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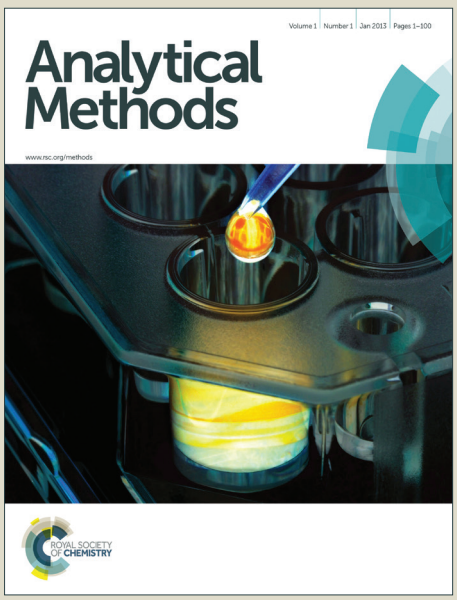

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Targeted quantitative analysis of anthraquinone derivatives by high-performance liquid chromatography coupled to tandem mass spectrometry to discriminate crude and processed rhubarb samples

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Abstract

Crude rhubarb has been used as drastic laxative for thousands years in China. To alleviate celialgia and moderate drastic effect, crude rhubarb is subjected to processing prior to clinical usage. In the present study, a targeted plant metabolomic method using liquid chromatography-tandem mass spectrometry in segmental multiple reaction monitoring mode was developed to simultaneously determine the 13 marker constituents (two anthrones, six anthraquinone glycosides and five anthraquinone aglycones) in rhubarb. With satisfactory linearity, precision and accuracy, the developed method was then applied to explore the content variations of these compounds in crude and processed rhubarbs. After processing, the total content of anthraquinone glycosides decreased significantly, which might result in mild purgative function. Moreover, based on the contents of the marker compounds, unsupervised principal component analysis was employed to differentiate 81 batches samples covering both crude and processed rhubarbs. The results indicated that targeted quantification of marker compounds by LC-MS/MS coupled with PCA would be a reliable strategy to discriminate crude and processed herbs.

Keywords Anthraquinone; Rhubarb; Targeted quantification; Processing; LC-MS/MS

1. Introduction

Unlike Western herbs which are generally used simply fresh or dried, many Chinese herbs are subjected to processing (*Paozhi*) before they are used as materia medica. Processing, any physical and/or chemical treatment of herbal medicine, can moderate drastic action, enhance efficacy, reduce toxicity and alleviate side effect by changing chemical composition of crude herbs[1]. Since crude and processed herbs are always used differently in clinic, the discrimination of them becomes extremely important.

Rhubarb is one of the earliest and best-known Chinese herbal medicines used for thousands of years in the history of Traditional Chinese Medicine (TCM). According to the processing method, crude rhubarb (*Shengdahuang*, DH) can be processed as *Jiudahuang* (JDH), *Shudahuang*(SDH), and *Dahuangtan* (TDH) [2].

Rhubarb has been widely used in the treatment of constipation, gastrointestinal diseases, cholestatic hepatitis, chronic renal failure, jaundice, and ulcers [3,4]. These activities are mainly attributed to the anthraquinone derivatives in rhubarb. Among them, sennosides (anthrones) and anthraquinone glycosides are considered as the main purgative components[5], while free anthraquinones possess anti-inflammatory[6], anticarcinogenic[7], hepatoprotective[8], antibacterial [9], antioxidant effects [10]. Therefore, anthraquinone derivatives are usually analyzed to control the quality of rhubarb products. By present, most established analytical methods such as thin layer chromatography[8], micellar electrokinetic chromatography[11], and liquid chromatography[12,13] with different detectors including mass spectrometry were capable of determining only free anthraquinones. Apparently, only identification and quantification of free anthraquinones are not sufficient since purgative effect is mainly attributed to sennosides and anthraquinone glycosides.

The published methods for quantification both aglycones and glycosides of anthraquinone derivatives suffer from the drawback of long analysis time and incomplete resolution[14, 15]. Therefore, a rapid and reliable method, which can quantify both free anthraquinones and their glycosides, is urged to control the quality of rhubarb. Moreover, little is known about the differences in the contents of anthraquinone derivatives between crude and processed rhubarb. Hence, in this study, we establish a targeted plant metabolomic method based on high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to simultaneous determination of thirteen anthraquinone derivatives, namely emodin (EM), rhein (RH), aloe-emodin (AL), chrysophanol (CH), physcion (PH), sennoside A (SA), sennoside B (SB), emodin-1-O-β-D-glucoside (EM-1-G), emodin-8-O-β-D-glucoside (EM-8-G), aloe-emodin-8-O-β-D-glucoside (AL-8-G), rhein-8-O-β-D-glucoside (RH-8-G), chryphanol-8-O-β-D-glucoside (CH-8-G) and physcion-8-O-β-D-glucoside (PH-8-G) (Fig. 1). The quantitative results were applied to compare the differences between crude and processed rhubarbs with the aid of unsupervised principal component analysis (PCA).

