δ 3.28–3.34 were excluded from the analysis due to the residual signals of D_2O and CD_3OD, respectively.

The rTLC V.1.0 program was used (http://shinyapps.ernaehrung.uni-giessen.de/rtlc/) for data matrix extraction from the HPTLC images [32]. The parameters used for data acquisition were the same as those used for sample application on the plates, with a pixel width of 128. The warping algorithm was selected to overcome possible R_f shift misalignments. All signals were normalised to the quality control signals in each plate. The extracted data from the grey channel was analysed by multivariate data analysis. OPLS modelling analysis and OPLS-DA were performed using SIMCA P software (version 15, Umetrics).

Supporting information

Information for the *O. japonicus* employed in this study is available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Verpoorte R, Choi YH, Kim HK. NMR-based metabolomics at work in phytochemistry. Phytochem Rev 2007; 6: 3–14

[2] Abreu IN, Choi YH, Sawaya AC, Eberlin MN, Mazzafera P, Verpoorte R. Metabolic alterations in different developmental stages of *Pilocarpus microphyllus*. Planta Med 2011; 77: 293–300

[3] Kim HK, Choi YH, Verpoorte R. NMR-based metabolomic analysis of plants. Nat Protoc 2010; 5: 536–549

[4] Wu W, Jiao C, Li H, Ma Y, Jiao L, Liu S. LC-MS based metabolic and metabonomic studies of *Panax ginseng*. Phytochem Anal 2018; 29: 331–340

[5] Chapagain BP, Wiesman Z, Tsror L. *In vitro* study of the antifungal activity of saponin-rich extracts against prevalent phytopathogenic fungi. Ind Crop Prod 2007; 26: 109–115

[6] Bahrami Y, Zhang W, Chataway T, Franco C. Structural elucidation of novel saponins in the sea cucumber *Holothuria lessoni*. Mar Drugs 2014; 12: 4439–4473

[7] Sobolewska D, Michalska K, Podolak I, Grabowska K. Steroidal saponins from the genus *Allium*. Phytochem Rev 2016; 15: 1–35

[8] Yaidikar L, Thakur S. Arjunolic acid, a pentacyclic triterpenoidal saponin of *Terminalia arjuna* bark protects neurons from oxidative stress associ-

ated damage in focal cerebral ischemia and reperfusion. Pharmacol Rep 2015; 67: 890–895

[9] Kizu H, Tomimori T. Studies on the constituents of *Clematis* species. I. On the saponin of the roots of *Clematis chinese* Osbeck. Chem Pharm Bull 1982; 30: 3340–3346

[10] Nasrollahi V, Mirzaie-Asl A, Piri K, Nazeri S, Mehrabi R. The effect of drought stress on the expression of key genes involved in the biosynthesis of triterpenoid saponins in liquorice (*Glycyrrhiza glabra*). Phytochemistry 2014; 103: 32–37

[11] Kim HK, Saifullah, Khan S, Wilson EG, Kricun SD, Meissner A, Goraler S, Deelder AM, Choi YH, Verpoorte R. Metabolic classification of South American *Ilex* species by NMR-based metabolomics. Phytochemistry 2010; 71: 773–784

[12] Rajendran S, Deepalakshmi PD, Parasakthy K, Devarajb H, Devaraj SN. Effect of tincture of *Crataegus* on the LDL-receptor activity of hepatic plasma membrane of rats fed an atherogenic diet. Atherosclerosis 1996; 123: 235–241

[13] Zheng L, Wang M, Ibarra-Estrada E, Wu C, Wilson EG, Verpoorte R, Klinkhamer PG, Choi YH. Investigation of chemomarkers of Astragali Radix of different ages and geographical origin by NMR profiling. Molecules 2015; 20: 3389–3405

[14] Uematsu Y, Hirata K, Saito K, Kudo I. Spectrophotometric determination of saponin in Yucca extract used as food additive. J AOAC Int 2000; 83: 1451–1454

[15] Wang Y, Zhao H, Liu Y, Guo W, Bao Y, Zhang M, Xu T, Xie S, Liu X, Xu Y. GC-MS-Based metabolomics to reveal the protective effect of gross saponins of *Tribulus terrestris* fruit against ischemic stroke in rat. Molecules 2019; 24: E793

[16] Oleszek WA. Chromatographic determination of plant saponins. J Chromatogr A 2002; 967: 147–162

