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## Research Article

# Metabolomic Study on the Halophyte *Suaeda salsa* in the Yellow River Delta

Plant metabolomics has been well established and applied across multiple fields including medicine, biotechnology, and environmental sciences in the post-genomic era. The Chenopodiaceae C<sub>3</sub> halophyte *Suaeda salsa* is the most important plant species in the vegetation of saline soil and even intertidal zone in the Yellow River Delta, which is economically consumed as food, widely used as a bioindicator of environmental stresses (salinity, drought, and pollution) and typically applied for the phyto-remediation of degraded wetland. However, no global studies have been focused on the metabolic profile of this halophyte which is widely applied in environment related research areas. In metabolomics, the first crucial step is the preparation of plant samples. In this work, several strategies of metabolite extraction from this C<sub>3</sub> halophytes *S. salsa* were evaluated and the metabolic profile was characterized by NMR-based metabolomics. Multiple replicates of plant tissues (approx. 250 mg whole fresh weight of leaves and stems) were homogenized by a high throughput automated Precellys 24 bead-based homogenizer and then extracted using the following solvent systems of varying polarities: methanol, water, methanol/water, methanol/chloroform/water, and acetonitrile. The hydrophilic metabolites were analyzed using one-dimensional proton NMR spectroscopy, and the subsequent NMR spectra were evaluated by principal components analysis (PCA). Each extraction protocol revealed unique metabolic profile from *S. salsa*. Overall, the quality of each extraction protocol was assessed based on the yield, reproducibility, ease, and speed, and we concluded that the solvent of methanol/water was preferable for the metabolite extraction due to its highest reproducibility, ease, and speed, and considerably high yield. Validation of methanol/water (1:1) solvent also showed a high degree of reproducibility with technical variation being considerably lower than biological variation in *S. salsa* samples. This demonstrated the quality and robustness of the methanol/water extraction method for NMR-based metabolomics studies.

**Keywords:** Metabolite extraction; Metabolomics; NMR; *Suaeda salsa*

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## 1 Introduction

Metabolomics, a system biology approach, has been defined the global profiling of metabolites, especially the low molecular weight organic metabolites contained in cells, tissues, biofluids, and even

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**Abbreviations:** CMO, choline monoxygenase; DMA, dimethylamine; DMG, dimethylglycine; MAE, microwave-assisted extraction; NCBI, National Center for Biotechnology Information; PC, principal component; PCA, principal components analysis; PLE, pressurized liquid extraction; PR, pattern recognition; SFE, subcritical-fluid extraction; TSE, traditional solvent extraction; TSP, 3-trimethylsilyl-2,2,3,3-d<sub>4</sub>-propionate; UE, ultrasonic extraction

whole organism [1, 2]. The components (metabolites) of the metabolome, as the end products of metabolisms represent the functional responses of a cell. The quantitative measurements of large numbers of cellular metabolites can provide a broad view of the biochemical status of an organism that can be used to monitor and assess gene function [3]. Therefore, metabolomics is useful to characterize the physiological and biochemical perturbations induced by both endogenous and exogenous factors [4–10]. This technique has been widely applied in environmental sciences with the term “environmental metabolomics” [11, 12]. Environmental metabolomics on the plant usually performs studies on the plant–environment interactions and for assessing plant function and health at the molecular level, and focuses widely on the metabolic responses to the environmental changes (drought, salinity, etc.), abiotic stresses (contaminations), and transgenic events [8, 9, 13, 14].

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The Chenopodiaceae C3 halophyte *Suaeda salsa* is an important and crucial halophyte in China and has important economical value because its seeds contain approximately 40% oil, rich in unsaturated fatty acids, which can be easily converted to chemical compounds for industrial and pharmaceutical use [15]. Since *S. salsa* is a dominant native halophyte in saline soil and even grows in the intertidal zone of the Yellow River Delta, where soil salt content is often higher than 3% [16], this halophyte has been widely applied in the environment-related areas including monitoring of environmental stresses and phyto-remediation of pollution in the degraded wetland [17, 18]. As a matter of fact, *S. salsa* is the pioneer plant in the intertidal zones of the Yellow River Delta. Due to the high tolerance to salinity and immobility, *S. salsa* has exhibited the potential as a bioindicator for the environmental monitoring of intertidal zones and saline soil compared with non-halophytes and animals. Therefore, it has been of great virtues in environmental sciences and applied for the monitoring of environmental stressors in the intertidal zones and phyto-remediation of degraded wetland with pollutions (heavy metals and oil) or increasing salinity [17–19]. For example, Zhu et al. [17] has reported the distribution and bioavailability of heavy metals (Cu, Zn, Pb, and Cd) in *S. salsa* and suggested that *S. salsa* was applicable for the phyto-remediation of heavy metal polluted soil. Additionally, the physiological and molecular responses of *S. salsa* and other high plants to environmental stressors such as salinity, water, and contaminants have been extensively studied [20–30]. To our knowledge, no application of metabolomics on *S. salsa* has been carried out, which limits the understanding of the biochemical responses induced by environmental stressors including increased salinity, drought, and contamination.