2. Experimental

2.1. Chemicals and herbal materials

Reference standards of EM, RH, AL, CH, PH and naringenin were purchased from the National Institutes for Food and Drug Control (Beijing, China). EM-1-G, EM-8-G, AL-8-G, RH-8-G, SA and SB were purchased from Shanghai Yilin Biotechnology Co., Ltd (Shanghai, China). CH-8-G was purchased from Chengdu MUST Biotechnology Co., Ltd (Sichuan, China). PH-8-G was purchased from Chengdu Chroma-Biotechnology Co., Ltd (Sichuan, China). The purity of reference standards was higher than 98% determined by HPLC-DAD. Methanol of

HPLC grade was purchased from Merck (Darmstadt, Germany). Formic acid (analytical reagent) was purchased from the First Chemical Company of Nanjing (Jiangsu, China). Deionized water was prepared by a Milli-Q system (Millipore, MA, USA).

Eighty-one batches of rhubarb products from various sources are listed in Table S1. All samples were authenticated according to the current standard of Chinese Pharmacopoeia. The voucher specimens were deposited at the Herbarium of China Pharmaceutical University, Nanjing, China.

2.2. Sample preparation

The rhubarb powder (0.5 g) was weighted accurately and ultrasonic-extracted with 25.0 mL methanol-water (80:20, v/v) for 30 min at room temperature. After extraction, methanol-water (80:20, v/v) was added into the flask to compensate for the lost weight during extraction. Then 10 μL naringenin solution (internal standard, IS, 4 μg/mL) was added into 200 μL extract and then filtered through a 0.22 μm syringe membrane filter for HPLC- MS/MS analysis.

2.3. LC-MS/MS analysis

The experiments were performed on a triple quadrupole TSQ Quantum mass spectrometer equipped with a Finnigan Surveyor LC pump, a Finnigan Surveyor autosampler and a computer system with Xcalibur data acquisition software (Thermo Fisher, Palo Alto, CA). Chromatographic separation was achieved on a Phenomenex Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm). The mobile phase was composed of A (acetonitrile) and B (0.1% formic acid) under gradient elution conditions: 82-30% B at 0-6 min, 30-5% B at 6-10 min, 5% B at 10-16 min. The flow rate was 0.2 mL/min. The MS/MS parameters were set as follows: electrospray ionization (ESI) in negative mode, spray voltage, 4.0 kV; capillary temperature, 300 $^{\circ}$C; scan width for MRM, 0.2 *m/z*; scan

time, 0.2 s. The peak width settings for both Q1 and Q3 were 0.7 *m/z*. The MRM ion pair transitions and collision energy levels of each component are listed in Table 1.

2.4. Method validation

An appropriate amount of each reference standard was dissolved with methanol. The concentrations of methanol stock solution were 20μg/mL for RH and EM, 10μg/mL for AL, CH, PH, EM-8-G, AL-8-G, CH-8-G, PH-8-G, SA and SB, and 2μg/mL for EM-1-G. A series of solutions was consecutively diluted with the stock solution to prepare the standard solutions for calibration (dilution factor = 1, 5, 10, 25, 50, 100, 200). The calibration graphs were plotted after weighted linear least-squares regression of the peak area ratios (peak area of analyte/peak area of IS) versus concentration. The quantitation of each marker compound was calculated based on its calibration curve.

The limits of detection (LOD) and quantification (LOQ) were determined by injecting a series of diluted solutions with known concentrations and defined as the concentrations giving signal-to-noise ratios of 3 (S/N=3) and 10 (S/N=10) respectively.

The intra- and inter-day precisions were determined on three consecutive days with three repetitions each[16].

The accuracy was estimated by recovery assays. The reference standards at three different concentration levels (approximately equivalent to 50%, 100% and 150% of the concentration in matrix) with three parallels at each level were added into a rhubarb sample. The recoveries were calculated by the following formula: recovery% = (amount of detected-original amount) / amount spiked×100.

