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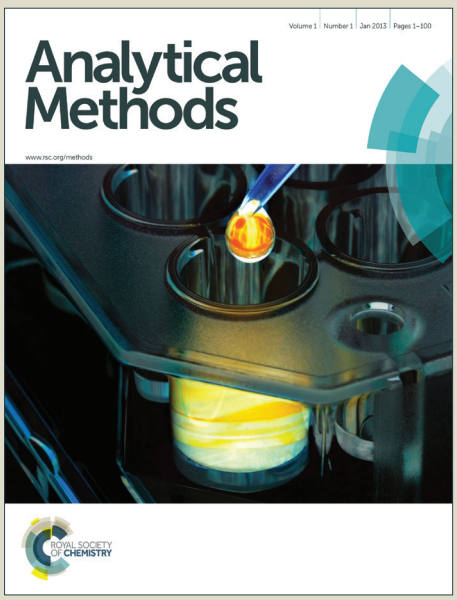

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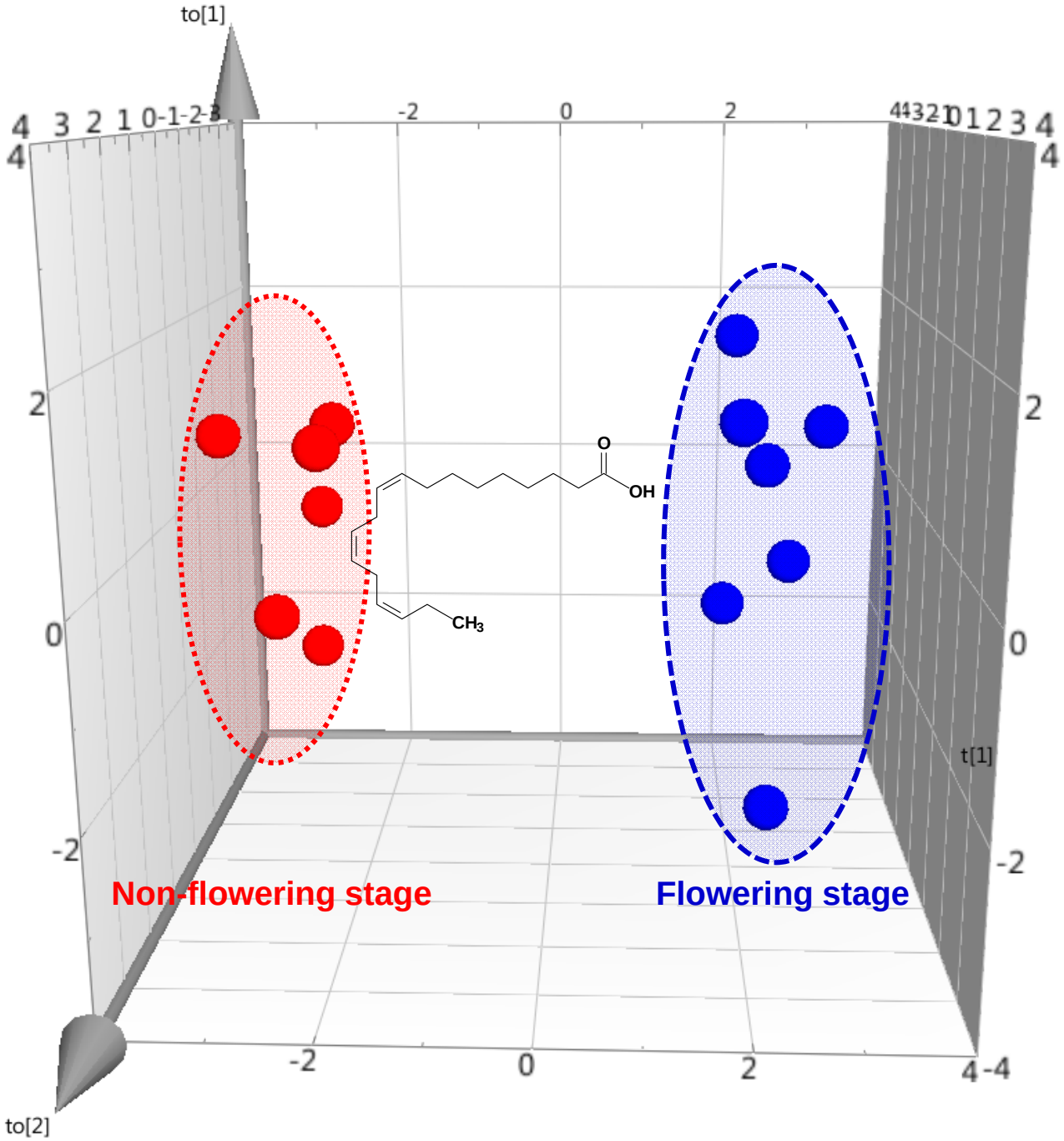
Graphic Abstract



Discrimination of *Pterocephalus hookeri* collected at flowering and non-flowering stages using GC-MS-based fatty acid profiling

Xiao-Jing Zhang[a], Huanxing Su[a], Cheng-Wei He[a], Yi-Tao Wang[a], Feng-Qing Yang[b,*], Jian-Bo Wan[a,*]

[a] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, PR China

[b] School of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400030, China

*Correspondences

Dr. Jian-Bo Wan,

Institute of Chinese Medical Sciences,

University of Macau, Taipa, Macao, China.

Tel: +853-397 4873; Fax: +853-28841 358

E-mail: jbwan@umac.mo

Dr. Feng-Qing Yang

School of Chemistry and Chemical Engineering,

Chongqing University, Chongqing 400030, China

Tel/Fax: +86-23-65106615

E-mail: fengqingyang@cqu.edu.cn

Abstract

Herbal medicine harvested at different growth stage affects its quality and efficiency. We hypothesized that fatty acid profiling might be used to discriminate herbal samples according to their growth stages. To test the hypothesis, fatty acids of *Pterocephalus hookeri* samples collected at flowering (FS) and non-flowering stages (NFS) were characterized and compared using gas chromatography-mass spectrometry (GC-MS) and followed by multivariate statistical analysis. A total of 14 fatty acids were identified and quantified in all *P. hookeri* samples. Both relative and absolute composition of 14 fatty acids varied greatly between FS and NFS groups, suggesting each group has its own fatty acid pattern. Orthogonal partial least squares discriminant analysis (OPLS–DA) and hierarchical clustering analysis (HCA) based on data sets of relative and absolute composition of fatty acids showed that 13 tested samples could be clearly classified into two clusters in terms of their growth stages. More importantly, α-linolenic acid, a plant-derived n-3 polyunsaturated fatty acid (PUFA), was identified as the potential fatty acid biomarker for its greatest contribution to group separation. In addition, to evaluate the quality of *P. hookeri* at FS and NFS, oleanolic acid (OA) and ursolic acid (UA) were determined by HPLC, as described in Chinese Pharmacopoeia (version 2010). Higher total concentration of OA and UA could be found in the *P. hookeri* samples at flowering stage, which suggested to be better quality. These findings demonstrated that GC-MS-based fatty acid profiling coupled with multivariate statistical analysis provides a reliable platform to discriminate the herb collected at different growth stages, which is helpful for ensuring its efficacy.