Usually, metabolomic analysis consists of three experimental steps including preparation of the sample, acquisition of data using analytical instruments, and data analysis by pattern recognition (PR) methods [31]. All steps are strongly interrelated, but the first step of metabolite extraction is very crucial since it is necessary to determine the quality of metabolome including the desired chemical components from the plant tissues for further analysis. Extraction of metabolites from plant tissues often is the most labor-intensive and rate-limiting step in metabolomics. In contrast, the most widely used analytical techniques in metabolomics, proton NMR spectroscopy, and MS, both are capable of high sample throughput and automated analysis [32, 33]. Therefore, there is a need to streamline and automate the sample extraction procedure so as to increase the overall sample throughput for metabolomics studies [34]. There are several extraction methods such as supercritical- and subcritical-fluid extraction (SFE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), ultrasonic extraction (UE), and traditional solvent extraction (TSE) in which TSE is the most popular method applied for the metabolite extraction [35, 36].

Due to the large variety of metabolites contained within plant tissues, all with highly differing physical and chemical properties, there is no one ideal way to simultaneously extract all classes of metabolites with high efficiency. There is at present no single solvent capable to extract all the metabolites (whole metabolome), and, therefore, the choice of extraction solvent limits the view on the metabolome. Currently, the solvent extraction systems mainly include methanol, water, ethanol, acetonitrile, chloroform, the mixed solvents, methanol/water, methanol/chloroform/water, and even the deuterated solvents [31]. There are several aspects to be considered for solvent extraction, such as the solvent characteristics, duration of extraction and temperature, and hence the subsequent

extraction yield, reproducibility, ease, and speed are the criteria for assessing the quality of an extraction protocol for metabolomics [34, 37]. In this work, the aim is to (1) characterize the metabolic profile of halophyte *S. salsa* and (2) compare several solvent systems for the extraction of metabolites from halophyte *S. salsa* tissues and then determine and validate a rapid, straightforward, and reproducible method to extract metabolites from *S. salsa* tissues using NMR-based metabolomics.

## 2 Materials and methods

### 2.1 Plant tissue samples and chemicals

Fresh leave with stem tissue samples of *S. salsa* were collected from seedlings with length ranging from 13 to 15 cm at the Yellow River Delta in Shandong Province. After harvesting, all the plant tissue samples were rinsed by de-ionized water and dried using clean tissue paper, then flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction. All the organic solvents for metabolite extraction,  $\text{D}_2\text{O}$  for NMR analysis and 3-trimethylsilyl-2,2,3,3- $\text{d}_4$ -propionate (TSP) for internal standard of NMR were purchased from Sigma Chemical Company (USA). All the organic solvents were of HPLC grade for metabolite extraction from *S. salsa* tissues.

### 2.2 Metabolite solvent extraction

Solvent systems for metabolite extraction methods were taken based on the varying polarities including methanol, water, methanol/water, methanol/chloroform/water, and acetonitrile. Frozen plant tissue samples were pooled and then ground together in a liquid  $\text{N}_2$ -cooled mortar. The homogenous tissue powder (weighing from 250 to 300 mg per sample) was divided into 25 tubes containing  $\sim 50$  ceramic beads with 1 mm diameter, and then homogenized with 3.33 mL/g of either (1) methanol, (2) water, (3) methanol/water (1:1), (4) methanol/chloroform/water (1:2:1), or (5) acetonitrile ( $n = 5$  each) using a high throughput homogenizer, Precellys 24 (Bertin, France). The volume of solvent and weight of plant tissues were determined by a preliminary experiment using several different ratios of solvent volume to tissue weight. Finally, a ratio 3.33 mL solvent to 1 g tissue was determined by the comparison between the NMR peak intensities of metabolites and 0.05 mM internal standard TSP in buffered 1.007 mM  $\text{D}_2\text{O}$  (data not shown). Specially, 3.33 mL/g chloroform were used in the extraction protocol methanol/chloroform/water to partition the possible macromolecules that bring broad resonances in NMR spectrum from *S. salsa* samples. After homogenization, samples were transferred to Eppendorf tubes and vortexed for 15 s three times. Following centrifugation ( $3000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ), the supernatant (for methanol, water, methanol/water, and acetonitrile extractions), or the entire upper layer (for methanol/chloroform/water extractions) were removed and then lyophilized prior to NMR analysis. It was subsequently resuspended in 600  $\mu\text{L}$  of 100 mM of phosphate buffer ( $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ , including 0.5 mM TSP, pH 7.0) in  $\text{D}_2\text{O}$ . The mixture was vortexed and then centrifuged at  $3000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The supernatant substance (550  $\mu\text{L}$ ) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

Additionally, plant tissues from ten individuals of *S. salsa* were divided into two groups ( $n = 5$ ) to obtain adequate weights of two mixed sample tissues which were ground separately under liquid nitrogen by mortar and pestle. The homogenous tissue powder of

each mixed sample was divided on 10 tubes and extracted with either (1) methanol, (2) water, (3) methanol/water (1:1), (4) methanol/chloroform/water (1:2:1), and (5) acetonitrile; each  $n = 2$ . All these plant tissue samples were then processed following the procedures mentioned above.