2.5. Statistical analysis

The data are expressed as means ± standard deviation (SD). Statistical significance was evaluated by Mann-Whitney U test and the significance level of $p<0.05$ was adopted for all statistical comparisons. PCA analysis was performed by SIMCA-P version 13.0 (Umetrics, Sweden) with the contents of 13 analytes as variables and 81 batches of samples as observations.

3. Results and Discussion

3.1. Targeted plant metabolomic analysis

Plant metabolomics deals with qualitative and quantitative analysis of components in plant and can mainly be divided in two categories, targeted and untargeted[17]. Targeted plant metabolomics focuses on the quantification of a specific set of analytes. The analytes to be monitored are the bioacitive constituents or differential compounds selected by untargeted approach. Previous untargeted plant metabolomic researches[18,19] have indicated that anthraquinone derivatives are the potential chemical markers to distinguish crude and processed rhubarb products. Besides, anthraquinone derivatives are the compounds responsible for the putative pharmacological action of rhubarb. Therefore, we selected anthraquinone derivatives as marker compounds in present targeted plant metabolomic study.

3.2. Optimization of extraction conditions

To obtain satisfactory extraction efficiency, the extraction conditions including extraction solvent (40%methanol, 60% methanol, 80% methanol, and 100% methanol), extraction time (10, 30, and 45 min), and extraction frequency (once and twice) were investigated by univariate test. The results indicated that ultrasonication with 25 ml of 80% methanol for 30 min once was sufficient for complete extraction of the marker compounds.

3.3. Method development for quantification of anthraquinone derivatives

Regarding the quantification of anthraquinone derivatives, UV detector is preferred with advantages of being simply and cost-effective, high robust and reproducible. Initially, we had attempted but failed to establish a rapid and sensitive HPLC-UV method, because of the following challenges: (1) rhubarb is a complex mixture containing a large number of constituents differing in molecular weight, structural class, and hydrophobicity[1,20]. It took more than 30 min to baseline separate anthraquinones in rhubarb even using ultra performance liquid chromatography[21]; (2) ultraviolet detection of anthraquinone derivatives is unspecific because it is based on the maximum absorbance at around 260 nm, where other co-existing compounds, such as tannins, also have significant ultraviolet absorption (Supplementary Fig. S1); (3) Sample preparation is the crucial first step in the chromatographic analysis of herbal medicines[22]. In our preliminary experiment, several conventional sample preparation techniques such as liquid-liquid extraction, pH-dependent liquid-liquid extraction and solid-phase extraction were conducted but failed to remove interferences from rhubarb. Finally, a liquid chromatography- mass spectrometry method was developed to simultaneous determination of anthraquinone derivatives in rhubarb.

Among LC-MS methods, LC-MS/MS in multiple reaction monitoring (MRM) mode is typically used as standard method for quantitative analysis due to its superior sensitivity and specificity, but when it applied to analyze multi-components in complex matrix, such as herbal medicines, the quality of data would be compromised[23]. In order to shorten the scan cycle and increase sensitivity, we arranged the MRM transitions into a segmented MRM (SMRM) program, where the detection duration was set according to the retention time of each compound (Fig. 2).

3.4. Validation of the LC-MS/MS method

The parameters from the calibration curve with R^2, linear range and regression equation,

LOD and LOQ of the thirteen marker compounds are listed in Table 1. Good linearity was observed with the correlation coefficients greater than 0.995. The RSD values for intra- and inter-day precision were in the ranges 1.06-4.96% and 1.32-4.98%, respectively (Supplementary Table S2). The recoveries of the marker compounds ranged from 93.56-104.87% (Supplementary Table S3). The results from validation of the method showed satisfactory linearity, sensitivity, precision, and recovery for simultaneous analysis of marker compounds.

3.5. Application

3.5.1. Quantitative analysis

The validated LC-SMRM-MS/MS method was applied to the simultaneous determination of the thirteen marker compounds in 81 batches of rhubarb (Supplementary Fig. S2). A typical LC-SMRM-MS/MS chromatogram of DH is shown in Fig. 2. Although baseline separations of some analytes with different masses were not achieved, SMRM transitions permitted unambiguous peak integrations for quantitative analysis. From Fig. 3, it can be seen that the contents of individual marker compound within the same type of rhubarb products varied in a wide range, which may be attributed to internal factors such as genetic variation and plant species as well as external factors including geographical origin, harvest time, storage condition, and processing procedure of the herb[24-26]. Rhein and emodin, with the content ranges of 0.111-0.673 and 0.112-0.512 mg/g respectively, are the most abundant constituents among the compounds analyzed. Since DHT was produced by frying DH till carbonized, which was a vigorous process, the contents of all the marker compounds except PH-8-G decreased significantly.