[17] Liu X, Ahlgren S, Korthout H, Salomé-Abarca LF, Bayona LM, Verpoorte R, Choi YH. Broad range chemical profiling of natural deep eutectic solvent extracts using a high performance thin layer chromatography-based method. J Chromatogr A 2018; 1532: 198–207

[18] Salomé-Abarca LF, van der Pas J, Kim HK, van Uffelen GA, Klinkhamer PGL, Choi YH. Metabolic discrimination of pine resin using multiple analytical platforms. Phytochemistry 2018; 155: 37–44

[19] Chen MH, Chen XJ, Wang M, Lin LG, Wang YT. *Ophiopogon japonicus* – A phytochemical, ethnomedicinal and pharmacological review. J Ethnopharmacol 2016; 181: 193–213

[20] Ge Y, Sun M, Salomé-Abarca LF, Wang M, Choi YH. Investigation of species and environmental effects on rhubarb roots: metabolome using ^{1}H NMR combined with high performance thin layer chromatography. Metabolomics 2018; 14: 137–148

[21] Chang X, Zhang J, Li D, Zhou D, Zhang Y, Wang J, Hu B, Ju A, Ye Z. Non-targeted metabolomics approach for the differentiation of cultivation ages of mountain cultivated ginseng leaves using UHPLC/QTOF-MS. J Pharmaceut Biomedical 2017; 141: 108–122

[22] Wu Y, Dong Z, Wu H, Ding W, Zhao M, Shi Q, Wang Q. Comparative studies on Ophiopogonis and Liriopes based on the determination of 11 bioactive components using LC–MS/MS and hierarchical clustering analysis. Food Res Int 2014; 57: 15–25

[23] Lin Y, Zhu D, Qi J, Qin M, Yu B. Characterization of homoisoflavonoids in different cultivation regions of *Ophiopogon japonicus* and related antioxidant activity. J Pharmaceut Biomed 2010; 52: 757–762

[24] Zhao M, Xu WF, Shen HY, Shen PQ, Zhang J, Wang DD, Xu H, Wang H, Yan TT, Wang L, Hao HP, Wang GJ, Cao LJ. Comparison of bioactive components and pharmacological activities of *Ophiopogon japonica* extracts from different geographical origins. J Pharmaceut Biomed 2017; 138: 134–141

[25] Ge LL, Kan LD, Zhuge ZB, Ma KE, Chen SQ. *Ophiopogon japonicus* strains from different cultivation regions exhibit markedly different properties on cytotoxicity, pregnane X receptor activation and cytochrome P450 3A4 induction. Biomed Rep 2015; 3: 430–434

[26] Hu CX, Wei H, Kong HW, Bouwman J, Gonzalez-Covarrubias V, van der Heijden R, Reijmers TH, Bao X, Verheij EW, Hankemeier T, Xu G, van der Greef J, Wang M. Linking biological activity with herbal constituents by systems biology-based approaches: effects of *Panax ginseng* in type 2 diabetic Goto-Kakizaki rats. Mol Biosyst 2011; 7: 3094–3103

[27] Qin Y, Chen L, Yang X, Tang Y, Li S, Liu C. Determination of 19 representative pesticides in traditional Chinese medicines by dispersive liquid-liquid microextraction and ultra high-performance liquid chromatography-tandem mass spectrometry. Chromatographia 2016; 79: 875–884

[28] Zhao X, Mu Y, Yang M. A simple multi-residue method for determination of plant growth retardants in *Ophiopogon japonicus* and soil using ultra-performance liquid chromatography-tandem mass spectrometry. Chemosphere 2018; 207: 329–336

[29] Teng HM, Fang MF, Cai X, Hu ZH. Localization and dynamic change of saponin in vegetative organs of *Polygala tenuifolia*. J Integ Plant Biol 2009; 51: 529–536

[30] Ma XQ, Shi Q, Duan JA, Dong TTX, Tsim KWK. Chemical analysis of Radix Astragali (Huangqi) in China: a comparison with its adulterants and seasonal variations. J Agric Food Chem 2002; 50: 4861–4866

[31] Peng HS, Wang J, Zhang HT, Duan HY, Xie XM, Zhang L, Cheng ME, Peng DY. Rapid identification of growth years and profiling of bioactive ingredients in *Astragalus membranaceus* var. mongholicus (*Huangqi*) roots from Hunyuan, Shanxi. Chinese Med 2017; 12: 14

[32] Fichou D, Ristivojevic P, Morlock GE. Proof-of-principle of rTLC, an open-source software developed for image evaluation and multivariate analysis of planar chromatograms. Anal Chem 2016; 88: 12494–12501