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Seaweed metabolomics and its applications

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Abstract

Japan is the highest producer and consumer of seaweed in the world. Seaweed are classified into 3 groups; red, brown, and green algae. A comprehensive profiling of metabolites of seaweed is not readily available in mass spectrometry-based (MS) methods. MS-based methods require only a small amount of sample, but sample pre-treatment is necessary. However, the effects of drying and extraction methods are not well established. In the first part of the study, the effects of different pre-treatment methods were initially evaluated on three Japanese brown algae species. Freeze-drying was able to retain a higher number of high concentrations of metabolites as compared to oven-drying, while extraction methods had no significant effect on the metabolites' concentrations. The second study then used the freeze-dried method to investigate the metabolites relationship between the various seaweed' groups collected from Tohuku area; four brown, five red, and two green algae. Two extraction methods were tested. Consequently, the results confirmed that species characterization was the main factor affecting the metabolites profiles, not extraction methods. Particularly, only sugar profiling was able to discriminate between the seaweed' groups. Mannitol is the main constituent in brown algae, while fructose, sucrose, and glucose are in green algae. For red algae, it mostly depends on the individual species. Finally, the effect of heat on individual species was examined as metabolites profiling is unique in each species. Two parts of *Undaria pinnatifida*, Wakame (leaf) and Mekabu (sporophyll), are normally blanched in boiling water for a certain period before consumption. In order to retain the high metabolites concentrations, Wakame and Mekabu were shown to prefer blanching times of less than 20 and 40 seconds, respectively. These comprehensive metabolites data can be used as a basis for further explorations in other fields of research; namely, pharmaceutical (drug) and nutraceutical (food).

Keywords: Seaweed, metabolites, mass spectrometry, pre-treatment methods, blanching

論文題目

海藻のメタボローム解析およびその応用

論文要旨

海藻は陸生植物と比較して特有の代謝経路を保持しており，食品および医薬品など他の産業にとっても重要である．質量分析法(MS)は核磁気共鳴法よりも少量の試料しか必要としないが，MS を用いた包括的な海藻メタボローム研究は極めて少ない．このため本研究では，質量分析計を用いたメタボロミクス解析のための前処理法を確立し，海藻における基礎および応用研究に活用した．まず，3 種類の食用褐藻類について異なる前処理方法の影響を評価した．凍結乾燥はオーブン乾燥と比較してより多くの物質を高濃度で検出できた．一方，抽出法の違いは乾燥方法の違いよりも構成される物質濃度に大きな影響を及ぼさなかった．次に，3 つの海藻グループである褐藻，紅藻，および緑藻について前述の凍結乾燥法を使用して分析を行うと共に，2 つの抽出法の違いによる影響も確認した．代謝産物プロファイルは抽出方法よりも種分類群によって特徴付けられていた．海藻の分類を識別するには糖類のみを用いることが有効であり，マンニトールは褐藻，フルクトース，スクロース，グルコースは緑藻に含まれていることから各藻類の特徴づけができた．紅藻類の場合は個々の種に糖類の特徴が依存していた．第三の研究として，ワカメ（*Undaria pinnatifida*）の 2 つの部位，包葉部（ワカメ）および胞子体（メカブ）に対する加熱効果を解析した．ワカメの構成成分濃度は加熱 20 秒以内に劇的に減少したが，メカブは 40 秒後に徐々に減少していた．この過程で，茶褐色から緑色への色の変化が観察された．多くの構成成分濃度は加熱時間の増加とともに減少した．これらの研究は食品あるいは水産関連産業といった様々な産業研究分野における探索に用いることができ，更なる発展的利用が期待される．

キーワード：海藻，代謝産物，質量分析，前処理法，加熱調理

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Chapter 1

Introduction

1.1 Background

Seaweed or marine macroalgae are photosynthetic non-flowering plant-like organisms that are divided into three major groups based on their dominant pigmentation: brown (Phaeophyceae, approximately 1755 species), red (Rhodophyta, approximately 6000 species), and green algae (Chlorophyta, approximately 1500 species) (West *et al.*, 2017; Gury & Guiry, 2018). Among all three seaweed groups, brown algae are phylogenetically distant from red and green algae. It is because brown algae are derived from the secondary endosymbiosis, while red and green algae are derived from the primary endosymbiosis of a prokaryotic photosynthesis (De Clerck *et al.*, 2012; Groisillier *et al.*, 2014). Seaweed is a unique organism as it is neither a plant nor an animal. Seaweed have neither leaves, roots, nor a specialized vascular system compared to terrestrial plants, yet, they nourish themselves by osmosis (Gupta & Abu-Ghannam, 2011).

The demand for seaweed is increasing every year. According to Global Market Insight research, the global commercial market size for seaweed in 2016 was estimated at over USD 45 billion and expected to rise by over 8 % in 2024 based on the compound annual growth rate (CAGR) (Pulidindi & Rajpathak, 2017). Seaweed utilization as food in human consumption and food additive are rated as the highest global seaweed demand compared to the other uses of seaweed in the commercial markets such as in paper and textile industry, fertilizer and others (Figure 1.1). China, Japan and Korea are the highest seaweed consumers and producers in the world (Pulidindi & Rajpathak, 2017). Generally, seaweeds are consumed for their high nutritional value, either dried or fresh. Sometimes, seaweed are also used for flavouring purpose as some seaweed have umami

characteristics (Holdt & Kraan, 2011; Roohinejad *et al*., 2017). The average seaweed consumption in Japan is estimated at 10 gram per person per day (Leri *et al.*, 2019). Okinawa's population is known to have longevity and good health as a consequence of their seaweed-rich diet (Fitton, 2003). Besides direct consumption, the seaweed-derived hydrocolloid is usually used as a food additive since it contains gelling, thickening and stabilizing agents. As for animal feed, seaweed are able to boost the health, fertility and milk production (Gómez-Ordóñez *et al.*, 2010; Kilinc *et al.*, 2013; Peinado *et al.*, 2014; Rodrigues *et al.*, 2015; Stengel & Connan, 2015; Tanna & Mishra, 2018).

Seaweed industry is one of the primary resources for Japan's economy besides fisheries (MAFF, 2016). It is estimated that more than 200 species of seaweed are found in Japan. This is because Japan is an island nation comprising of a total of 6852 islands which is surrounded by the Pacific Ocean, the Sea of Okhotsk, the Sea of Japan and the East China Sea. Japan has also a temperate climate, with four distinct seasons, that contributes to the diversity of the seaweed species (MFAJ, 2014). The production of seaweed in Japan was drastically reduced after the Great East Japan Earthquake in 2011. However, it has since reached up to 75 % of the pre-2011 levels (24,799 tones) in 2015 (MAFF, 2016). The high variety of seaweed species in Japan encourages research in seaweed taxonomy and naming of new species of seaweed. However, seaweed metabolomics research is still lacking.

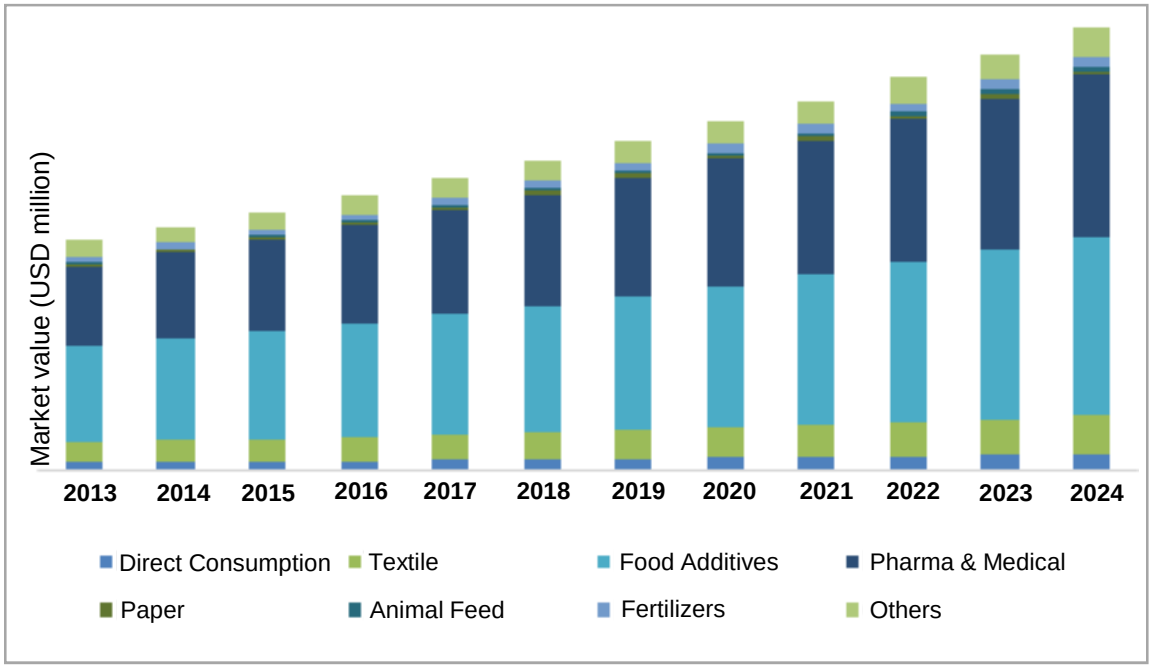

Figure 1.1 The global commercial seaweed market demand based on end-user application.

Metabolomics is an emerging research area which provides the latest insights in systems biology as it reflects the immediate biological information from genomics and transcriptomics. It is also a tool to advance the understanding of primary and secondary metabolism (Gupta *et al.*, 2014). There are many analytical platforms which could generate extensive metabolites information (Heyman & Dubery, 2016).

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most popular methods for metabolites profiling. Commonly, MS-based analysis is coupled with gas chromatography (GC-MS) or liquid chromatography (LC-MS) or capillary electrophoresis (CE-MS) (Gupta *et al*., 2014; Gu *et al.*, 2015; de Raad *et al.*, 2016). Although NMR offers precise structural information, it requires a larger sample volume for quantification purposes compared to MS.

Among the MS-based methods, GC-MS is only suitable to analyze volatile metabolites and requires sample derivatisation (Hattab *et al.*, 2007; Goulitquer *et al.*, 2012; Andrade *et al.*, 2013). On the other hand, LC-MS is useful to analyze the metabolites without derivatisation (Bedair & Sumner, 2008; Zhang *et al.*, 2014). However, LC-MS requires chromatographic column for metabolites separation purpose. Generally, non-polar metabolites are separated using reverse-phase column such as C18 and C8, while, for polar metabolites, hydrophilic interaction liquid chromatography (HILIC) column is needed for better separation (Yoshida *et al.*, 2007; Ikegami *et al.*, 2008). Alternatively, CE-MS is a powerful tool to analyze charged metabolites. (Soga & Heiger 2000; Soga *et al.*, 2002; Ramautar *et al.*, 2009; Hirayama *et al.*, 2014; Wakayama *et al.*, 2015).This is because, most of the primary metabolism metabolites such as amino acids, carboxylic acid, sugar phosphate, and nucleic acid are charges; either cation or anion. The separation of metabolites using CE principally depends on the metabolite charge and size. The unit used is mass to charge ratio (*m/z*) and it is detected using MS. CE-MS only requires a small amount of sample to identify a comprehensive metabolites profile.

However, the extraction process is crucial (Cai & Henion, 1995; Ramautar *et al.*, 2009; Wakayama *et al.*, 2015). Solvent extraction method is the most common method among the extraction methods, especially for MS-based analysis. However, there is not a single solvent which is able to extract all the metabolites simultaneously, thus an optimization process is required (Sekiyama *et al.*, 2010; Kim & Verpoorte, 2010; Ernst *et al.*, 2014; Kadam *et al.*, 2015). Solvent mixture containing methanol and other solvents

such as chloroform is often used to enhance the extraction of polar and non-polar compound concurrently (Bligh & Dyer, 1959; Kim & Verpoorte, 2010; Ernst *et al.*, 2014).

Seaweed contain various bioactive components such as proteins, minerals, vitamins, soluble dietary fibres, antioxidants, phytochemicals and polyunsaturated fatty acids (Holdt & Kraan, 2011; Gupta & Abu-Ghannam, 2011; Amorim *et al.*, 2012; Brown *et al.*, 2014; Pal *et al.*, 2014; Hamed *et al.*, 2015). Seaweed are also valuable as a renewable economic resource of the oceans as they have different morphological and physiological characteristics compared to terrestrial plants. The reason for this is that seaweed inhabit a harsh aquatic environment and experience a range of ecological diversity. Thus, the chemical components in seaweed vary with species, habitat, salinity, temperature, light intensity and other environmental conditions (Goulitquer *et al.*, 2012; Kumar *et al.*, 2016; Werner *et al.*, 2016; Palanisamy *et al.*, 2018). Besides that, seaweed also experience stress due to the threat from invasive species and pathogens (La Barre *et al.*, 2004; Jamers *et al.*, 2009; Liu & Pang, 2010; Kaaria *et al.*, 2015; Kumar *et al.*, 2016).

Therefore, it is important for us to have a better understanding of seaweed metabolites profiling. Previously, many studies were carried out on seaweed chemical components (Robledo & Freile Pelegrín, 1997; Tabarsa *et al.*, 2012; Date *et al.*, 2012; Rodrigues, *et al.*, 2015; Palanisamy *et al.*, 2018). Nevertheless, only specific species were focused on in those studies. For example, the studies on the whole genome sequence data of red algae, *Chondrus crispus* (Collén *et al.*, 2013) and brown algae *Cladosiphon okamuranus* (Nishitsuji *et al.*, 2016), and *Ectocarpus siliculosus* (Cock *et al.*, 2010; Michel *et al.*, 2010; Rousvoal *et al.*, 2011). Other studies only concentrated on specific compounds such as lipids and their derivatives (Thompson, 1996; Sánchez-Machado *et al.*, 2004; Harwood & Guschina, 2009; Brett & Kainz, 2013; Miyashita *et al.*, 2013; Gerasimenko & Logvinov, 2016), as well as mono- and polysaccharides of various seaweed (Percival, 1979; Skriptsova *et al.,* 2010; Wijesekara *et al.*, 2011; Rioux & Turgeon, 2015; Garcia-Vaquero *et al.*, 2017; Robin *et al.*, 2017). However, the comprehensive metabolomics studies for marine macroalgae are still lacking especially in various seaweed groups.

In comparison, there are more than 50,000 terrestrial plant metabolites data in KNApSAck database (http://kanaya.naist.jp/KNApSAcK/) (Nakamura *et al.*, 2014). Whereas, only 1110 seaweed metabolites are contained in Seaweed Metabolites Database

(SWMD; http://www.swmd.co.in/) and most of the data are from one species, the red alga, *Laurencia sp.* (Davis & Vasanthi, 2011).

1.2 Objectives

Seaweed are one of the economically important crops used as food and non-food application. They have various bioactive components that are beneficial for human and other organisms. The harsh living environment and the unique physiology characteristics of seaweed make them of particular interest for further exploration especially in the metabolite components. In comparison to terrestrial plant, the information on seaweed metabolites is limited. Thus, by using metabolomics-based approaches, the comprehensive metabolites from seaweed could be attained.

The aim of this study is to comprehensively profile seaweed metabolites using Japanese seaweed via MS-based methods (CE-MS and LC-MS). This study is divided into two parts: basic research and applied research. The basic research is covered in Chapter 2, while applied research in Chapter 3 and Chapter 4. In Chapter 2, the seaweed pre-treatment methods, drying and extraction, were established using three species of brown algae. In Chapter 3, by using the unified pre-treatment methods, the metabolome profiling of brown, red and green algae were conducted. In Chapter 4, the effect of heat treatment, such as blanching, on the metabolites' concentration on one specific species, *Undaria pinnatifida,* was investigated. Blanching is commonly applied in daily life before the consumption of seaweed. The overall study framework carried out is illustrated as Figure 1.2.

This study provides an insight into seaweed metabolomics especially using MS-based tools. The comprehensive data generated from this study could be utilized as resource guide to further explore the uses of seaweed in foods and health-related fields, such as in nutraceuticals.

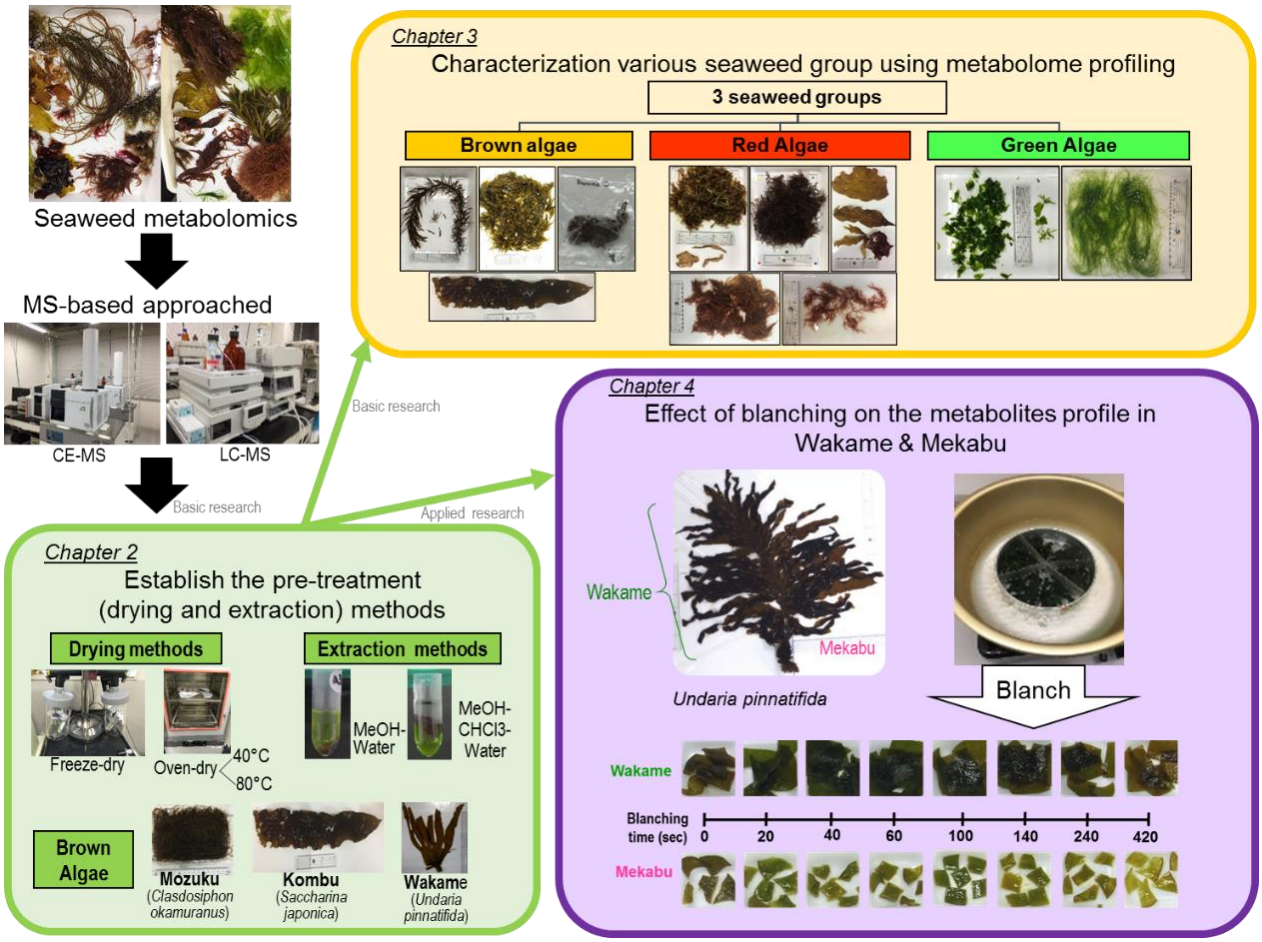

Figure 1.2 Research framework.

Chapter 2

Drying and extraction effects on three edible brown algae for metabolomics

2.1 Introduction

The brown algae, which comprise the class Phaeophyceae, are the largest group of multicellular algae with most of its species are edible (Wijesekara *et al*., 2011; Gupta & Abu-Ghannam, 2011). Brown algae like *Cladosiphon okamuranus* (Mozuku), *Saccharina japonica* (Kombu), and *Undaria pinnatifida* (Wakame) are commonly consumed in Asian countries such as Japan and Korea (Fitton, 2003). The nutritional and pharmaceutical benefits of these algae have been extensively studied. They contain fucoxanthin and fucoidan, which form part of a complex of polysaccharides with anticancer properties (Ale & Meyer, 2013; Zorofchian Moghadamtousi *et al*., 2014). Previous studies have been focusing on the variations in the specific properties of these compounds within and between species and throughout the seasons and geographic regions. Different chemicals used for extraction of metabolites have also been studied (Harnedy & FitzGerald, 2011). However, there are comparatively few studies on the effects of drying on seaweed compounds (Chan *et al.*, 1997). Therefore, it is essential to comprehensively evaluate the effects of various drying and extraction methods on seaweed metabolites.

Drying decreases water activity, delays microbial growth, preserves desirable qualities, and reduces storage volume of seaweed (Gupta & Abu-Ghannam, 2011). Newly harvested wet seaweed must be dried before processing or else their quality will deteriorate quickly. Crude extracts of wet seaweed do not contain the desirable gel (Chan *et al*., 1997; Wong & Cheung, 2001). For consumption and long-term storage, seaweed are sun-dried, freeze-dried, or oven-dried (Chan *et al*., 1997). The choice of seaweed drying method is significant because it affects the nutritional composition as a whole. In

general, seaweeds are sun-dried by spreading them over a flat surface or by hanging them. In some places, a rotary heating dryer is used (Gupta & Abu-Ghannam, 2011). The oven-dried method uses hot air, which reduces drying time. Previous studies have investigated the effects of oven-drying, at different temperatures, on the nutritional properties of brown algae. The algae were dried at 45 °C (Kaehler & Kennish, 1996), 60 °C (Chan *et al*., 1997), and 105 °C (Robledo & Freile Pelegrín, 1997). Unlike oven-drying, freeze-drying minimises physical damage, oxidation, and thermal reactions and preserves the characteristic chemical compositions of the seaweed because in freeze-drying, the seaweed are frozen overnight at −80 °C then dried directly by sublimation (Wong & Cheung, 2001). In most of the previous studies on seaweed drying, one specific temperature for different species was used. In consequence, the effects of drying at various temperatures on seaweed metabolite concentrations remain unclear.

Several comprehensive metabolite profiling methods are available in analyzing metabolite species such as NMR and MS-based methods (Gupta *et al*., 2014; de Raad *et al*., 2016; Heyman & Dubery, 2016). NMR is a high-throughput method that identifies and quantifies metabolites without destroying them (Lin & Shapiro, 1997; Gupta *et al.*, 2013). Nevertheless, NMR requires larger sample volumes than MS-based methods. In contrast, although MS-based methods work with relatively lower sample concentrations, the metabolites must first be extracted before analysis. Extraction is an essential step in MS analysis and must be performed swiftly in order to stop biochemical reactions in the tissues (Ernst *et al*., 2014). In previous studies of seaweed metabolite profiles by NMR and MS, the identification of particular groups like lipids and their derivatives was focused upon. These studies put emphasis on the stress-induced production of metabolites such as cyclosporine-like amino acids and halogenated compounds (Goulitquer *et al*., 2012; Gupta *et al*., 2014). However, the metabolite profiles of seaweed which are subjected to different drying and extraction treatments have not been comprehensively investigated.

Ordinarily, solvent extraction is used in MS analysis. It is vital, before conducting MS analysis, to optimise solvent selection in order to extract as many metabolites as possible. Unfortunately, no single solvent can extract all metabolites simultaneously. Often, a mixture of solvents is required to be able to extract all metabolites of interest (Ernst *et al*., 2014). In general, methanol or a solvent mixture containing it is used to

extract polar compounds. On the other hand, chloroform is used to extract lipophilic (non-polar) compounds and mixtures of polar and non-polar compounds (Van Hal *et al.*, 2014). In this study, the effects of two extraction methods, methanol-water with and without chloroform, were compared to improve the understanding of the metabolite profiles of brown algae.

Comprehensive studies on the effects of drying and extraction on edible brown algae are limited (Buschmann *et al*., 2017). Therefore, water-soluble metabolite quantification with CE-MS and LC-MS, after freeze-drying or oven-drying at 40 °C or 80 °C, was thoroughly evaluated. Two extractions methods, namely, methanol-water with and without chloroform, were compared. The materials used consisted of Mozuku, Kombu, and Wakame. The multivalent and statistical analyzes derived from this study will increase the understanding of the effects of pre-treatment drying and extraction on metabolite concentration profiles in different brown algae species.

2.2 Materials and methods

Three species of blanched brown algae, *Cladosiphon okamuranus* (Mozuku), *Saccharina japonica* (Kombu) and *Undaria pinnatifida* (Wakame) were purchased at a local supermarket in Tsuruoka City, Yamagata, Japan in December 2016 because they were abundant at the time this study was commencing. Those three species of seaweed were collected and shipped from Okinawa, Iwate, and Miyagi prefectures (Japan), respectively.

2.2.1 Sample drying

Wakame and Kombu samples were cut to a uniform size (~1 cm^2). Mozuku samples were more brittle than those of Wakame and Kombu and were divided into ~10 g lots. All samples were weighed on an analytical balance (AUW220D; Shimadzu Corp., Kyoto, Japan). They were then either freeze-dried or oven-dried, at 40 °C or 80 °C. For freeze-drying, the samples were initially frozen in liquid nitrogen and stored in a −80 °C freezer (MDF-U482-PJ, Panasonic Corp., Kadoma, Osaka, Japan) for 24 h. Before placing the samples in the freeze-dryer, its freeze-trap (Freeze Trap VA-800R, Taitec Corp., Saitama-ken, Japan) was set to −70 °C and <20 Pa. The samples were then moved into the freeze-dryer vessels and maintained at < −55 °C and <50 Pa with a vacuum pump (GLD-137CC

ULVAC, Taitec Corp., Saitama, Japan) for 72 h. The freeze-dryer pressure was then stabilized at <0.5 Pa. The samples were reweighed and stored in a freezer at −80 °C until extraction. The rest of the samples were dried in one of two separate ovens (Program Oven, Panasonic Corp., Kadoma, Osaka, Japan) for 48 h at 40 °C and 80 °C, respectively. After oven-drying, the samples were reweighed. The dry weight percentage of all dried seaweed samples was then calculated.

Dry weight percentage (%) = (weight after drying (g)) × 100 / (weight before drying (g)).

2.2.2 Chemicals and reagents

All reagents used in this study were either the analytical or the higher-grade types. Standard reagents were purchased and blended in-house for the quantification of each metabolite. To quantify the metabolites accurately, the following internal standards dissolved in methanol were used- Anion: 2-(*N*-morpholino) ethanesulfonic acid (MES) (No. 349-01623; Dojindo Laboratories, Kumamoto, Japan) and D-camphor-10-sulfonic acid (CSA) (No. 037-01032; Wako Pure Chemical Industries, Ltd., Osaka, Japan); Cation: L-methionine sulfone (No. 502-76641; Wako Pure Chemical Industries, Ltd., Osaka, Japan); Sugar: $^{13}C_6$-glucose (No. 404624; Sigma-Aldrich Corp., St. Louis, MO, USA). All of these were prepared as 200 μM stock solutions. In order to correct the CE-MS migration times, 200 μM trimesate (No. 206-03641; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 3-aminopyrrolidine (No. 404624; Sigma Aldrich Corp., St. Louis, MO, USA) dissolved in Milli-Q water were used.

2.2.3 Sample extractions

Fifty milligram lots of the dried samples were collected in 13-mL disruption tubes and disrupted without solvent at 1,500 rpm for 60 s in a cell disruptor (Shake Master Neo, BMS, Tokyo, Japan) fitted with a 1 cm × 1 cm diameter metal cylinder. Three replicates of powdered samples were then weighed out (~5 mg each) for extraction purposes.

For the methanol-water with chloroform extraction method, the samples were mixed with 500 μL of 200 μM internal standards-methanol solution in a micro-mixer

(Micromixer E-36, Taitec Corp., Saitama, Japan) for 3 min to which 500 µL chloroform and 200 µL Milli-Q water was added (Wakayama *et al*., 2015).

For the methanol-water extraction method, the samples were added to 500 µL of 200 µM internal standards-methanol mixture and 500 µL of Milli-Q water (Wakayama *et al*., 2010).

For both extraction methods, the samples were subjected to vortex for 3 min then centrifuged at 12,000 × g and 4 °C for 10 min in the MX-307 centrifuge (Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation, 300 µL of the aqueous top layer was transferred to ultrafiltration tubes (MW 5000 kDa; HMT, Inc., Tsuruoka, Japan) and centrifuged at 4 °C and 9,100 × g for 3 h. Then 30 µL of the filtrate was transferred to the LC vial for sugar analysis and stored in a freezer at −80 °C. The remaining 100 µL of the filtrate was evaporated at 4 °C in a refrigerated spin dryer (CentriVap Concentrator, Labconco Corp., Kansas City, MO, USA). The evaporated samples were stored at −80 °C until the time for CE-MS analysis. In order to normalise CE-MS migration time, the samples were dissolved in 20 µL of 200 µM trimesate and 3-aminopyrrolidine in Milli-Q water. For the cationic and anionic metabolite determinations, 10 µL aliquots of the solution were transferred to CE-MS sample vials and subjected to CE-MS analyzes.

2.2.4 Quadrupole LC-MS/MS mass spectrometry conditions for free sugar analysis

LC-MS/MS measurements were performed with an Agilent 1100 series column oven (Agilent Technologies, Santa Clara, CA, USA), an LC binary pump, and an autosampler fitted with the API 3000 Quadrupole LC-MS/MS tandem mass spectrophotometer system (Sciex, Framingham, MA, USA). The sample solutions were separated in the HILIC amino column (Asahipak NH2P-4E; 4.6 mm inner diameter × 250 mm length; 5 µm; Showa Denko K.K., Tokyo, Japan). The initial mobile phase was 80 % acetonitrile and 20 % Milli-Q water, and the flow rate was 0.8 mL min^{-1}. The acetonitrile gradient profile was 70 %, 60 %, and 80 % at 23 min, 35 min, and 40 min, respectively. The duration of each sample analysis was 40 min. The temperature of the column oven was set to 30 °C. One-microliter sample of aliquots was injected into the column. The nebuliser, curtain, and collision gas pressures, the ion spray voltage, and the ion source temperature were 15

psi, 11 psi, 8 psi, −4500 V, and 500 °C, respectively. Turbo-spray mode was selected. For MS analysis, the negative ion and MRM modes were selected.

2.2.5 Capillary electrophoresis-mass spectrometry (CE-MS) analysis of free amino acids, organic acids, and charged metabolites

All CE-MS analyzes were performed on an Agilent capillary electrophoresis system consisting of an Agilent G6220A LC-MSD TOF, an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Santa Clara, CA, USA).

2.2.6 Cationic metabolite analysis

Cationic metabolites like amino acids and amines were separated with fused-silica capillaries (50 μm inner diameter × 100 cm length) filled with 1 M formic acid as an electrolyte (Soga and Heiger, 1998). To stabilize ESI sprayer ionisation, a sheath liquid (methanol-water (50 % v/v) containing 0.1 μM Hexakis(2, 2-difluoroethoxy)phosphazene was used. It was delivered at 10 μL min^{-1} via an isocratic HPLC pump.