### 2.3 NMR analysis

Extracts of *S. salsa* tissue were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K). One-dimensional (1D)  $^1\text{H-NMR}$  spectra were obtained using a 11.9  $\mu\text{s}$  pulse, 6009.6 Hz spectral width, and mixing time 0.1 and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, with 128 transients collected into 16 384 data points. Datasets were zero-filled to 32 768 points and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All  $^1\text{H-NMR}$  spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were assigned following tabulated chemical shifts [38] and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada). Some of the metabolites were confirmed by the 2D NMR method, *J*-resolved NMR spectra were acquired using 8 transients per increment for a total of 128 increments, which were collected into 8-k data points using spectral widths of 5 kHz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). The relaxation delay of 1.5 s was used. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to Fourier transformation, and tilted by 45°, symmetrized about F1, and then calibrated, using TopSpin (version 2.1, Bruker).

### 2.4 Spectral pre-processing and multivariate data analysis

1D proton NMR spectra were converted to a format for multivariate analysis using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA). Each spectrum was segmented into 0.005 ppm bins between 0.2 and 10.0 ppm with bins from 4.72 to 4.96 ppm (water) excluded from all the NMR spectra. Bins between 7.76 and 7.96 ppm, between 7.66 and 7.72 ppm, between 7.36 and 7.60 ppm, between 7.12 and 7.17 ppm, and between 6.44 and 6.50 ppm containing pH-sensitive NMR peaks were compressed into single bins. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with transformation parameter  $\lambda = 6.4 \times 10^{-8}$  [39, 40] to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks (Fig. 2).

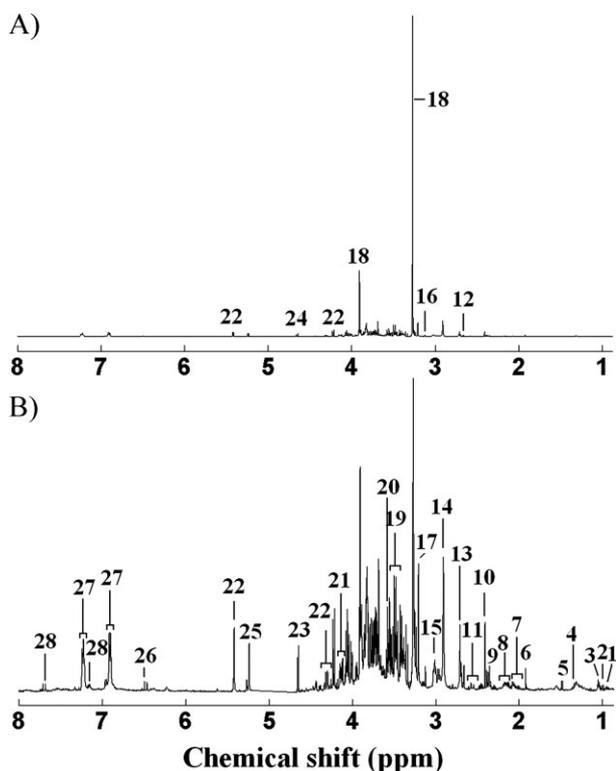
Principal components analysis (PCA) was used in this work for the separation of sample groups with various extractions of solvent systems. PCA is an exploratory unsupervised PR method since it detects inherent variation within the dataset and takes no account of class membership. The algorithm of this PR method calculates the highest amount of correlated variation along principal component (PC1), with subsequent PCs containing correspondingly smaller amounts of variance. For each model built, the loading vector for the PC was examined to identify the metabolites which contributed to the clusters [41].