Keywords: Fatty acid profiling; Gas chromatography–mass spectrometry; Multivariate statistical analysis; *Pterocephalus hookeri*; Flowering stage.

1. Introduction

Fatty acids, originally considered the energy source and structural components of cell membrane, have attracted tremendous interest because of their impacts on human health and diseases. In the recent years, fatty acid profiling has the great potential in differentiating the healthy controls from several diseases, such as nonalcoholic steatohepatitis,[1] type II diabetes mellitus,[2] Alzheimer disease,[3] and chemically induced liver injury.[4] Furthermore, fatty acid characteristics have been widely used in the identification of microbial species, including bacteria and fungi.[5, 6] Our previous study also demonstrated that fatty acid profiling could clearly discriminate three *Panax* species, including *P. ginseng*, *P. notoginseng* and *P. quinquefolius*.[7] Given the fact that fatty acids, as primary metabolites of plant, greatly vary at different growth stages, especially in the flowering and seed formation stages, [8, 9] we hypothesized that fatty acid profiling might be used to discriminate the herb collected at its different growth stages.

The whole plant of *Pterocephalus hookeri* (C. B. Clarke) Hoeck, a commonly-used Tibetan herb, has been prescribed in the treatment of cold, flu, inflammation, rheumatoid arthritis and enteritis in China. [10] Phytochemical studies revealed that triterpenoid saponins, such as ursolic acid (UA) and oleanolic acid (OA), were main bioactive components of *P. hookeri*,[11] and quality control markers suggested in Chinese Pharmacopoeia (version 2010).[12] In folk medicine, the harvest of *P. hookeri* is preferred during its flowering stage. However, according to Chinese Pharmacopoeia (version 2010), *P. hookeri* was recommended to be collected in late summer and early autumn, which duration covered the flowering and non-flowering stages.[12] For the effective use of this Tibetan medicine, the discrimination of *P. hookeri* in different stages, as well as comparison of their qualities, are quite important. Therefore, in the present study, fatty acids of *P. hookeri* harvested at flowering and non-flowering stages were profiled and compared using GC-MS followed by multivariate statistical analysis. In addition, the quality of *P. hookeri* in different stages was also evaluated by their total concentration of OA and UA, as suggested in Chinese Pharmacopoeia.

2. Materials and methods

2.1 Herbal materials and chemicals

A total of thirteen batches of commercial *P. hookeri*, including seven batches in the flowering stage (FS1–FS-7) and six batches in the non-flowering stage (NFS-1–NFS-6), were purchased from different drugstores in Sichuan and Qinghai provinces, China. All samples were collected in the late summer or early autumn according to requirements of Chinese Pharmacopoeia. The botanical origin

and their growth stages were authenticated carefully by microscopic identification. All voucher specimens were deposited at 4 °C in Institute of Chinese Medical Sciences, University of Macau, Macao, China.

HPLC-grade methanol, *n*-hexane, boron trifluoride (BF_3) - methanol solution (14%), and the internal standard tricosanoic acid (C23:0, ≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GLC-461 reference standard, consisting of 32 fatty acid methyl esters (FAMEs), was purchased from Nu-Chek Prep (Elysian, MN, USA), which specifically included the following: 13 saturated chain fatty acids (SFA, C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C24:0); 7 monounsaturated fatty acids (MUFA, C14:1 n-9, C16:1 n-7, C17:1 n-7, C18:1 n-9, C20:1 n-9, C22:1 n-9 and C24:1 n-9); 7 n-6 polyunsaturated fatty acids (n-6 PUFA, C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C22:2 n-6 and C22:4 n-6); and 5 n-3 PUFA (C18:3 n-3, C20:3 n-3, C20:5 n-3, C22:5 n-3 and C22:6 n-3). OA and UA in HPLC analysis were isolated and purified previously from *P. hookeri* by repeated silica gel column chromatography and preparative high performance liquid chromatography (pre-HPLC). Their structures were elucidated by comparison of spectral data (MS, ^{1}H-NMR and ^{13}C-NMR) with reference.[13] The purities of OA and UA were determined to be higher than 98% by normalization of the peak areas detected by HPLC-UV. Deionized water was purified by a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2 Microscopic identification

The dried powder of sample was passed through a 250 μm sieve, and treated with chloral hydrate. At least three microscopic slices from each sample were mounted. All features were carefully observed, and the representative microscopic characteristics were recorded using Nikon eclipse Ni-E microscope (Nikon, Tokyo, Japan).

2.3 Sample preparation

Sample preparation for fatty acid analysis was conducted according to the previous method.[14] Briefly, 50.0 mg of air-dried and pulverized sample was accurately weighed, transferred to a glass methylation tube, and then mixed with hexane (1.5 mL), 14% BF_3/methanol (1.5 mL) and tricosanoic acid (30 μg, internal standard). After blanketed with nitrogen, the mixture was heated at 100 °C in a MK200-2 dry bath incubator (AoSheng, Hangzhou, China) for 1 h. Methyl esters were extracted in hexane followed by the addition of 1 mL water and then centrifuged for 5 min at 3000 rmp. The upper hexane layer was transferred and concentrated under nitrogen gas. Residue was re-dissolved in

200 μL hexane, and subsequently subjected to GC–MS analysis.

For HPLC analysis of UA and OA, samples were prepared according to Chinese Pharmacopoeia with minor modifications. A portion of fine sample powder (1.0 g) was placed into extraction tube containing 15 mL of methanol/ethyl acetate (95:5) and the capped tube was shaken for 1 min before ultrasonication for 30 min at room temperature (25 ℃) in an ultrasonic bath (Transsonic T700/H, Lab-Line instrument, Inc., USA). The supernatant was transferred into a 25 mL volumetric flask which was brought up to its volume, and filtered through 0.45 μm membrane filters (Tianjin automatic science instrument Co. Ltd, Tianjin, China) prior to HPLC analysis

2.4 GC–MS analysis

GC-MS analysis was conducted on an Agilent GC-MS system (Agilent Technologies, Palo Alto, CA) consisting of an Agilent 6890 gas chromatography and an Agilent 5973 mass spectrometer. Fatty acid methyl esters were separated on an Omegawax^{TM} 250 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Supelco, Belletonte, PA) under the optimized oven temperature program: initial temperature set at 180 ℃ and held for 3 min; then ramped to 240 ℃ at 2.5 ℃ /min and held at 240 ℃ for 3 min. High purity helium (99.999 %) was employed as carrier gas with the flow rate at 1.5 mL/min. An autosampler injected 2.0 μL of sample solution with a split ratio of 1:15 at the injector temperature of 250 ℃. The spectrometer was operated in electron-impact (EI) mode with ionization voltage at 70 eV, accompanied by scan ranging from 35 to 550 atomic mass unit (amu) between 2 and 30 min and scan rate at 0.34 s per scan. The temperatures of quadrupole and ionization source were 150 ℃ and 280 ℃, respectively.