For each analysis, the sample solution was injected at 50 hPa for 5 s (3 nL) and 30 kV. The capillary temperature was maintained at 20 °C, and the temperature of the sample tray was kept at <5 °C. Time-of-flight mass spectrometry (TOF-MS) was conducted in the positive ion mode. The capillary, fragmentor, skimmer, and OCT RF voltages were set at 4,000 V, 75 V, 50 V, and 500 V, respectively. Ionisation was stabilized in MS by maintaining the drying nitrogen gas flow rate at 10 L min^{-1} and the heater temperature at 300 °C. The nebuliser gas pressure was set to 7 psi (48.2 kPa). Each acquired spectrum was automatically recalibrated with reference masses of sheath liquid ($[2MeOH+H_2O+H]^+$, *m/z* 66.06306) and protonated Hexakis ($[M+H]^+$, *m/z* 622.02896). Exact mass data were acquired at the rate of 1.5 cycles/second over a 50–1,000 *m/z* range.

2.2.7 Anionic metabolite analysis

Anionic metabolites like organic carboxylic acids and sugar phosphates were separated in a cationic polymer coated COSMO (+) capillary (50 μm i.d. × 105 cm lengths) (Nacalai Tesque, Kyoto, Japan) (Soga *et al.*, 2009). The electrolyte used for CE separation was 50 mM ammonium acetate (pH 8.5). New capillaries were first flushed over a 10 min period with running electrolyte, then with 50 mM acetic acid (pH 3.4), and again with the electrolyte. The capillary temperature thermostat was set to 20 °C, and the sample tray was cooled to <5 °C. A mixture of 10 μL min^{-1} of 5 mM ammonium acetate in 50 % (v/v) methanol-water containing 0.1 μM Hexakis was delivered to the CE interface with an Agilent 1100 series isocratic pump fitted with a 1:100 ratio splitter. This mixture was used as a sheath liquid around the outside of the CE capillary to provide a stable electrical connection between the capillary tip and the grounded electrospray needle.

Sample solutions were injected at 50 hPa for 30 s (30 nL), and −30 kV was applied. Before each injection, the capillary was flushed with 50 mM acetic acid (pH 3.4) for 2 min then with running electrolytes for 5 min. The MS capillary voltage was set to −3,500 V and the fragmentor, skimmer, and Oct RF voltages were set to 100 V, 50 V, and 500 V, respectively. MS ionisation was stabilized by maintaining the drying nitrogen gas flow rate to 10 L min^{-1} and the heater temperature to 300° C. The nebuliser gas pressure was set to 7 psi (48.2 kPa). Each acquired spectrum was automatically recalibrated with reference masses ([^{13}C isotopic ion of deprotonated acetic acid dimer ($2CH_3COOH$-H)]$^-$, *m/z* 120.03834) and ([Hexakis + deprotonated acetic acid (CH_3COOH-H)]$^-$, *m/z* 680.03554). Exact mass data were acquired at the rate of 1.5 spectra/second over a 50–1,000 *m/z* range.

2.2.8 Metabolite peak and statistical analyzes

Raw data for sugars obtained from LC-MS/MS were analyzed with Analyst v. 1.4.2. Cationic and anionic metabolite data derived from CE-TOF-MS were collected using Agilent Mass Hunter v. B06.00 software. Metabolite peaks were analyzed in Masterhands v. 2.17.3.17 propriety software from Keio University (Sugimoto *et al.*, 2012). All data

were then merged using an in-house macro program run in Microsoft Excel v. 2013 (Microsoft Corp, Redmond, WA, USA).

All metabolite concentrations were calculated by dry weight and were analyzed by JMP v. 13.2.1 (SAS Corp., Cary, NC, USA). Tukey-HSD analysis (Tables 2.2–2.4 and S2.10–S2.12), principal component analysis (PCA) (Figure 2.1 and S2.1), and hierarchical clustering analysis (HCA) (Figure 2.2) were performed on all data sets. HCA was performed using Ward's methods (Murtagh & Legendre, 2014). Two-way clusterisation was performed on metabolite concentrations and sample conditions. To elucidate any trends in metabolite concentrations, HCA was performed according to species and PCA was performed according to species, drying method, and extraction method.

2.3 Results

2.3.1 Dry weight percentage

The dry weight percentage of Mozuku, Kombu, and Wakame were determined by freeze-drying and by oven-drying at 40 °C and 80 °C (Table 2.1). The order of the dry weight percentage was Wakame > Kombu > Mozuku (9 %, 5 %, and 4 %, respectively) and was the same for all three drying methods. The difference in dry weight percentage among the three drying methods was < 1 %.

Table 2.1 Dry weight percentage of the samples after being dried using different drying methods.

	Freeze-dry (%)	Oven-dry (40 °C) (%)	Oven-dry (80 °C) (%)
C. okamuranus **(Mozuku)**	4.73	4.93	4.43
S. japonica **(Kombu)**	5.54	5.72	5.43
U. pinnatifida **(Wakame)**	9.58	9.64	9.31

2.3.2 Metabolite profiles of the three brown algae species

To identify any trends in metabolite composition, multivalent analyzes on all data sets were performed. The PCAs for the three dried and extracted brown algae revealed clear separations of the metabolite components. A variance of 34.8 % was observed in PC1 (Figures 2.1 and S2.1). The metabolite separations in PC1 were species-dependent. The Mozuku samples are located on the positive PC1 plots whereas the Kombu and Wakame samples are located on the negative PC1 plots. Positive PC1 loading plots for the Mozuku samples indicated high contribution rates of metabolites like 2-isopropylmalate, isethionate, and orotate (Figure 2.2, S2.1; Table S2.1). In contrast, negative PC1 loading plots indicated high contribution rates of metabolites like Asp, Glu, and Asn. PC2 represents the drying and extraction methods and revealed no clear separation variance among samples. PC3 revealed a separation between freeze-dried and oven-dried samples at a low 8.74 % variance (Figure S2.1; Table S2.1). PC4 represents separations of the extraction methods at a low variance of 6.21 % (Figure S2.1; Table S2.1). Similar trends were found in the PCA for the different drying methods (Figures S2.2–S2.4; Tables S2.2–S2.4) and for the extraction methods (Figures S2.5–S2.6; Tables S2.5–S2.6). Although the drying and extraction methods differed, the metabolite concentrations depended mainly on the brown algae species.

HCA was also performed on the dried and extracted seaweed. A total of 114 metabolites at various concentrations were detected and expressed as a heat map (Figure 2.2). The heat map shows that the relative metabolite distribution and clusterisation were based on metabolite concentrations whereas sample conditions were mainly species-dependent. HCA also demonstrated that Kombu and Wakame were clustered together and Mozuku was separated from them.

Drying methods also affected clusterisation; freeze-drying and oven-drying were clearly grouped. The extraction method using methanol-water with or without chloroform was the third clusterisation factor for seaweed species. More significant numbers of highly concentrated metabolites were found primarily in the Mozuku samples (100/114) followed by the Wakame (82/114) and Kombu (80/114) samples (Figures 2.2, 2.4, 2.5, 2.6; Tables and Figures S2.10–S2.12).

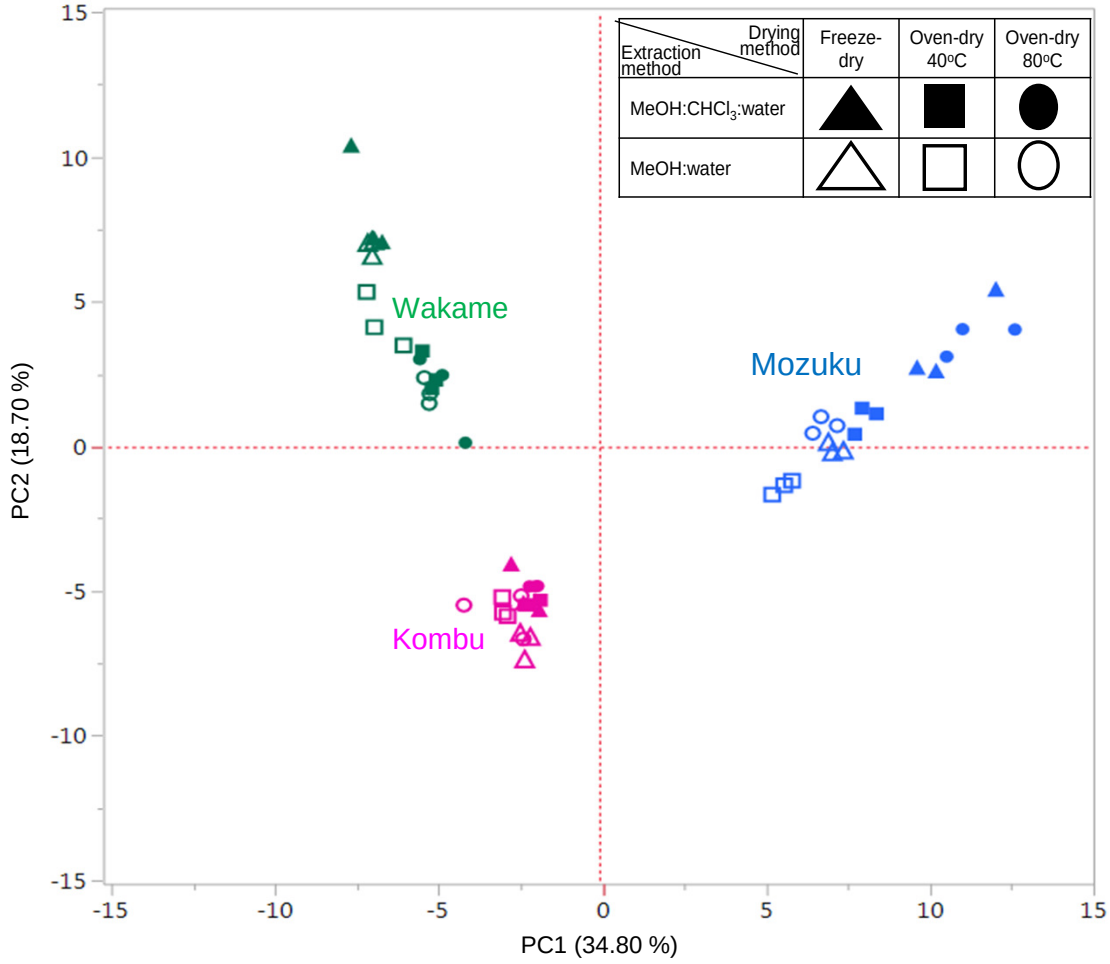

Figure 2.1 PCA score plot of metabolite concentrations in three brown algae after all drying and extractions methods.

The blue, pink, and green colored labels indicate Mozuku, Kombu, and Wakame, respectively. Filled and unfilled colors indicate the methanol-water extractions with chloroform or without chloroform, respectively. Triangle, square, and circle shapes indicate freeze-dry, oven-dry at 40˚ C, and oven-dry at 80˚ C, respectively. The horizontal axis value indicates PC1 separation variance and the vertical axis value indicates PC2 separation variance. Precise PCAs (PC1 to PC5) are shown in Figure S2.1. PCAs separation variances are shown in Table S2.1.

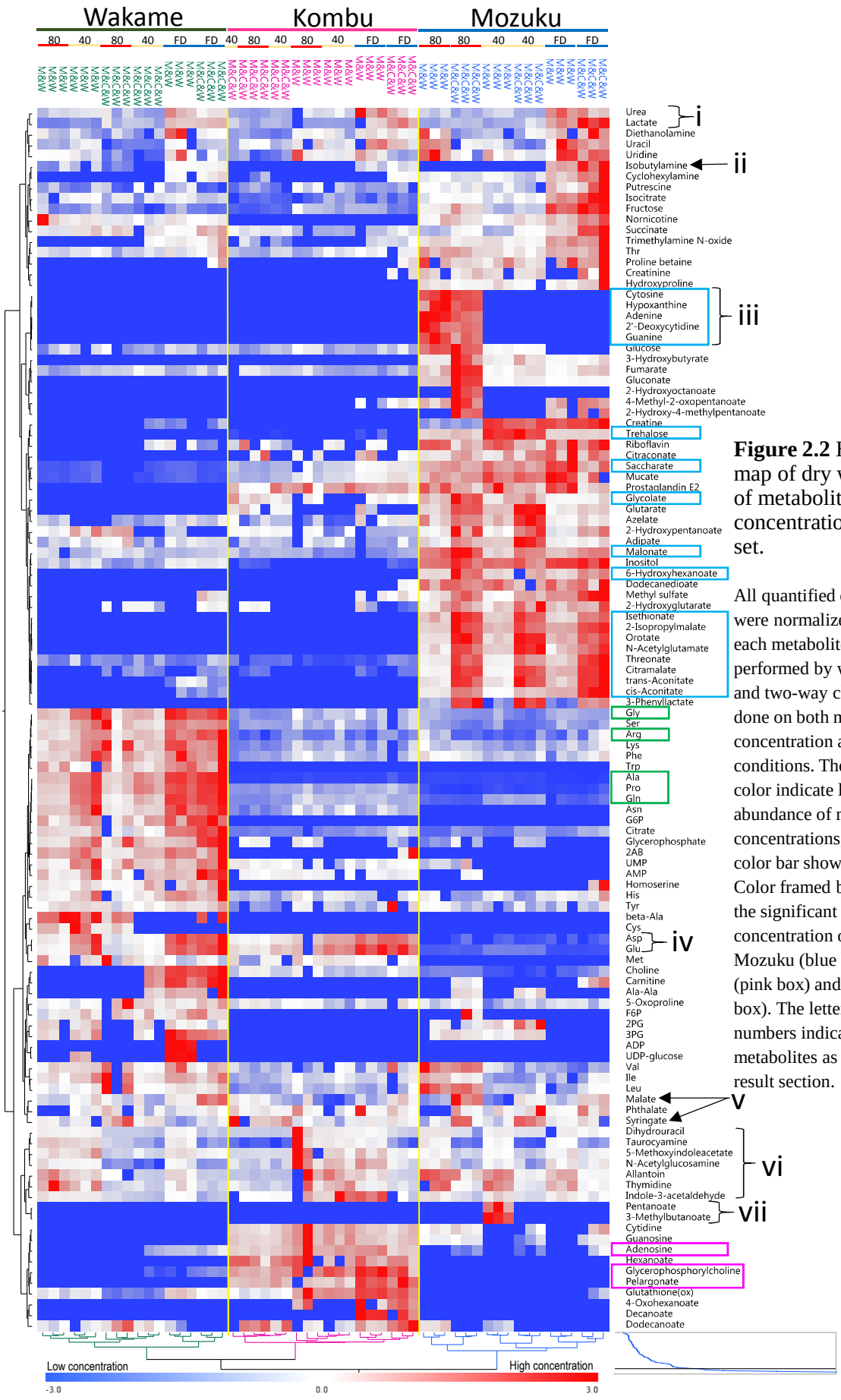

Figure 2.2 HCA and heat map of dry weight based of metabolite concentrations in all data set.

All quantified concentrations were normalized to Z-scores for each metabolite. HCA were performed by ward methods and two-way clusterization was done on both metabolites concentration and sample conditions. The blue and red color indicate low and high-abundance of metabolites concentrations, respectively as color bar shown in bottom. Color framed boxes indicated the significant high concentration of metabolite in Mozuku (blue box), Kombu (pink box) and Wakame (green box). The letter of roman numbers indicated the groups of metabolites as mentioned in the result section.

2.3.3 Independent species multivariate analyzes

In order to examine the effects of drying and extraction on each species more closely, multivariate analyzes were performed using PCA (Figures 2.3 and S2.7–S2.9; Tables S2.7–S2.9) and HCA (Figures 2.4–2.6). For PCA, the separation variances of the extraction methods were located in PC1 for Mozuku and Kombu (Figures 2.3a–b) and in PC2 for Wakame (Figure 2.3c).

The results showed that methanol-water extractions with and without chloroform mainly affected the Mozuku and Kombu metabolites. Their PC1 separation variances were 27.7 % and 23.1 %, respectively (See PC1: Figures 2.3a–b and S2.7–S2.8). PC2 for Mozuku and Wakame showed that the separation variance was attributed to the drying methods (25.4 % and 18.7 %, respectively). The positive plots of both PC2 for Mozuku and Wakame were separated by freeze-drying whereas the negative plots were separated by oven-drying (See PC2: Figures 2.3a–b and S2.7–S2.8). Wakame showed a 35.6 % separation variance in PC1 which could be explained primarily by the drying method. The freeze-drying method is located on the positive PC1 whereas the oven-drying method is situated on the negative plot (See PC1: Figures 2.3c and S2.9). PC2 for Wakame indicated that the extraction methods accounted for the separation variances (See PC2: Figures 2.3c and S2.9).

HCA for each seaweed species showed similar tendencies in terms of metabolite concentrations. They were associated with different drying and extraction methods (Figures 2.4–2.6).

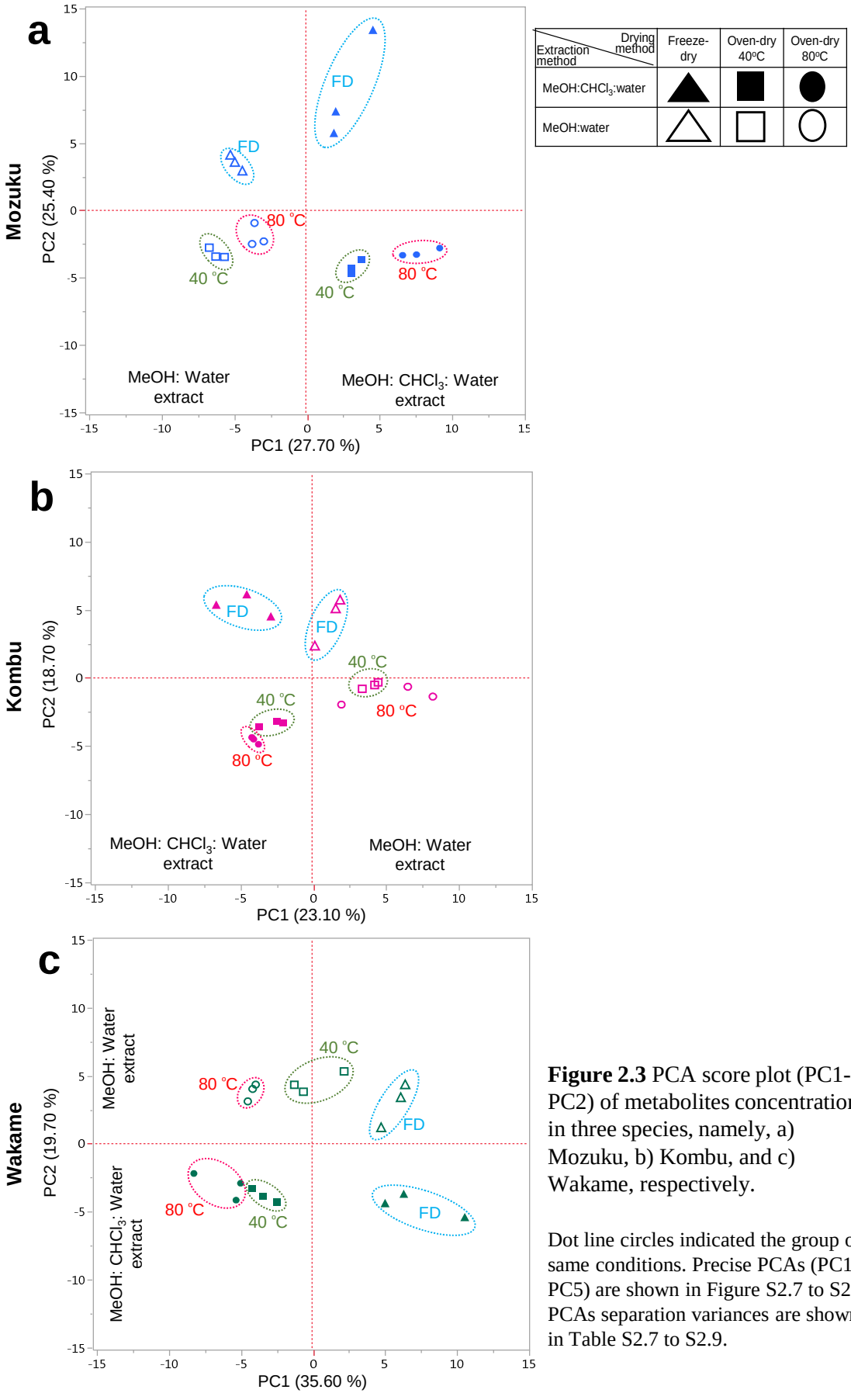

Figure 2.3 PCA score plot (PC1-PC2) of metabolites concentrations in three species, namely, a) Mozuku, b) Kombu, and c) Wakame, respectively.

Dot line circles indicated the group of same conditions. Precise PCAs (PC1 to PC5) are shown in Figure S2.7 to S2.9. PCAs separation variances are shown in Table S2.7 to S2.9.

2.3.4 Characteristics of Mozuku

The overall heat map of the dried and extracted brown algae illustrated that Mozuku contained more metabolites than Kombu or Wakame (Figure 2.2). The following metabolites were found in significantly higher concentrations in Mozuku than in Kombu or Wakame: malanoate, isethionate, 6-hydroxyhexanoate, threonate, citramalate, orotate, pelargonate, trans-aconitate, cis-aconitate, 2-isopropylmalate, *N*-acetylglutamate, gluconate, saccharate, and trehalose (See blue box in Figure 2.2; Table S2.10). In contrast, the Asp content in Mozuku was significantly lower than it was in Kombu or Wakame according to Tukey's HSD test (Table 2.2).

Drying method also affected the metabolite concentrations in Mozuku. High concentrations of urea, isobutylamine, lactate, isocitrate, cyclohexylamine, trimethylamine-*N*-oxide, fructose, putrescine, and inositol were detected in freeze-dried Mozuku (Figure 2.4i). On the other hand, only 2PG and Ala were found at high levels in oven-dried Mozuku (Figure 2.4v), which was extracted by either method. 3PG, Ala-Ala, syringate, glycerolphosphate, glutarate, adipate, and azelate were measured at high levels in oven-dried Mozuku extracted by methanol-chloroform-water (Figure 2.4vi). High concentrations of Gln, Glu, and trehalose were found in Mozuku oven-dried at 40° C (Figure 2.4iv). High levels of cytosine, hypoxanthine, glucose, Val, adenine, 2′-deoxycytidine, guanine, and Leu were detected in Mozuku oven-dried at 80°C (Figure 2.4viii). However, only low concentrations of creatine were noted in these samples (Figure 2.4iii). Glycolate, 3-hydroxybutyrate, fumarate, gluconate, 2-hydroxyoctanoate, malanoate, 5-oxoproline, 4-methyl-2-oxopentanoate, 2-hydroxy-4-methylpentanoate, and 6-hydroxyhexanoate were found at high levels in Mozuku oven-dried at 80° C and subjected to extraction with methanol-chloroform-water (Figure 2.4ix).

Extraction method also affected the metabolite concentrations in the Mozuku samples. Gly, Ser, uracil, uridine, 5-methoxyindoleacetate, riboflavin, *N*-acetylglucosamine, and dodecanedionate were detected at high levels in Mozuku samples subjected to extraction with methanol-water (Figure 2.4ii). Further, 2-hydroxypentanoate, isethionate, 3-phenyllactate, malate, orotate, *N*-acetylglutamate, threonate, 2-isopropylmalate, citramalate, trans-aconitate, citrate, 2-hydroxyglutarate, and phthalate

were measured at high concentrations in Mozuku subjected to extraction with methanol-chloroform-water (Figure 2.4vii).

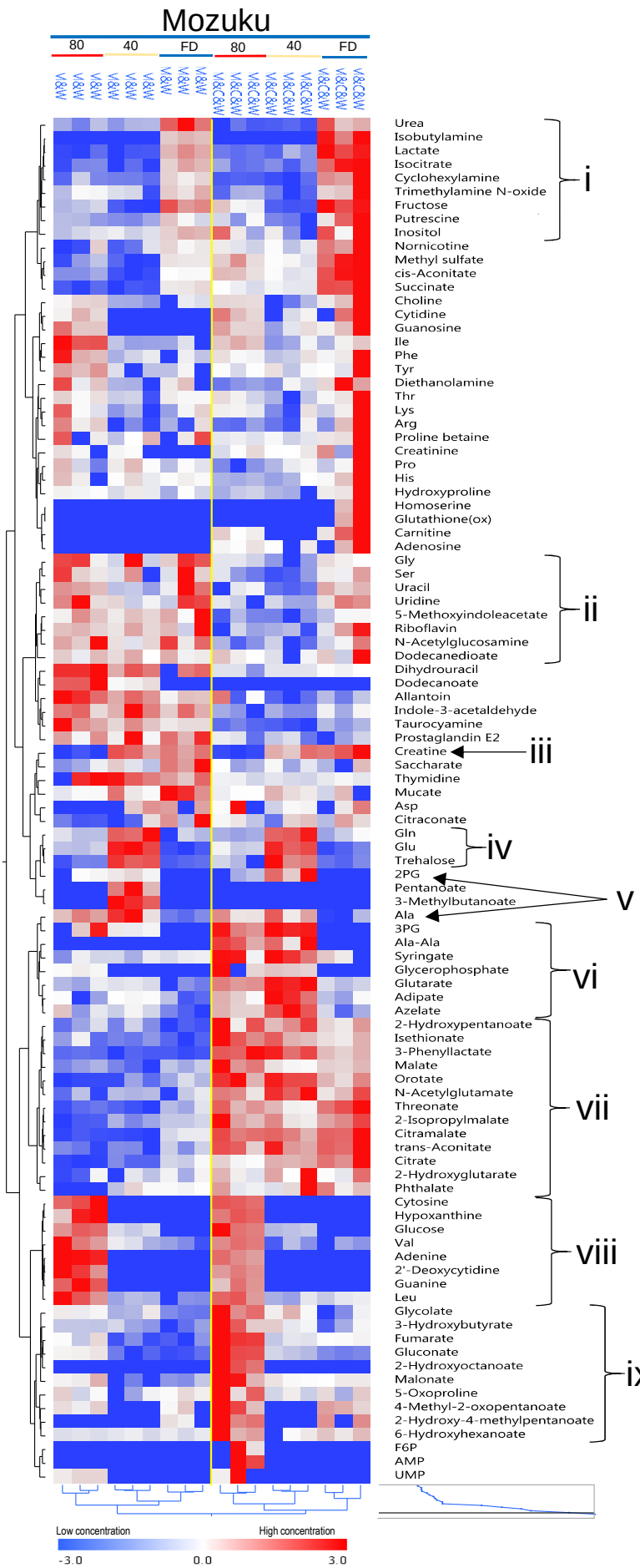

Figure 2.4 HCA and heat map of dry weight based of metabolite concentrations in Mozuku samples.

The letter of roman numbers indicated the groups of metabolites as mentioned in the result section.

Table 2.2 Free amino acid concentrations in Mozuku samples after various drying and extractions methods.

		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
	Amino Acids	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**
Essentials Amino Acids	Histidine	9.81±4.91^{abc}	3.61±1.87^{c}	2.76±0.18^{c}	6.11±0.67^{bc}	6.23±1.78^{bc}	8.14±1.68^{bc}
	Isoleucine	7.21±3.38^{abcd}	3.92±2.09^{cd}	10.50±0.64^{bcd}	4.15±1.67^{d}	3.25±0.74^{d}	14.64±1.08^{abc}
	Leucine	8.89±1.48^{cd}	6.51±2.46^{cd}	22.63±2.11^{ab}	3.78±0.20^{d}	5.70±0.75^{cd}	24.27±3.50^{a}
	Lysine	12.24±5.80^{bcd}	7.44±3.46^{d}	9.72±1.10^{bcd}	9.18±2.80^{bcd}	6.03±1.22^{d}	11.47±4.37^{bcd}
	Methionine	ND	ND	ND	ND	ND	ND
	Phenylalanine	10.60±3.72^{cdef}	7.07±2.26^{def}	8.65±1.07^{cdef}	5.01±2.72^{f}	6.13±1.08^{ef}	12.21±2.04^{bcdef}
	Threonine	87.13±20.56^{a}	58.76±6.88^{abcde}	74.85±8.34^{abc}	76.09±1.81^{abc}	60.90±7.28^{abcde}	78.83±6.46^{ab}
	Tryptophan	ND	ND	ND	ND	ND	ND
	Valine	7.94±4.24^{d}	9.20±4.11^{d}	42.65±1.64^{ab}	7.87±3.13^{d}	15.68±3.43^{bcd}	45.34±7.91^{a}
Non-Essentials Amino Acids	Alanine	52.26±19.42^{e}	129.39±15.46^{e}	122.06±11.77^{e}	55.70±4.45^{e}	140.99±21.58^{e}	131.37±6.82^{e}
	Arginine	9.96±5.84^{d}	4.81±2.16^{d}	3.91±0.39^{d}	5.48±2.68^{d}	3.50±0.51^{d}	9.65±2.37^{d}
	Asparagine	ND	ND	ND	ND	ND	ND
	Aspartate	26.98±9.73^{e}	13.96±3.94^{e}	29.94±19.76^{e}	29.95±0.36^{e}	28.13±4.88^{e}	ND
	Cysteine	ND	ND	ND	ND	ND	ND
	Glutamine	14.06±5.73^{ghi}	40.69±2.89^{efhgh}	9.39±0.78^{hi}	12.51±2.45^{i}	40.56±3.79^{efgh}	13.04±2.46^{ghi}
	Glutamate	43.02±6.67^{f}	154.93±7.30^{f}	74.70±7.90^{f}	37.57±2.76^{f}	162.30±6.86^{f}	67.33±3.10^{f}
	Glycine	31.95±1.55^{d}	17.48±3.77^{d}	26.10±1.26^{d}	38.85±3.84^{d}	30.72±10.63^{d}	38.02±5.25^{d}
	Proline	13.69±7.71^{fg}	9.08±1.74^{g}	5.75±0.94^{g}	8.83±1.39^{g}	12.36±2.37^{fg}	9.79±4.78^{g}
	Serine	24.04±5.14^{c}	11.62±1.93^{c}	14.73±3.22^{c}	27.16±9.87^{c}	19.35±10.48^{c}	28.32±6.14^{c}
	Tyrosine	10.23±6.76^{ab}	6.90±2.68^{ab}	6.59±1.31^{ab}	6.13±2.92^{ab}	4.78±0.93^{ab}	12.44±0.94^{ab}
	Total Free Amino Acids	**370.01 ± 112.64**	**485.36 ± 65.02**	**464.94 ± 62.42**	**334.37 ± 43.73**	**546.62 ± 78.34**	**504.86 ± 58.89**

The values indicate as mean ±SD (nmol g^{-1}) of dry weight (n = 3). ND, not detected. Different character indicates the significant differences ($P<0.05$) between each type of sample by Tukey-HSD analysis. Statistical analysis were compared between all data set (species and treatments (Table 2.2–2.4)).