## 3 Results and discussion

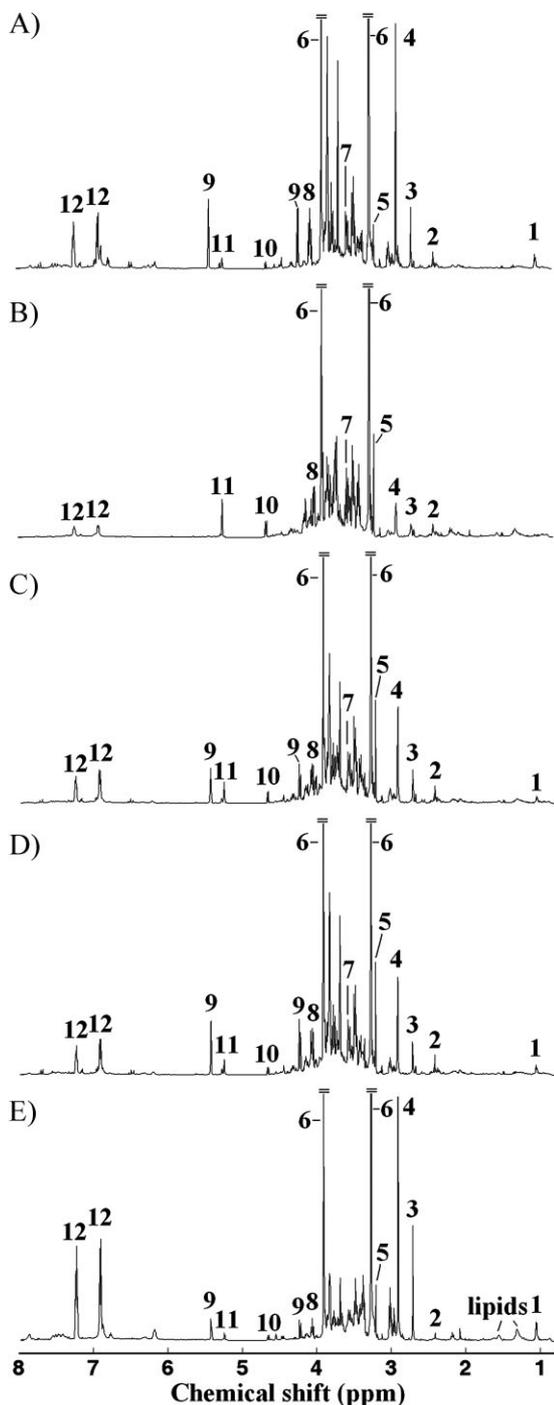
### 3.1 Metabolic profiles of *Suaeda salsa* extracted using various solvent extraction systems

As a representative halophyte in the vegetation of saline soil and even intertidal zone in the Yellow River Delta, *S. salsa* has been attractive to researchers [16, 20, 42, 43]. The tolerance to the salinity has been extensively studied by Zhang's and Wang's groups [20, 23, 24, 42]. The expressed sequence tags from NaCl-treated *S. salsa* cDNA library have been published and uploaded to the public database of National Center for Biotechnology Information (NCBI) [20]. To our knowledge, the metabolic profile of *S. salsa* has not been characterized yet, which limits the understanding of metabolic pathways of this species and to elucidate the metabolic mechanisms to the environmental stressors on a global view of metabolisms.

1D  $^1\text{H-NMR}$  spectrum (in both original and glog-transformed forms) from *S. salsa* using methanol/water extraction system is shown in Fig. 1 with the labels of identified metabolites (assigned in Tab. 1), and those representative  $^1\text{H-NMR}$  spectra of extracts from *S. salsa* using various solvent extraction systems are shown in Fig. 2. Following PCA, the separations between each extraction protocol were clearly observed from the PC1 versus PC2 scores plot (Fig. 3A). The differential metabolic fingerprints between various extraction



**Figure 1.** A representative 1D 500 MHz  $^1\text{H-NMR}$  spectrum of tissue extracts from *S. salsa* using extraction solvent system of methanol/water (1:1) in original (A) and generalized log transformed (with  $\lambda = 6.36 \times 10^{-8}$ ) (B) forms. Metabolite assignments: (1) leucine, (2) isoleucine, (3) valine, (4) lactate, (5) alanine, (6) acetate, (7) glutamate, (8) glutamine, (9) malate, (10) succinate, (11) citrate, (12) aspartate, (13) DMA, (14) DMG, (15)  $\gamma$ -aminobutyric acid (GABA), (16) malonate, (17) choline, (18) betaine, (19) myo-inositol, (20) glycine, (21) fructose, (22) sucrose, (23)  $\beta$ -glucose, (24) residual water, (25)  $\alpha$ -glucose, (26) fumarate, (27) tyrosine, and (28) beta-cyanin pigment (unconfirmed metabolite).



**Figure 2.** Representative 1D  $^1\text{H-NMR}$  spectra of the extracts from *S. salsa* obtained by the five extraction methods: (A) methanol, (B) water, (C) methanol/water (1:1), (D) methanol/chloroform/water (1:2:1), and (E) acetonitrile. Metabolite assignments: (1) valine, (2) succinate, (3) DMA, (4) DMG, (5) choline, (6) betaine, (7) glycine, (8) fructose, (9) sucrose, (10)  $\beta$ -glucose, (11)  $\alpha$ -glucose, and (12) tyrosine.

strategies were also detected according to the PC loadings plots (Fig. 3B and C). Overall, all the  $^1\text{H-NMR}$  spectra were dominated by an organic osmolyte and betaine, which is a main compound to maintain the osmotic balance and was approx. 10–100 times higher than other metabolites in the NMR spectral intensities (Fig. 1A). As a

matter of fact, betaine usually serves as organic osmolyte, substances synthesized, or taken up from the environment by cells for protection against osmotic stress, drought, high salinity, or high temperature in biological systems including both plants and animals (especially marine invertebrates) [44]. The pathway of betaine synthesis is short and straightforward: Choline monooxygenase (CMO) converts choline (a detectable metabolite in *S. salsa*, Fig. 1) to betaine aldehyde, and betaine aldehyde dehydrogenase converts this product to betaine [45–47]. In higher plants, such as *S. salsa*, betaine is an important secondary metabolite of alkaloid for the protection of osmotic stresses [44, 46]. As a halophyte, *S. salsa* is native to the saline soil containing a high saline up to 3% salinity, therefore, the organic osmolyte and betaine plays important physiological roles in osmotic regulation and hence was detected at a high level in *S. salsa* tissues.