Fatty acid methyl esters were identified by three means: (*i*) searching potential compounds from NIST MS Search 2.0 database, (*ii*) comparing retention time with those of reference compounds eluted under the identical chromatographic condition, and (*iii*) comparing their mass spectra with those of authentic standards. Normalization of individual peak areas as the percentages of total fatty acids was applied to calculate relative composition of fatty acids, and their absolute concentrations were quantified by comparing their peak areas to that of the internal standard (tricosanoic acid, C23:0).

2.5 HPLC analysis

In order to determine the concentrations of UA and OA, a HPLC analysis was carried out on an Agilent 1260 infinity system (Agilent Technologies, Palo Alto, CA). An Agilent Zorbax SB-C_{18}

column (250 × 4.6 mm, 5 μm) and a Zorbax SB-C_{18} guard column (12.5 × 4.6 mm, 5 μm) were used for separation. The isocratic 85% aqueous methanol was used as mobile phase at flow-rate of 0.9 mL/min at 30 °C. The detection wavelength was set at 214 nm, and the injection volume was 10 μL. The calibration curves of OA and UA were constructed by plotting the peak areas versus the concentrations of each analyte.

2.6 Data processing and multivariate statistical analysis

All data were expressed as mean ± standard deviation (SD). After verifying normal distribution of dependent variables by Kolmogorov-Smirnov test, one-way analysis of variance (ANOVA) was carried out by using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA) to assess statistical difference in the concentration of each fatty acid, as well as OA and UA, between flowering and non-flowering stages. A value of $p < 0.05$ and $p \leq 0.001$ were considered significant and extreme significant, respectively. The relative and absolute concentrations of fatty acids from GC-MS were separately imported into SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. Unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS–DA) were carried out to examine the distributions and discriminations between groups according to the difference in fatty acid pattern. In addition, to evaluate correlation of 13 tested samples, hierarchical cluster analysis (HCA) was used to generate the dendrogram by SIMCA-P based on their fatty acids characteristics. A method named as Ward, a very efficient method for analysis of variance between clusters, was chosen as measurement. Pearson's correlation analysis between the level of fatty acid biomarker, α-linolenic acid, and total concentration of OA and UA were evaluated by using GraphPad Prism 5.0 (La Jolla, CA, USA).

3. Results

3.1 Microscopic characteristics

To verify botanical origin and growth stage of *P. hookeri* samples, microscopic identification was performed by using light microscopy and polarized light microscopy. All samples presented representative microscopic features of *P. hookeri*, including non-glandular hairs, clusters of calcium oxalate and reticulated or spiral vessels (**Fig. 1**). According to the description of microscopic characteristics of *P. hookeri* in Chinese Pharmacopoeia,[12] all batch samples were verified as whole plant of *P. hookeri* (C. B. Clarke) Hoeck. Among them, seven batches were identified as flowering samples, due to existence of bright yellow pollen grain which is the typical feature of flowering

stage.

3.2 Validation of GC-MS Method

To guarantee the reliability of analytical results, the developed GC-MS method, including instrumental analysis and sample preparation, has been validated by using precision, stability and reproducibility tests. To examine intra-day precision of GC-MS analysis, the FAMEs mixed standard was measured in succession for six times, and 14 investigated fatty acids were selected to monitor the instrumental drift. Overall, the retention time and composition variations (RSD) of fatty acids were less than 0.24% and 4.7%, respectively (data not shown), suggesting excellent instrumental performance during whole analytical run. Due to oxidative susceptibility of fatty acids, particularly PUFA, the stability of methylated fatty acids was tested. Freshly prepared NFS-1 was analyzed at different time intervals of 0h, 2h, 4h, 6h, 8h and 10h. As shown in **Table 1**, FAMEs derived from the tested samples were stable for at least 10 hours at ambient room temperature with overall variation of 0.30%-4.71%. In addition, to test the repeatability of methylation, sample of NFS-1 was divided into six aliquots and derivatized under the methylation conditions, and then analyzed by GC-MS. The repeatability of each fatty acid in the tested sample was less than 4.68%. In conclusion, the developed GC-MS method was robust with good precision, stability and repeatability.

3.3 GC-MS analysis of fatty acids in *P. hookeri* at flowering and non-flowering stages

Representative GC-MS total ion chromatograms (TIC) of the mixed standard containing 32 FAMEs and *P. hookeri* samples at flowering (FS) and non-flowering stages (NFS) were shown in **Fig. 2**, the peaks corresponding to fatty acids were well separated on an Omegawax 250 column within 30 min. A total of 14 fatty acids in their methyl esters form, including 8 SFA (e.g. C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C24:0), 4 MUFA (e.g. C16:1 n-7, C17:1 n-7, C18:1 n-9, and C20:1 n-9) and 2 PUFA (e.g. C18:2 n-6 and C18:3 n-3), were detected consistently and characterized in *P. hookeri* samples, mainly by comparison of their retention times and mass spectra with those obtained from reference compounds under the identical chromatographic conditions. It was difficult to discriminate *P. hookeri* samples in FS and NFS by visual observation of the fatty acid profiles detected by GC-MS, as the fatty acid types between samples were similar.

Relative (%) and absolute composition (mg/g) of 14 investigated fatty acids in *P. hookeri* at two stages were summarized in **Table 2**. The concentrations of SFA with ≤18 carbon chain, including myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), and stearic acid (C18:0),

were significantly higher in FS group, when compared with NFS group. In contrast, SFA with ＞18 carbon chain, including arachidic acid (C20:0), behenic acid (C22:0) and lignoceric acid (C24:0), were much abundant in NFS group. The relative concentrations of MUFA, except for C17:1 n-7, in FS group were significantly lower than those in NFS group. In addition, the flowering samples showed higher level of n-3 PUFA (e.g. α-linolenic acid), and lower level of n-6 PUFA (e.g. linoleic acid) and n-6/n-3 PUFA ratio. Taken together, although there was no significant difference in total absolute concentration of fatty acids between two stages, the individual fatty acid concentration, especially for the relative concentration, varied greatly between FS and NFS, which suggested that each stage has its own fatty acid pattern.