2.3.5 Characteristics of Kombu

Kombu contained significantly higher concentrations of glycerophosphorylcholine, adenosine, and pelargonate (See pink box in Figure 2.2; Table S2.11) than Mozuku and Wakame. Urea, pelargonate, decanoate (Figure 2.5i), Asp, Glu, and glycerophosphorylcholine (Figure 2.5ii) were detected at significantly higher concentrations in freeze-dried Kombu than they were in oven-dried Kombu (Tables 2.3 and S2.11).

High concentrations of dihydrouracil, indole-3-acetaldehyde, taurocyamine, *N*-acetylglucosamine, 5-methylindolacetate, thymidine, allantoin, nornicotine, Gly, Lys, Ser, Ile, Leu, His, Phe, Met, Asn, Thr, putrescine, glucose, fructose, uridine, cytidine, guanosine, adenosine, prostaglandin E2, and isocitrate were detected in methanol-water-extracted Kombu (Figure 2.5iv). In contrast, methanol-chloroform-water extraction yielded high levels of glycolate, 2-hydroxypentanoate, malanoate, threonate, syringate, succinate, and UMP (Figure 2.5iii).

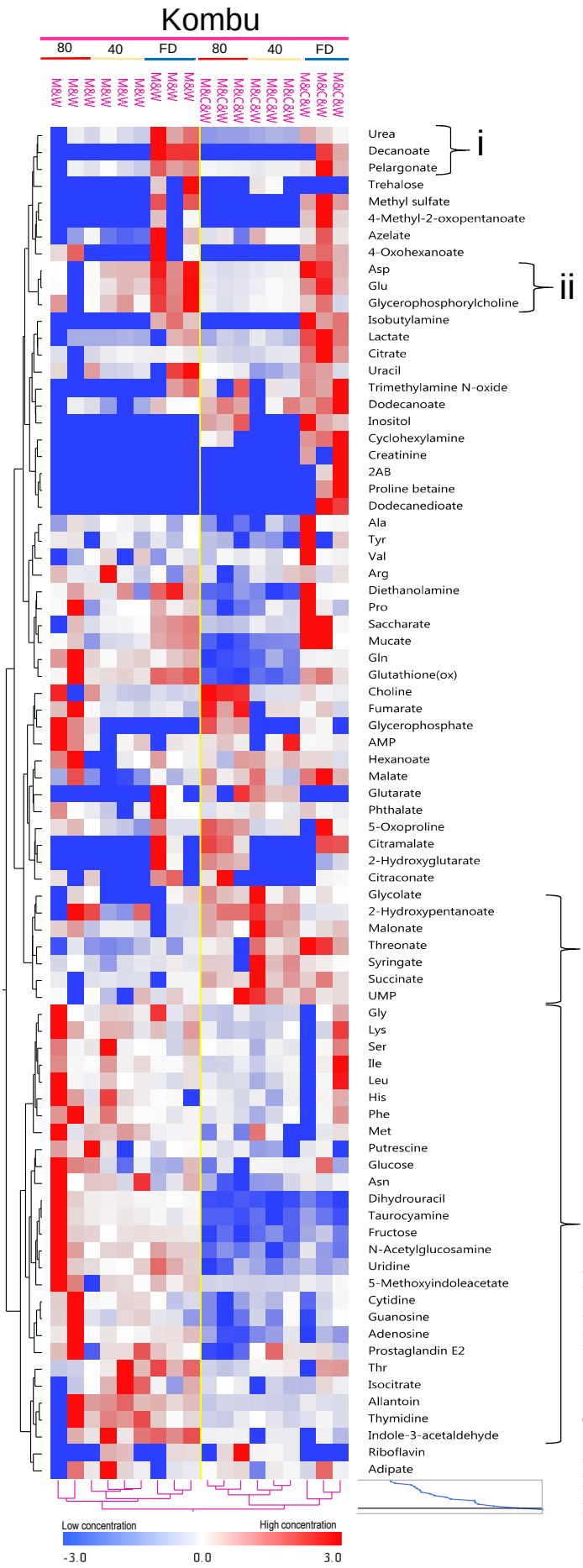

Figure 2.5 HCA and heat map of dry weight based of metabolite concentrations in Kombu samples.

The letter of roman numbers indicated the groups of metabolites as mentioned in the result section.

Table 2.3 Free amino acid concentrations in Kombu samples after various drying and extractions methods.

		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
	Amino Acids	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**
Essentials Amino Acids	Histidine	6.23±1.41^{c}	3.41±1.17^{c}	3.94±0.56^{c}	4.58±0.06^{c}	7.01±1.99^{abc}	7.08±3.23^{abc}
	Isoleucine	8.44±3.06^{abcd}	4.76±1.28^{abcd}	4.08±0.57^{bcd}	5.80±0.82^{abcd}	7.13±1.10^{abcd}	6.45±1.87^{abcd}
	Leucine	7.51±4.12^{cd}	3.79±1.69^{d}	3.79±0.32^{d}	4.68±1.42^{cd}	5.93±1.02^{cd}	6.80±3.34^{cd}
	Lysine	8.22±5.02^{d}	5.00±1.44^{d}	4.56±0.99^{d}	7.79±2.81^{d}	7.95±2.26^{cd}	9.65±2.84^{bcd}
	Methionine	1.07±0.33^{ab}	1.49±0.47^{ab}	0.57±0.52^{ab}	1.01±0.12^{ab}	1.53±0.12^{ab}	1.50±0.70^{ab}
	Phenylalanine	6.95±2.33^{f}	4.21±1.64^{f}	4.43±0.38^{f}	5.42±0.82^{f}	7.15±1.71^{def}	8.67±2.02^{cdef}
	Threonine	42.16±0.27^{ef}	32.64±3.23^{def}	20.68±1.76^{f}	44.56±3.99^{cdef}	41.84±8.32^{def}	23.88±6.70^{f}
	Tryptophan	ND	ND	ND	ND	ND	ND
	Valine	170.72±102.80^{abcd}	90.37±11.41^{cd}	85.18±6.27^{cd}	108.53±13.07^{cd}	109.88±3.96^{bcd}	116.85±33.62^{d}
Non-Essentials Amino Acids	Alanine	170.72±102.80^{e}	90.37±11.41^{e}	85.18±6.27^{e}	108.53±13.07^{e}	109.88±3.96^{e}	116.85±33.62^{e}
	Arginine	5.65±1.40^{d}	5.05±1.29^{d}	3.23±1.09^{d}	4.97±1.88^{d}	5.98±3.01^{d}	5.26±1.33^{d}
	Asparagine	27.16±4.84^{de}	18.13±3.35^{de}	12.93±4.77^{de}	27.79±11.41^{de}	38.08±16.08^{cd}	41.16±15.11^{cd}
	Aspartate	357.42±30.46^{ab}	238.91±10.35^{bcd}	212.64±9.45^{cd}	365.37±22.32^{ab}	321.62±1.75^{abc}	281.63±3.32^{d}
	Cysteine	ND	ND	ND	ND	ND	ND
	Glutamine	42.94±3.00^{efg}	30.47±2.91^{efghi}	26.01±0.96^{fghi}	51.26±5.04^{ef}	48.86±3.84^{ef}	60.14±9.95^{e}
	Glutamate	1134.16±96.30^{ab}	835.96±46.29^{abcde}	724.10±27.91^{bcde}	1198.06±68.02^{a}	1023.43±32.47^{abcd}	913.43±29.98^{de}
	Glycine	47.46±12.71^{d}	24.99±1.32^{d}	26.06±5.62^{d}	50.48±20.39^{d}	47.71±3.94^{d}	48.37±23.70^{d}
	Proline	71.31±17.92^{e}	56.89±4.87^{efg}	49.12±3.06^{efg}	71.15±4.66^{e}	62.24±1.66^{ef}	68.48±20.16^{e}
	Serine	101.21±109.70^{c}	20.81±6.08^{c}	27.24±1.14^{c}	28.72±9.98^{c}	36.64±15.94^{bc}	35.31±9.90^{bc}
	Tyrosine	32.43±16.91^{ab}	3.11±0.31^{b}	4.39±0.38^{ab}	6.48±1.98^{ab}	6.15±1.81^{ab}	14.39±0.65^{ab}
	Total Free Amino Acids	**2033.26 ± 331.47**	**1393.46 ± 102.61**	**1223.55 ± 68.33**	**1997.43 ± 175.04**	**1802.78 ± 102.22**	**1666.37 ± 181.82**

The values indicate as mean ±SD (nmol g^{-1}) of dry weight (n = 3). ND, not detected. Different character indicates the significant differences ($P < 0.05$) between each type of sample by Tukey-HSD analysis. Statistical analysis was compared between all data set (species and treatments (Table 2.2–2.4)).

2.3.6 Characteristics of Wakame

Wakame contained Gly, Pro, Arg, Ala, and Gln at significantly higher concentrations than Mozuku and Kombu (See green box in Figure 2.2; Table 2.4). All amino acids except for Met and Cys were detected in freeze-dried Wakame samples. Met was found in all oven-dried Wakame sample, but Cys was detected only in Wakame oven-dried at 80 °C and subjected to extraction with methanol-chloroform-water (Figures 2.2 and 2.6; Table 2.4). Relatively higher concentrations of urea, lactate, trans-aconitate, cis-aconitate, Trp, azelate, trimethylamine-*N*-oxide, adenosine, choline, carnitine, Ala-Ala, creatine, isobutylamine, citrate, cyclohexylamine, Thr, Arg, malate, and homoserine were measured in freeze-dried Wakame than in oven-dried Wakame (Figure 2.6i).

Comparatively higher levels of ADP-glucose and UDP-glucose were found in freeze-dried Wakame extracted by methanol-water (Figure 2.6iii). In contrast, proline betaine, glycerophosphorylcholine, and methyl sulfate were detected at relatively higher concentrations in freeze-dried Wakame subjected to extraction with methanol-chloroform-water (Figure 2.6ii). Methanol-water also extracted high concentrations of dihydrouracil, indole-3-acetaldehyde, taurocyamine, *N*-acetylglucosamine, 5-methylindolacetate, thymidine, allantoin, and nornicotine (Figure 2.6v) whereas chloroform-water extracted high levels of citramalate and 2-hydroxyglutarate (Figure 2. 6iv).

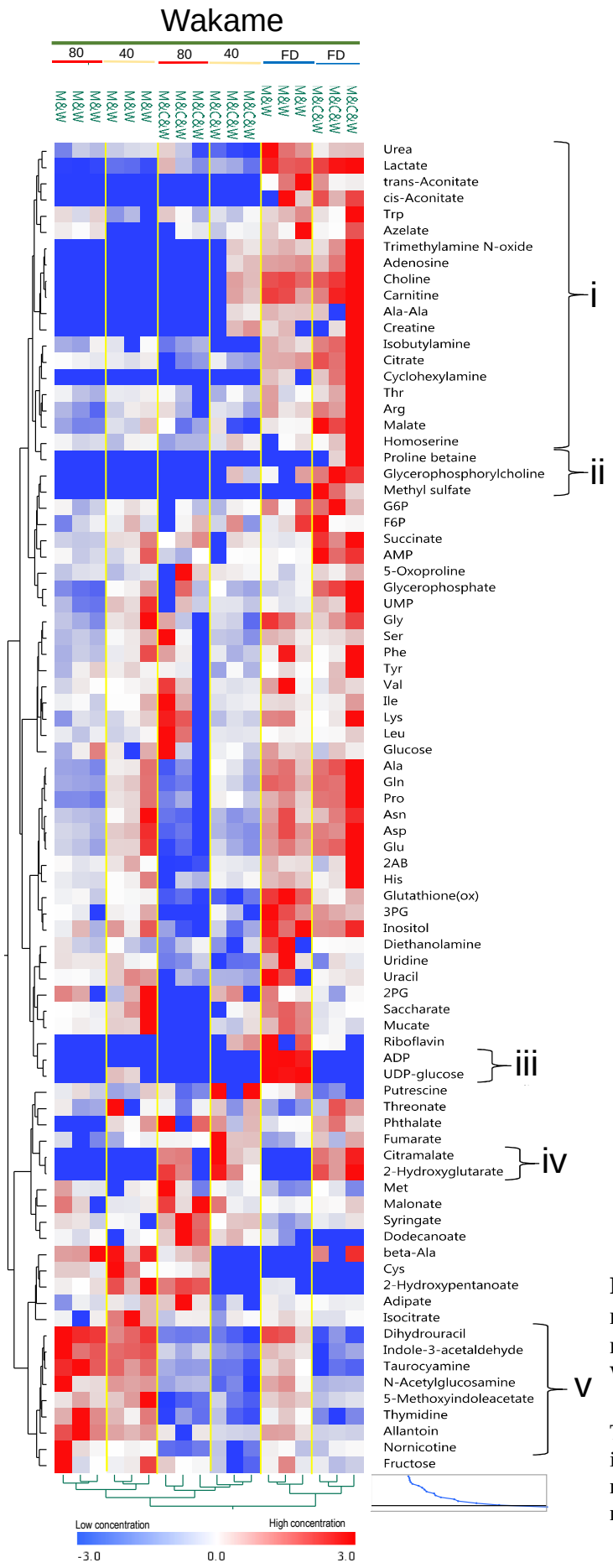

Figure 2.6 HCA and heat map of dry weight based of metabolite concentrations in Wakame samples.

The letter of roman numbers indicated the groups of metabolites as mentioned in the result section.

Table 2.4 Free amino acid concentrations in Wakame samples after various drying and extractions methods.

		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
	Amino Acids	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**	**Freeze-Dry**	**Oven-Dry 40 °C**	**Oven-Dry 80 °C**
Essentials Amino Acids	Histidine	6.23±1.41^{c}	3.41±1.17^{c}	3.94±0.56^{c}	4.58±0.06^{c}	7.01±1.99^{abc}	7.08±3.23^{abc}
	Isoleucine	8.44±3.06^{abcd}	4.76±1.28^{abcd}	4.08±0.57^{bcd}	5.80±0.82^{abcd}	7.13±1.10^{abcd}	6.45±1.87^{abcd}
	Leucine	7.51±4.12^{cd}	3.79±1.69^{d}	3.79±0.32^{d}	4.68±1.42^{cd}	5.93±1.02^{cd}	6.80±3.34^{cd}
	Lysine	8.22±5.02^{d}	5.00±1.44^{d}	4.56±0.99^{d}	7.79±2.81^{d}	7.95±2.26^{cd}	9.65±2.84^{bcd}
	Methionine	1.07±0.33^{ab}	1.49±0.47^{ab}	0.57±0.52^{ab}	1.01±0.12^{ab}	1.53±0.12^{ab}	1.50±0.70^{ab}
	Phenylalanine	6.95±2.33^{f}	4.21±1.64^{f}	4.43±0.38^{f}	5.42±0.82^{f}	7.15±1.71^{def}	8.67±2.02^{cdef}
	Threonine	42.16±0.27^{ef}	32.64±3.23^{def}	20.68±1.76^{f}	44.56±3.99^{cdef}	41.84±8.32^{def}	23.88±6.70^{f}
	Tryptophan	ND	ND	ND	ND	ND	ND
	Valine	170.72±102.80^{abcd}	90.37±11.41^{cd}	85.18±6.27^{cd}	108.53±13.07^{cd}	109.88±3.96^{bcd}	116.85±33.62^{d}
Non-Essentials Amino Acids	Alanine	170.72±102.80^{e}	90.37±11.41^{e}	85.18±6.27^{e}	108.53±13.07^{e}	109.88±3.96^{e}	116.85±33.62^{e}
	Arginine	5.65±1.40^{d}	5.05±1.29^{d}	3.23±1.09^{d}	4.97±1.88^{d}	5.98±3.01^{d}	5.26±1.33^{d}
	Asparagine	27.16±4.84^{de}	18.13±3.35^{de}	12.93±4.77^{de}	27.79±11.41^{de}	38.08±16.08^{cd}	41.16±15.11^{cd}
	Aspartate	357.42±30.46^{ab}	238.91±10.35^{bcd}	212.64±9.45^{cd}	365.37±22.32^{ab}	321.62±1.75^{abc}	281.63±3.32^{d}
	Cysteine	ND	ND	ND	ND	ND	ND
	Glutamine	42.94±3.00^{efg}	30.47±2.91^{efghi}	26.01±0.96^{fghi}	51.26±5.04^{ef}	48.86±3.84^{ef}	60.14±9.95^{e}
	Glutamate	1134.16±96.30^{ab}	835.96±46.29^{abcde}	724.10±27.91^{bcde}	1198.06±68.02^{a}	1023.43±32.47^{abcd}	913.43±29.98^{de}
	Glycine	47.46±12.71^{d}	24.99±1.32^{d}	26.06±5.62^{d}	50.48±20.39^{d}	47.71±3.94^{d}	48.37±23.70^{d}
	Proline	71.31±17.92^{e}	56.89±4.87^{efg}	49.12±3.06^{efg}	71.15±4.66^{e}	62.24±1.66^{ef}	68.48±20.16^{e}
	Serine	101.21±109.70^{c}	20.81±6.08^{c}	27.24±1.14^{c}	28.72±9.98^{c}	36.64±15.94^{bc}	35.31±9.90^{bc}
	Tyrosine	32.43±16.91^{ab}	3.11±0.31^{b}	4.39±0.38^{ab}	6.48±1.98^{ab}	6.15±1.81^{ab}	14.39±0.65^{ab}
	Total Free Amino Acids	**2033.26 ± 331.47**	**1393.46 ± 102.61**	**1223.55 ± 68.33**	**1997.43 ± 175.04**	**1802.78 ± 102.22**	**1666.37 ± 181.82**

The values indicate as mean ±SD (nmol g^{-1}) of dry weight (n = 3). ND, not detected. Different character indicates the significant differences ($P < 0.05$) between each type of sample by Tukey-HSD analysis. Statistical analysis was compared between all data set (species and treatments (Table 2.2–2.4)).

2.3.7 Effects of drying on metabolite concentration

Freeze-drying and oven-drying at 40 °C and 80 °C were compared for all three brown algae species. The characteristics of the metabolite profiles differed with species. The drying methods also contributed to the variation in metabolite concentrations (Figure 2.2). HCA heat map showed that freeze-dried seaweed yielded the highest metabolite concentrations. For example, urea and lactate were detected at higher concentrations in freeze-dried samples of all three species than the oven-dried samples (Figure 2.2i). Asp and Glu were detected at higher concentrations in freeze-dried Kombu and Wakame than in freeze-dried Mozuku (Figure 2.2iv). Isobutylamine was measured at a high concentration only in freeze-dried Mozuku (Figure 2.2ii). However, other metabolites were only found in oven-dried samples. Cytosine, hypoxanthine, adenine, 2′-deoxycytidine, and guanine were only detected in Mozuku oven-dried at 80 °C (Figure 2.2iii) whereas pentanoate and 3-methylbutanoate were only found in Mozuku oven-dried at 40 °C (Figure 2.2vii).

2.3.8 Effects of extraction solvent on metabolite concentration

In this study, extraction was performed with methanol-water and with methanol-chloroform-water. HCA heat map shows that dihydrouracil, taurocyamine, 5-methoxyindoleacetate, *N*-acetylglucosamine, allantoin, thymidine, and indole-3-acetaldehyde (Figure 2.2vi) were detected at high concentrations in the methanol-water extracts of all three seaweed species. On the other hand, only malate and syringate were detected at high concentrations in the methanol-chloroform-water extracts of the three seaweed species (Figure 2.2v).

Metabolite extraction efficiency was correlated mainly with seaweed species rather than extraction method. Many metabolites at high concentrations were detected in methanol-water extracted from Kombu and Wakame (Figures 2.2, 2.5, and 2.6). For Mozuku, however, high levels of these metabolites were only found in the methanol-chloroform-water extracts (Figures 2.2 and 2.4).

2.4 Discussion

2.4.1 MS-based metabolomics for seaweed

Previously, metabolite profiling studies were performed by NMR. Those reports focused on the chemical profiles of various seaweed, chemical defences in red seaweed, and seasonal metabolite changes in brown algae (Nylund *et al.*, 2011; Date *et al*., 2012; Gerasimenko & Logvinov, 2016).

In all of those studies, however, the seaweed samples required complex preparation and derivatisation. Plus, an estimated ~200 mg of samples was needed per analysis. In contrast, only 5 mg of dried sample was used in this present study, and the methodology was comparatively simple. Nevertheless, an extensive amount of data on amino acids, organic acids, and sugars were collected and analyzed by CE-MS and LC-MS. Therefore, study purpose, reproducibility, robustness, extraction method, and sample volume must be considered in the selection of the tools to be used for metabolite analysis.

The choice of extraction solvent is crucial in MS-based metabolomics. Sample homogenization before extraction is essential because it reduces experimental variation and increases reproducibility (Ernst *et al*., 2014). Methanol and its mixtures are commonly used as the extraction solvents in plant metabolomics analyzes. On the other hand, chloroform is used to extract lipophilic (non-polar) compounds like chlorophyll and fatty acids from lipid-rich samples (Kim & Verpoorte, 2010; Ernst *et al*., 2014; Hirayama *et al*., 2014). Chloroform separates the sample solution into two phases. The lower chloroform layer contains dissolved lipid compounds whereas the upper methanol-water layer contains polar compounds. Combining the use of methanol and chloroform for extraction is known as the Bligh-Dyer method (Bligh & Dyer, 1959).

In this study, the Bligh-Dyer method was modified such that only the upper layer containing polar compounds was collected. The substances extracted with methanol-water were also analyzed. Methanol-water and methanol-chloroform-water extractions were compared because most seaweed contain major lipids and fatty acids,

but their quantities are highly variable and generally lower than those detected in animals (Sanina *et al.*, 2004; Sánchez-Machado *et al.*, 2004; Dawczynski *et al.*, 2007).

Remarkably, high concentrations of numerous metabolites were detected in Kombu and Wakame after methanol-water extraction. In contrast, many metabolites were found in Mozuku subjected to extraction with methanol-chloroform-water. Mozuku has more significant amounts of free fatty acids than other brown algae species (Terasaki & Itabashi, 2003). The lipids were dissolved in the chloroform layer after the seaweed had been subjected to extraction with methanol-chloroform-water method.

For CE-MS and LC-MS, only the water-soluble metabolites in the polar layer were used. Removal of the lipid layer increased the signal-to-noise ratio. Consequently, the metabolite contents detected in the methanol-chloroform-water extracts of Mozuku were high.

2.4.2 Species characteristics of metabolomics

Notably, the PCA metabolite separation was determined by species identity rather than drying or extraction method (Figure 2.1). In comparison to Kombu and Wakame (Figure 2.1; negative PC1), Mozuku (Figure 2.1; positive PC1) was clearly separated in terms of metabolite characteristics mainly because of phylogeny.

Mozuku is in the order Ectocarpales whereas Wakame and Kombu are in the order Laminariales. According to AlgaeBase website (Guiry & Guiry, 2018), these classifications are based on advancements in molecular characterisation (Rousseau *et al.*, 2007). PCA of this study showed that species identity also determined metabolite concentrations (Figure 2.1).

Previous NMR metabolomics studies of various seaweed indicated that PCA score plots for metabolites were clustered according to taxonomical differences (Date *et al*., 2012). Metabolite profiling could further elucidate differences in species taxonomy.

Mozuku has a higher number of highly concentrated metabolites than Kombu or Wakame (Figure 2.2; Tables 2.2–2.4). However, no amino acids were found among these metabolites. Glu and Asp are the most commonly occurring amino acids in seaweed (Fleurence, 2010). Glu is responsible for the taste essence known as umami. It is found mostly in Kombu (Ninomiya, 2002). In the present study, only free amino acids were

detected rather than protein hydrolysates. It has been reported that Glu and Asp occur at higher concentrations in Wakame followed by Kombu, then Mozuku (Figure 2.2iv). Previous studies indicated similar tendencies for protein-hydrolysed amino acids (Dawczynski *et al.*, 2007; Amorim *et al*., 2012). Therefore, Wakame maintains higher free amino acid levels than Kombu.

Cys and Met are sulfur-containing amino acids. With CE-MS, the detection limits for Cys and Met were low compared to those for the other amino acids. Only a trace amount of Cys was detected in Wakame oven-dried at 80 °C (Tables 2.2–2.4). Met was found at low concentrations in Wakame and Kombu but not at all in Mozuku. A previous study reported that high amounts of Cys and Met only occur in red seaweed and not in green or brown algae (Qasim, 1991). Only small quantities of Cys and Met were measured in the present study since only brown algae were analyzed.

Chapter 3

Metabolomic profiling of various seaweed species discriminates between brown, red, and green algae

3.1 Introduction

More than 3000 different compounds have been identified in seaweed, indicating a diversity that is linked to their polyphyletic origin and the different marine environments in which they thrive (Leal *et al*., 2013; Belghit *et al*., 2017). Studies reported that among all the seaweed, the brown algae are phylogenetically distant from the red and green algae (De Clerck *et al*., 2012; Groisillier *et al*., 2014). Therefore, many morphological and metabolic properties of brown algae differ greatly from those of red and green algae. For instance, brown algae have plastids with four membranes and a specific cell wall composition associated with unique metabolic pathways (Cavalier-Smith, 2000; Rousvoal *et al*., 2011).

Previous studies already indicated that the chemical composition of seaweed varies with the species, habitat, salinity, temperature, light intensity, and other environmental conditions such as stress due to the threat from an invasive species or pathogen (Robledo & Freile Pelegrín, 1997; La Barre *et al.*, 2004; Date *et al.*, 2012; Tabarsa *et al.*, 2012; Peinado *et al*., 2014; Endo *et al*., 2015; Rodrigues *et al.*, 2015; Collins *et al.*, 2016; Palanisamy *et al.*, 2018). However, those studies were mostly focused on select species using the whole genome sequence data of, for example, *Chondrus crispus* (red algae) and *Ectocarpus siliculosus* (brown algae) (Cock *et al*., 2010; Collén *et al.*, 2013; Nishitsuji *et al.*, 2016). Some other studies investigated specific molecule classes, such as lipids and their derivatives (Goulitquer *et al.*, 2012; Kumari *et al*., 2013),

or targeted compounds synthesized as defense response, such as mycosporine-like amino acids and halogenated compounds (Laturnus, 1996; Hartmann *et al.*, 2015), or examined mono- and polysaccharide extracts of various seaweed (Percival, 1979; Costa *et al.*, 2010; Skriptsova *et al*., 2010; Wu *et al*., 2014; Hamed *et al.*, 2015; Robin *et al.*, 2017; Garcia-Vaquero *et al*., 2017). Those studies were often limited to either a few algae species or certain treatment effects on several species. However, a comprehensive analysis which compares the chemical profiles of various seaweed is still lacking.

Metabolomics is an emerging field that greatly contributes to the latest insights in systems biology as it captures the immediate biological information generated by the genomic and transcriptomic activities. Most studies had focused on select metabolites involved in amino acid and/or lipid metabolism (Wahbeh, 1997; Hwang *et al.*, 2013; Zhou *et al*., 2015; Parjikolaei *et al.*, 2016; Astorga-España *et al*., 2016). More than 50,000 metabolites of terrestrial plants have been deposited in the KNApSAck database (http://kanaya.naist.jp/KNApSAcK/) (Nakamura *et al.*, 2014), whereas the information on seaweed metabolites is still limited; the seaweed metabolite database (SWMD; http://www.swmd.co.in/) only contains 1110 metabolites mostly from the red algae *Laurencia* sp. (Davis & Vasanthi, 2011).

Several analytical platforms are being used in metabolomics (Lei *et al*., 2011; Heyman & Dubery, 2016), including a variety of NMR spectroscopy and mass spectrometry (MS) methods, such as MS coupled with gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) (Gupta & Abu-Ghannam, 2011; Gu *et al*., 2015; de Raad *et al*., 2016).

Although NMR analysis generates precise structural information, it requires larger sample volumes with higher concentrations for quantification than MS-based methods. Using MS, small sample amounts are sufficient to obtain comprehensive metabolite analyzes (Cai & Henion, 1995; Ramautar *et al*., 2009; Hirayama *et al*., 2014), but sample preparation typically includes drying and extraction steps. Presently, there is no comprehensive extraction and drying technique to recover all compound classes with high yield and reproducibility. Thus, to simultaneously compare several seaweed species, an optimal method (drying and extraction) had to be identified.

Previous studies on seaweed metabolites or nutrients used different extraction and drying methods (Chan *et al*., 1997; Wong & Cheung, 2001a; Michalak & Chojnacka,

2015). For example, to examine amino acids in seaweed, some studies targeted only free amino acids (Norziah & Ching, 2000; Omar *et al.*, 2017), but others investigated amino acids in acid-hydrolyzed materials (Qasim, 1991; Dawczynski *et al.*, 2007; Astorga-España *et al*., 2016). However, due to the differences in sample processing methods, it is difficult to compare the metabolite concentrations among the studies. Various extraction and drying methods on edible blanched brown algae were previously compared and some differences in metabolite concentrations were observed, depending on the pre-treatment (Hamid *et al.*, 2018). Thus, to conduct a comparability metabolomics analysis of the three seaweed groups, we needed to develop an optimized extraction and drying procedure that would generate comparable results with samples from different seaweed species.

In the present study, comprehensive profiling of water-soluble metabolites of red, brown, and green algae was conducted, such as free amino acids, organic acids, and sugars. In order to assess the effect of the extraction conditions on the profiling results, different extraction methods, including methanol-water extraction with or without chloroform, were compared using CE-MS and LC-MS. This study would provide guidance on seaweed species characterization based on metabolite profiling using only a single pre-treatment procedure.

3.2 Materials and methods

3.2.1 Sample collection

Various seaweed were collected at two different sites in the northern part of Japan, the Shonai coastal area in the Yamagata Prefecture (Sea of Japan) and Muroran in Hokkaido (Pacific Ocean), between February and August 2017. The differences in metabolite profiles, based on seaweed taxonomy, were assessed using samples of four brown algae species, *Sargassum micracanthum* (Togemoku; Japanese name), *Sphaerotrichia firma* (Ishimozuku), *Papenfussiella kuromo* (Kuromo), and *Saccharina japonica* (Kombu); samples of five red algae species, *Pyropia pseudolinearis* (Uppurui Nori), *Gelidium elegans* (Makusa), *Neodilsea yendoana* (Akaba), *Dasya sessilis* (Enashi Dajia), and *Botryocladia wrightii* (Taoyagisou); and samples of two green algae species, *Ulva australis* (Ana Aosa) and *Chaetomorpha moniligera* (Tamajuzumo) (Figure 3.1, Table 3.1). After the samples had been collected, the seaweed materials were washed using filtered seawater, and the excess water removed using paper towels. The fresh seaweed specimens were weighed and divided into five replicate samples (n = 5). Each sample was placed into a polyethylene-polypropylene non-woven fabric bag (11.0 × 10.5 cm, Dashi bag, DAISO, Hiroshima, Japan) and quenched in liquid nitrogen before storage in a −80 ºC freezer (MDF-U482-PJ, Panasonic Corp., Kadoma, Osaka, Japan).

Figure 3.1 The photograph of analyzed samples.

Each of the species in all 3 seaweed groups; brown, red and green algae used in this study. Details of each seaweed species is described in Table 3.1. Scale bars indicate 5 cm length of the original sample.

Table 3.1 Sample information; the species name, collection date, location and drying percentage after freeze-drying.