The other representative organic osmolytes, dimethylglycine (DMG), and dimethylamine (DMA) showed relatively high abundances in all the samples (Fig. 1). DMG is an amino acid (glycine) derivative like betaine found in the cells of all plants and animals. In several mammals, betaine can be demethylated by betaine-homocysteine S-methyltransferase (EC 2.1.1.5) [48] to DMG [49, 50]. However, betaine cannot be actively catabolized in plants [51], so that the DMG could not be related to betaine and independently play the role of osmotic regulation in *S. salsa*. DMA is an organic secondary amine commonly found in mammalian animals. It also serves as an osmolyte to regulate the osmotic balance. In our case, the relatively high level of DMA could be related to osmotic regulation as well in *S. salsa* though the function needs further studies.

Sucrose, as a compound of energy storage in plant was much higher than fructose and glucose and was approx. 5–10% of betaine in the NMR spectral intensities from *S. salsa* samples (Fig. 1). Tyrosine is the most abundant amino acid in the *S. salsa* compared with other amino acids such as glycine, glutamate, valine, leucine, and isoleucine. In plants, tyrosine is produced via prephenate, an intermediate on the shikimate pathway. Prephenate is oxidatively decarboxylated with retention of the hydroxyl group to give *p*-hydroxyphenyl pyruvate, which is transaminated using glutamate as the nitrogen source to give tyrosine and  $\alpha$ -ketoglutarate. A tyrosine residue also plays an important role in chloroplasts (photosystem II); it acts as an electron donor in the reduction of oxidized chlorophyll [52].

There are other several classes of metabolites observed from the NMR spectra with varying intensities due to the various extraction protocols of solvent systems, including branched chain amino acids (valine, isoleucine, and leucine), organic acids (acetate, malate, and fumarate), energy storage compounds (fructose,  $\alpha$ -glucose, and  $\beta$ -glucose), Krebs intermediates (succinate and citrate), etc.

### 3.2 Evaluation of metabolite extraction strategies for *Suaeda salsa*

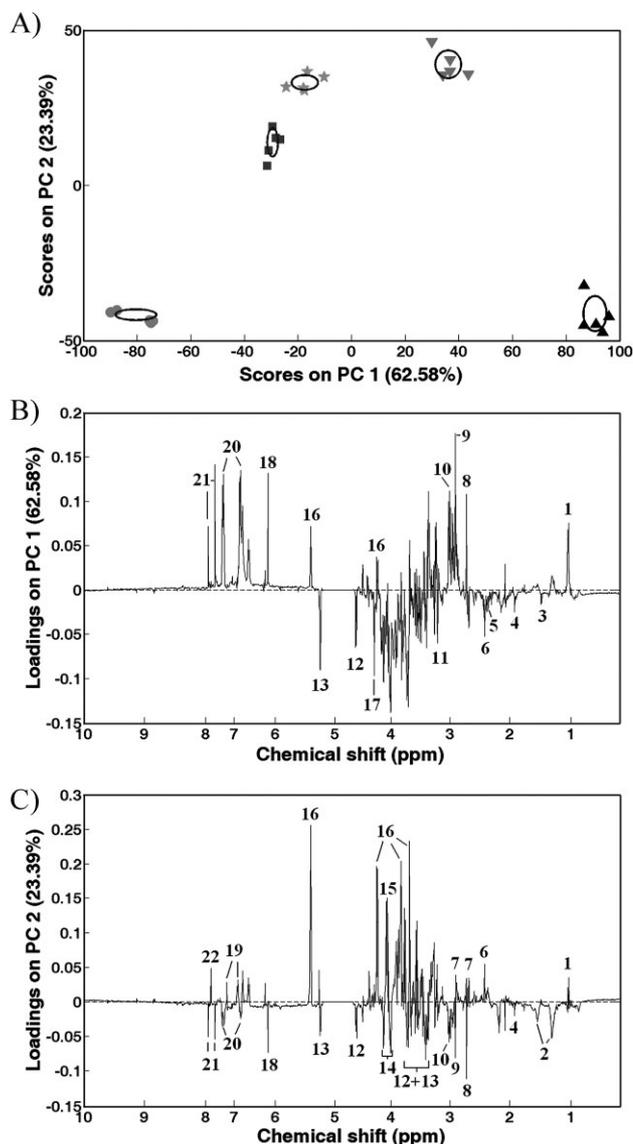
The metabolome, especially plant metabolome, consists of a wide variety of compounds at various levels and with distinct polarities. At present, there is no single solvent capable to dissolve the whole range of compounds. Solvents can be selected based on their physical and chemical properties such as polarity, toxicity, and inertness. Polarity of the solvents plays a critical role as solvents will extract metabolites following the “like-dissolves-like” principle [31]. However, metabolite extraction from a biological sample cannot be simplified according to purely physical and chemical properties, it also depends on the mechanism of release of metabolites from the biological matrix. In general, metabolomic studies should be