3.3 Multivariate statistical analysis of fatty acid profiles

With the data sets of absolute and relative compositions, PCA, an unsupervised statistical technique, was firstly applied to investigate whether FS and NFS of *P. hookeri* samples could be separated according to their differences in fatty acid profiles. After unit variance (UV) scaling and mean-centering, the two-dimensional PCA score plots showed a tendency to separate the FS and NFS of *P. hookeri* samples (**Fig. 3**). The first two principal components from PCA models constructed by absolute composition (81.7%) explained more systematic variation than that by relative composition (73.6%). It is plausible that the relative composition might narrow the difference in fatty acids among different species by normalizing peak areas as percentages of total fatty acids, in comparison with absolute composition. OPLS-DA, a supervised statistical modeling method to cluster multivariate data, was subsequently conducted to sharpen the separation between the groups in PCA. As shown in **Fig. 4**, the score plots analysis according to both relative and absolute compositions of fatty acids demonstrated that all tested samples were clearly classified into two clusters i.e. FS and NFS. The obtained OPLS-DA models were then further analyzed using analysis of variance of sevenfold Cross-Validation predictive residual (CV-ANOVA). Several parameters, including R^2 and Q^2, are commonly used to evaluate the quality and reliability of OPLS-DA model, and their values close to 1.0 indicate an excellent fitness for the model.[15] All observations fell within the Hotelling T2 (0.95) ellipse, where the model fit parameters were 0.965 of R^2Y and 0.936 of Q^2 in the score plot using relative composition as variations (**Fig. 4A**), and 0.977 of R^2Y and 0.865 of Q^2 using absolute composition (**Fig. 4B**), indicating that the constructed OPLS-DA model has the excellent fitness and predictive capability. In addition, based on absolute and relative concentrations of 14 fatty acids, HCA of the *P. hookeri* samples was carried out to visualize the differences and/or similarities among samples through linkage distances. The HCA dendrograms, derived from both absolute and relative

concentrations, respectively, showed that *P. hookeri* samples derived from FS and NFS could be divided into two main clusters (**Fig. 5**), each stage corresponding to a cluster. These results suggested that based on both absolute and relative concentrations of fatty acids, the *P. hookeri* samples collected at FS and NFS could be clearly discriminated using GC-MS analysis and multivariate statistical analysis, such as OPLS-DA and HCA.

To identify potential fatty acid biomarker contributing most to group separations, extended multivariate analyses were employed to generate an S-plot, in which each point represented a variable. S-plot integrates covariance and loading plot of OPLS-DA, in which the X-axis and Y-axis represent variable contribution and variable confidence, respectively. The further the fatty acid point departs from zero of X-axis and Y-axis, the more the fatty acid contributes to group separation.[7] As shown in **Fig. 6**, the variable most contributing to discrimination of different stages in group separations derived from both relative and absolute concentrations was identified as α-linolenic acid (C18:3 n-3), which had the remarkably higher level in flowering stage. To validate the potential discriminative capacity of α-linolenic acid, HCA dendrograms were also conducted using either relative or absolute composition of α-linolenic acid as the single variable, and obtained similar results with those using 14 fatty acid profiles as variables (Data not shown).

3.4 Quantification of OA and UA

To evaluate the quality of *P. hookeri* of flowering and non-flowering stages, HPLC method was employed to determine the total concentration of OA and UA, as suggested in Chinese Pharmacopoeia. As shown in **Table 3,** the total concentration of OA and UA was significantly higher in FS group (4.16–9.16 mg/g) when compared to NFS group (1.06–3.43 mg/g), attributing to dramatically higher concentration of UA, rather than OA, in the FS group. Our results demonstrated that although *P. hookeri* samples were harvested in the time specified in Chinese Pharmacopoeia, their qualities varied considerably, and the quality of sample collected at its flowering stage was obviously higher than that in non-flowering stage. In addition, Pearson's correlation analysis was conducted to examine correlation between the relative level of identified fatty acid biomarker (e.g. α-linolenic acid) with the total concentration of UA and OA. As shown in **Fig.7**, the correlation coefficient (r) was 0.796 with p value$<$0.001, indicating that the level of α-linolenic acid were positively correlated with the production of UA and OA in *P. hookeri*.

4. Discussions

In the past decade, plant metabolomics, including NMR-based and UPLC/Q-TOFMS-based, have been also successfully applied for the discrimination of Chinese medicines that derived from different origin,[16] cultivation age,[17] grown region[18] and processing methods,[19] according to their differences in the global plant metabolites, especially secondary metabolites. These approaches are very complicated and require the expensive equipment. In our study, a simple GC-MS based fatty acid profiling was proposed to discriminate *P. hookeri* samples collected at flowering and non-flowering stages. As the primary metabolites of plant, fatty acids are mainly responsible for energy source and structural components of plant cell membrane. Unlike specific bioactive components of herbs, such as alkaloid, triterpenoid, flavone, etc., fatty acids are present in almost all herbs. Actually, fatty acid profiling has been successfully applied for differentiating of herbal species.[7] Our present findings also demonstrated that *P. hookeri* samples collected at flowering and non-flowering stages could be clearly discriminated according to either absolute or relative compositions of fatty acids. Therefore, fatty acid profiling provided a convenient tool to discriminate the herbs collected at different growth stages.

Flowering is an important stage of sexual reproduction of plant. During this stage, under the catalysis of 9-specific lipoxygenase, α-linolenic acid could be metabolized to α-ketol linolenic acid which strongly induce plant flowering.[20] Our results demonstrated that *P. hookeri* in flowering stage presents significantly higher level of α-linolenic acid when compared to non-flowering stage. Furthermore, several desaturase enzymes, including Δ-9, Δ-6 and Δ-15 desaturases, have been implicated in the biosynthesis of α-linolenic acid, and their activities are commonly estimated from the ratio of C18:1 n-9/C18:0, C18:2 n-6/C18:1 n-9, and C18:3 n-3/C18:2 n-6, respectively.[21-23] As shown in **Table 2**, the lower C18:1 n-9/C18:0 ratio and higher C18:3 n-3/C18:2 n-6 ratio were observed, suggesting the decreased Δ-9 and elevated Δ-15 desaturase activity in flowering stage. Therefore, in different growth stages, the composition and level of fatty acid might be varied greatly to meet the requirement of plant growth, which provides a foundation for discrimination of different growth stages using fatty acid profiling.

It is well known that PUFA have been considered to be important precursors of eicosanoids that serve as signaling molecules, and play an essential role in maintaining human health. However, the eicosanoids derived from n-6 and n-3 PUFA are functionally distinct, and exhibit the pro-inflammatory and anti-inflammatory actions, respectively.[24] The compelling evidence indicates that α-linolenic acid, a plant-derived n-3 PUFA, is associated with decreased incidence and severity of several chronic diseases, including myocardial infarction,[25] atherosclerosis,[26] hyperlipidemia,[27]

rheumatoid arthritis[28] and cancer.[29] Fatty acids in Chinese medicines might substantially contribute to the whole beneficial effects of herbs, besides their secondary metabolites. Our results demonstrated that *P. hookeri* in flowering stage showed the higher level of α-linolenic acid and lower level of linoleic acid which belongs to n-6 PUFA, when compared to non-flowering stage, suggesting the greater nutritional value of *P. hookeri* collected at flowering stage. Along with the higher total concentration of OA and UA, *P. hookeri* was suggested to be harvested in its flowering stage.