Scientific name	Japanese name	Phylum	Class	Family	Collection date	Collection location	GPS Location	Drying percentage (%)
Brown algae								
Sargassum micracanthum	Togemoku	Ochrophyta	Phaeophyceae	Sargassaceae	16-Feb-17	Wasada beach, Shonai Coastal area, Yamagata	38°34'48.2"N 139°33'25.2"E	21.68
Sphaerotrichia firma	Ishimozuku	Ochrophyta	Phaeophyceae	Chordariaceae	12-Jun-17	Wasada beach, Shonai Coastal area, Yamagata	38°34'48.2"N 139°33'25.2"E	13.06
Papenfussiella kuromo	Kuromo	Ochrophyta	Phaeophyceae	Chordariaceae	3-Jul-17	Wasada beach, Shonai Coastal area, Yamagata	38°34'48.2"N 139°33'25.2"E	15.00
Saccharina japonica	Kombu	Ochrophyta	Phaeophyceae	Laminariaceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	20.30
Red algae								
Pyropia pseudolinearis	Uppurui Nori	Rhodophyta	Bangiophyceae	Bangiaceae	16-Feb-17	Wasada beach, Shonai Coastal area, Yamagata	38°34'48.2"N 139°33'25.2"E	19.66
Gelidium elegans	Makusa	Rhodophyta	Florideophyceae	Gelidiales	13-Jul-17	Kobato port, Shonai Coastal area, Yamagata	38°41'30.8"N 139°38'37.0"E	39.35
Neodilsea yendoana	Akaba	Rhodophyta	Florideophyceae	Dumontiaceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	27.14
Dasya sessilis	Enashi Dajia	Rhodophyta	Florideophyceae	Dasyaceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	12.11
Botryocladia wrightii	Taoyagisou	Rhodophyta	Florideophyceae	Rhodymeniaceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	5.94
Green algae								
Ulva australis	Ana aosa	Chlorophyta	Ulvophyceae	Ulvaceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	20.54
Chaetomorpha moniligera	Tamajuzumo	Chlorophyta	Ulvophyceae	Cladophoraceae	25-Aug-17	Denshi beach, Muroran Marine Station, Hokkaido	42°18'50.2"N 140°58'05.1"E	10.46

3.2.2 Sample preparation by freeze drying

To eliminate the effect of moisture content on metabolite quantification, all samples were preserved using a freeze-drying method (Hamid *et al*., 2018). Prior to being loaded into the freeze-dryer, the freeze-trap (Freeze Trap VA-800R, Taitec Corp., Saitama, Japan) was set to −70 °C and <20 Pa. Then, the completely frozen samples (−80 ºC, >24 h) were placed into freeze-dryer vessels. The temperature and pressure were maintained at < −55 °C and <50 Pa, respectively, using a vacuum pump (GLD-137CC ULVAC, Taitec Corp., Saitama, Japan) for 7 days. Then, once the freeze-dryer pressure was stabilized at <0.5 Pa for more than 24 h, the samples were removed from the vessels and reweighed. The dried samples were stored in a −80 °C freezer until further processing.

3.2.3 Chemicals and reagents

Analytical reagents or higher-grade purity were used. The standard reagents were purchased and prepared for identification and quantification of each metabolite. To normalize the quantification, the following internal standard solutions (IS1) were prepared as 200 μM stock solutions in methanol: anionic standard, 2-(*N*-morpholino) ethanesulfonic acid (MES) (No. 349-01623; Dojindo Laboratories, Kumamoto, Japan) and $_{D}$-camphor-10-sulfonic acid (CSA) (No. 037-01032; Wako Pure Chemical Industries, Ltd., Osaka, Japan); cationic standard, $_{L}$-methionine sulfone (No. 502-76641; Wako Pure Chemical Industries, Ltd., Osaka, Japan); and sugar standard, $^{13}C_6$-glucose (No. 404624; Sigma-Aldrich Corp., St. Louis, MO, USA). To have a standard for CE-MS migration time corrections, Milli-Q water was used to prepare 200 μM stock solutions of trimesate (No. 206-03641; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 3-aminopyrrolidine (No. 404624; Sigma Aldrich Corp., St. Louis, MO, USA).

3.2.4 Sample extractions

Aliquots of approximately 100 mg of each of the five freeze-dried sample replicates were placed into 13 mL disruption tubes. The sample materials were mechanically disrupted in a cell disruptor (Multi beads shocker, Yasui-Kikai, Osaka, Japan) without solvent using a 1 cm height × 1 cm diameter metal cylinder at 1,500 rpm for 120 seconds. Aliquots of

the powdered samples were recovered and weighed (~10 mg each) before being extract using two methods: (A) methanol-water and (B) methanol-chloroform-water.

For the methanol-water extraction method (A), the samples were suspended in 500 µL of 200 µM IS1 in methanol using a micro-mixer (Micromixer E36, Taitec Corp., Saitama, Japan) for 3 min and diluted with 500 µL Milli-Q water (Wakayama *et al*., 2010).

For the methanol-chloroform-water extraction method (B), the samples were mixed with 500 µL of 200 µM IS1 in methanol, followed with 500 µL chloroform and 200 µL Milli-Q water (Wakayama *et al*., 2015).

Then, all extraction samples were subjected to vortex for 3 min and centrifuged at 120,000 × g for 10 min at 4 °C in the MX-307 centrifuge (Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation, the aqueous solution of the top layer (~400 µL) was transferred into ultra-filtration tubes (MW 5000 kDa; HMT, Inc., Tsuruoka, Japan) and centrifuged at 9,100 × g and 4 °C for 3 h. Filtrate aliquots (30 µL) were collected and transferred into LC vials for sugar analysis. Sample vials were stored in the freezer at −80 °C until LC-MS analysis. An aliquot of 100 µL of each remaining filtrate was evaporated at 4°C in a refrigerated spin dryer (CentriVap Concentrator, Labconco Corp., Kansas City, MO, USA). Until CE-MS analysis, the dried samples were stored in a freezer at −80°C. The dried samples were dissolved in Milli-Q water mixed with 20 µL of 200 µM trimesate and 3-aminopyrrolidine (migration time marker). Then, two aliquots of 10 µL per sample were dispensed into two separate CE-MS vials for cationic and anionic metabolite analysis using CE-MS.

3.2.5 Free sugar analysis by quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The analysis was performed using an API 3000 Quadrupole LC-MS/MS system (Sciex, Framingham, MA, USA) fitted with an Agilent 1100 series column oven (Agilent Technologies, Santa Clara, CA, USA), LC binary pump, and an autosampler. Hydrophilic interaction chromatography (HILIC) amino column (Asahipak NH2P-4E; 4.6 mm inner diameter × 250 mm lengths; 5 µm; Showa Denko K.K., Tokyo, Japan) for sample separation was used.

At the start, the mobile phase was set to 80 % acetonitrile and 20 % Milli-Q water with 0.8 mL min^{-1} of flow rate. The acetonitrile gradient was changed to 70 %, 60 %, and 80 % after a run time of 23 min, 35 min, and 40 min, respectively. The duration of each sample analysis was 40 min; the column oven temperature was set to 30 °C. Only 0.3 µL aliquot per sample was injected into the system. Using the system in turbo spray mode, the pressure in the nebulizer, the curtain, the collision gas chamber, the ion spray voltage, and the ion source temperature was set at 15 psi, 11 psi, 8 psi, −4500 V, and 500 °C, respectively. The negative-ion and MRM modes were selected for MS analysis.

3.2.6 CE-MS analysis of free amino acids, organic acids, and charged metabolites

All CE-MS analyzes were performed using an Agilent CE-MS system consisting of an Agilent G6220A LC/MSD TOF, a G1603A Agilent CE-MS adapter kit, a G1607A Agilent CE-ESI-MS sprayer kit, and an Agilent 1100 series isocratic HPLC pump (Agilent Technologies, Santa Clara, CA, USA).

3.2.7 Cationic metabolite analysis

Cationic metabolites, i.e., amino acids and amines, were analyzed using an uncoated fused silica capillary (50 µm inner diameter × 100 cm lengths) filled with 1 M formic acid as an electrolyte (Soga & Heiger, 1998; Hirayama *et al.*, 2014). The sheath liquid (methanol-water, 50 % v/v) containing 0.1 µM hexakis(2,2-difluoroethoxy)phosphazene was delivered at 10 µL min^{-1} via a HPLC pump in an isocratic mode to stabilize the ESI sprayer ionisation. 3 nL of the sample was injected into the system at 50 hPa for 5 s. During the CE, the voltage was kept at +30 kV, the capillary temperature thermostat was set at 20 °C, and the sample tray temperature was cooled using a cooler system (TRL 108H Circulation type handy, Thomas, Tokyo, Japan). Time-of-flight mass spectrometry (TOF-MS) was conducted in positive ion mode. The capillary, fragmentor, skimmer, and OCT RFV voltages were set at 4,000 V, 75 V, 50 V, and 500 V, respectively. The MS ionisation was stabilized by maintaining the drying nitrogen gas flow rate at 10 L min^{-1}, the heater temperature at 300 °C, and the nebulizer gas pressure at 7 psi (48.2 kPa). The exact mass data of spectrum were acquired at a rate of 1.5 cycles/second over a 50–1,000

m/z range and automatically recalibrated using the reference masses of the sheath liquid ($[2MeOH+H2O+H]^+$, *m/z* 66.06306) and protonated hexakis(2,2-difluoroethoxy)phosphazene ($[M+H]^+$, *m/z* 622.02896).

3.2.8 Anionic metabolite analysis

Anionic metabolites, i.e., organic carboxylic acids and sugar phosphates, were analyzed using a cationic polymer coated COSMO (+) capillary (50 μm i.d. × 105 cm lengths) (Nacalai Tesque, Kyoto, Japan) (Soga *et al*., 2009) filled with 50 mM ammonium acetate (pH 8.5) as electrolyte. Prior to using a new capillary, the pre-treatment included a 10 min flushing step with an electrolyte solution, followed with 50 mM acetic acid (pH 3.4), and again with the electrolyte solution. The capillary temperature thermostat was set at 20 °C, and the sample tray was cooled using the cooler system. The sheath liquid of 5 mM ammonium acetate in methanol-water (50 % v/v) containing 0.1 μM hexakis(2,2-difluoroethoxy)phosphazene was delivered to the electrospray interface at 10 μL min^{-1} with an Agilent 1100 series isocratic pump fitted with a 1:100 splitter. The sheath liquid circulated around the outside of the electrospray and CE capillary was to provide a stable electrical connection between the capillary tip and the grounded electrospray needle. Before starting each injection, the capillary was flushed with 50 mM acetic acid (pH 3.4) for 2 min followed with an electrolyte solution for 5 min. The samples were injected at 50 hPa for 30 s (30 nL). After the injection, the CE voltage was maintained at −30 kV. The MS capillary voltage was set to −3,500 V and the fragmentor, skimmer, and OCT RFV voltages were set to 100 V, 50 V, and 500 V, respectively. MS ionisation was stabilized by maintaining the drying nitrogen gas flow rate at 10 L min^{-1} and the heater temperature at 300°C. The nebulizer gas pressure was set to 7 psi (48.2 kPa). Each acquired spectrum was automatically recalibrated with two reference masses, [^{13}C isotopic ion of deprotonated acetic acid dimer $(2CH_3COOH-H)]^-$, *m/z* 120.03834, and [hexakis (2,2-difluoroethoxy)phosphazene + deprotonated acetic acid $(CH3COOH-H)]^-$, *m/z* 680.03554. Exact mass data were acquired at a rate of 1.5 cycles/second over a 50–1,000 *m/z* range.

3.2.9 Statistical analysis

For the sugar analysis by LC-MS/MS, the Analyst v. 1.4.2 software was used for peak annotation and quantitation. To analyze the cationic and anionic metabolites by CE-TOF-MS, the Agilent Mass Hunter v. B06.00 software was used for data collection and Masterhands v. 2.17.3.17, original software from Keio University (Sugimoto *et al*., 2012), was used for peak annotation and peak area integration. The data sets were merged using in-house created macros in Microsoft Excel v. 2013 (Microsoft Corp, Redmond, WA, USA). Multivalent statistical analysis was performed on all data using JMP v. 13.2.1 (SAS Corp., Cary, NC, USA) to evaluate the metabolite profiles of different seaweed (Figures 3.2–3.8, S3.1–S3.23, Table 3.1–3.2, S3.1). Tukey's honestly significant difference (HSD) analysis, principal component analysis (PCA), and hierarchical clustering analysis (HCA) were performed based on both the fresh and dry weight. Both PCA and HCA were used to summarize the statistical metabolite profile variations among species of each major seaweed group and between the brown, red, and green algae. The PCA data were visualized using a score and loading plot. The score plot shows the sample separation distribution based on metabolite concentration variations in the data set, whereas the loading plot provides the information on the spectral position of metabolites corresponding to the score plot. HCA was performed by two-way clustering using Ward's method (Murtagh & Legendre, 2014) presents the metabolite characteristics in terms of hierarchical or tree-like structure and heat map. The heat map shows the relative concentrations of each metabolite. It indicated the tendencies in each species of all seaweed groups in two extraction methods were separated according metabolites concentration of species identity.

3.3 Results

3.3.1 Dry weight

The dry weight percentage was different for each algae species (Table 3.1). The highest dry weight percentage of 39 % was observed in *G. elegans*, whereas the lowest percentage of approximately 5.9 % was observed in *B. wrightii*. Both species were red algae. The dry weight percentage range was larger in red algae (5.9–39 %) than in green algae (13–21 %) and brown algae (10–20 %). The average dry weight percentage in brown, red, and green algae was 20.84 %, 17.51 %, and 15.50 %, respectively.

3.3.2 Overview of the metabolite profiles

To evaluate the general tendencies of metabolites in seaweed and to assess the species-specific differences between their profiles as well as the effect of the extraction methods on the results, the entire data set was subjected to a multivalent analysis (11 species, 2 extraction methods) (Figures 3.2–3.8, S3.1–S3.23). PCA indicated that the seaweed species was the dominating factor for the metabolite concentrations (Figure 3.2a, S3.1: PC1–PC5). The separation variance of PC1 (22 %) and PC2 (13.2 %) were associated with the metabolite characteristics of *P. pseudolinearis* (red algae) and *U. australis* (green algae), respectively (Figure 3.2a: positive plot PC1–PC2, Figure S3.1). A clear separation variance between the brown, red, and green algae was identified in the PC2 and PC3 plots (Figure 3.2b, S3.1: PC2–PC3). Furthermore, the plots from PC1 to PC5 indicated that the separation patterns were mostly species-specific and not linked to the extraction methods (Figure 3.2, S3.1).

HCA was performed on 110 individual samples derived from 11 seaweed species to simultaneously determine the concentrations of 175 metabolites (Figure 3.3, S3.8–S3.12). Notably, the main factor in hierarchical clustering of metabolites was the species (Figure 3.3, see color boxes). Interestingly, the species did not cluster into the three seaweed groups of algae; instead, they were clustered in several clades (Figure 3.3, S3.2) that depended on the taxonomic class or family. For example, *S. firma* and *P. kuromo* of the brown algae family Chordariaceae were clustered closely by metabolites

profiling. Furthermore, if metabolite profiles were compared within each species, the two extraction methods were clustered together (Figure 3.3, S3.2).

The fresh weight of metabolite concentrations for evaluation by PCA and HCA was also calculated (Figures S3.14–S3.23). Similar to dry weight metabolite concentration data, the PCA separation and HCA cluster patterns depended mainly on the species and not on the extraction method. The fresh weight of metabolite concentrations in the samples of some species, such as *B. wrightii*, was relatively low to perform HCA (Figure S3.20, S3.22), and the clustering tendencies were difficult to interpret. Thus, only the dry weight of metabolite concentrations was used for further evaluation of the metabolite profiles of those samples.

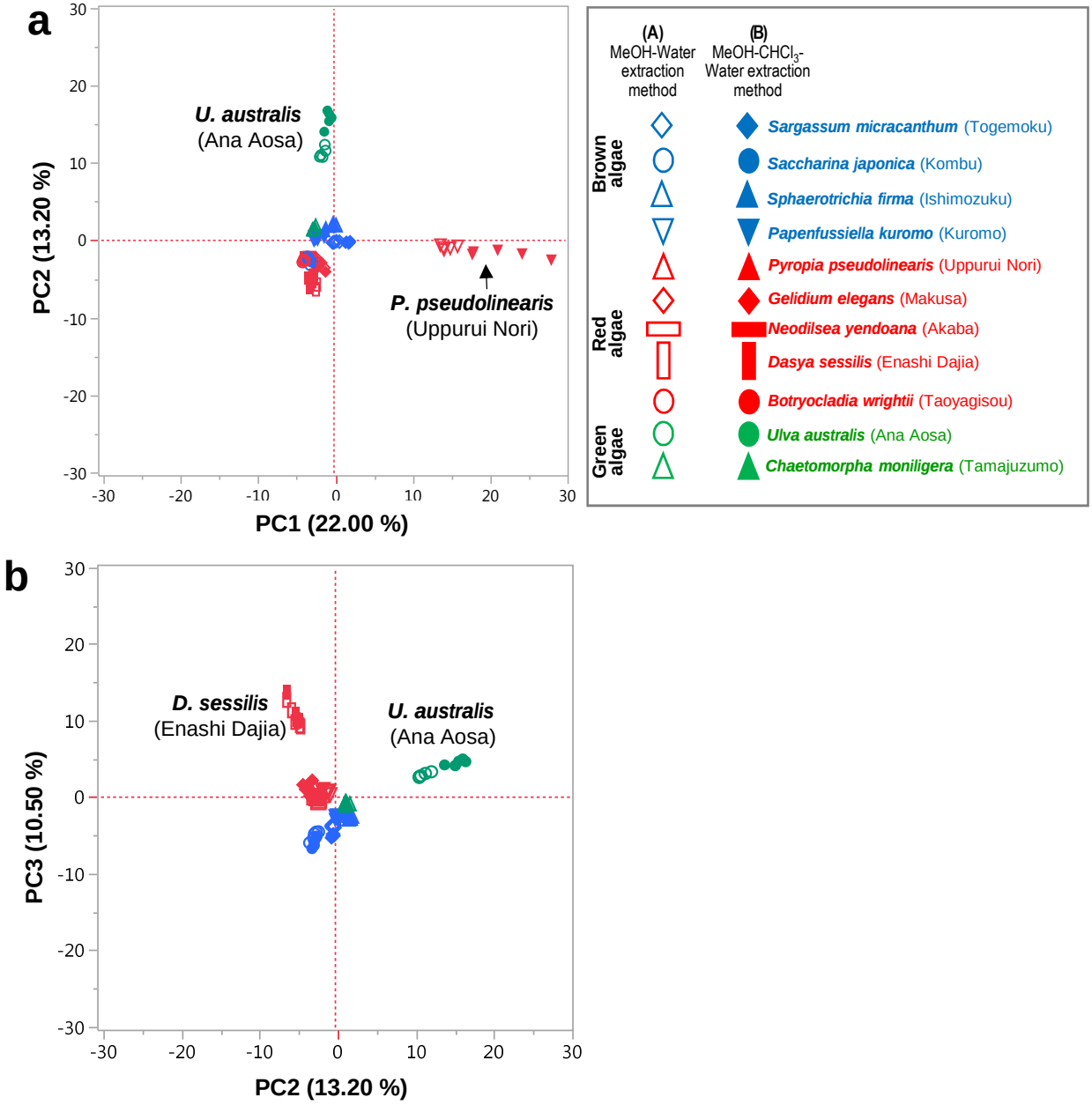

Figure 3.2 PCA score plots of dry weight based metabolites concentrations in all data set of this research a) PC1-PC2 plot and b) PC2-PC3 plots.

Four brown algae are indicated by blue color symbols; *Sargassum micracanthum* (Togemoku), *Saccharina japonica* (Kombu), *Sphaerotrichia firma* (Ishimozuku) and *Papenfussiella kuromo* (Kuromo), five red algae are indicated by red color symbols; *Pyropia pseudolinearis* (Uppurui Nori), *Gelidium elegans* (Makusa), *Neodilsea yendoana* (Akaba), *Dasya sessilis* (Enashi-Dajia) and *Botryocladia wrightii* (Taoyagisou), and two green algae are indicated by green color symbols; *Ulva australis* (Ana Aosa) and *Chaetomorpha moniligera* (Tamajuzumo). Filled and unfilled color indicate the methanol-water extractions without chloroform and with chloroform, respectively. The detail of each color and markers were listed on the figure legend. The percentage of each axis indicated separation variance. Precise PCAs (PC1 to PC5) including score and loading plots are shown in Figure S3.1.

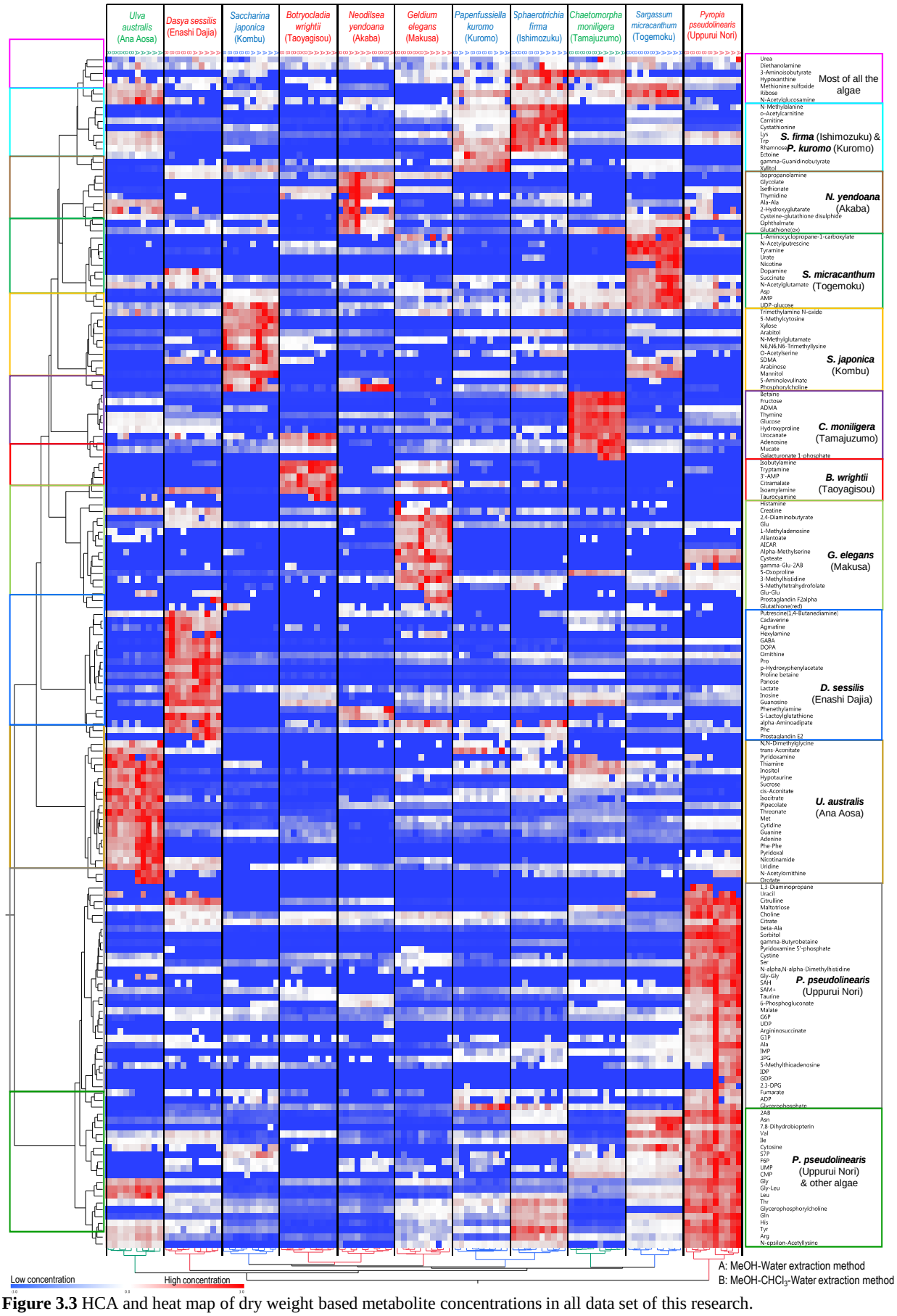

Figure 3.3 HCA and heat map of dry weight based metabolite concentrations in all data set of this research.

All quantified concentrations were normalized to Z-scores for each metabolite. HCA was performed by Ward methods and two-way clusterization was done on both metabolites concentration and each algae species after 2 extraction methods. Alphabet A indicates the methanol-water extraction method; alphabet B indicates the methanol-chloroform-water extraction method. The blue and red color indicate low and high-abundance of metabolites concentrations, respectively as color bar shown in the bottom. Blue, red and green fonts indicate the seaweed species groups; brown, red and green algae respectively. Color framed boxes indicated the significant high concentration of each seaweed species.

3.3.3 Effect of extraction methods

Two extraction methods, the methanol-water and the methanol-chloroform-water, were compared. An extraction method separation variance could not be clearly identified using all metabolite concentration data in PCA (Figure 3.2, S3.1). Instead, by restricting the data to seaweed groups, PCA separation variances were identified by performing the PC1–PC5 multivalent analysis (Figure 3.4, S3.3–S3.5).

In brown algae, a separation difference that aligned with the extraction methods was found in PC4 with a 5.46 % variance (Figure S3.3: PC4, see color rectangular); in red algae, it was found in PC5 with a 3.36 % variance (Figure S3.4: PC5, see color eclipse); and in green algae, the separation variance was 8.1 % in PC2 (Figure S3.5: PC2). Using the methanol-water extraction method, only one out of 175 metabolites, i.e., 1, 3-diaminopropane, was not detected (Figure S3.11, Table S3.1), whereas using the methanol-chloroform-water extraction method, nine out of 175 metabolites, i.e., nicotine, taurocyamine, Glu-Glu, orotate, 2, 3-DPG, galacturonate 1-phosphate, prostaglandin, IDP, and GDP, were not detected (Figure S3.12).

The efficiency of the two extraction methods was compared for several major metabolites, such as amino acids, TCA and glycolysis organic acids, and sugars, by plotting each of the extraction methods on x-y axis plots (Figure S3.13). The graph shows that most metabolite concentrations were not affected by either extraction method. However, some metabolites, such as Ala and Glu, had 2 to 3 times higher concentrations in samples processed by methanol-water extraction as compared to the concentrations in the other samples (Figure S3.13a), and peaks of 2, 3-DPG (Figure S3.13b) were only detectable in methanol-water extraction samples. The PCA scores and loading plots of the extraction methods did not differ significantly. Thus, although the extraction methods were different, the metabolite profiles were mainly determined by the seaweed species (Figure 3.5, S3.6–S3.7).

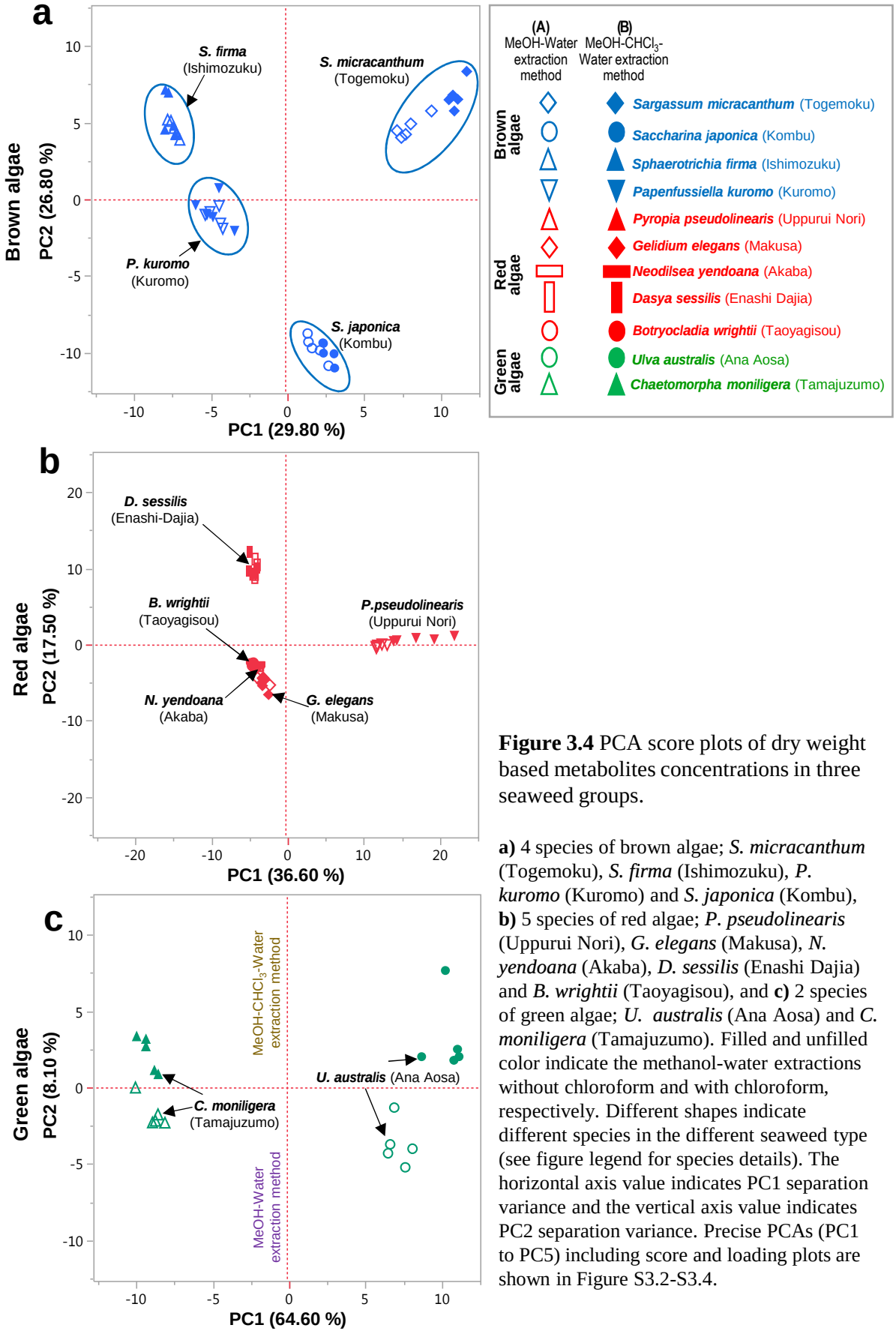

Figure 3.4 PCA score plots of dry weight based metabolites concentrations in three seaweed groups.

a) 4 species of brown algae; *S. micracanthum* (Togemoku), *S. firma* (Ishimozuku), *P. kuromo* (Kuromo) and *S. japonica* (Kombu), **b)** 5 species of red algae; *P. pseudolinearis* (Uppurui Nori), *G. elegans* (Makusa), *N. yendoana* (Akaba), *D. sessilis* (Enashi Dajia) and *B. wrightii* (Taoyagisou), and **c)** 2 species of green algae; *U. australis* (Ana Aosa) and *C. moniligera* (Tamajuzumo). Filled and unfilled color indicate the methanol-water extractions without chloroform and with chloroform, respectively. Different shapes indicate different species in the different seaweed type (see figure legend for species details). The horizontal axis value indicates PC1 separation variance and the vertical axis value indicates PC2 separation variance. Precise PCAs (PC1 to PC5) including score and loading plots are shown in Figure S3.2-S3.4.