**Table 1.** Characteristic  $^1\text{H}$  chemical shifts (ppm) and coupling constant (Hz) of hydrophilic metabolites obtained from 1D and 2D  $J$ -resolved NMR spectra in 99.9%  $\text{D}_2\text{O}$  ( $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$  buffer, pH 7.0)

Metabolite	Chemical shift (ppm) and coupling constant (Hz)
Leucine	0.94 (t, $J = 5.9$ )
Isoleucine	0.98 (d, $J = 7.0$ )
Valine	1.05 (d, $J = 7.1$ )
Lactate	1.33 (d, $J = 6.6$ )
Alanine	1.48 (d, $J = 7.3$ )
Acetate	1.92 (s)
Glutamate	2.10 (m), 2.35 (m), 3.75 (t, $J = 6.0$ )
Glutamine	2.15 (m), 2.45 (m)
Malate	2.38 (dd, $J = 15.4, 10.1$ ), 2.68 (dd, $J = 15.4, 2.9$ ), and 4.29 (dd, $J = 10.1, 2.8$ )
Succinate	2.41 (s)
Citrate	2.54 (d, $J = 15.2$ ) and 2.71 (d, $J = 15.2$ )
Dimethylamine	2.71 (s)
Dimethylglycine	2.91 (s)
$\gamma$ -Aminobutyric acid	2.30 (t, $J = 7.2$ ) and 3.01 (dd, $J = 8.4, 6.3$ )
Malonate	3.13 (s)
Choline	3.20 (s)
Betaine	3.27 (s) and 3.91 (s)
Glycine	3.57 (s)
Fructose	4.10 (d, $J = 8.7$ Hz)
Sucrose	4.21 (d, $J = 8.5$ ) and 5.42 (d, $J = 3.9$ )
$\beta$ -Glucose	4.64 (d, $J = 7.9$ Hz)
$\alpha$ -Glucose	5.24 (d, $J = 3.9$ Hz)
Fumarate	6.52 (s)
Tyrosine	6.88 (m) and 7.20 (m)

designed to detect as many metabolites as possible in an organism, and solvents which can extract diverse groups of metabolites should be chosen. Thus, the evaluation of metabolite extraction strategies needs to be performed to determine whether a selected solvent is applicable to extract a maximum amount of metabolites with detectable levels. Fortunately, there is less limitation in solvent choice for NMR analysis, a mid-polar solvent system such as methanol/water is often used. Bligh and Dyer [53] proposed a methanol/water protocol combined chloroform for the metabolite extraction from the animal tissue samples which usually contain a relatively high level lipids or lipoproteins. For the plant samples, the application of chloroform in the solvent systems is not mandatory due to the low abundance of lipids or lipoproteins in plant tissues such as leaf, stem, or root. In this work, the solvent systems methanol/water/chloroform (1:2:1) was evaluated together with others including methanol, methanol/water (1:1), water, and acetonitrile.

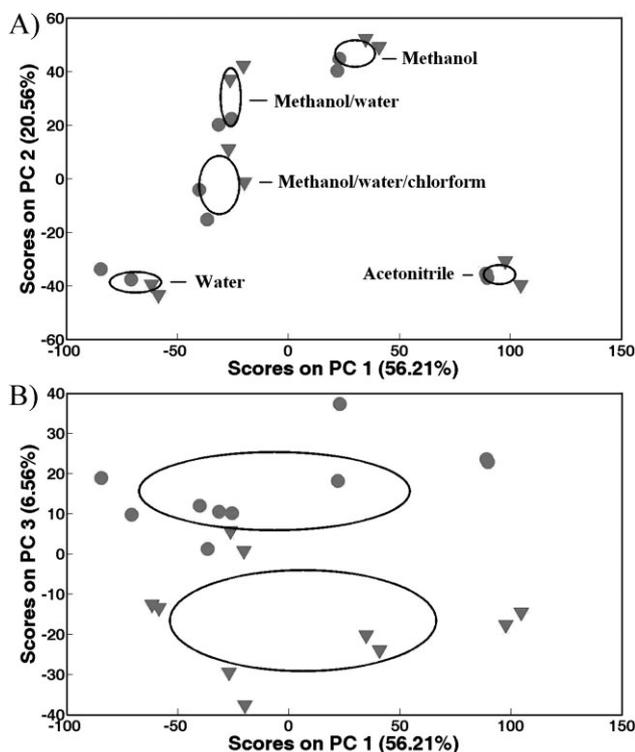
Basically, the PC scores plot indicated highly significant separations ( $p < 0.01$ ) between extraction protocols, which meant the huge amount of differences from the various extractions (Fig. 3A). To compare the variation of extraction with that of biological samples, two groups of *S. salsa* tissues (replicates,  $n = 10$ , respectively), were extracted using the five extraction protocols as mentioned above, and PCA was performed on the NMR datasets from the tissue extracts. Figure 4 shows the PC scores plots, PC1 versus PC2, and PC1 versus PC3. Apparently, Fig. 4A indicated the highly significant separation between the extraction protocols but between the two biological samples with total variation of 76.77% on PC1 and PC2. However, the biological variation between various 2 *S. salsa* samples was only 6.56% along PC3 (Fig. 4B), which implied that the major variation ( $\sim 76.77\%$ ) was caused by the extraction methods.