5. Conclusions

In the present study, a GC-MS based fatty acid profiling followed by multivariate statistical analysis was proposed to discriminate *P. hookeri* samples collected at flowering and non-flowering stages. A plant-derived n-3 PUFA, α-linolenic acid, was identified as the potential fatty acid biomarker contributing most to their differences. Our findings also demonstrated that the quality of *P. hookeri* sample collected at its flowering stage was obviously better than that in non-flowering stage, according to their total concentration of OA and UA, which suggested *P. hookeri* to be harvested in its flowering stage. In summary, based on absolute and relative composition of fatty acid profiling, this approach provides reliable platform to classify the herb collected at different growth stages, which is helpful for ensuring their safety and efficacy.

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Tables

Table 1. Methylation repeatability and FAME stability data of 14 investigated fatty acids in *P. hookeri* (NFS-1) analyzed by GC-MS.

Fatty Acid	Repeatability Test (n=6)				Stability Test (n=6)			
	Relative composition (%)		Absolute composition (mg/g)		Relative composition (%)		Absolute composition (mg/g)	
	mean±SD	RSD(%)	mean±SD	RSD(%)	mean±SD	RSD(%)	mean±SD	RSD(%)
C14:0	1.42±0.04	3.12	0.07±0.00	1.79	1.44±0.05	3.43	0.07±0.00	2.45
C15:0	0.82±0.03	4.25	0.04±0.00	3.44	0.86±0.02	2.05	0.04±0.00	2.62
C16:0	23.35±0.39	1.65	1.20±0.04	3.07	23.57±0.07	0.30	1.19±0.02	1.74
C16:1,7	1.07±0.03	2.87	0.06±0.00	2.84	1.14±0.04	3.55	0.06±0.00	3.86
C17:0	0.82±0.03	3.41	0.04±0.00	3.23	0.85±0.02	1.87	0.04±0.00	3.29
C17:1,7	0.49±0.02	3.51	0.03±0.00	2.51	0.52±0.02	3.45	0.03±0.00	4.01
C18:0	6.28±0.09	1.36	0.32±0.01	3.01	6.36±0.06	0.92	0.32±0.01	2.48
C18:1,9	10.41±0.14	1.33	0.53±0.02	2.72	10.46±0.11	1.07	0.53±0.01	2.28
C18:2,6	12.44±0.24	1.91	0.64±0.01	1.61	12.73±0.10	0.77	0.64±0.01	1.32
C18:3,3	3.35±0.08	2.48	0.17±0.01	3.58	3.44±0.03	0.79	0.17±0.00	1.78
C20:0	16.02±0.27	1.66	0.82±0.03	3.45	16.16±0.11	0.66	0.81±0.01	1.57
C20:1,9	0.86±0.02	2.63	0.04±0.00	1.43	0.91±0.04	4.01	0.05±0.00	4.71
C22:0	10.12±0.27	2.62	0.52±0.02	4.06	9.17±0.35	3.81	0.46±0.02	3.93
C24:0	12.54±0.57	4.58	0.64±0.03	4.68	12.39±0.17	1.39	0.62±0.01	2.18

Table 2. The relative and absolute compositions of fatty acids in *P. hookeri* samples collected at flowering stage (FS, n=7) and non-flowering stage (NFS, n=6)

Peak No.	Fatty acids		Relative composition (%)		Absolute composition (mg/g)	
	Common name	Symbol	NFS	FS	NFS	FS
1	Myristic acid	C14:0	1.09±0.28	1.45±0.14**	0.06±0.01	0.09±0.01**
2	Pentadecanoic acid	C15:0	0.70±0.13	0.96±0.17**	0.04±0.01	0.06±0.01***
3	Palmitic acid	C16:0	18.10±2.40	24.22±3.74**	1.07±0.11	1.45±0.16***
4	Palmitoleic acid	C16:1 n-7	0.91±0.17	0.57±0.07***	0.05±0.01	0.03±0.01***
5	Heptadecanoic acid	C17:0	1.69±0.33	1.58±0.33	0.10±0.02	0.10±0.02
6	Heptadecenoic acid	C17:1 n-7	0.36±0.19	0.48±0.06	0.02±0.01	0.03±0.00
7	Stearic acid	C18:0	6.45±0.93	9.63±1.75**	0.38±0.04	0.58±0.11***
8	Oleic acid	C18:1 n-9	10.95±0.84	7.29±0.41***	0.65±0.08	0.44±0.05***
9	Linoleic acid	C18:2 n-6	12.58±2.20	9.47±0.69**	0.75±0.17	0.57±0.08*
10	α-Linolenic acid	C18:3 n-3	3.88±0.47	14.00±3.01***	0.23±0.02	0.85±0.19***
11	Arachidic acid	C20:0	15.04±1.20	12.46±1.40**	0.89±0.10	0.71±0.14*
12	Eicosenoic acid	C20:1 n-9	0.59±0.08	0.53±0.16**	0.04±0.01	0.03±0.01
13	Behenic acid	C22:0	9.56±0.57	7.53±1.47**	0.59±0.06	0.46±0.12*
14	Lignoceric acid	C24:0	18.10±3.40	9.84±3.90**	1.08±0.27	0.57±0.27**
Saturated fatty acid (SFA)			64.18±3.49	67.67±2.60*	4.19±0.38	4.14±0.58
Monounsaturated fatty acid (MUFA)			15.63±0.70	8.87±0.43***	0.76±0.07	0.54±0.06***
Polyunsaturated fatty acid (PUFA)			20.20±3.37	23.47±2.69***	0.98±0.19	1.46±0.25**
n-6/n-3 ratio			**3.28±0.65**	**0.71±0.19*****		
C18:1 n-9/C18:0			1.72±0.22	0.78±0.18***		
C18:2 n-6/C18:1 n-9			0.12±0.24	0.13±0.07		
C18:3 n-3/C18:2 n-6			0.32±0.06	0.15±0.39***		
Total					5.93±0.54	6.13±0.79

NFS *vs* FS * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 3. The concentrations (mg/g) of oleanolic acid (OA) and ursolic acid (UA) in *P. hookeri* samples collected at the flowering (FS) and non-flowering stages (NFS)

No.	FS	NFS
OA	0.90±0.10	0.78±0.22
UA	5.05±1.65	1.22±1.07***
OA+UA	5.94±0.70	1.99±0.96***

NF *vs* NFS*** $p<0.001$

Figure captions

Fig. 1. The representative microscopic characteristics of *Pterocephalus hookeri* collected at flowering and non-flowering stage, including nonglandular hairs (**A**), reticulated or spiral vessels (**B**), crystals of calcium oxalate (**C**) and pollen grain (**D**).