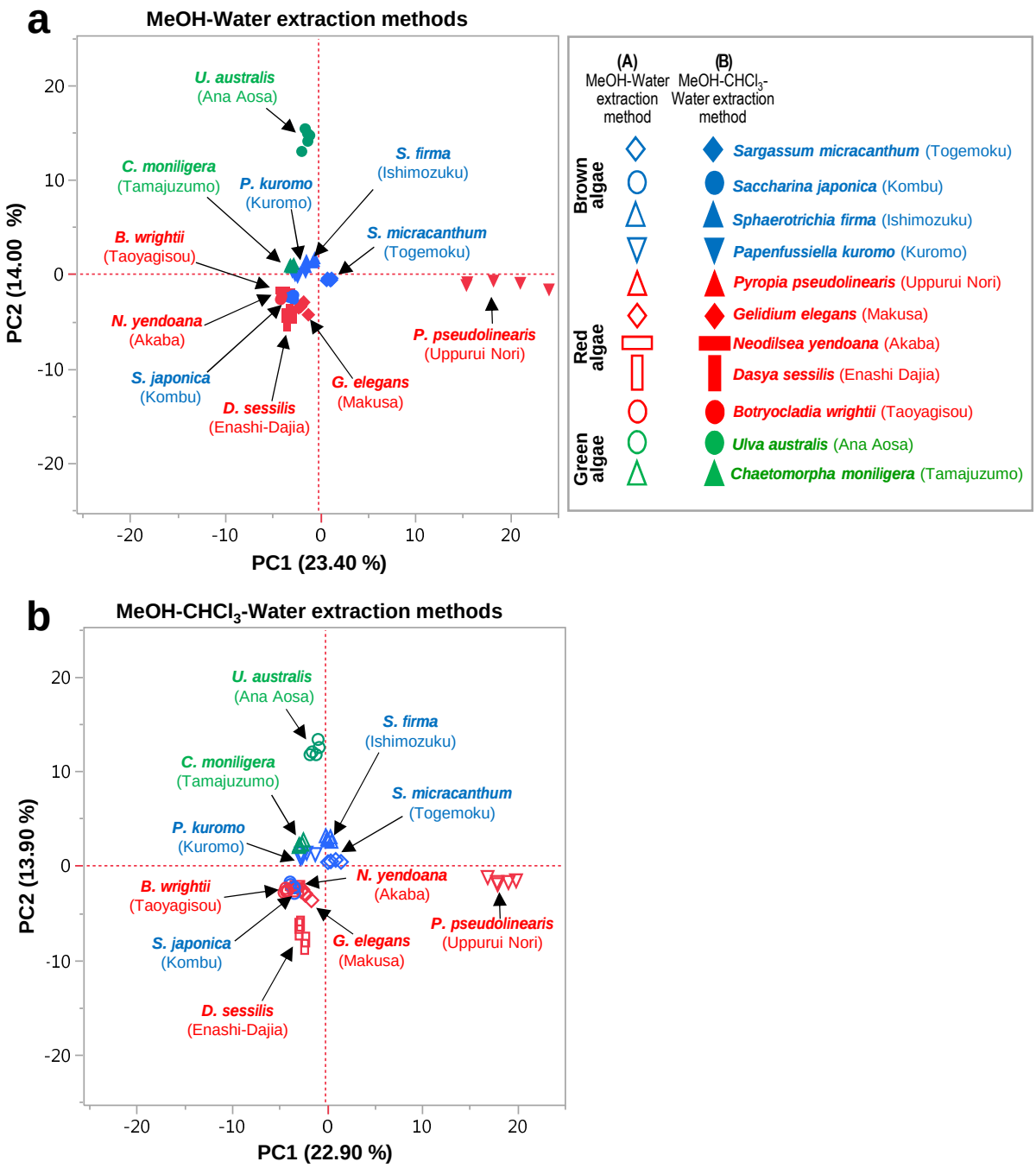

Figure 3.5 PCA score plots of dry weight based metabolites concentrations in all data set after two extraction methods. **a)** Methanol-water extractions methods and **b)** methanol-water with chloroform methods.

Different shapes indicate different species of in the different seaweed type (see figure legends for species details). The horizontal axis value indicates PC1 separation variance and the vertical axis value indicates PC2 separation variance. Precise PCAs (PC1 to PC5) including score and loading plots are shown in Figure S3.5-S3.6.

3.3.4 Metabolite characteristics of three group of seaweed

The PCA of the entire data set indicated a clear separation variance between the three seaweed groups in the PC2 and PC3 plots (Figure 3.2b, S3.1). The PC2 positive plot is dominated by the high concentrations of certain metabolites identified in green algae, such as sucrose (Figure S3.1: PC2). The positive plot of PC3 is dominated by high metabolite concentrations in red algae, whereas the negative PC3 plot is dominated by the brown algae. Significantly high concentrations of the major metabolites such as proline betaine, panose, *p*-hydroxyphenylacetate, and DOPA were detected in certain red algae species, for instance *D. sessilis* (Figure S3.1: positive plot PC3), whereas mannitol and arabitol were dominant in all brown algae (Figure S3.1: negative plot PC3).

3.3.5 Metabolite characteristics in brown algae

The concentrations of mannitol (at least 3 times) and cystathionine (at least 5 times) were significantly higher in brown algae than in red or green algae (Table 3.2, S3.1). Some of the metabolites were only detected in certain species, e.g., arabinose in *S. micracanthum* and *S. japonica* or 5-methylcytosine in *S. japonica*. Significantly high concentrations of tyramine, succinate, urate, and AMP were detected in *S. micracanthum* samples and carnitine in *S. firma* samples. Twenty-one of 175 metabolites, including sorbitol, maltotriose, and panose, were not detected in any brown algae species (Figures 3.3, S3.8, Table S3.1).

The PCA separation variances and HCA clusters demonstrated that the metabolites profiles of the four brown algae species displayed significant differences (Figures 3.4a, S3.3). The PC1 plot illustrates the separation variance of 29.8 % between the Chordariaceae family (*S. firma* and *P. kuromo*) (Figure 3.4a: negative plot PC1, Figure S3.3) and the Sargassaceae (*S. micracanthum*) and Laminariaceae (*S. japonica*) families (Figure 3.4a: positive plot PC1, Figure S3.3). *S. firma* and *P. kuromo,* from the same family, were clustered because they shared some metabolites with different concentrations, such as rhamnose, betaine, Trp, Phe and Thr (Figure 3.4a, S3.3). The positive PC1 plot separated *S. micracanthum* and *S. japonica* from *S. firma* and *P. kuromo*.

3.3.6 Metabolite characteristics in red algae

In red algae, each species displayed a characteristic metabolite profile. The concentrations of some metabolites were significantly higher in certain red algae species than in brown or green algae, e.g., isoamylamine (except *P. pseudolinearis*), citrulline (except *G. elegans*), and isethionate (except *D. sessilis*) (Table 3.2, S3.1). Among the 175 profiled metabolites, ten were not detected in red algae, i.e., 5-methylcytosine, nicotine, Phe-Phe, orotate, urate, galacturonate 1-phosphate, ribose, arabinose, xylose and rhamnose (Figure 3.3, S3.9). Some metabolites were detected only in certain species. For example, sorbitol, GDP, UDP, and IDP were detected only in *P. pseudolinearis*, glycolate and UDP only in *N. yendoana*, panose and *p*-hydroxyphenyl acetate only in *D. sessilis*, and 3'-AMP only in *B. wrightii* (Figure 3.3, S3.9, Table S3.1).

The PCA of the five red algae species, *P. pseudolinearis*, *G. elegans*, *N. yendoana*, *D. sessilis*, and *B. wrightii*, shows that the PC1-4 plots mainly illustrate species-specific variations (Figure 3.4b, S3.4), whereas the PC5 plot indicates the effect of the extraction methods (Figure S3.4: PC5). The clade of *P. pseudolinearis* was clustered distantly (Figure 3.3; Figure 3.2: positive plot PC1; Figure 3.4b, S3.4) from the other red algae species with a separation variance of 36.6 % (Figure 3.2: negative plot PC1; Figure 3.4b, S3.4) in alignment with a different taxonomic classification. *P. pseudolinearis* belongs to Bangiophyaceae, whereas the other red algae species are classified as Florideophycae (Figure 3.2: positive plot PC1; Figure 3.3, 3.4b, S3.4). Loading plot of PC1 indicates *P. pseudolinearis* with high concentrations of amino acids (Trp, Asn, Arg, Gly-Gly, and Gly-Leu), CMP, and gamma-butyrobetaine (Figure S3.4: positive plot PC1). These metabolites were significantly concentrated in *P. pseudolinearis* but not in the other red algae species. The PC2:PC4 plot provides a clear separation of each red algae species (Figure S3.4a: see the circle in PC2:PC4).

3.3.7 Metabolite characteristics in green algae

The concentrations of thymine (at least 2 times), fructose (at least 15 times), glucose (at least 2 times), sucrose (at least 4 times), and inositol (at least 1.3 times) were significantly higher in green algae than in brown or red algae (Table 3.2, S3.1). Among the profiled metabolites, orotate and Phe-Phe were specific for *U. australis*. High concentrations of several metabolites, such as pipecolate, Met, guanine, uridine, threonate and cis-aconitate, were detected at high concentrations in *U. australis* but only one metabolite, betaine, in *C. moniligera*. There were 42 out of 175 profiled metabolites that were not detected in green algae (Figure 3.3, S3.10, Table S3.2).

PCA indicated a clear separation variance between the two green algae species in relation to the two extraction methods (Figure 3.4c, S3.5). The PC1 plot shows the separation of *U. petusa* and *C. monilligera* with a separation variance of 64.6 % (Figure 3.4c: PC1; S3.5). The positive and negative PC2 plot shows the separation by extraction methods using methanol-water with and without chloroform, respectively, at a separation variance of 8.1 % (Figure 3.4c: PC2; Figure S3.5).

3.3.8 Amino acid and sugar profiling in various seaweed

Most of the amino acids were detected at different concentrations in all seaweed with the exception of Cys (Figure 3.6, Table 3.2). However, cystine, which is a dimer of Cys, was detected in most seaweed except in *S. micracanthum*, *S. japonica*, *D. sessilis*, and *C. moniligera*. There were other amino acids that could not be detected in some algae: Met (*P. kuromo*, *N. yendoana*, and *D. sessilis*), Asn (*B. wrightii*), Lys (*D. sessilis*), and Trp (*N. yendoana*, *D. sessilis,* and *B. wrightii*). Moreover, in all seaweed' samples, the Asp concentration was higher than that of Asn (Table 3.2). High concentrations of Ala were detected in all species, and Pro was detected at the highest concentration in *D. sessilis*. In green and red algae (except in *P. pseudolinearis*), the concentration of Glu was higher than that of Gln. In brown algae (except in *S. japonica*), Gln concentration was higher than that of Glu. The variations in the amino acid concentrations were mainly related to the individual species in this algae group, and were not significantly affected by the different extraction methods (Figure 3.6, Table 3.2).

To determine the characteristic properties of major metabolite groups (i.e., amino acids, TCA and glycolysis organic acids, and sugars) in seaweed, each metabolite group was subjected to HCA (Figure 3.6–3.8). Importantly, sugar profiling by HCA determined clear differences that aligned with the taxonomy of seaweed, i.e., the brown, red, and green algae (Figure 3.8). The same cannot be demonstrated in of amino acids profiling (Figure 3.6) as well as TCA and glycolysis organic acids (Figure 3.7).

Mannitol was found at significantly high concentrations in brown algae, whereas the concentrations of fructose, glucose, and sucrose were significantly high in green algae (Figure 3.8, Table 3.2). Among species-specific metabolites found in red algae, sorbitol was detected at high concentrations only in *P. pseudolinearis,* and panose was detected only in *D. sessilis*. Arabinose was only detected in the brown algae *S. micracanthum* and *S. japonica*. Depending on the seaweed groups, some sugars were not detectable; ribose and rhamnose were not detected in red algae, whereas maltotriose was not detected in brown algae (Figure 3.8, Table 3.2).

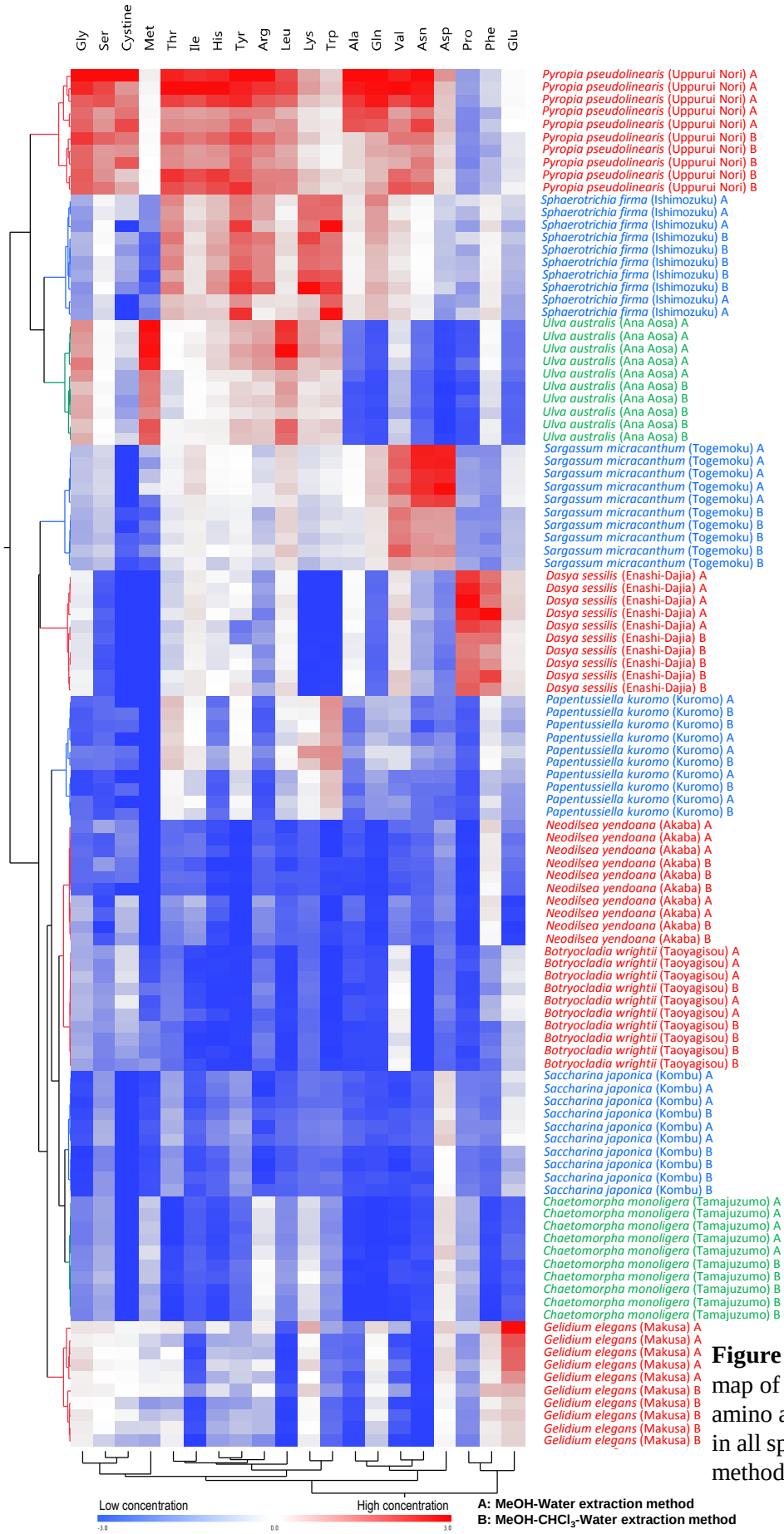

Figure 3.6 HCA and heat map of dry weight based on amino acids concentrations in all species and extraction methods.

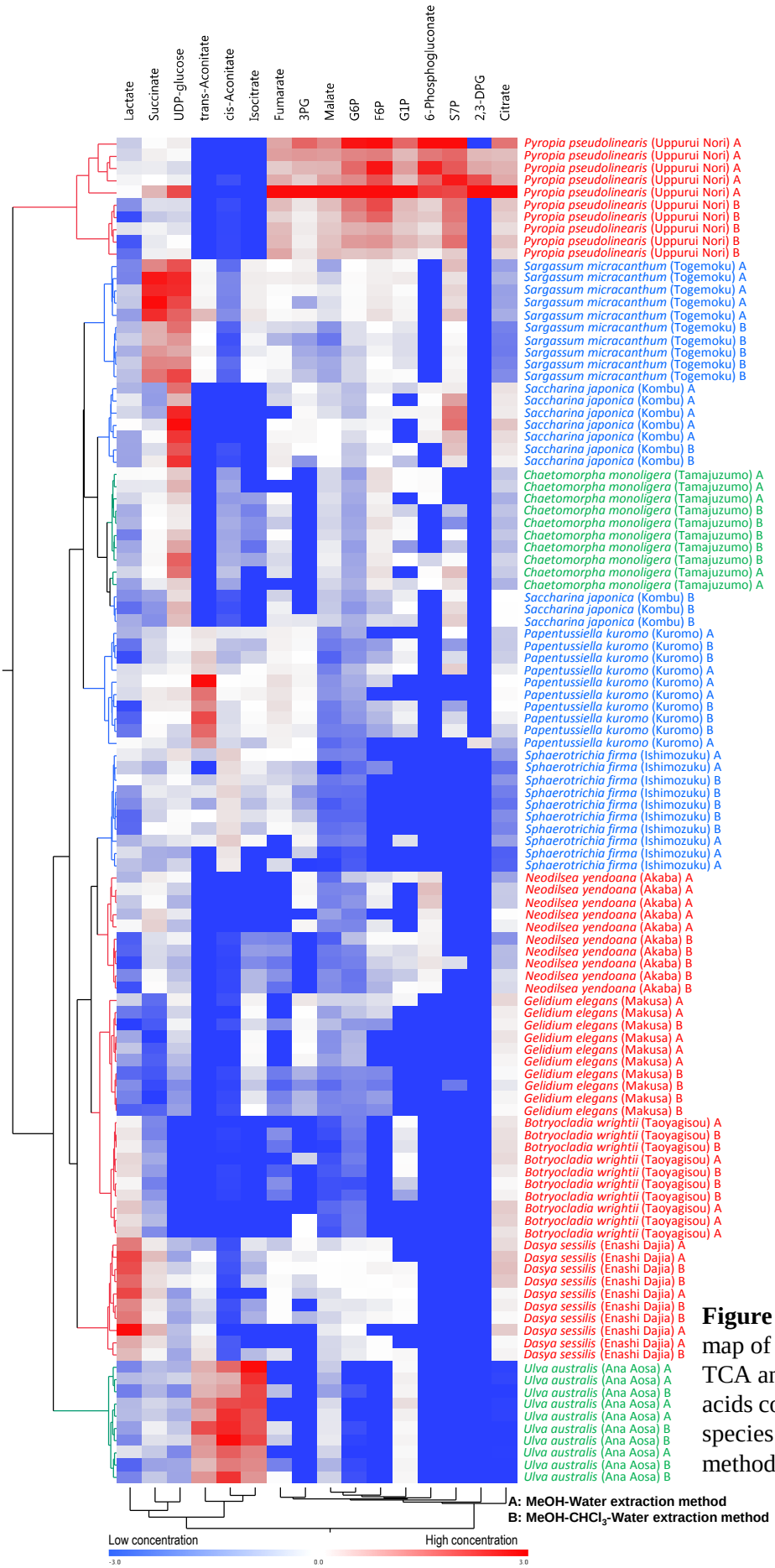

Figure 3.7 HCA and heat map of dry weight based on TCA and glycolysis organic acids concentrations in all species and extraction methods.

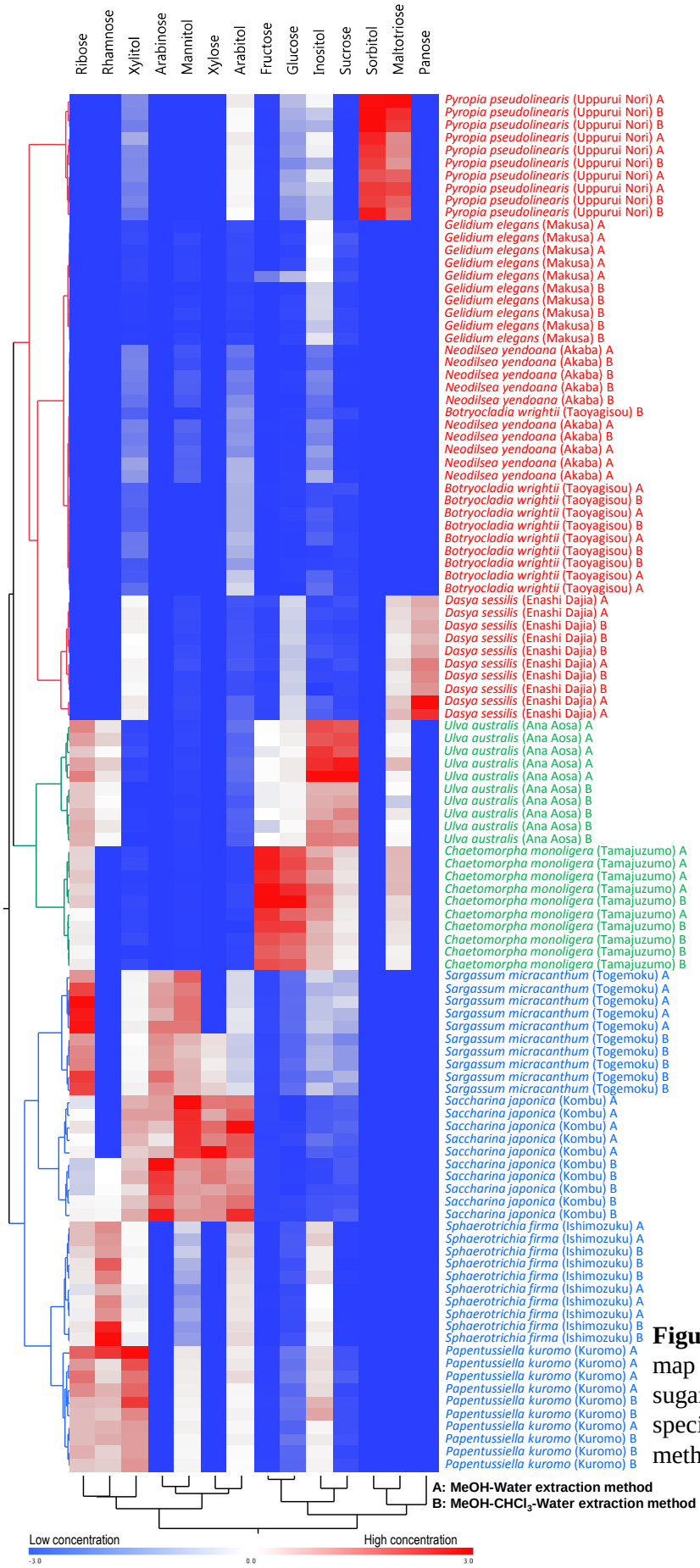

Figure 3.8 HCA and heat map of dry weight based on sugars concentrations in all species and extraction methods.

Table 3.2 Amino acids, sugars and major organic acids of 11 seaweeds after methanol-water extraction method.