Based on the chemical structures, the thirteen marker compounds can be divided into three chemical classes, i.e. anthrones, anthraquinone glycosides and free anthraquinones. The relative

contents of each class of compounds were calculated and presented in Supplementary Fig. S3. The contents of anthrones and anthraquinone glycosides decreased significantly after processing. Anthrones were hardly detected in SDH and TDH. Besides, the relative content of total anthraquinone glycosides was only 25.8% in TDH samples, which might lead to lost of purgative function.

3.5.2. Discrimination of crude and processed rhubarbs

In this study, PCA was further carried out to provide more information about the chemical variations of different rhubarb products. PCA is the most preferred unsupervised multivariate technique to provide an overview of class separation and clustering[18,27,28]. The first two principal components (PCs) accounted for 54.7% of total variance. As can be seen from the scores plot (Fig. 4), the crude and processed samples were classified into two groups obviously. The DH samples were also clustered in one region but within a larger sphere, indicating the quality of the commercial crude products needs to be controlled more strictly. The samples of JDH and SDH were not clearly demarcated, which was consistent with our previous report[19]. Although there were some overlaps among the SDHs and JDHs, most samples were clearly clustered in the score plot. The results of PCA revealed that the processing was the dominant factor causing the obvious differentiation.

4. Conclusion

This study developed and validated an HPLC-SMRM-MS/MS method for targeted quantitative analysis of 13 marker compounds in rhubarb. A significant decrease in the contents of anthrones and anthraquinone glycosides might induce weak purgative efficacy of processed products. Unsupervised PCA was performed to discriminate different rhubarb products. Targeted

plant metabolomic analysis based on HPLC-SMRM-MS/MS is a promising method for the quality control, particularly discrimination between crude and processed herbs.

Acknowledgements

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Figure Captions

Fig. 1. Chemical structures of marker compounds. Glc: glucose.

Fig. 2. The representative LC-SMRM-MS/MS chromatogram for the marker compounds in DH. 1: SB; 2: SA; 3: AL-8-G; 4: RH-8-G; 5: EM-1-G; 6: EM-8-G; 7: CH-8-G; 8: IS; 9: PH-8-G; 10: AL; 11: RH; 12: EM; 13: CH; 14: PH.

Fig. 3. The contents of marker compounds in different rhubarb products. Results are mean +standard deviation. (*, $p < 0.05$, compared with crude samples)

Fig. 4. Score plot from principal component analysis of crude and processed rhubarb products.

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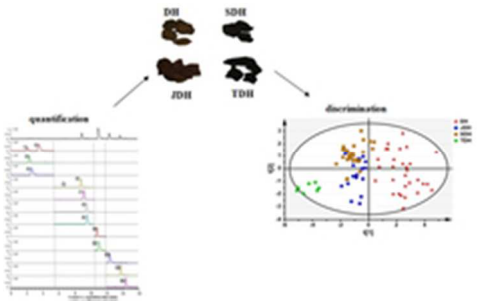
Table 1 MS/MS detection parameters, calibration curves, Linear range, limits of detection (LOD) and quantification (LOQ) for the 13 marker compounds of rhubarb.

Compounds	$[M-H]^-$ (*m/z*)	MRM transitions	Collison energy(V)	Regression equations [a]	r^2	Linear range (μg/mL)	LOQ (ng/mL)	LOD (ng/mL)
RH	283.0	283.0→238.9	15	y=431.4x+0.4827	0.9977	0.1-20	2	1
EM	269.0	269.0→224.9	27	y=1876x-0.04264	0.9954	0.1-20	2	1
AL	269.0	269.0→239.9	23	y=106.5x+4.112	0.9955	0.05-10	2	1
CH	253.0	253.0→224.9	30	y=335.6x-37.86	0.9962	0.05-10	50	20
PH	283.0	283.0→239.9	27	y=77.43x+93.16	0.9954	0.05-10	50	20
RH-8-G	445.0	445.0→238.9	34	y=150.1x-0.1580	0.9952	0.05-10	10	5
EM-1-G	431.0	431.0→269.0	30	y=1876 x-0.02863	0.9965	0.01-2	2	1
EM-8-G	431.0	431.0→269.0	30	y=1105x-0.5437	0.9976	0.05-10	2	1
AL-8-G	431.0	431.0→269.0	13	y=337.8x-1.284	0.9954	0.05-10	10	5
CH-8-G	415.1	415.1→252.9	28	y=3.145x-0.1119	0.9961	0.05-10	2	1
PH-8-G	445.0	445.0→283.0	30	y=326.0x-0.06854	0.9952	0.05-10	10	5
SA	861.1	861.1→386.1	37	y=159.2x-1.079	0.9959	0.05-10	50	20
SB	861.1	861.1→386.1	41	y=122.9x-10.08	0.9967	0.05-10	50	20