Fig. 2. Representative total ion chromatograms of the mixed standards (**A**), and the methyl esters of fatty acids in *Pterocephalus hookeri* samples collected at the flowering (**B**) and non-flowering stage (**C**). The mixed standards contain 32 fatty acid methyl esters as described in the section of Chemicals. Fatty acid represents as the corresponding methyl ester. **1**, C14:0; **2**, C15:0; **3**, C16:0; **4**, C16:1 n-7; **5**, C17:0; **6**, C17:1 n-7; **7**, C18:0; **8**, C18:1 n-9; **9**, C18:2 n-6; **10**, C18:3 n-3; **11**, C20:0; **12**, C20:1 n-9; **13**, C22:0; **14**, C24:0; **15**, C23:0 (internal standard).

Fig. 3. PCA score plots based on relative composition (**A**) and absolute composition (**B**) of fatty acids in *Pterocephalus hookeri* samples collected at flowering (●FS, n=7) and non-flowering stage (▲NFS, n=6).

Fig. 4. OPLS-DA score plots based on relative composition (**A**) and absolute composition (**B**) of fatty acids in *Pterocephalus hookeri* samples collected at flowering (●FS, n=7) and non-flowering stage (▲NFS, n=6).

Fig. 5. HCA dendrograms resulting from relative composition (**A**) and absolute composition (**B**) of 14 fatty acids in *Pterocephalus hookeri* samples collected at flowering (●FS, n=7) and non-flowering stage (▲NFS, n=6)

Fig. 6. S-plots in OPLS-DA model constructed according to relative (**A**) and absolute compositions (**B**) of 14 fatty acids in *Pterocephalus hooker* samples.

Fig. 7. Pearson's correlation analysis between the level of α-linolenic acid (C18: 3 n-3) with total concentration of oleanolic acid (OA) and ursolic acid (UA).