		Methanol-Water extraction methods										
		Brown algae				Red algae					Green algae	
	Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Amino acids	Gly	736.39 ± 36.75^{ef}	546.45 ± 51.57^{fgh}	285.24 ± 81.90^{ghi}	185.57 ± 24.37^{hi}	4278.75 ± 481.72^{a}	1886.07 ± 263.85^{d}	476.36 ± 204.87^{fghi}	1029.74 ± 81.06^{e}	674.16 ± 50.38^{ef}	3339.20 ± 205.82^{b}	412.57 ± 22.29^{fghi}
	Ala	14777.00 ± 746.18^{e}	29971.00 ± 1754.79^{c}	4247.00 ± 757.72^{g}	2446.00 ± 303.52^{g}	100818.00 ± 9381.19^{a}	2771.00 ± 656.15^{g}	2423.00 ± 535.45^{g}	16216.00 ± 1797.01^{de}	1891.00 ± 134.09^{g}	3176.00 ± 230.91^{g}	1146.00 ± 61.30^{g}
	Ser	930.06 ± 27.61^{cdef}	1101.54 ± 122.06^{cdef}	318.94 ± 73.07^{f}	616.10 ± 86.36^{def}	6949.48 ± 1493.02^{a}	1835.74 ± 533.41^{c}	336.12 ± 178.09^{f}	277.26 ± 23.16^{f}	536.97 ± 43.23^{ef}	1520.16 ± 139.94^{cd}	630.42 ± 30.92^{def}
	Pro	1275.90 ± 187.83^{cdef}	1686.30 ± 451.19^{c}	384.10 ± 75.06^{defg}	882.10 ± 109.63^{cdefg}	1092.70 ± 153.36^{cdefg}	1354.30 ± 410.95^{cde}	125.20 ± 20.11^{g}	28052.50 ± 1644.27^{a}	267.80 ± 65.87^{fg}	401.60 ± 138.05^{defg}	1488.30 ± 92.38^{c}
	Val	2807.73 ± 227.46^{a}	1651.99 ± 178.00^{b}	595.42 ± 190.45^{defg}	199.01 ± 39.81^{fg}	3009.32 ± 505.94^{a}	318.46 ± 176.83^{efg}	320.11 ± 107.77^{efg}	1631.52 ± 185.45^{b}	1065.13 ± 208.76^{c}	825.68 ± 121.55^{cd}	146.20 ± 9.54^{g}
	Thr	1050.34 ± 30.37^{def}	2379.17 ± 258.36^{b}	1702.41 ± 329.50^{c}	638.24 ± 61.62^{efghi}	3044.47 ± 483.78^{a}	1169.27 ± 317.08^{d}	431.62 ± 82.55^{ghi}	854.71 ± 76.99^{defgh}	420.40 ± 35.50^{ghi}	1113.48 ± 137.32^{de}	208.83 ± 14.19^{i}
	Ile	663.02 ± 55.10^{bc}	723.81 ± 51.56^{b}	417.14 ± 119.55^{ef}	54.22 ± 14.14^{g}	1309.73 ± 250.03^{a}	64.05 ± 53.08^{g}	127.59 ± 63.27^{g}	590.89 ± 43.44^{bcde}	34.55 ± 17.87^{g}	459.00 ± 71.71^{def}	57.91 ± 5.67^{g}
	Leu	1180.95 ± 91.64^{c}	1035.06 ± 112.64^{c}	553.46 ± 176.12^{de}	104.52 ± 26.74^{f}	1926.48 ± 301.44^{ab}	29.90 ± 24.86^{f}	71.19 ± 33.72^{f}	890.72 ± 93.12^{cd}	15.97 ± 5.43^{f}	2130.03 ± 374.04^{a}	223.08 ± 8.14^{ef}
	Asn	9982.58 ± 389.77^{a}	2089.68 ± 391.19^{d}	504.57 ± 194.98^{ef}	269.73 ± 41.80^{ef}	9712.72 ± 1185.88^{a}	25.35 ± 8.85^{f}	326.32 ± 35.86^{ef}	917.51 ± 100.64^{e}	ND^{f}	349.14 ± 55.10^{ef}	126.25 ± 24.25^{f}
	Asp	18619.30 ± 713.38^{a}	2993.90 ± 755.02^{gh}	1706.40 ± 392.09^{hijk}	9043.70 ± 615.23^{d}	11068.90 ± 1301.71^{c}	8594.30 ± 926.78^{de}	2184.50 ± 254.56^{ghi}	1791.00 ± 107.65^{hij}	1646.00 ± 167.74^{ijk}	602.60 ± 47.73^{jk}	8874.60 ± 552.88^{d}
	Gln	21320.80 ± 735.62^{c}	27317.50 ± 3873.72^{b}	4699.10 ± 1335.76^{fg}	793.40 ± 209.32^{ghi}	43085.80 ± 4055.55^{a}	11803.30 ± 4156.67^{e}	245.00 ± 21.92^{hi}	1507.00 ± 70.99^{ghi}	578.70 ± 79.32^{hi}	739.50 ± 126.76^{ghi}	83.10 ± 9.73^{i}
	Glu	5930.20 ± 245.67^{de}	3756.70 ± 947.02^{ef}	3726.20 ± 800.70^{ef}	6154.90 ± 1244.38^{de}	7901.90 ± 829.56^{d}	38041.60 ± 6103.94^{a}	1168.00 ± 849.53^{f}	17649.20 ± 2151.44^{b}	5713.00 ± 431.26^{de}	1558.80 ± 110.65^{f}	1151.80 ± 211.16^{f}
	Lys	306.15 ± 27.78^{fgh}	995.82 ± 204.64^{b}	609.26 ± 244.88^{de}	91.82 ± 11.75^{ghi}	791.82 ± 162.33^{bcd}	602.07 ± 203.88^{de}	64.42 ± 17.90hi	ND^{i}	74.43 ± 8.70^{ghi}	867.49 ± 98.13^{bc}	290.86 ± 15.56^{fgh}
	Met	53.76 ± 25.10^{def}	32.08 ± 3.89^{ef}	ND^{f}	7.31 ± 3.35^{f}	166.06 ± 29.54^{c}	103.84 ± 50.71^{cde}	ND^{f}	ND^{f}	9.01 ± 2.20^{f}	898.67 ± 114.92^{a}	66.29 ± 7.58^{def}
	His	150.29 ± 13.38^{ef}	291.01 ± 30.71^{c}	52.09 ± 15.71^{ij}	56.13 ± 6.44^{hij}	481.48 ± 87.78^{a}	97.43 ± 20.22^{fghi}	37.29 ± 5.39^{ij}	126.58 ± 16.39^{efg}	29.11 ± 2.10^{ij}	245.40 ± 23.20^{cd}	32.00 ± 1.30^{ij}
	Phe	345.82 ± 20.99^{hij}	1301.04 ± 152.78^{bc}	759.16 ± 185.89^{efg}	269.93 ± 28.56^{ij}	579.33 ± 76.25^{fghi}	1469.49 ± 282.03^{b}	1360.87 ± 121.87^{bc}	2816.21 ± 336.65^{a}	413.80 ± 67.59^{ghij}	930.56 ± 132.52^{def}	115.15 ± 6.14^{ij}
	Arg	350.43 ± 39.57^{efg}	848.58 ± 205.81^{cd}	129.21 ± 35.14^{hi}	89.60 ± 18.81^{i}	1330.19 ± 217.92^{a}	365.55 ± 138.08^{ef}	157.33 ± 49.42^{ghi}	199.66 ± 22.88^{fghi}	139.02 ± 18.88^{hi}	1009.54 ± 102.30^{bc}	370.67 ± 22.66^{ef}
	Tyr	300.36 ± 18.26^{c}	784.52 ± 95.35^{a}	300.26 ± 67.24^{c}	114.41 ± 16.89^{efg}	862.76 ± 104.08^{a}	169.85 ± 27.08^{cdef}	1.61 ± 1.19^{g}	237.00 ± 104.57^{cde}	4.16 ± 0.71^{g}	562.59 ± 87.93^{b}	47.79 ± 5.12^{fg}
	Trp	96.69 ± 7.78e	414.00 ± 52.04a	285.57 ± 39.84^{b}	25.69 ± 5.46^{g}	203.11 ± 34.88^{cd}	29.62 ± 12.04^{g}	ND^{g}	ND^{g}	ND^{g}	245.08 ± 33.48^{bc}	39.51 ± 3.57^{fg}
	Cystine	ND^{c}	3.52 ± 4.81^{c}	0.48 ± 1.08^{c}	ND^{c}	113.83 ± 24.06^{a}	16.11 ± 4.53^{c}	5.20 ± 2.06^{c}	ND^{c}	9.23 ± 1.30^{c}	8.38 ± 1.86^{c}	ND^{c}
Sugars	Ribose	3993.82 ± 565.94^{a}	1745.22 ± 726.43^{e}	3143.74 ± 413.92^{bc}	1437.75 ± 356.81^{ef}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	2924.37 ± 367.30bc	2063.63 ± 363.73de
	Arabinose	1495.59 ± 211.76^{b}	ND^{d}	ND^{d}	1224.12 ± 326.15^{c}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	Xylose	ND^{c}	ND^{c}	ND^{c}	118.51 ± 29.40^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
	Xylitol	292.67 ± 22.13^{c}	213.63 ± 43.52^{c}	887.67 ± 154.73^{a}	608.08 ± 73.85^{b}	71.36 ± 16.84^{d}	10.77 ± 1.85^{d}	70.20 ± 14.13^{d}	317.85 ± 33.80^{c}	35.09 ± 10.46^{d}	11.18 ± 3.44^{d}	9.04 ± 2.89^{d}
	Arabitol	202.65 ± 10.97^{ef}	566.35 ± 101.81^{c}	431.39 ± 92.28^{cd}	1203.48 ± 142.29^{a}	395.43 ± 55.68^{d}	21.52 ± 1.19^{h}	106.83 ± 32.95^{fgh}	37.59 ± 9.35^{gh}	156.40 ± 24.47^{fgh}	53.26 ± 6.04^{gh}	15.67 ± 2.12^{h}
	Rhamnose	ND^{f}	66.55 ± 6.67^{b}	56.67 ± 24.29^{bc}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	33.78 ± 10.08de	NDf
	Fructose	316.00 ± 29.64^{d}	73.00 ± 11.67^{d}	158.00 ± 21.51^{d}	249.00 ± 23.13^{d}	150.00 ± 18.10^{d}	552.00 ± 1143.43^{d}	60.00 ± 14.85^{d}	347.00 ± 125.71^{d}	58.00 ± 16.01^{d}	11336.00 ± 1830.66^{c}	106271.00 ± 3962.32^{a}
	Glucose	1040.50 ± 24.31^{cde}	446.80 ± 146.29^{cde}	1122.10 ± 229.07^{cde}	146.00 ± 75.51^{e}	2594.40 ± 357.92^{cde}	790.70 ± 1257.70^{cde}	112.00 ± 32.31^{e}	3977.00 ± 282.88^{c}	120.90 ± 43.33^{e}	11799.20 ± 1980.38^{b}	43512.50 ± 3144.48^{a}
	Sorbitol	ND^{b}	ND^{b}	ND^{b}	ND^{b}	198.32 ± 15.08^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
	Mannitol	275498.00 ± 11393.90^{b}	27678.00 ± 8818.40^{g}	103188.00 ± 7287.40^{e}	347984.00 ± 14768.70^{a}	121.00 ± 22.80^{h}	1520.00 ± 652.30^{h}	7797.00 ± 1699.20^{h}	2948.00 ± 647.50^{h}	287.00 ± 37.70^{h}	1533.00 ± 212.70^{h}	655.00 ± 80.70^{h}
	Inositol	172.20 ± 17.07^{fgh}	352.27 ± 93.37^{cd}	398.09 ± 43.56^{c}	60.98 ± 9.45^{ij}	230.39 ± 23.61^{ef}	286.00 ± 16.90^{de}	110.92 ± 23.99^{ghij}	49.86 ± 12.48^{j}	61.38 ± 10.77^{ij}	841.79 ± 56.78^{a}	634.77 ± 51.54^{b}
	Sucrose	704.50 ± 124.81^{e}	10.30 ± 5.51^{e}	97.00 ± 34.98^{e}	162.00 ± 30.53^{e}	6.90 ± 5.70^{e}	81.40 ± 33.72^{e}	13.70 ± 4.65^{e}	69.30 ± 24.27^{e}	53.60 ± 21.62^{e}	11705.00 ± 1306.03^{a}	4020.10 ± 700.63^{c}
	Maltotriose	ND^{e}	ND^{e}	ND^{e}	ND^{e}	715.37 ± 127.23^{a}	ND^{e}	ND^{e}	377.58 ± 53.97^{bc}	ND^{e}	269.58 ± 106.65^{cd}	436.32 ± 48.24^{b}
	Panose	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	44.55 ± 15.13^{a}	ND^{c}	ND^{c}	ND^{c}
Major organic acids	Lactate	1572.33 ± 128.19^{c}	1287.87 ± 715.57^{c}	772.68 ± 143.46^{c}	1189.72 ± 203.22^{c}	1051.14 ± 537.64^{c}	850.93 ± 223.49^{c}	773.87 ± 172.93^{c}	8532.86 ± 1477.16^{a}	3947.28 ± 605.71^{b}	1024.36 ± 164.11^{c}	1323.23 ± 190.35^{c}
	Fumarate	111.26 ± 26.06^{cde}	117.31 ± 14.33^{bcde}	263.41 ± 55.73^{bc}	173.17 ± 86.72^{bcd}	509.73 ± 67.68^{a}	36.54 ± 11.32^{de}	38.77 ± 10.20^{de}	146.25 ± 25.98^{bcde}	33.55 ± 11.35^{de}	89.63 ± 37.48^{de}	111.50 ± 24.54^{cde}
	Succinate	1435.31 ± 149.60^{b}	524.14 ± 78.50^{efgh}	622.41 ± 152.68^{defg}	439.22 ± 240.47^{fghi}	521.03 ± 62.75^{efgh}	133.23 ± 29.02^{i}	568.56 ± 112.64^{defgh}	871.65 ± 107.09^{cd}	270.23 ± 31.55^{hi}	436.30 ± 101.86^{fghi}	679.74 ± 45.23^{def}
	Malate	411.00 ± 69.17^{c}	268.45 ± 31.80^{c}	303.39 ± 35.14^{c}	764.02 ± 208.14^{c}	3885.64 ± 305.17^{b}	375.37 ± 105.98^{c}	317.87 ± 60.77^{c}	779.64 ± 128.14^{c}	184.22 ± 23.50^{c}	621.55 ± 142.07^{c}	717.18 ± 62.85^{c}
	trans-Aconitate	204.37 ± 33.66^{bc}	119.30 ± 36.84^{bc}	676.60 ± 271.33^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	193.50 ± 106.02^{bc}	ND^{c}	709.60 ± 155.34^{a}	ND^{c}
	cis-Aconitate	66.84 ± 23.00^{c}	1454.89 ± 136.61^{b}	363.56 ± 63.75^{c}	41.11 ± 10.72^{c}	19.78 ± 3.28^{c}	26.31 ± 10.60^{c}	19.94 ± 5.87^{c}	36.01 ± 12.47^{c}	12.59 ± 4.91^{c}	3860.52 ± 852.51^{a}	248.29 ± 11.93^{c}
	3PG	41.39 ± 9.24^{cd}	44.95 ± 14.54^{cd}	78.55 ± 8.88^{bcd}	35.53 ± 38.74^{cd}	195.94 ± 23.70^{b}	44.31 ± 18.41^{cd}	ND^{d}	38.02 ± 39.26^{cd}	ND^{d}	ND^{d}	ND^{d}
	Isocitrate	122.80 ± 10.98^{cd}	89.77 ± 14.10^{cdef}	112.81 ± 29.34^{cde}	ND^{f}	ND^{f}	105.37 ± 20.32^{cdef}	54.96 ± 15.20^{def}	70.76 ± 14.14^{def}	ND^{f}	786.54 ± 117.83^{a}	41.55 ± 11.27^{def}
	Citrate	805.46 ± 87.55^{fg}	844.00 ± 182.66^{fg}	2381.47 ± 574.59^{ef}	3731.79 ± 774.12^{cde}	6054.64 ± 561.76^{b}	3410.95 ± 677.47^{de}	1778.86 ± 519.10^{efg}	5028.01 ± 1522.49^{bcd}	4586.35 ± 709.24^{bcd}	ND^{g}	1798.41 ± 203.45^{efg}
	G1P	59.29 ± 7.14^{cde}	ND^{e}	61.62 ± 7.94^{cde}	62.77 ± 16.23^{cde}	253.51 ± 32.28^{b}	ND^{e}	66.57 ± 20.54^{cde}	91.49 ± 14.24^{cde}	56.96 ± 21.11^{cde}	113.21 ± 5.52^{cd}	63.07 ± 25.74^{cde}
	F6P	142.06 ± 13.86^{def}	ND^{g}	71.89 ± 11.30^{efg}	110.40 ± 28.52^{efg}	581.48 ± 128.53^{b}	46.69 ± 8.42^{fg}	102.91 ± 28.00^{efg}	169.09 ± 35.26^{cde}	ND^{g}	ND^{g}	257.56 ± 27.73^{cd}
	G6P	509.49 ± 38.78^{cd}	142.55 ± 29.60^{d}	238.91 ± 32.84^{cd}	357.70 ± 80.57^{cd}	3953.17 ± 765.54^{b}	223.92 ± 24.61^{cd}	221.22 ± 105.25^{cd}	670.21 ± 97.88^{cd}	122.00 ± 49.53^{d}	38.03 ± 18.10^{d}	307.83 ± 18.93^{cd}
	2,3-DPG	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
	6-Phosphogluconate	ND^{e}	ND^{e}	ND^{e}	ND^{e}	317.27 ± 89.06^{b}	ND^{e}	188.06 ± 48.05^{c}	ND^{e}	ND^{e}	ND^{e}	11.94 ± 26.70^{de}
	S7P	348.59 ± 44.77^{def}	ND^{g}	159.56 ± 54.48^{fg}	572.34 ± 150.89^{de}	1045.93 ± 205.50^{b}	11.68 ± 26.11^{g}	38.83 ± 86.82^{fg}	ND^{g}	ND^{g}	ND^{g}	194.59 ± 95.75^{fg}
	UDP-glucose	320.65 ± 34.29^{abc}	148.24 ± 22.48^{fg}	149.61 ± 30.96^{fg}	297.97 ± 70.98^{abc}	140.92 ± 35.82^{fg}	77.37 ± 37.85^{gh}	103.66 ± 23.42^{fg}	92.44 ± 31.76^{gh}	ND^{h}	79.04 ± 26.75^{gh}	255.83 ± 63.54^{cde}

The values indicate as mean ±SD (nmol g^{-1}) of the metabolite concentration of dry weight (n = 5). ND, not detected. Different superscript letters indicate the significant differences (P < 0.05) between each type of species by Tukey HSD analysis. Statistical analysis was compared between all data set (species and extraction methods (Table 3.2, S3.1)).

Table 3.3 Amino acids, sugars and major organic acids of 11 seaweeds after methanol-chloroform-water extraction method.

		Methanol-Chloroform-Water extraction methods										
		Brown algae				Red algae					Green algae	
	Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Amino acids	Gly	649.19 ± 50.60^{efg}	604.76 ± 81.26^{fg}	271.30 ± 69.60^{ghi}	153.04 ± 14.83^{i}	4067.37 ± 226.47^{a}	1634.75 ± 200.07^{d}	422.10 ± 165.07^{fghi}	1028.04 ± 59.17^{e}	618.43 ± 41.44^{fg}	2810.42 ± 196.89^{c}	444.18 ± 30.94^{fghi}
	Ala	9563.00 ± 574.74^{f}	20834.00 ± 1239.88^{d}	2978.00 ± 412.96^{g}	1673.00 ± 269.34^{g}	36888.00 ± 2705.91^{b}	1598.00 ± 426.63^{g}	1575.00 ± 300.67^{g}	11530.00 ± 1360.28^{ef}	1323.00 ± 84.23^{g}	1946.00 ± 101.17^{g}	964.00 ± 84.82^{g}
	Ser	824.73 ± 50.44^{def}	1253.71 ± 126.52^{cde}	309.13 ± 73.43^{f}	529.13 ± 67.12^{ef}	5971.16 ± 660.81^{b}	1410.88 ± 460.50^{cde}	312.06 ± 163.86^{f}	270.57 ± 26.45^{f}	502.03 ± 57.51^{ef}	1318.60 ± 139.41^{cde}	677.43 ± 54.33^{def}
	Pro	1150.60 ± 147.18^{cdefg}	1603.80 ± 425.46^{c}	369.90 ± 56.46^{defg}	797.00 ± 112.93^{cdefg}	1011.70 ± 99.66^{cdefg}	1035.20 ± 195.79^{cdefg}	104.10 ± 15.24^{g}	22173.10 ± 1026.78^{b}	230.70 ± 52.51^{fg}	312.70 ± 89.63^{efg}	1427.40 ± 138.32^{cd}
	Val	2673.50 ± 235.13^{a}	1641.00 ± 133.14^{b}	609.99 ± 189.77^{cdef}	175.70 ± 40.10^{fg}	2639.62 ± 346.44^{a}	262.85 ± 150.79^{efg}	282.17 ± 75.61^{efg}	1664.29 ± 221.77^{b}	1029.53 ± 146.37^{cd}	673.15 ± 88.26^{cde}	166.45 ± 28.30^{fg}
	Thr	971.82 ± 64.09^{def}	2598.55 ± 174.90^{ab}	1719.45 ± 325.96^{c}	592.01 ± 63.34^{fghi}	2902.00 ± 303.81^{a}	1039.94 ± 345.48^{def}	392.66 ± 46.15^{hi}	889.15 ± 96.24^{defg}	392.75 ± 29.04^{hi}	963.69 ± 115.36^{def}	220.44 ± 20.18^{i}
	Ile	533.05 ± 20.86^{cdef}	632.13 ± 28.07^{bcd}	379.89 ± 123.06^{f}	45.27 ± 9.52^{g}	1185.06 ± 146.76^{a}	49.56 ± 49.61^{g}	100.65 ± 43.50^{g}	548.25 ± 44.90^{bcdef}	26.52 ± 7.63^{g}	448.96 ± 53.76^{def}	56.47 ± 8.30^{g}
	Leu	1205.99 ± 105.68^{c}	1075.56 ± 93.48^{c}	587.64 ± 195.62^{d}	95.48 ± 22.51^{f}	1724.71 ± 131.00^{b}	23.43 ± 26.71^{f}	76.88 ± 33.54^{f}	945.21 ± 130.36^{c}	15.50 ± 2.89^{f}	1721.81 ± 279.48^{b}	239.97 ± 34.73^{ef}
	Asn	6583.31 ± 402.54^{c}	2221.94 ± 135.92^{d}	404.43 ± 186.20^{ef}	185.19 ± 61.59^{ef}	7682.69 ± 553.53^{b}	20.67 ± 10.31^{f}	262.79 ± 21.78^{ef}	890.03 ± 102.40^{e}	ND^{f}	275.76 ± 36.54^{ef}	101.17 ± 19.49^{f}
	Asp	12758.80 ± 641.30^{b}	3387.80 ± 219.34^{g}	1496.10 ± 244.78^{ijk}	6038.70 ± 679.84^{f}	8837.60 ± 681.66^{d}	5776.20 ± 640.58^{f}	1688.70 ± 204.29^{hijk}	1583.50 ± 176.75^{ijk}	1300.70 ± 174.46^{ijk}	455.40 ± 33.28^{k}	7496.50 ± 675.27^{e}
	Gln	15865.20 ± 601.33^{d}	24715.90 ± 1847.37^{bc}	4131.10 ± 1000.94^{fgh}	631.90 ± 137.07^{hi}	25520.10 ± 1648.95^{b}	7313.50 ± 2291.48^{f}	187.90 ± 21.06^{hi}	1361.70 ± 142.78^{ghi}	463.00 ± 61.34^{hi}	565.90 ± 77.60^{hi}	90.40 ± 35.43^{i}
	Glu	4004.40 ± 158.09^{ef}	3706.50 ± 322.80^{ef}	3059.50 ± 491.27^{ef}	4211.50 ± 1101.22^{def}	5679.40 ± 368.06^{de}	20319.70 ± 2402.72^{b}	855.50 ± 617.49^{f}	13806.80 ± 1424.25^{c}	4301.90 ± 334.87^{def}	1094.10 ± 51.51^{f}	931.30 ± 136.02^{f}
	Lys	253.21 ± 28.49^{fghi}	1271.70 ± 123.86^{a}	487.54 ± 172.68^{ef}	81.11 ± 9.25^{ghi}	711.14 ± 101.03^{cde}	470.28 ± 146.85^{ef}	53.94 ± 14.05^{hi}	2.23 ± 0.82^{i}	78.50 ± 7.71^{ghi}	654.48 ± 57.11^{cde}	331.03 ± 43.24^{fg}
	Met	21.05 ± 9.55^{f}	11.71 ± 3.04^{f}	ND^{f}	6.39 ± 0.87^{f}	126.76 ± 11.88^{cd}	74.93 ± 46.30^{def}	ND^{f}	ND^{f}	30.11 ± 6.37^{ef}	704.18 ± 67.59^{b}	54.02 ± 8.17^{def}
	His	117.67 ± 15.39^{fgh}	361.64 ± 24.97^{b}	52.47 ± 13.17^{ij}	44.38 ± 3.69^{ij}	445.57 ± 61.44^{a}	81.36 ± 19.04^{ghij}	29.67 ± 2.31^{j}	127.14 ± 16.16^{efg}	28.11 ± 1.46^{ij}	184.66 ± 13.79^{de}	33.13 ± 4.18^{i}
	Phe	323.59 ± 30.75^{hij}	1131.48 ± 71.60^{bcd}	690.27 ± 174.59^{fgh}	222.45 ± 13.67^{ij}	487.07 ± 47.22^{ghi}	1430.44 ± 298.19^{bc}	1085.50 ± 128.29^{cde}	2504.23 ± 314.48^{a}	251.21 ± 43.80^{ij}	691.27 ± 70.69^{fgh}	97.96 ± 12.89^{ij}
	Arg	300.38 ± 41.51^{efgh}	1199.24 ± 68.25^{ab}	112.81 ± 22.98^{hi}	91.33 ± 23.00^{i}	1184.39 ± 73.09^{ab}	286.01 ± 90.42^{efghi}	141.66 ± 30.53^{hi}	210.11 ± 30.08^{fghi}	152.95 ± 21.50^{ghi}	792.56 ± 64.51^{d}	443.90 ± 56.75^{e}
	Tyr	267.59 ± 9.57^{cd}	816.59 ± 60.29^{a}	304.65 ± 65.23^{c}	111.23 ± 9.19^{efg}	825.56 ± 50.68^{a}	147.64 ± 25.21^{def}	1.58 ± 1.39^{g}	272.92 ± 127.93^{cd}	3.82 ± 1.36^{g}	504.43 ± 53.12^{b}	55.43 ± 6.00^{fg}
	Trp	84.87 ± 5.81^{ef}	378.07 ± 30.08^{a}	279.75 ± 50.89^{b}	23.59 ± 4.18^{g}	170.30 ± 26.06^{d}	23.67 ± 9.97^{g}	4.97 ± 0.76^{g}	1.02 ± 0.47^{g}	1.02 ± 0.67^{g}	205.31 ± 23.55^{cd}	39.55 ± 4.42^{fg}
	Cystine	1.02 ± 0.60^{c}	5.16 ± 0.91^{c}	2.16 ± 0.25^{c}	ND^{c}	89.95 ± 23.57^{b}	9.80 ± 3.14^{c}	3.37 ± 2.49^{c}	ND^{c}	5.81 ± 1.39^{c}	6.22 ± 1.81^{c}	ND^{c}
Sugars	Ribose	3468.96 ± 451.91^{ab}	1921.73 ± 159.20^{de}	2551.00 ± 111.09^{cd}	1056.72 ± 342.31^{f}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	2544.94 ± 174.21^{cd}	1819.01 ± 300.14^{e}
	Arabinose	1642.00 ± 149.04^{b}	ND^{d}	ND^{d}	2321.15 ± 235.85^{a}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	Xylose	44.27 ± 9.97^{b}	ND^{c}	ND^{c}	103.11 ± 10.81^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
	Xylitol	274.75 ± 29.84^{c}	249.77 ± 41.55^{c}	770.65 ± 129.59^{a}	544.33 ± 88.88^{b}	58.69 ± 7.98^{d}	5.43 ± 0.84^{d}	54.50 ± 5.69^{d}	265.96 ± 37.38^{c}	33.91 ± 10.79^{d}	4.11 ± 0.95^{d}	9.72 ± 3.23^{d}
	Arabitol	180.39 ± 14.35^{fg}	576.00 ± 46.90^{c}	351.22 ± 50.42^{d}	1034.95 ± 188.75^{b}	341.02 ± 38.46^{de}	15.60 ± 1.77^{h}	78.21 ± 19.55^{fgh}	27.85 ± 3.46^{h}	124.46 ± 14.93^{fgh}	40.57 ± 4.45^{gh}	14.68 ± 3.69^{h}
	Rhamnose	ND^{f}	86.95 ± 18.79^{a}	48.71 ± 6.63^{cd}	18.01 ± 2.74^{e}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	22.73 ± 5.18^{e}	ND^{f}
	Fructose	311.00 ± 16.22^{d}	68.00 ± 15.57^{d}	175.00 ± 20.08^{d}	253.00 ± 24.25^{d}	123.00 ± 11.63^{d}	28.00 ± 5.18^{d}	45.00 ± 7.42^{d}	263.00 ± 48.19^{d}	59.00 ± 12.27^{d}	9000.00 ± 1521.81^{c}	96088.00 ± 9764.49^{b}
	Glucose	951.70 ± 91.98^{cde}	480.00 ± 116.48^{cde}	1093.60 ± 159.19^{cde}	158.50 ± 69.33^{de}	2228.70 ± 399.78^{cde}	192.20 ± 51.28^{de}	92.30 ± 30.06^{e}	3725.70 ± 183.70^{cd}	118.50 ± 14.36^{e}	9691.70 ± 1313.73^{b}	43757.80 ± 5779.09^{a}
	Sorbitol	ND^{b}	ND^{b}	ND^{b}	ND^{b}	206.28 ± 15.21^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
	Mannitol	189028.00 ± 8076.60^{d}	27376.00 ± 6452.20^{g}	84350.00 ± 5324.60^{f}	225793.00 ± 9377.40^{c}	101.00 ± 18.10^{h}	1177.00 ± 657.80^{h}	6126.00 ± 1579.90^{h}	2412.00 ± 495.80^{h}	255.00 ± 19.40^{h}	1057.00 ± 272.40^{h}	674.00 ± 83.10^{h}
	Inositol	147.27 ± 22.80^{fghij}	361.35 ± 31.69^{cd}	433.84 ± 130.19^{c}	44.84 ± 5.84^{j}	161.92 ± 12.83^{ghi}	194.23 ± 16.38^{efg}	86.85 ± 11.61^{hij}	42.17 ± 7.82^{j}	48.03 ± 14.05^{j}	616.61 ± 64.11^{b}	565.01 ± 63.39^{b}
	Sucrose	479.60 ± 103.41^{e}	10.90 ± 4.32^{e}	75.20 ± 18.57^{e}	127.60 ± 27.37^{e}	5.10 ± 2.35^{e}	46.60 ± 16.60^{e}	7.00 ± 2.17^{e}	53.40 ± 13.40^{e}	42.10 ± 12.22^{e}	7863.80 ± 990.57^{b}	3041.70 ± 515.42^{d}
	Maltotriose	ND^{e}	ND^{e}	ND^{e}	ND^{e}	710.86 ± 107.34^{a}	ND^{e}	ND^{e}	265.27 ± 25.03^{cd}	ND^{e}	164.00 ± 64.50^{d}	276.24 ± 45.72^{cd}
	Panose	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	34.10 ± 4.43^{b}	ND^{c}	ND^{c}	ND^{c}
Major organic acids	Lactate	1572.33 ± 128.19^{c}	1287.87 ± 715.57^{c}	772.68 ± 143.46^{c}	1189.72 ± 203.22^{c}	1051.14 ± 537.64^{c}	850.93 ± 223.49^{c}	773.87 ± 172.93^{c}	8532.86 ± 1477.16^{a}	3947.28 ± 605.71^{b}	1024.36 ± 164.11^{c}	1323.23 ± 190.35^{c}
	Fumarate	111.26 ± 26.06^{cde}	117.31 ± 14.33^{bcde}	263.41 ± 55.73^{bc}	173.17 ± 86.72^{bcd}	509.73 ± 67.68^{a}	36.54 ± 11.32^{de}	38.77 ± 10.20^{de}	146.25 ± 25.98^{bcde}	33.55 ± 11.35^{de}	89.63 ± 37.48^{de}	111.50 ± 24.54^{cde}
	Succinate	1435.31 ± 149.60^{b}	524.14 ± 78.50^{efgh}	622.41 ± 152.68^{defg}	439.22 ± 240.47^{fghi}	521.03 ± 62.75^{efgh}	133.23 ± 29.02^{ij}	568.56 ± 112.64^{defgh}	871.65 ± 107.09^{cd}	270.23 ± 31.55^{hi}	436.30 ± 101.86^{fghi}	679.74 ± 45.23^{def}
	Malate	411.00 ± 69.17^{c}	268.45 ± 31.80^{c}	303.39 ± 35.14^{c}	764.02 ± 208.14^{c}	3885.64 ± 305.17^{b}	375.37 ± 105.98^{c}	317.87 ± 60.77^{c}	779.64 ± 128.14^{c}	184.22 ± 23.50^{c}	621.55 ± 142.07^{c}	717.18 ± 62.85^{c}
	trans-Aconitate	204.37 ± 33.66^{bc}	119.30 ± 36.84^{bc}	676.60 ± 271.33^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	193.50 ± 106.02^{bc}	ND^{c}	709.60 ± 155.34^{a}	ND^{c}
	cis-Aconitate	66.84 ± 23.00^{c}	1454.89 ± 136.61^{b}	363.56 ± 63.75^{c}	41.11 ± 10.72^{c}	19.78 ± 3.28^{c}	26.31 ± 10.60^{c}	19.94 ± 5.87^{c}	36.01 ± 12.47^{c}	12.59 ± 4.91^{c}	3860.52 ± 852.51^{a}	248.29 ± 11.93^{c}
	3PG	41.39 ± 9.24^{cd}	44.95 ± 14.54^{cd}	78.55 ± 8.88^{bcd}	35.53 ± 38.74^{cd}	195.94 ± 23.70^{b}	44.31 ± 18.41^{cd}	ND^{d}	38.02 ± 39.26^{cd}	ND^{d}	ND^{d}	ND^{d}
	Isocitrate	122.80 ± 10.98^{cd}	89.77 ± 14.10^{cdef}	112.81 ± 29.34^{cde}	ND^{f}	ND^{f}	105.37 ± 20.32^{cdef}	54.96 ± 15.20^{def}	70.76 ± 14.14^{def}	ND^{f}	786.54 ± 117.83^{a}	41.55 ± 11.27^{def}
	Citrate	805.46 ± 87.55^{fg}	844.00 ± 182.66^{fg}	2381.47 ± 574.59^{ef}	3731.79 ± 774.12^{cde}	6054.64 ± 561.76^{b}	3410.95 ± 677.47^{de}	1778.86 ± 519.10^{efg}	5028.01 ± 1522.49^{bcd}	4586.35 ± 709.24^{bcd}	ND^{g}	1798.41 ± 203.45^{efg}
	G1P	59.29 ± 7.14^{cde}	ND^{e}	61.62 ± 7.94^{cde}	62.77 ± 16.23^{cde}	253.51 ± 32.28^{b}	ND^{e}	66.57 ± 20.54^{cde}	91.49 ± 14.24^{cde}	56.96 ± 21.11^{cde}	113.21 ± 5.52^{cd}	63.07 ± 25.74^{cde}
	F6P	142.06 ± 13.86^{def}	ND^{g}	71.89 ± 11.30^{efg}	110.40 ± 28.52^{efg}	581.48 ± 128.53^{b}	46.69 ± 8.42^{fg}	102.91 ± 28.00^{efg}	169.09 ± 35.26^{cde}	ND^{g}	ND^{g}	257.56 ± 27.73^{cd}
	G6P	509.49 ± 38.78^{cd}	142.55 ± 29.60^{d}	238.91 ± 32.84^{cd}	357.70 ± 80.57^{cd}	3953.17 ± 765.54^{b}	223.92 ± 24.61^{cd}	221.22 ± 105.25^{cd}	670.21 ± 97.88^{cd}	122.00 ± 49.53^{d}	38.03 ± 18.10^{d}	307.83 ± 18.93^{cd}
	2,3-DPG	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
	6-Phosphogluconate	ND^{e}	ND^{e}	ND^{e}	ND^{e}	317.27 ± 89.06^{b}	ND^{e}	188.06 ± 48.05^{c}	ND^{e}	ND^{e}	ND^{e}	11.94 ± 26.70^{de}
	S7P	348.59 ± 44.77^{def}	ND^{g}	159.56 ± 54.48^{fg}	572.34 ± 150.89^{de}	1045.93 ± 205.50^{b}	11.68 ± 26.11^{g}	38.83 ± 86.82^{fg}	ND^{g}	ND^{g}	ND^{g}	194.59 ± 95.75^{fg}
	UDP-glucose	320.65 ± 34.29^{abc}	148.24 ± 22.48^{fg}	149.61 ± 30.96^{fg}	297.97 ± 70.98^{abc}	140.92 ± 35.82^{fg}	77.37 ± 37.85^{gh}	103.66 ± 23.42^{fg}	92.44 ± 31.76^{gh}	ND^{h}	79.04 ± 26.75^{gh}	255.83 ± 63.54^{cde}

The values indicate as mean ±SD (nmol g^{-1}) of the metabolite concentration of dry weight (n = 5). ND, not detected. Different superscript letters indicate the significant differences (P < 0.05) between each type of species by Tukey HSD analysis. Statistical analysis was compared between all data set (species and extraction methods (Table 3.2, S3.1)).

3.4 Discussion

3.4.1 Taxonomic differences of metabolite profiles

In this study, PCA and HCA generated characteristic clusters from metabolite profiles that were typical for each species, not the extraction method (Figures 3.2, 3.3). By focusing on the clades of the HCA clusters, several taxonomic characteristics were identified by metabolite profiling (Figure 3.3). Among the red algae, *P. pseudolinearis* was not clustered with its taxonomic group in the HCA (Figure 3.3, S3.9) and the PCA score plots (Figures 3.2, 3.4b, S3.1, S3.3). *P. pseudolinearis* belongs to class Bangiophyaceae, whereas the other red algae species are from class Florideophycae. Among the four brown algae, *S. firma* and *P. kuromo* were closely clustered by metabolite profiling, matching to their taxonomic classification in the same family, Chordariaceae (Figures 3.3, 3.4a, S3.3, S3.8). Metabolic profiling on various seaweed from Norway, including 11 brown algae, 7 red algae, and 3 green algae, was performed in a previous study (Belghit *et al*., 2017).