**Figure 3.** PCA scores plot (A), PC1 loadings plot (B), and PC2 loadings plot (C) from the analysis of the 1D  $^1\text{H}$ -NMR spectra of the *S. salsa* extracted using methanol ( $\blacktriangledown$ ), water ( $\bullet$ ), methanol/water (1:1) ( $\blacksquare$ ), methanol/chloroform/water (1:2:1) ( $\blackstar$ ), and acetonitrile ( $\blacktriangle$ ). Ellipses represent mean  $\pm$  SD for each extraction method. Metabolite assignments: (1) valine, (2) lipids, (3) alanine, (4) acetate, (5) malate, (6) succinate, (7) aspartate, (8) DMA, (9) DMG, (10)  $\gamma$ -aminobutyric acid (GABA), (11) choline, (12)  $\beta$ -glucose, (13)  $\alpha$ -glucose, (14) fructose, (15) unknown 1 (4.07 ppm), (16) sucrose, (17) tartrate, (18) unknown 2 (6.26 ppm), (19) betacyanin pigment (unconfirmed metabolite), (20) tyrosine, (21) benzonate, and (22) unknown 3 (7.69 ppm).

Therefore, the assessment of extraction protocols was extremely necessary to determine a preferable one with good quality for the metabolite extraction from *S. salsa*.

The criteria for assessing the quality of a tissue extraction strategy for metabolomics include yield, reproducibility, ease, and speed, each of which is addressed below (Tabs. 2 and 3). Since low molecular weight metabolites are often extracted simultaneously with macromolecules such as lipids and lipoproteins which produce broad resonances in 1D NMR spectra, the net yield of extracts (i.e., the



**Figure 4.** PCA scores plots (A: PC1 vs. PC2 and B: PC1 vs. PC3) from the analysis of  $^1\text{H-NMR}$  spectra of the extracts from both *S. salsa* (sample 1,  $\blacktriangledown$  and 2,  $\bullet$ ) that were extracted using methanol, water, methanol/water (1:1), methanol/chloroform/water (1:2:1), and acetonitrile. Ellipses represent mean  $\pm$  SD for each extraction method in (A) as well as for the mean  $\pm$  SD for samples 1 and 2 considering all methods together in (B).

total peak area of the 1D NMR spectra) can only provide an approximate tool for comparing metabolite yields from different extraction protocols. Total NMR spectral area of each extraction was normalized to TSP signal area and to tissue mass in order to compare yields between extractions. Further, total spectral areas were normalized around methanol to a yield of 1 (Tab. 2). According to the NMR data, methanol extraction produced highest yield, while water, methanol/water (1:1), methanol/chloroform/water (1:2:1), and acetonitrile showed 96.8, 96.0, 93.9, and 39.9% yield of methanol. Obviously,

**Table 2.** Comparison of the yield and reproducibility of several metabolite extraction methods

Extraction methods	Yield <sup>a)</sup> (mean $\pm$ SD)	Reproducibility <sup>b)</sup>
Methanol	1.000 $\pm$ 0.104	22.334
Water	0.968 $\pm$ 0.170	13.844
Methanol/water (1:1)	0.960 $\pm$ 0.024	9.284
Methanol/chloroform/ water (1:2:1)	0.939 $\pm$ 0.037	12.969
Acetonitrile	0.399 $\pm$ 0.016	24.660

a) Mean  $\pm$  SD determined from the ratios of total spectral area/TSP/tissue mass. The yields were normalized relative to the basic extraction solvent system methanol with a yield of 1.

b) Determined from PCA scores plots by multiplying SD along PC1 by SD along PC2 for each extraction method, where the smaller the value, the better the reproducibility.

the extraction of acetonitrile produced lowest yield for metabolite extraction, while the other four extraction protocols seemed to be similar in the extraction yields.

The reproducibility of one extraction protocol is essential for the sample preparation. We have determined the reproducibility of each extraction strategy using PCA scores plots, which provide an unbiased assessment of the similarities and differences between the metabolic fingerprints of multiple replicates of one biological sample. Specifically, reproducibility was determined by the area of the one standard deviation ellipse (i.e., derived from the standard deviation of the replicate samples along PC1 multiplied by the standard deviation along PC2), using the data from Fig. 3 (Tab. 2). In general, it was found that acetonitrile and methanol extractions yielded approx. twice the variability of the water, methanol/water, and methanol/chloroform/water extractions. And the extraction protocol methanol/water (1:1) produced lowest variation between the replicates hence the highest reproducibility (the smaller area of standard deviation ellipse, the higher reproducibility). Considering reproducibility of extraction protocols, methanol/water based extraction protocols (methanol/water and methanol/chloroform/water) could be preferable for the metabolite extraction from *S. salsa*. However, the origin of the differences between the reproducibility of extraction protocols was unclear.