[a] y is the peak area ratio of mass detection (peak area of analyte/peak area of IS), x is the compound concentration injected and r^2 is the correlation coefficient of the

equation.



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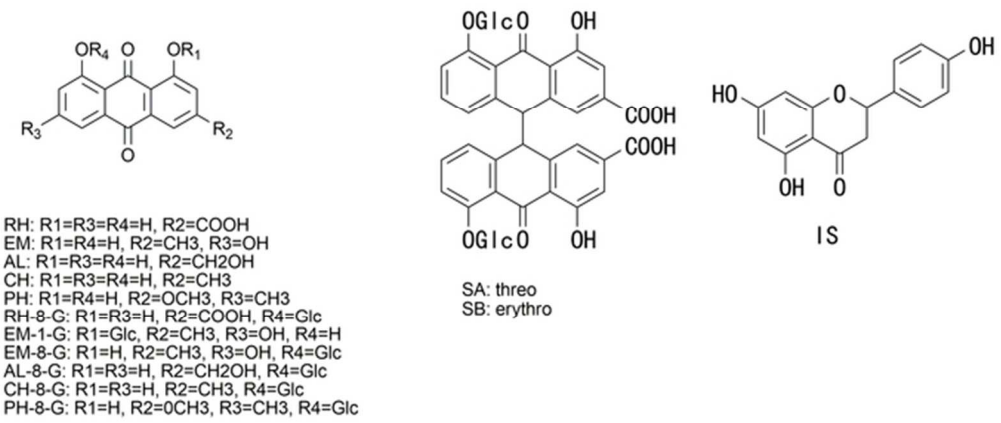

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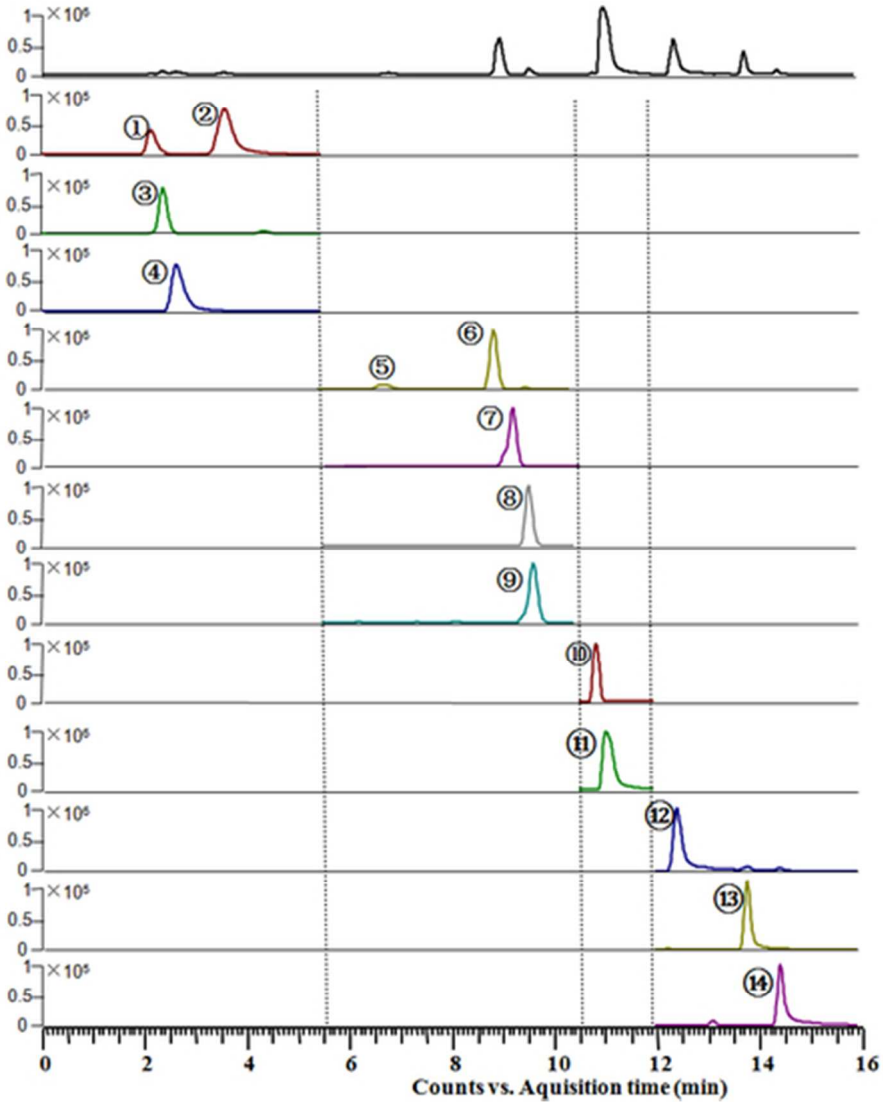