Noteworthy, by using HCA, 391 metabolites were clustered independently in the brown and red algae clades, but there were only two clades of green algae. Although two red algae classes, Bangiophyaceae and Florideophyceae, were included, the red algae species clustered under one clade. In the HCA of the entire data set, the three seaweed groups were not clustered together (Figure 3.3). PC1 was mainly affected by the metabolite characteristics of *P. pseudolinearis*. However, the PCA score plot (PC2–PC3) suggests that characteristic metabolite profiles existed in three seaweed groups (Figure 3.2b, Figure S3.1: PC2–PC3). The main contributing factor of PCA separation in three groups was the high concentration of some metabolites, such as mannitol (brown algae) and sucrose (green algae).

The formation of distinct clades linked to the algae species had been observed in related studies. A fatty acid profiling study on various tropical marine algae indicated clear trends within the respective phyla in the PCA separation variance and HCA (Kumari *et al*., 2013). Endosymbiotic relationships mainly contributed to the fatty acid profiles because red and green algae evolved from primary endosymbiosis of cyanobacteria whereas brown algae from secondary endosymbiosis of red algae (Ryall *et al*., 2003; Chan

et al., 2012; Kumari *et al*., 2013). These relationships may have caused the differences of the clade arrangements.

Among all metabolite profiling studies of various seaweed including this work, several data set indicate clear PCA clusters (Figure 3.2b) or HCA (Figure 3.8), but some data set do not (Figure 3.2a, Figure 3.3), depending on the selection of the metabolites and algae species. Because the type of data affected the cluster patterns generated by PCA and HCA, the metabolite types (sugars and amino acids) selected for HCA were also discussed.

3.4.2 Sugar profiling

Monosaccharides play important roles in food storage, macromolecule synthesis and energy transfer from phototropic to heterotrophic pathways that are essential for living organisms (Ortiz-Tena *et al*., 2016). The sugar profiles (Figure 3.8) formed a taxonomic tree representing the three seaweed groups, the brown, red and green algae, which were not created using amino acids and major organic acids (Figure 3.6) involved in TCA and glycolysis (Figure 3.7). The sugar characteristics mainly contributed to the formation of the seaweed groups (Figure 3.8).

Mannitol is the main constituent in brown algae, which is consistent with other studies (Rousvoal *et al*., 2011; Groisillier *et al.*, 2014; Belghit *et al*., 2017), whereas fructose, sucrose, and glucose are characteristic in green algae (Thompson, 1996; Kim *et al.*, 2016). The characteristic metabolites in green algae are similar to those in land plants due to the evolutionary relationship; green plants originated from green algae. Thus, the taxonomic clades were closely clustered (De Clerck *et al*., 2012). In red algae, the sugar profiles depended on the species. Sorbitol is significant in *P. pseudolinearis* and panose in *D. sessilis* (Figure 3.8). The red algae sugar profiles have a similar tendency as the monosaccharides study on algae from the Eastern Mediterranean Sea. The results showed that the red algae species has the largest variation in monosaccharide diversity as compared to those in brown and green algae (Robin *et al*., 2017). This study suggests that the characteristic profiles are closely linked to the species.

3.4.3 Amino acid profiling

Remarkably, most amino acids were found in all species; however, individual concentrations depended mainly on the species and varied between the algae groups (Figure 3.6). It is well established that both essential and non-essential amino acids are available at different concentrations throughout the year, though depending on the season and the environmental conditions (Wong & Cheung, 2001b). Among the amino acids, the Ala concentration was the highest in all the algae species (Figures 3.3, 3.6, Table 3.2). Unlike in terrestrial or freshwater plants, many marine algae experienced changing water levels due to the high and low tides that generate an oxygen flux, which stimulates changes in the metabolism to increase the Ala concentration (Narsai *et al*., 2011). Glu and Asp, the acidic amino acids, are linked to the umami taste of the seaweed and also play an essential role in the nitrogen transport system and macromolecule storage (Bolton, 2009; Fleurence, 2010; Biancarosa *et al.*, 2017). Studies conducted by other research groups showed that brown algae have a higher level of Glu and Asp as compared to those in red and green algae (Lourenço *et al.*, 2002; Diniz, 2011; Biancarosa *et al*., 2017). However, in this study, the levels of Glu and Asp (Figures 3.3, 3.6, Table 3.2) were species-dependent and not based on the seaweed group.

In term of nutritional aspect, nine essential amino acids (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val) are necessary for human life which cannot be produced by the human body. Among the various edible seaweed, *P. pseudolinearis* has the highest concentrations of five essential amino acids (Thr, Ile, His, Leu, and Val) and eight non-essential amino acids (Cystine, Gly, Ser, Tyr, Arg, Ala, Gln, and Asn). While high concentrations of Met and Phe are contained in *U. pertusa* and *G. elegans*, respectively. In Japan, the dried *Pyropia sp*. is commonly consumed as Nori (Taboada *et al.*, 2013). The high nutritional value contained in this edible seaweed is not only able to promote good health, but also to develop into new functional food ingredient.

3.4.4 Effect of the extraction methods on the metabolite profiles

To identify an optimized extraction method, sample processing was performed by extraction in methanol-water with or without chloroform. No significant differences in the metabolic profiles were found in all the species that could be linked to the extraction method (Figures 3.2, 3.3, 3.4, 3.5). However, some effects of the extraction method on the metabolite concentrations were observed in the individual seaweed groups, the brown algae (Figure S3.3: PC4), the red algae (Figure S3.4: PC5), and the green algae (Figure S3.5: PC2); but the effect on the outcome was minimal.

In summary, the study showed that species-specific differences had the most effect on the metabolite profiles in various seaweed (Figure 3.2, 3.3, S3.1). This study generated a similar result, in which species characteristics were the main contributor to differences in the metabolite profiles followed by different pre-treatment methods (Hamid *et al.*, 2018).

3.4.5 Metabolite characteristics of each species

The specimens analyzed in this study were dried before further processing to avoid bias in metabolite quantification due to the difference in moisture content (Gupta *et al*., 2014). The results showed that the dry weight percentage varied significantly between the species (5 %–47 % [w/w] dry weight), but it did not depend on the seaweed groups (Table 3.1). These results were consistent with study previously done on three edible brown algae, which indicated that species identity was the main factor affecting the dry weight percentage (Hamid *et al.*, 2018). The dry weight percentage also contributed to the diversity of the metabolite profiles of each algae species. In addition to the dry weight percentage, previous research showed that the metabolite diversity may also be related to the variation in the environmental conditions that affect the metabolite profile of an organism, such as the season, sample collection time, and location (Kim & Verpoorte, 2010; Gupta *et al*., 2013; Manns *et al*., 2014; Ernst *et al*., 2014; Hamid *et al*., 2018).

Seaweed studies such as on *Undaria pinnatifida* (Zhou *et al*., 2015), *Fucus spiralis* (Paiva *et al*., 2018) and some seaweed from Mediterranean Sea coast, Egypt (El-Said & El-Sikaily, 2013) showed that the chemical composition such as total nitrogen, amino acid, and antioxidant properties of seaweed differed due to seasonal variability,

growth condition, location, plant parts, and environmental changes. In the case of amino acids, this data showed that no seaweed group characteristics were observed. Instead, the distinct seaweed group characteristics were found in sugar compounds, even though the samples were collected at different locations, seasons and time. By focusing on the environmental conditions, further metabolomics studies would contribute to the knowledge about the effects on metabolite profiles.

Chapter 4

Effect of blanching on the metabolites profiling in Wakame and Mekabu

4.1 Introduction

Blanching is one of the most important processes used in food preparation and preservation. By heating for a certain period of time, enzymes present in the food are inactivated, thereby improving the texture, color, and flavor of the food making it more suitable for eating or preservation. The most common and simple blanching methods are either by immersion of the raw food in hot water or by steaming. Other methods for blanching include microwaving and infrared heating (Fellows, 2000; Reyes De Corcuera *et al.*, 2004). During the blanching process, the temperature, blanching time, the heating method, and the food type and size all affect the food quality (Fellows, 2000). Previous studies have evaluated the effects of blanching on the color, taste, texture, and nutrients among different blanching methods, such as the use of hot water, microwave, steam, and infrared heating (Jamali *et al.*, 2018; Lane *et al.*, 1984; Quarcoo, 2016; Ruiz-Ojeda & Peñas, 2013) in various types of vegetables (Chhe *et al.*, 2018; Jain *et al.*, 2013; Jamali e *et al.*, 2018; Lo *et al.*, 2002; Mosha *et al.*, 1995; Sugimoto *et al.*, 2010; Tijskens *et al.*, 2001).

In Japan, consuming shellfish and seaweed as daily foods has been reported in the literature from as far back as A.D. 701 (Nisizawa *et al.*, 1987). Common edible seaweed such as *Porphyra sp.* (Nori), *Laminaria sp.* (Kombu), and *Undaria pinnatifida* (Wakame and Mekabu) are consumed either after drying, salting, or blanching in hot boiling water (Kurokura, 2004; Nisizawa *et al.*, 1987). Among all the edible seaweed, the leafy parts of *U. pinnatifida,* (Wakame) and the sporophyll parts (Mekabu) are highly consumed in Japan and Korea in soups or salads (Fung *et al.*, 2013; Pang *et al.*, 2015).

For salads, the raw Wakame or Mekabu are immersed in hot boiling water until the color changes from brown to green.

U. pinnatifida is also considered to be one of the healthiest marine vegetables because of its high nutritional content; essential amino acids, vitamins, fibers, and trace minerals (Nisizawa *et al.*, 1987; Taboada *et al.*, 2013). Most *U. pinnatifida* studies have focused on its bioactive compounds such as carotenoids, fatty acids, and phytosterols that have high anti-oxidant (Kolb *et al.*, 2004; Kumar *et al.*, 2008), anti-inflammatory (Ale & Meyer, 2013; Jaspars & Folmer, 2013; Wang *et al.*, 2018) and anti-cancerous properties (Synytsya *et al.*, 2010; Wang *et al.*, 2018; Zhang *et al.*, 2014; Zorofchian Moghadamtousi *et al.*, 2014). *U. pinnatifida* is rich in fucoxanthin (Miyashita *et al.*, 2013; Prabhasankar *et al.*, 2009) and is able to reduce blood pressure (Kumar *et al.*, 2008) and increase the quality and shelf-life of food (Dawczynski *et al.*, 2007; Liu *et al.*, 2016). However, a comprehensive metabolite study, after different times of blanching of the two parts of *U. pinnatifida*, Wakame and Mekabu, has not yet been conducted.

Metabolomics is an emerging technology that is used to comprehensively understand metabolite profiles in biological systems at specific time points. By using this metabolomics approach, this study aimed to investigate the effect of different blanching times on the concentration of metabolites remaining in the Wakame and Mekabu samples. The preferred blanching time that is able to retain the highest compound of metabolites in both Wakame and Mekabu will be provided to the consumer as well as to the Wakame and Mekabu-based food industry. This comprehensive study of the effect of heat on metabolite concentration contributes to a deeper understanding of the blanching process on the sensory and nutritional qualities.

4.2 Materials and methods

4.2.1 Seaweed samples

Fresh *Undaria pinnatifida*, Wakame (the leafy top part) and Mekabu (the sporophyte bottom part) were purchased from a supermarket in Tsuruoka City, Yamagata, Japan. Both samples were collected from Yura Port (38°43'02.7"N 139°40'48.6"E), located near the Shonai coastal area in the Yamagata Prefecture (Sea of Japan).

4.2.2 Blanching process

The mid-rib part of the purchased Wakame and Mekabu were removed, cut, and weighed approximately 1 g each. For replicates, six samples (1 g each) were exposed to the same heat treatment using a sieve partitioned into six compartments. Before blanching, 2 L of Milli-Q water was boiled using an alumite pot. The samples contained in the sieve were immersed into 2 L of boiling Milli-Q water for 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 240, 300, and 420 s. Five of the replicate samples were used for metabolome analysis, and one was used for sensory evaluation. Samples without blanching were used as the 0 s sample. After each blanching process, the samples for the metabolome analysis were immediately placed into a polyethylene-polypropylene non-woven fabric bag (11.0 × 10.5 cm, Dashi bag, Daiso, Hiroshima, Japan) and quenched in liquid nitrogen to stop any further biochemical processes. After being freeze-dried, the samples were stored in a −80 ºC freezer (MDF-U482-PJ, Panasonic Corp., Kadoma, Osaka, Japan) for over 24 h.

4.2.3 Sample drying

To avoid the interference of any moisture content with metabolite quantification, all samples were freeze-dried (Hamid *et al.*, 2018). In advance, the freeze-trap (Freeze Trap VA-800R, Taitec Corp., Saitama, Japan) was set to −70 °C and < 20 Pa, before the completely frozen samples (−80 ºC, >24 h) were placed into the freeze-dryer vessels. A vacuum pump (GLD-137CC ULVAC, Taitec Corp., Saitama, Japan) was used to maintain the temperature and pressure. After 4 days, and when the pressure stabilized at < 0.5 Pa for more than 24 h, the freeze-drying process was stopped and the dried samples were collected from the vessels and weighed. Drying ratio were calculated as following equation.

Drying ratio (%) = (Freeze-dried sample weight (g)) × 100 / (Raw sample weight (g))

4.2.4 Color analysis

Images of the Wakame and Mekabu after blanching were obtained using a digital camera and calibrated using a standard color scale, CASMATCH (Bear Medic Corp. Tokyo, Japan) (Suehara *et al.*, 2017). The image color and size were adjusted using the CASMATCH and the image editing software program (Adobe Photoshop; Adobe Systems Inc. San Jose, CA, USA). Even though CASMATCH has a nine color section on the sheet, the color correction can be achieved using three achromatic colors; white, grey, and black (Suehara *et al.*, 2017). Accordingly, this study performed the color correction using these three colors. The color changes after blanching for different time intervals in boiling water were then evaluated (Figure 4.1, 4.2). For each time point, five replicates were analyzed for color using ImageJ (ImageJ 1.52e, Wayne Rasband, NIH, USA). The color levels were evaluated using the International Commission on Illumination (CIE). The color in the digital image are measured based on the lightness, L^* [from black (darkest black = 0) to white (brightest white = 100)], redness, a^* [from green (negative direction) to red (positive direction)], and yellowness, b^* [from blue (negative direction) to yellow (positive direction)].

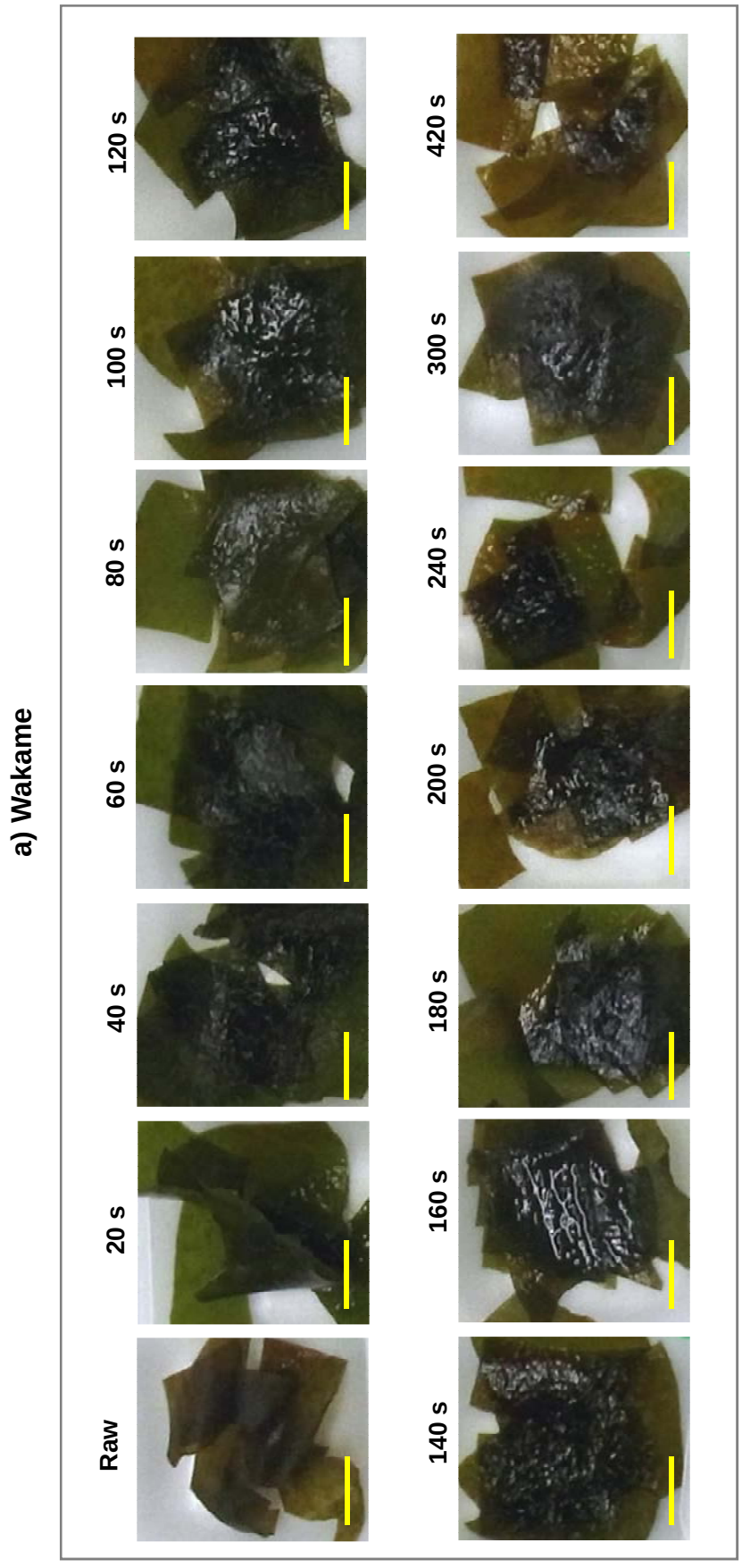

Figure 4.1a The photograph of Wakame samples after different blanching time.

Each sample were blanched in boiling water at 0, 20, 40, 60, 80, 100, 140, 160, 180, 200, 240, 300, and 420 s. Scale bars indicate 1 cm length of the original sample.

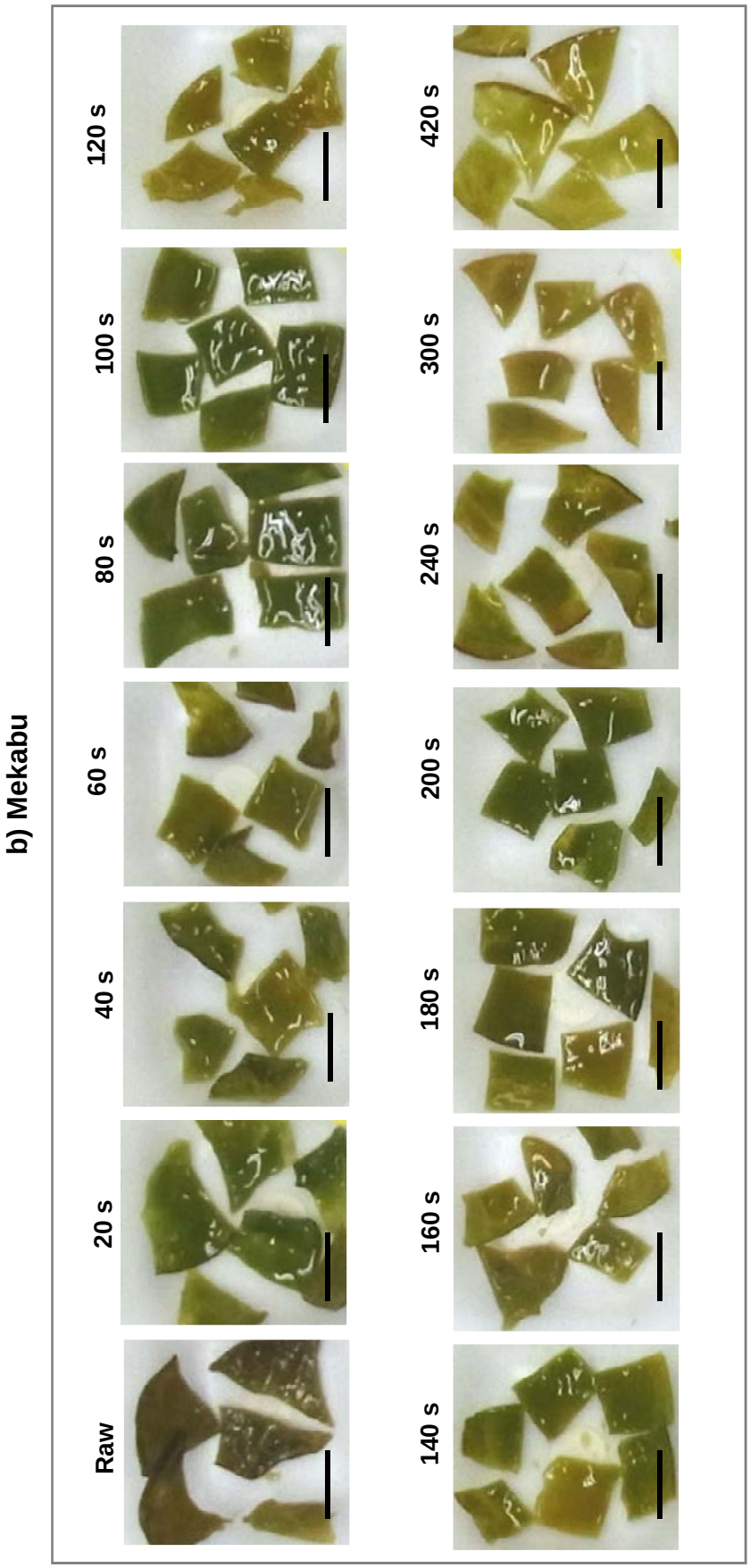

Figure 4.1b The photograph of Wakame samples after different blanching time.

Each sample were blanched in boiling water at 0, 20, 40, 60, 80, 100, 140, 160, 180, 200, 240, 300, and 420 s. Scale bars indicate 1 cm length of the original sample.

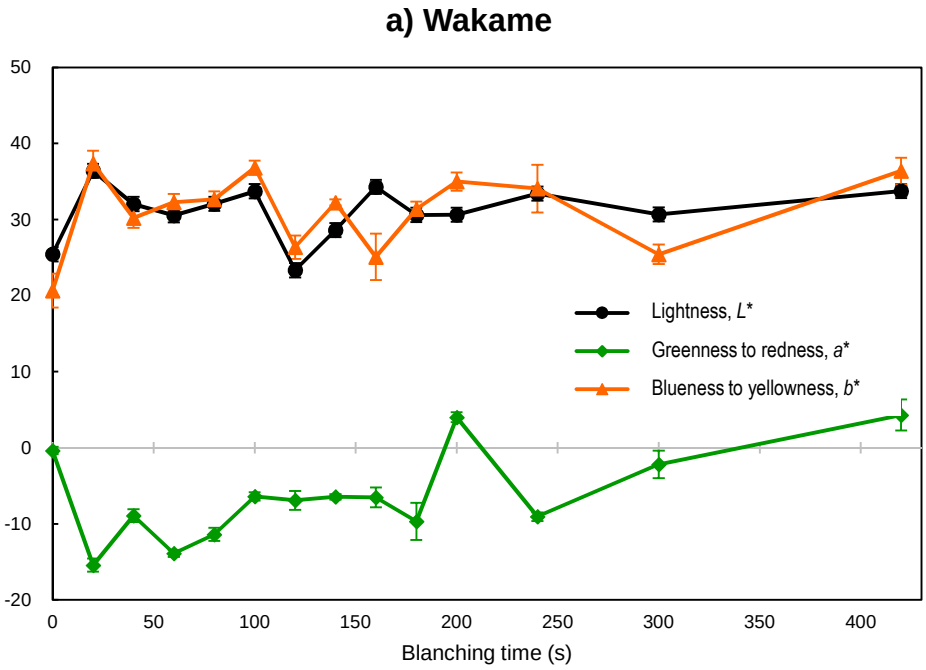

Figure 4.2a Color analysis of Wakame using ImageJ.

The color levels were evaluated by International Commission on Illumination (CIE); the color in the digital image are measured based on the lightness, *L** [from black (darkest black=0) to white (brightest white = 100)], redness, *a** [from green (negative direction) to red (positive direction)] and yellowness, *b** [from blue (negative direction) to yellow (positive direction)]. The values indicate the average ±SD, *n=5*.

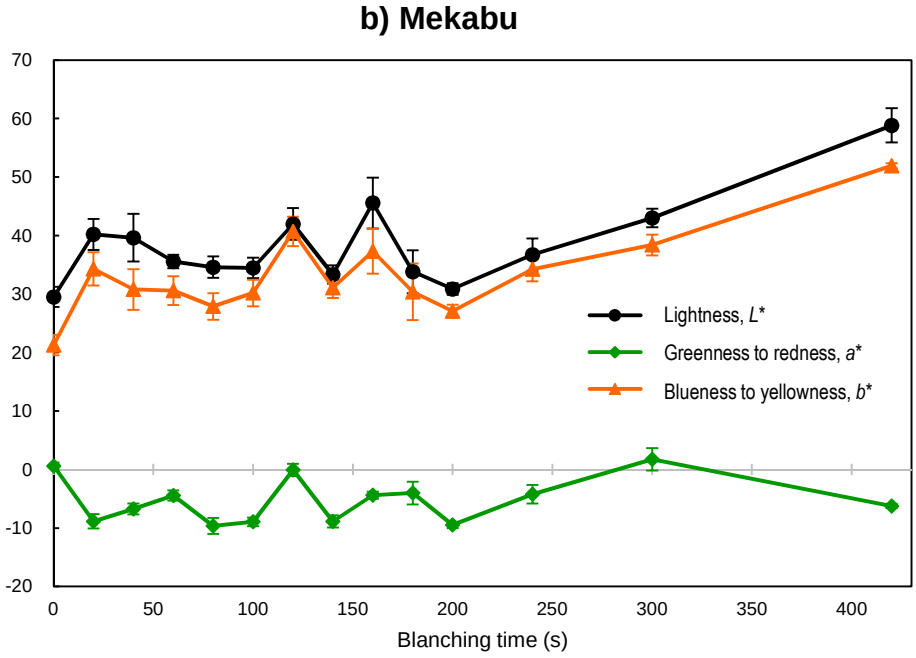

Figure 4.2b Color analysis of Mekabu using ImageJ.

The color levels were evaluated by International Commission on Illumination (CIE); the color in the digital image are measured based on the lightness, *L** [from black (darkest black=0) to white (brightest white = 100)], redness, *a** [from green (negative direction) to red (positive direction)] and yellowness, *b** [from blue (negative direction) to yellow (positive direction)]. The values indicate the average ±SD, *n=5*.

4.2.5 Sample selection for metabolome analysis using sensory evaluation

For metabolome analysis, the samples were selected based on the sensory evaluation. The sensory evaluation of the blanched *U. pinnatifida* samples was carried out by six panelists. The panelists did not undergo any specialized training but were familiar with the seaweed. Each sample was graded on its appearance (green color), odor (seawater smell), umami strength, and texture (chewiness and stickiness). The grading of the odor, umami strength and texture were based on a five hedonic scale with scores ranging from five (strong) to one (weak). The green color was graded from five (dark green) to one (brown). Based on the preferences of the six panelists, evaluation of the metabolite profiles was limited to eight different blanching times; 0 (raw), 20, 40, 60, 100, 140, 240, and 420 s.

4.2.6 Chemicals and reagents

All reagents used in this study were of analytical or higher grade of purity. For the identification and quantification of each metabolite, a standard reagent was purchased and mixed for standard solution. The following internal standard solution (IS1) was prepared to normalize the quantification. An IS1 stock solution (200 μM) in methanol was made by mixing 2-(*N*-morpholino) ethanesulfonic acid (MES) (Cat. No. 349-01623; Dojindo Laboratories, Kumamoto, Japan) and D-camphor-10-sulfonic acid (CSA) (Cat. No. 037-01032; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and was used as the anionic standard solvent. L-methionine sulfone (Cat. No. 502-76641; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the cationic standard solvent, whereas $^{13}C_6$-glucose (No. 404624; Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the sugar standard solvent. To standardize the capillary electrophoresis mass spectrometry (CE-MS) migration time, 200 μM stock solutions of trimesate (Cat. No. 206-03641; Wako Pure Chemical Industries, Ltd.), and 3-aminopyrrolidine (Cat. No. 404624; Sigma Aldrich Corp) were prepared by diluting in Milli-Q water.

4.2.7 Sample extractions

Approximately 100 mg of the freeze-dried sample was weighed into a 13 mL disruption tube and the sample disrupted using a cell disruptor (Multi beads shocker, Yasui-Kikai, Osaka, Japan) equipped with metal corn (1 cm height × 1 cm diameter metal cylinder) and run at 1,500 rpm for 60 s without solvent. Following this, 10 mg of the powdered sample was weighed into a 2 mL tube, 500 µL of 200 µM IS1 in methanol was added and the sample mixed using a micro-mixer (Micromixer E36, Taitec Corp., Saitama, Japan) for 3 min before being diluted with 500 µL of Milli-Q water (Wakayama *et al.*, 2010). The samples were vortexed for 3 min and centrifuged at 120,000 × *g* for 10 min at 4 °C in a MX-307 centrifuge (Tomy Seiko Co. Ltd., Tokyo, Japan). The aqueous top layer (~400 µL) was transferred into ultra-filtration tubes (MW 5000 kDa; HMT, Inc., Tsuruoka, Japan) after centrifugation. The filled ultra-filtration tubes were then centrifuged at 9,100 × g at 4 °C for 3 h. The filtrate (~30 µL) was collected and aliquoted into LC vials for sugar analysis. The LC vials were kept in a freezer at −80 °C until analysis. The remaining filtrate (~100 µL), was aliquoted into 1.5 mL tubes and evaporated at 4 °C until dry using a refrigerated spin dryer (CentriVap Concentrator, Labconco Corp., Kansas City, MO, USA). Following this, the dried samples were stored at −80 °C until CE-MS analysis. To normalize the CE-MS migration time, the dried evaporated samples were dissolved in 20 µL of Milli-Q water mixed with 200 µM trimesate and 3-aminopyrrolidine. For each sample 10 µL was analyzed by CE-MS to measure cationic and anionic metabolites.

4.2.8 Free sugar analysis by liquid chromatography-quadrupole mass spectrometry (LC-MS/MS)

For free sugar analysis, an API 3000 quadrupole mass spectrometry (MS/MS) system (Sciex, Framingham, MA, USA) fitted with an Agilent 1100 series liquid chromatography (LC) system; a column oven (Agilent Technologies, Santa Clara, CA, USA), an LC binary pump, and an autosampler was used. For sugar and sugar alcohol separation, a hydrophilic interaction chromatography (HILIC) amino column (Asahipak NH2P-4E; 4.6 mm inner diameter × 250 mm length; 5 µm; Showa Denko K.K., Tokyo, Japan) was used. The gradients, injection volume, and column oven temperature used were similar to those reported in a previous study (Hamid *et al.*, 2019). Using the system in turbo spray mode,

the pressure in the nebulizer, curtain, collision gas chamber, ion spray voltage, and ion source temperature were set at 15 psi, 11 psi, 8 psi, −4500 V, and 500 °C, respectively. For enhancing the detection limits and selectivity of each sugar, multiple reaction monitoring (MRM) modes were selected for MS analysis.