Fig. 1

Fig. 2

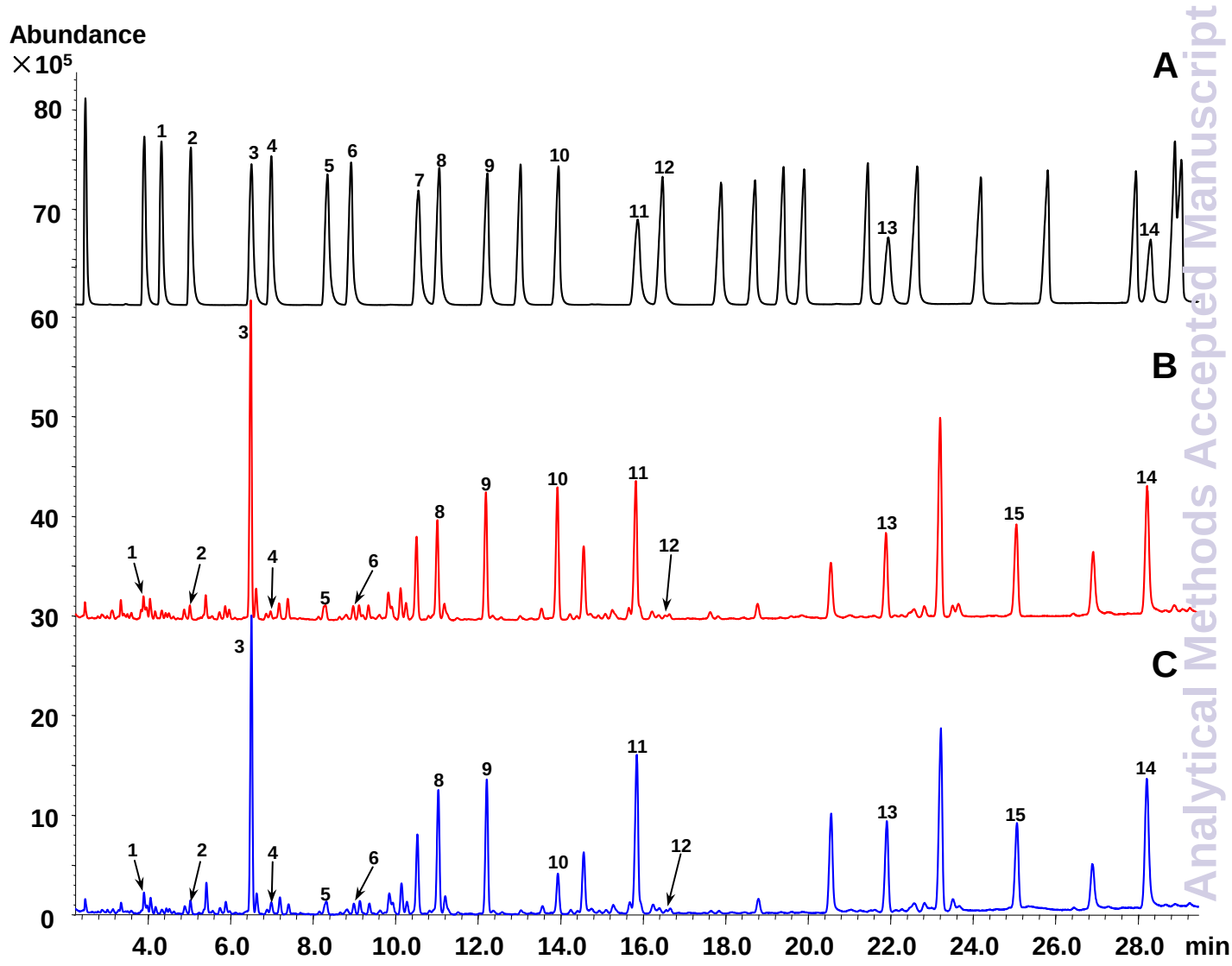


Fig. 3

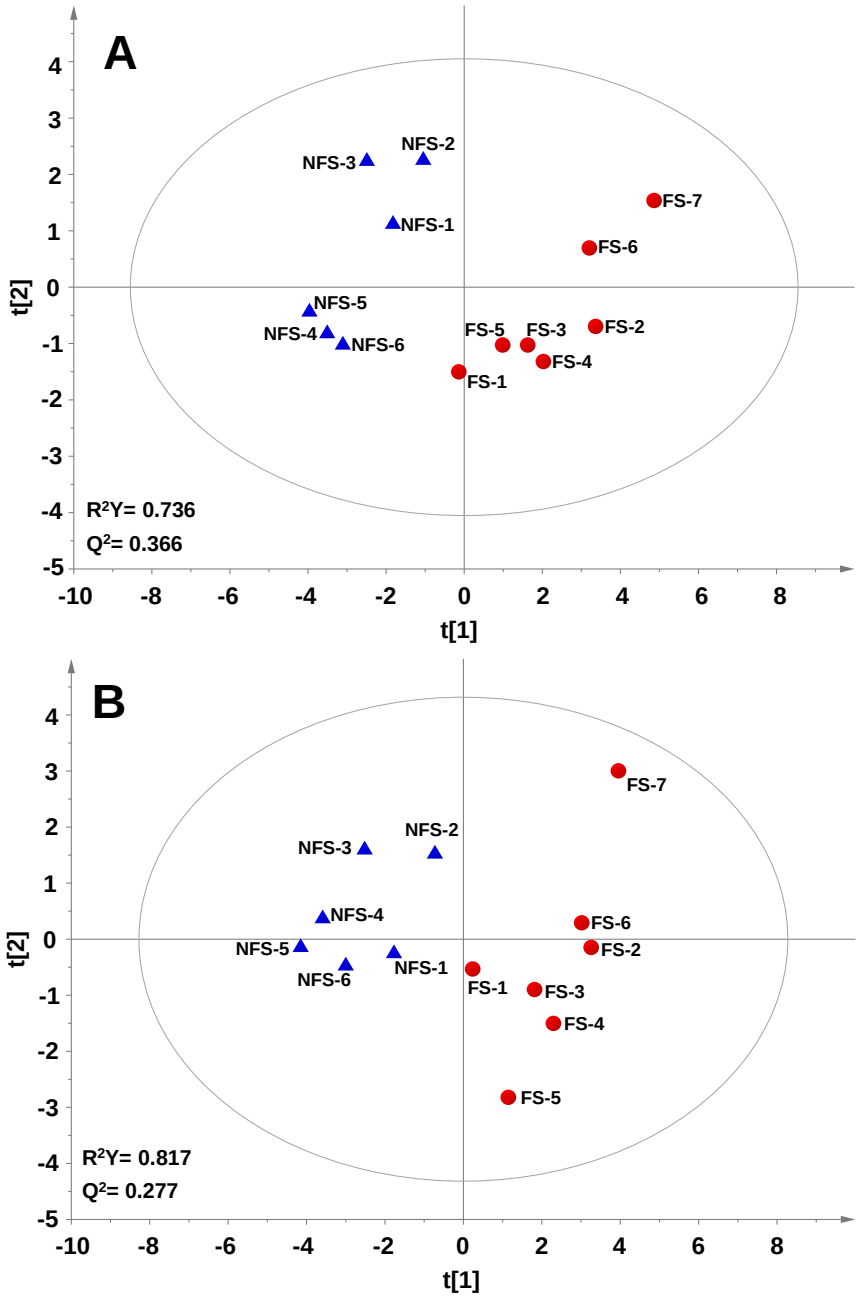
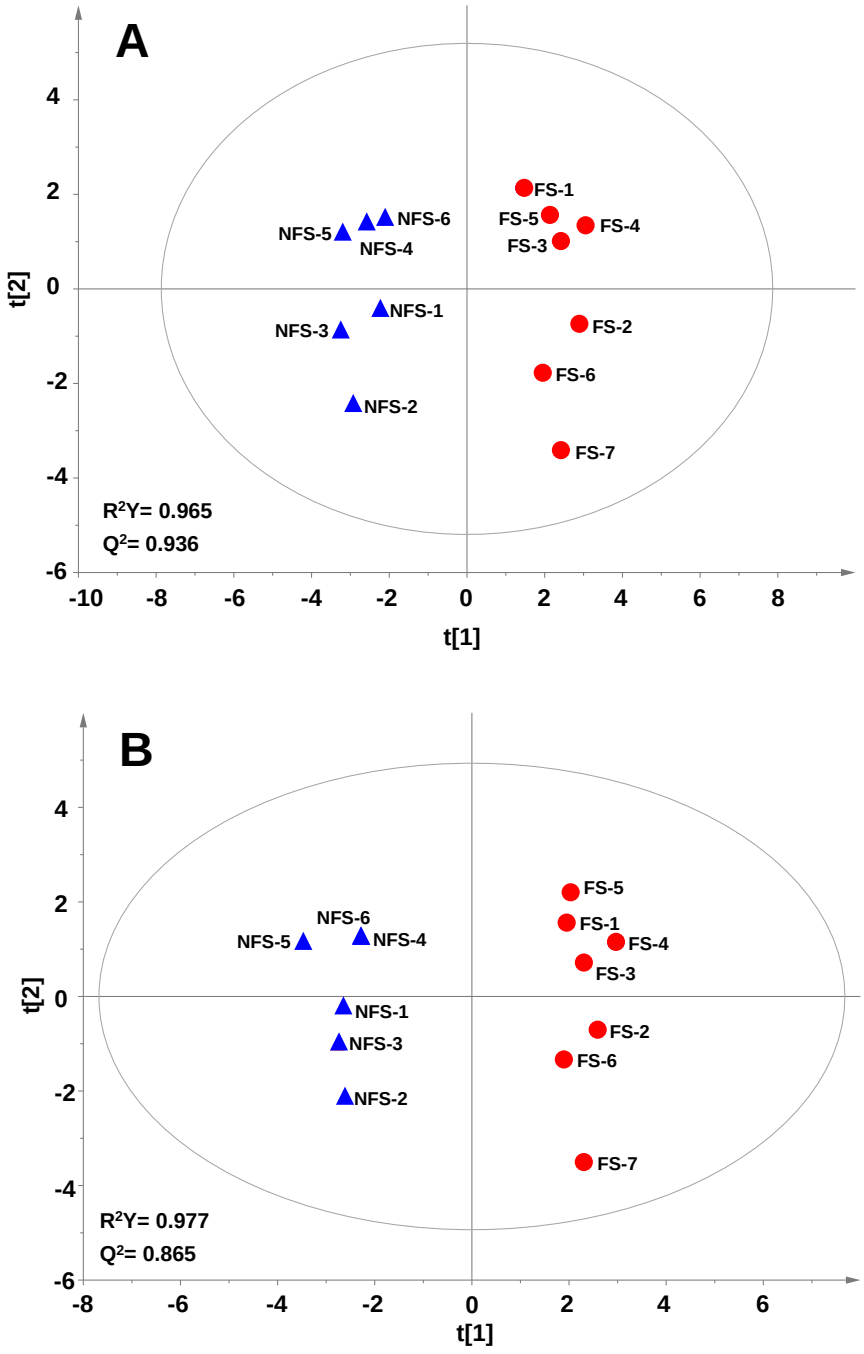