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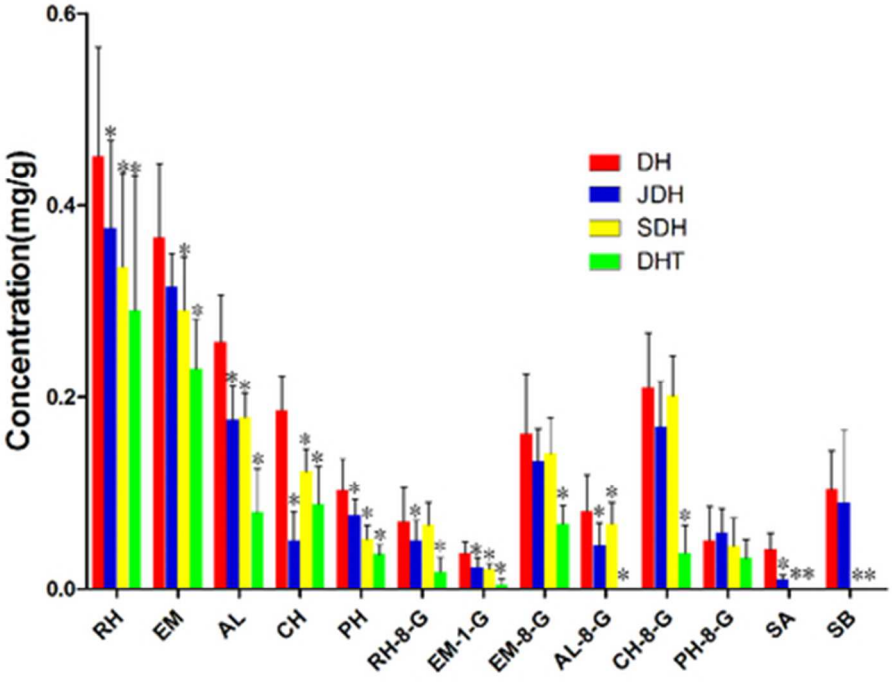

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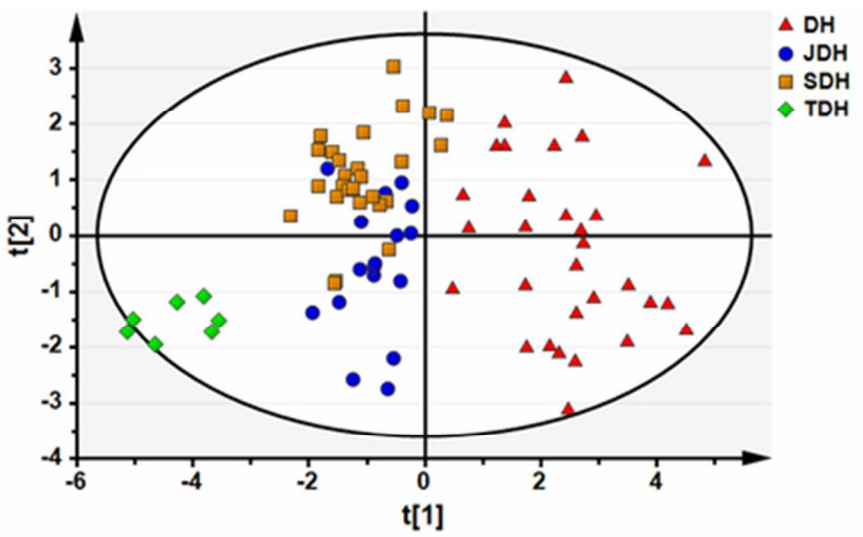

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