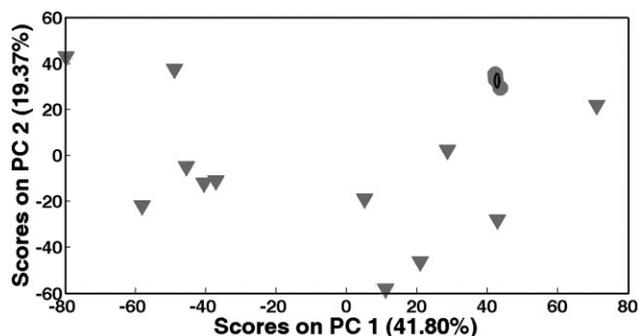
Since the different solvent systems have different polarities, it is expected that differential metabolomes will be extracted with differing efficiencies. For example, this is evident when considering the extraction of metabolites of a range of polarities. The PC1 loadings plot (Fig. 3B) showed that water with the highest polarity, preferentially extracted more polar metabolites with the chemical groups of  $-\text{COOH}$  and  $-\text{OH}$  such as organic acids and glucose (malate, succinate, fructose,  $\alpha$ -glucose, and  $\beta$ -glucose), but acetonitrile extracted metabolites with relatively low polarities or solubility such as tyrosine and sucrose (50% of the solubility of fructose). Along PC2, the solvent systems with 50% water extracted relatively high level of glucose, fructose, etc. Overall, the solvent system of methanol/water (1:1) was capable to extract most number of metabolites with relatively high levels (although not highest) such as sucrose, glucose, fructose, DMG, DMA, tyrosine, valine, etc. Therefore, we recommended that methanol/water (1:1) was preferable for the extraction of a diverse range of metabolites from *S. salsa* based on the high yield, reproducibility, ease, and speed.

### 3.3 Validation of selected extraction method of methanol/water by NMR metabolomics

Usually, there are two types of variance in metabolomics datasets: technical (or analytical) variance created by the experimental procedure, including sample preparation and instrumental analysis, and inherent biological variance existing within a population. To reveal underlying biological knowledge of a population, it is crucial to ensure any technical variance smaller than biological variance. Here we evaluated the variability associated with selected extraction method of methanol/water (1:1) and analysis of *S. salsa* samples compared with biological variability in the natural population of *S. salsa* collected from the Yellow River Delta. For validation of our extraction method for NMR metabolomics, the variation between three replicates of one *S. salsa* tissue sample was compared with the variation across 12 different individual *S. salsa* samples. The resulting PCA scores plot shows that the three replicates clustered tightly

**Table 3.** Summary of metabolite solvent extraction methods

Extraction methods	Yield	Reproducibility	Ease/speed	Other advantages
Methanol	+		+	
Water	+		+	
Methanol/water (1:1)	+	+	+	
Methanol/chloroform/water (1:2:1)	+			Obtain hydrophobic fraction
Acetonitrile			+	



**Figure 5.** PCA scores plot from analysis of the  $^1\text{H-NMR}$  spectra of three replicates of one *S. salsa* sample (●, where each replicate was extracted separately) and 12 different *S. salsa* samples (▼), all extracted using the methanol/water (1:1) method. The small ellipse is drawn at one standard deviation of the technical replicates along PC1 and PC2.

together and distinct from the 12 biologically distinct samples (Fig. 5). This confirmed that individual metabolic differences were larger than differences arising from sample preparation and NMR analysis and the solvent system of methanol/water was applicable for the metabolite extraction from *S. salsa* samples.

## 4 Conclusions

The metabolome of *S. salsa* was characterized by NMR-based metabolomics. There were several classes of metabolites including both primary and secondary metabolites, such as organic osmolytes (betaine, DMG, etc.), intermediates of Krebs cycle (succinate and citrate), compounds of energy storage (glucose, sucrose, etc.), and amino acids (valine, tyrosine, etc.). Five different strategies for the metabolite extraction from *S. salsa* including methanol, water, methanol/water (1:1), methanol/chloroform/water (1:2:1), and acetonitrile have been evaluated in terms of speed and ease of the protocol, reproducibility, and metabolite yield. The most rapid extraction solvent systems were those single solvents (methanol, water, and acetonitrile) which showed their own deficits for the metabolite extraction such as the lowest metabolite yield for acetonitrile, less extracted number of metabolites for both methanol and water. The solvent system of methanol/chloroform/water was the most tedious extraction protocol with lower reproducibility than methanol/water (1:1). Overall, the preferable extraction method for NMR metabolomics in terms of sample throughput, reproducibility, and extraction yield was the methanol/water (1:1) solvent system. Validation of the methanol/water (1:1) method was conducted using NMR metabolomics as well showing a high degree of reproducibility with technical (or analytical) variation being considerably lower than biological variation in *S. salsa* samples. This demonstrated the quality and

robustness of the methanol/water extraction method for NMR-based metabolomics studies.

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