Fig. 4



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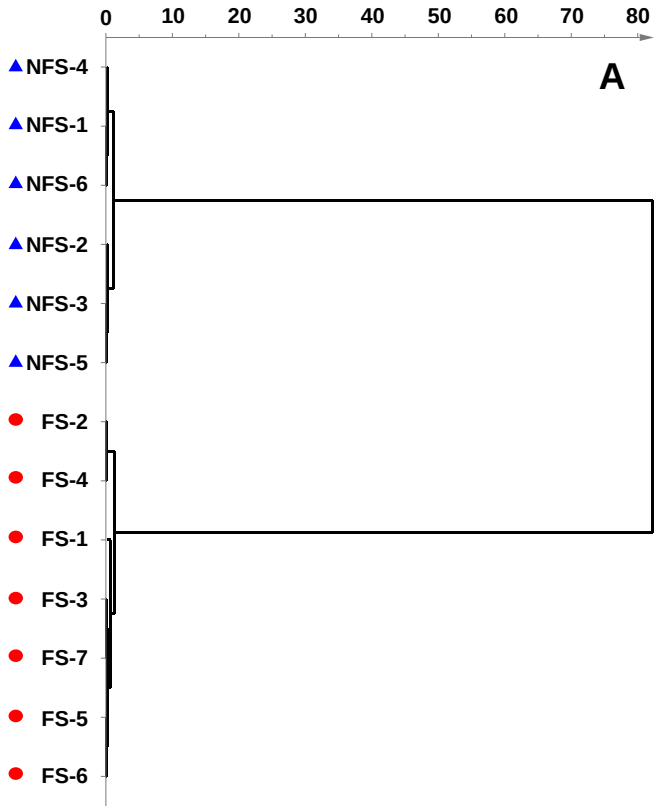


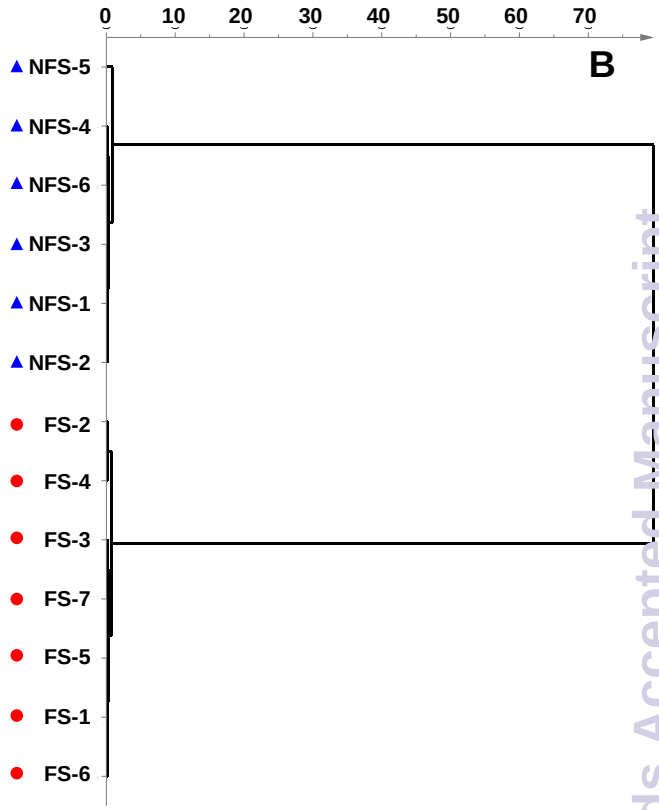


Fig. 6

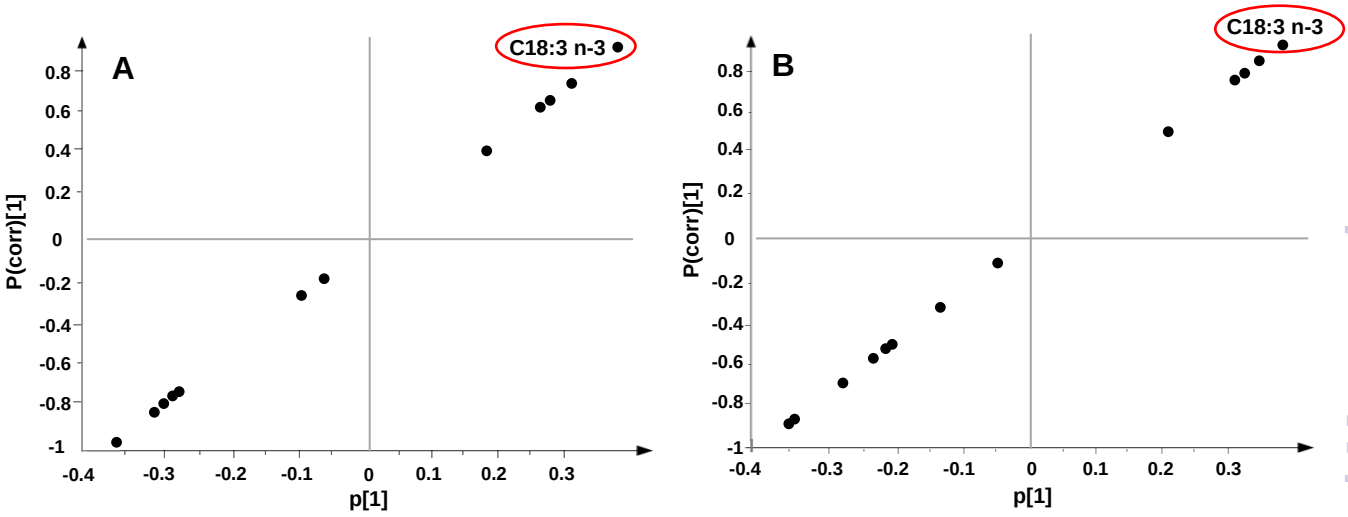
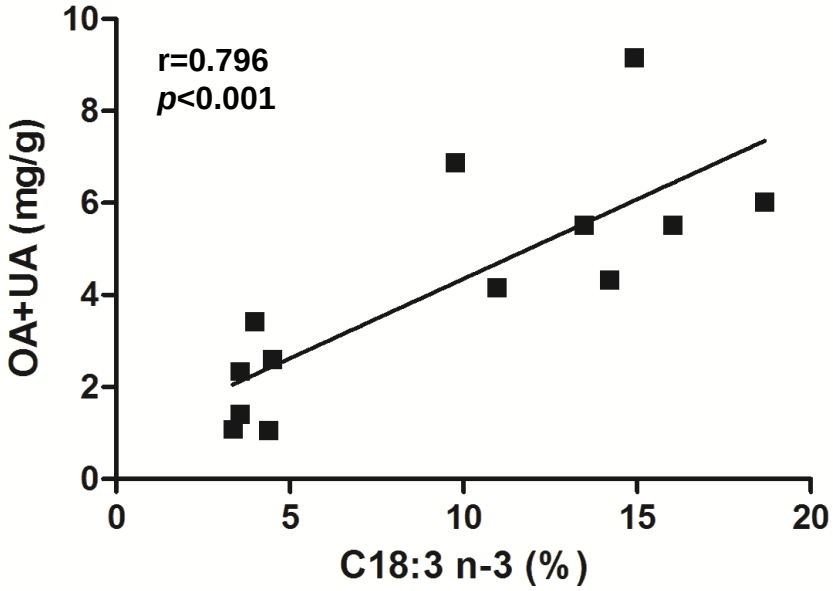


Fig. 7



Graphic Abstract