4.2.9 Analysis of free amino acids, organic acids, and charged metabolites by capillary electrophoresis- mass spectrometry (CE-MS)

All CE-MS analyzes were performed using a G7100A Agilent CE-MS system consisting of an Agilent G6220A LC/MSD TOF, an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies).

4.2.10 Cationic metabolite analysis

An uncoated fused silica capillary (50 μm inner diameter × 100 cm length) filled with 1 M formic acid as an electrolyte (Hirayama *et al.*, 2014; Soga & Heiger, 1998) was used to analyze cationic metabolites such as amino acids and amines. To stabilize the ESI sprayer ionization, a sheath solution (methanol-water, 50 % v/v) containing 0.1 μM hexakis(2,2-difluoroethoxy)phosphazene was delivered at 10 μL min^{-1} via an HPLC pump in isocratic mode. Then, approximately 3 nL of the sample was injected into the system at 50 hPa for 5 s. The CE voltage was maintained at +30 kV, the capillary temperature thermostat was set at 20° C, and the sample tray temperature was cooled using a cooler system (TRL 108H Circulation type handy, Thomas, Tokyo, Japan). Time-of-flight mass spectrometry (TOF-MS) was conducted in positive ion mode. The capillary, fragmentor, skimmer, and OCT RFV voltages were set at 4,000 V, 75 V, 50 V, and 500 V, respectively. To stabilize the MS ionization, the drying nitrogen gas flow rate was set at 10 L min^{-1}, the heater temperature was set at 300° C, and the nebulizer gas pressure was set at 7 psi (48.2 kPa). The spectrum exact mass data were acquired at 1.5 cycles s^{-1} over the range 50–1,000 *m/z* and recalibrated automatically using the reference masses of the sheath liquid ($[2MeOH+H_2O+H]^+$, *m/z* 66.06306) and protonated hexakis(2,2-difluoroethoxy)phosphazene ($[M+H]^+$, *m/z* 622.02896).

4.2.11 Anionic metabolite analysis

A cationic polymer coated COSMO (+) capillary (50 µm i.d. × 105 cm length) (Nacalai Tesque, Kyoto, Japan) (Soga *et al.*, 2009) filled with 50 mM ammonium acetate (pH 8.5) was used as an electrolyte to analyze anionic metabolites such as organic carboxylic acids and sugar phosphates. The capillary temperature thermostat was set at 20°C and the sample tray was kept cool using a cooler system. A sheath solution of 5 mM ammonium acetate in methanol-water (50 % v/v) containing 0.1 µM hexakis(2,2-difluoroethoxy)phosphazene was delivered at a rate of 10 µL min^{-1} to the electrospray interface fitted with a 1:100 splitter using an Agilent 1100 series isocratic pump. The circulated sheath liquid surrounding the outside of the electrospray and CE capillary allowed for a stable electrical connection between the capillary tip and the grounded electrospray needle. The capillary was flushed with 50 mM acetic acid (pH 3.4) for 2 min followed with an electrolyte solution for 5 min before starting each sample injection. Samples (30 nL) were injected at 50 hPa for 30 s. CE voltage was maintained at −30 kV after each injection. The MS capillary, fragmentor, skimmer, and OCT RFV voltages were set to −3,500 V, 100 V, 50 V, and 500 V, respectively. To stabilize the MS ionization, the drying nitrogen gas flow rate was set at 10 L min^{-1}, the heater temperature was set at 300 °C and the nebulizer gas pressure was set to 7 psi (48.2 kPa). The spectrum exact mass data were acquired at 1.5 cycles s^{-1} over the range 50–1,000 *m/z* and automatically recalibrated with two reference masses, [^{13}C isotopic ion of deprotonated acetic acid dimer ($2CH_3COOH\text{-}H)]^-$, *m/z* 120.03834, and [hexakis(2,2-difluoroethoxy)phosphazene + deprotonated acetic acid ($CH_3COOH\text{-}H)]^-$, *m/z* 680.03554.

4.2.12 Peak quantification and statistical analysis

Raw data for sugars obtained from LC-MS/MS were analyzed with Sciex Analyst v. 1.4.2. Cationic and anionic metabolite data derived from CE-TOF-MS were collected using Agilent Mass Hunter v. B06.00 software. Metabolite peaks were analyzed with Masterhands v. 2.17.3.17 a propriety software from Keio University (Sugimoto *et al.*, 2012). All data were merged using an in-house macro program run in Microsoft Excel v. 2013 (Microsoft Corp, Redmond, WA, USA). All metabolite concentrations were calculated on the basis of dry weight and were analyzed by JMP v. 13.2.1 (SAS Corp., Cary, NC, USA). Principal component analysis (PCA) (Figure S4.2), and hierarchical

clustering analysis (HCA) (Figure S4.3) were performed on all data set. HCA was performed using Ward's methods (Murtagh & Legendre, 2014). Two-way clustering was performed on metabolite concentrations and sample conditions. To elucidate any trends in metabolite concentrations, HCA was performed separately on the Wakame and Mekabu derived samples based on the blanching time (Figures 4.4–4.5, S4.4–S4.5).

4.3 Results

4.3.1 The effect on blanching time on the color of Wakame and Mekabu

The color of Wakame and Mekabu changed over the different blanching times (Figure 4.1). These color changes were analyzed from photos using the International Commission on Illumination (CIE) and ImageJ software. Within 20 s of blanching, lightness (L^*) and yellowness (b^*) in both Wakame and Mekabu were drastically increased, whereas redness (a^*) decreased to a negative value (Figure 4.2). A negative a^* indicates that a greener color was observed in the samples (Figure 4.1). For Wakame, the greenest value was seen at 20 s of blanching compared to the other blanching times (Figure 4.2a). Up to 200 s of blanching, L^*, a^*, and b^* were found to vary in both Wakame and Mekabu. After 240 s of blanching, a^* continuously increased in Wakame with the color changing from green to light brown (Figure 4.1a). Similarly, L^* and b^* increased in Mekabu (Figure 4.2b) resulting in sample discoloration or fading (Figure 4.1b).

4.3.2 Selection of samples for the metabolome analysis using a sensory test

In order to select the samples to be used for the metabolome analysis, each blanched sample was evaluated for several sensory indices by a group of six panelists. The tendency for color to change from brown (raw) to green (20 s of blanching) by the color analysis (Figures 4.1, 4.2) was also found by panelists (Figure S4.1). The panelists felt that a greener color could be seen in Wakame compared to Mekabu after 20 s of blanching. The panelists felt that the Wakame color was browner after 60 s of blanching, and the color faded in Mekabu after 200 s of blanching (Figure S4.1). In Wakame, the score for crunchiness was the highest at 40 s of blanching, before it decreased gradually. In contrast, in Mekabu, crunchiness increased after 80 s of blanching. For stickiness, the highest score was observed in Mekabu sample at 20 s of blanching but decreased gradually as the blanching time increasing. For both Wakame and Mekabu, the panelists agreed that the umami strength and the odor of seawater decreased with longer blanching times (Figure S4.1). From these sensory evaluation test, it appeared that an early blanching time is crucial for color changes (Figures 4.1, 4.2) and good taste attributes (Figure S4.1). Thus, for metabolome analysis, the following blanching times for Wakame and Mekabu samples were selected: 0 (raw), 20, 40, 60, 100, 140, 240, and 420 s.

4.3.3 Moisture analysis

After blanching, all Wakame and Mekabu samples were quenched rapidly in liquid nitrogen and freeze-dried. From the sample weights before/after freeze drying, the drying ratios were calculated. In both Wakame and Mekabu, the raw samples showed the maximum drying ratio compared to the other blanching times (Figure S4.2). Before 300 s, the Mekabu drying ratios were higher than for Wakame. In Wakame, the drying ratio drastically decreased within 20 s whereas in Mekabu, the drying ratio tended to be decrease gradually. A small increase in the drying ratio was found in Mekabu at 100 s, and in Wakame at 80 and 240 s. The drying ratios in both Wakame and Mekabu became almost constant after 300 s (Figure S4.2).

4.3.4 Overview of metabolites profiles in Wakame and Mekabu

In order to evaluate general changes in metabolites between Wakame and Mekabu after different blanching times, a principal component analysis (PCA) was performed on the entire data set (Figures S4.3, S4.4). The PCA indicated that the blanching time mainly affected metabolite concentrations as shown by PC1 (63.2 %) (Figure S4.3a; see the arrows). The differences between Wakame and Mekabu affected by the metabolite concentration were shown in PC2 (14.2 %) (Figure S4.3a see the eclipse: green indicates Wakame and pink indicates Mekabu). In the loading plot, most metabolites were located at the positive side of PC1 (Figure S4.3b; PC1 positive side). This shows that high metabolite concentrations were observed in both of the raw samples (Wakame and Mekabu) as well as in Mekabu after short blanching times (20 to 140 s).

To understand the changes in metabolites tendency in each sample, a PCA was performed on Wakame independently (Figures 4.3a, S4.5). The score plots for PC1 showed 79.7 % separation variance, which revealed a clear separation between the raw Wakame, 20 s blanched, and more than 20 s blanched samples (Figures 4.3a, S4.5). The PC1 loading plot showed that most of the metabolites, including amino acids (Ile, Asn, Ala, Gln, Ser, His, Glu and Leu) were found on the positive side of the plot (Figure S4.5b). This indicates that these metabolites were detected at high concentrations in the raw sample. After blanching for more than 20 s, metabolites such as organic acids (dodecanoate, hexanoate, decanoate, octanoate, and 4-oxopentanoate) were found on the negative side of the PC1 plot (Figure S4.5b).

For Mekabu, the PCA showed that the metabolites were clustered into three groups; i) raw to 40 s, ii) 60 s to 140 s, and iii) 240 s to 420 s with a separation variance of 60.5 % in PC1 (Figures 4.3b, S4.6). Similar to Wakame, in the loading plots, most of the metabolites were high on the PC1-positive side compare to the negative side (Figures 4.3b, S4.6).

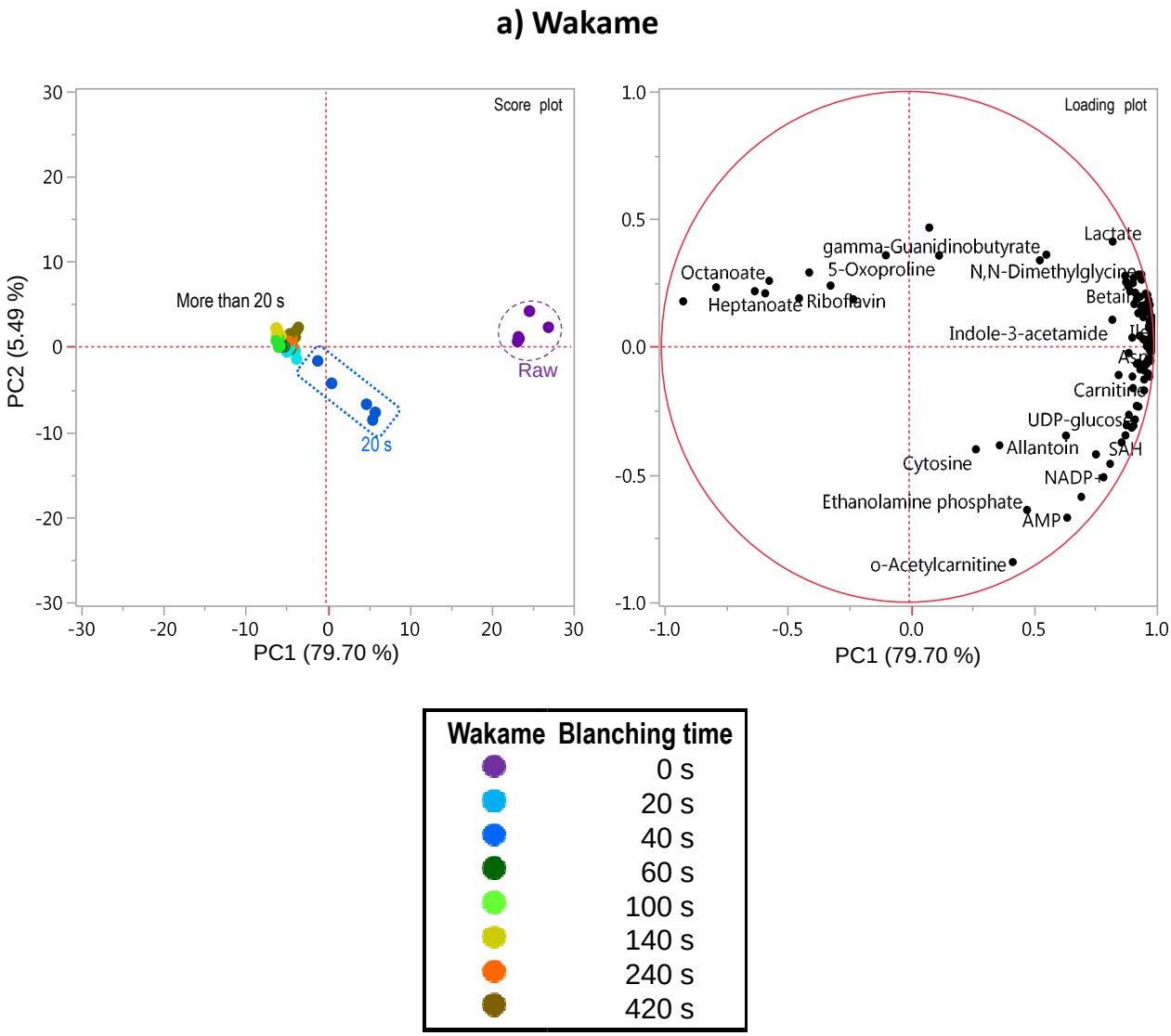

Figure 4.3a PCA of Wakame dry weight based metabolite concentrations after blanching.

For eight different times; 0 (purple), 20 (dark blue), 40 (light blue), 60 (dark green), 100 (light green), 140 (yellow), 240 (orange), and 420 s (olive brown). The horizontal axis value indicates the PC1 separation variance and the vertical axis value indicates the PC2 separation variance. Precise PCAs (PC1 to PC5) including the score and loading plots are shown in Figure S4.5-S4.6.

b) Mekabu

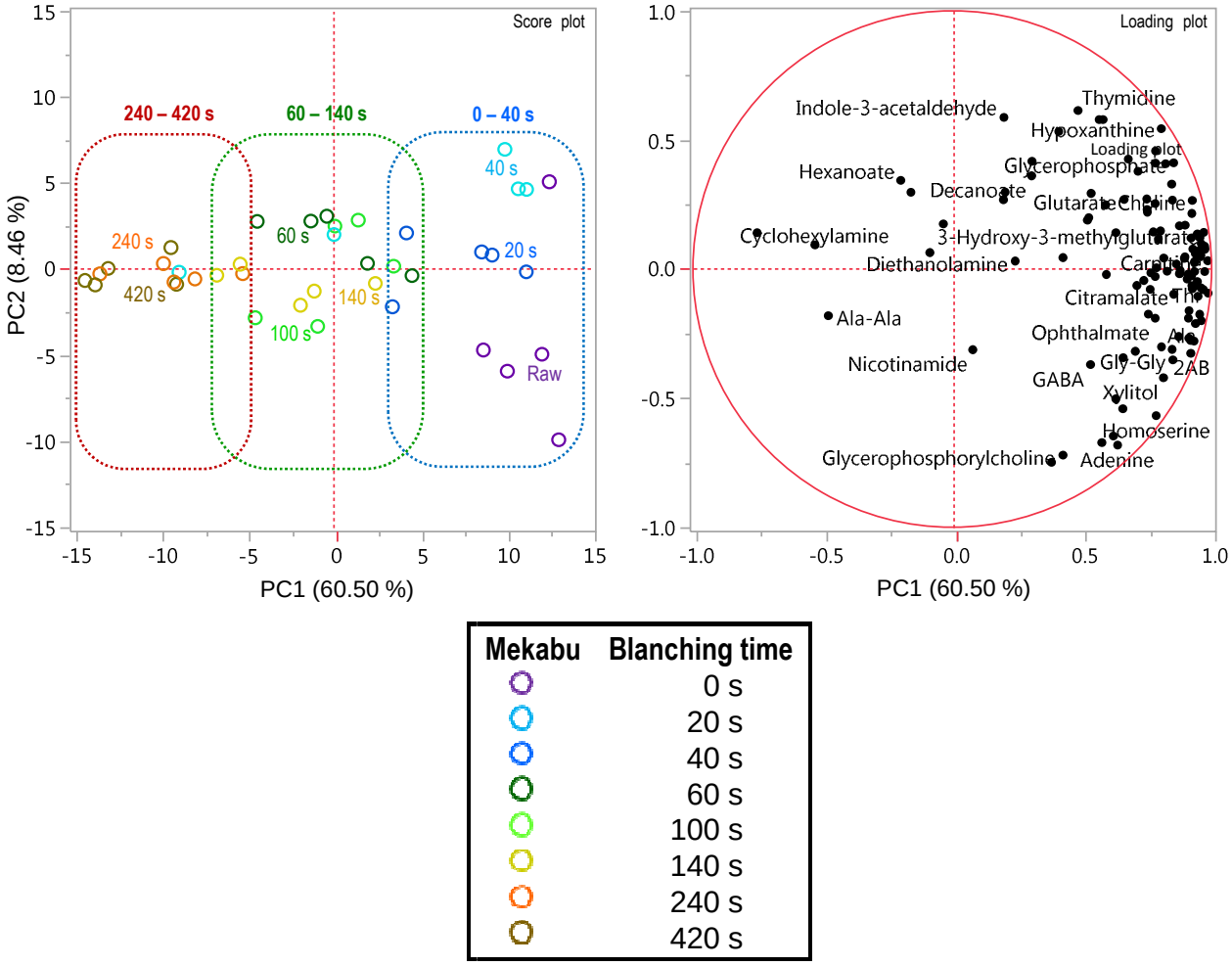

Figure 4.3b PCA of Mekabu dry weight based metabolite concentrations after blanching.

For eight different times; 0 (purple), 20 (dark blue), 40 (light blue), 60 (dark green), 100 (light green), 140 (yellow), 240 (orange), and 420 s (olive brown). The horizontal axis value indicates the PC1 separation variance and the vertical axis value indicates the PC2 separation variance. Precise PCAs (PC1 to PC5) including the score and loading plots are shown in Figure S4.5-S4.6.

4.3.5 Hierarchical clustering analysis and heat map metabolites profiles in Wakame and Mekabu

A hierarchical clustering analysis (HCA) and was performed on the five replicate samples replicates for each of the eight different blanching times of Wakame and Mekabu after extraction using the methanol-water method, which simultaneously generated 125 metabolites (Figure S4.4). The HCA heat map was mainly characterized by the two different seaweed sample types, Wakame and Mekabu. High concentrations of amino acids and organic acids were detected in the raw and 20 s blanched Wakame samples as well as in the Mekabu raw and less than 140 s blanched samples (Figure S4.4).

In Wakame, a total of 123 metabolites were detected, with most of the metabolite concentrations being drastically reduced after 20 s of blanching (Figure 4.4). Some metabolites (isoamylamine, *N,N*-dimethylglycine, 3-hydroxybutyrate, adipate, glutarate, and 2-oxoglutarate (Figure 4.4; blue frame)) were only present in the raw sample. In contrast, after 20 s of blanching, 11 metabolites (cyclohexylamine, heptanoate, octanoate, decanoate, hexanoate, fdodecanoate, *N*-acetylglucosamine, riboflavin, thymidine, diethanolamine and 4-oxopentanoate) were detected (Figure 4.4; pink frame). Three metabolites (nicotinamide, 5-oxoproline, and gamma-guanidinobutyrate) were detected in both the raw and after 240 s of blanching (Figure 4.4; green frame).

In Mekabu, 116 metabolites were detected, with high metabolite concentrations present in the raw, and the 20 and 40 s blanched samples (Figure 4.5). Nevertheless, as the blanching process time increased, most of the metabolite concentrations were gradually reduced (Figure 4.5). Among all these metabolites, seven metabolites (cyclohexylamine, octanoate, ala-ala, hexanoate, heptanoate, nicotinamide, and 2-furoate) were detected after blanching at 140 s (Figure 4.5; pink frame). Nine metabolites (uridine, inosine, adipate, dodecanoate, UMP, ethanolamine phosphate, AMP, SAH, and riboflavin) that were detected in Wakame were not detected in Mekabu. In contrast, two metabolites (indole-3-acetaldehyde and 2-furoate) were only found in Mekabu, not in Wakame (Figure 4.5).

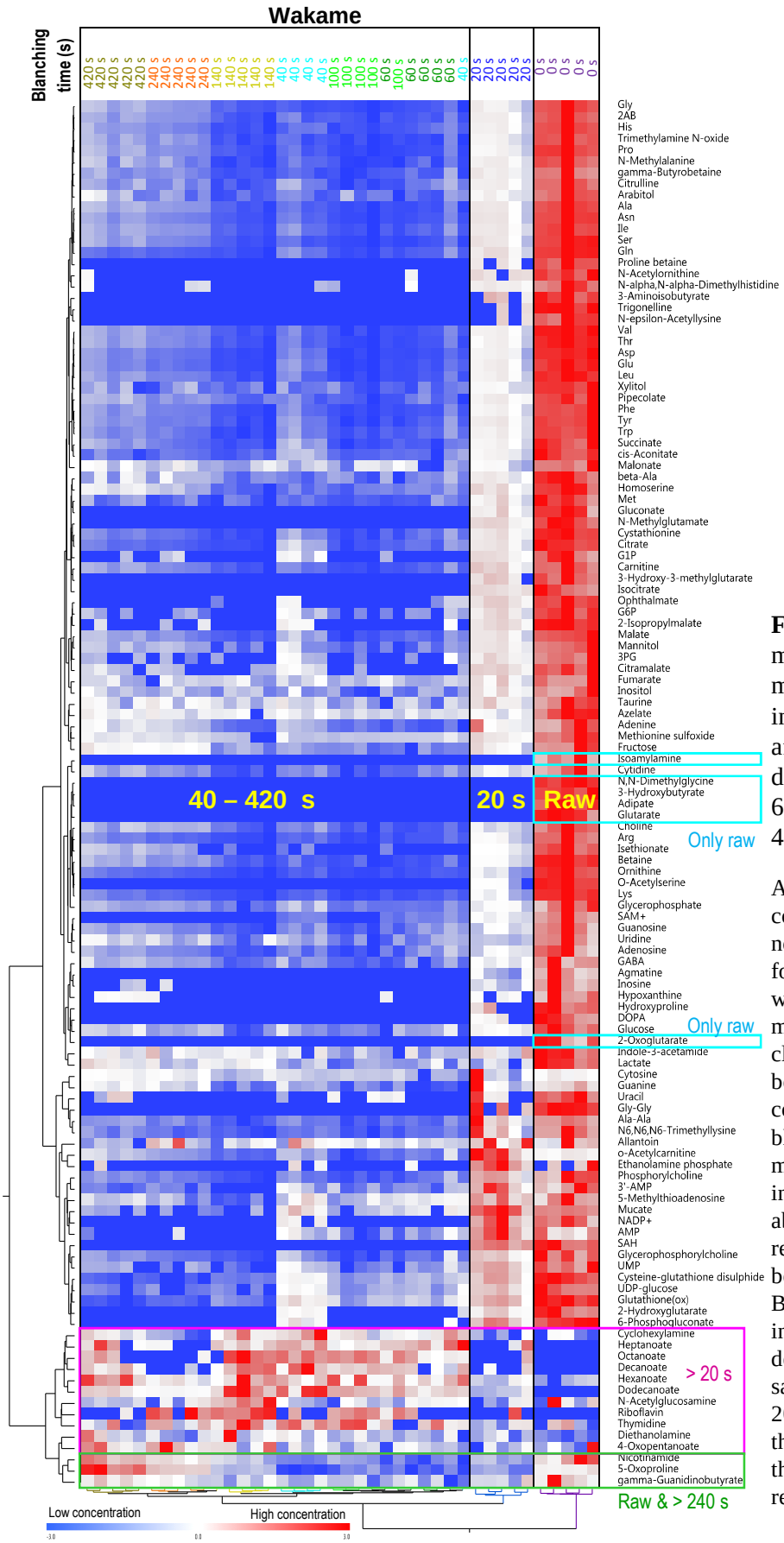

Figure 4.4 HCA and heat map of dry weight based metabolite concentrations in the Wakame sample after blanching for eight different times; 0, 20, 40, 60, 100, 140, 240, and 420 s.

All quantified concentrations were normalized to the *Z*-scores for each metabolite. HCA was performed by the Ward method, and two-way clustering was performed on both metabolite concentration and different blanching times. In the heat map, the blue and red colors indicate low and high-abundance metabolites, respectively as shown on the bottom of the color bar. Blue, pink, and green fonts indicate metabolites detected in; i) the raw sample only, ii) in more than 20 s of blanching, and iii) in the raw samples and in more than 40 s of blanching, respectively.

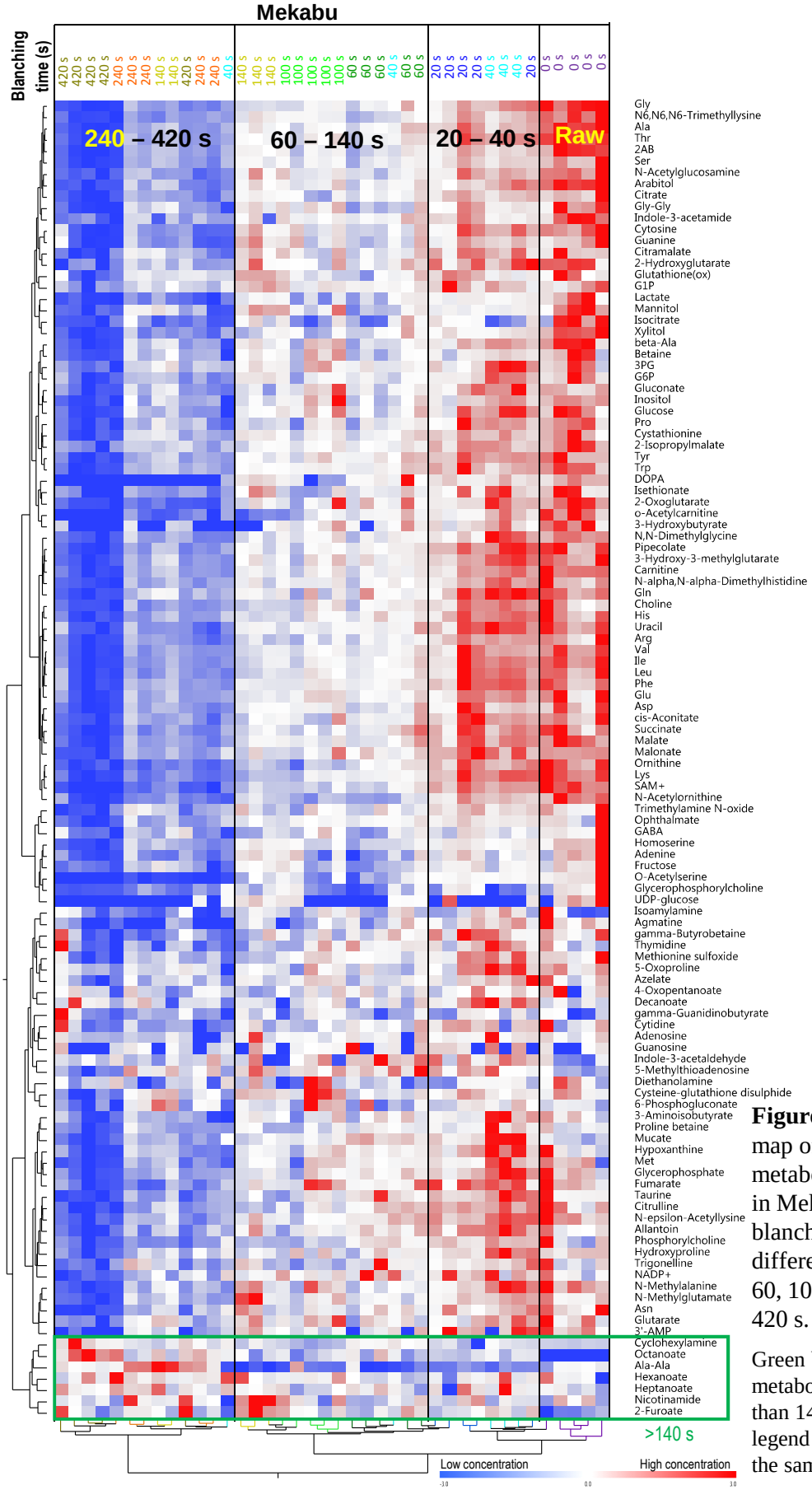

Figure 4.5 HCA and heat map of dry weight based metabolite concentrations in Mekabu samples after blanching for eight different times; 0, 20, 40, 60, 100, 140, 240, and 420 s.

Green box indicate metabolites detected more than 140 s of blanching. The legend and abbreviations are the same as in Figure 4.4.

4.3.6 Metabolite changing tendency during blanching process

The concentration of most metabolites, including amino acids, organic acids, sugars, and other metabolites decreased during the blanching process (Figures 4.6, S4.7–S4.8). In general, the concentration of metabolites was drastically reduced within 20 s of blanching in Wakame whereas they gradually decreased in Mekabu. Although the amino acid concentrations were decreased by the blanching process in both Wakame and Mekabu (Figures 4.6, S4.7a, b), the amino acid composition (Figures S4.7c, d) was not significantly affected. In general, the decrease in the Mekabu metabolite concentration was significant before 60 s. In Mekabu, most sugars (except xylitol) (Figure 4.6b), some amino acids (Asn, Gln, Glu, and Asp) (Figure 4.6a) and other types of metabolites (betaine, gluconate, and adenine) (Figure 4.6d) were slightly increased at 100 to 120 s of blanching. This showed a similar tendency with the drying ratio graph (Figure S4.2). 5-oxoproline concentrations were increased from 100 s in Wakame and were constant in Mekabu (Figure 4.6d).

4.3.7 Amino acids, sugars and other organic acids components of Wakame and Mekabu

Among the 19 amino acids detected, most amino acids were found at significantly higher concentrations in Mekabu than Wakame, including Ala (> 1.7 times), Glu (> 2.2 times), Gln (> 3 times), and Asp (> 4 times). Some amino acids (Met, His and Trp) were detected at low concentrations in both Wakame and Mekabu (Figure 4.6a). Six sugar compounds were also detected at higher concentrations in Mekabu than in Wakame, including mannitol (> 1.3 times), glucose (> 25 times), inositol (> 6.4 times), fructose (> 3.5 times) and arabitol (> 4.6 times) (Figure 4.6b). Xylitol was detected at low concentrations in both, Wakame and Mekabu samples. Among organic acids, Mekabu had higher concentrations compared to Wakame for lactate (> 5.5 times) and citrate (> 1.25 times) (Figure 4.6c).

a) Amino acids

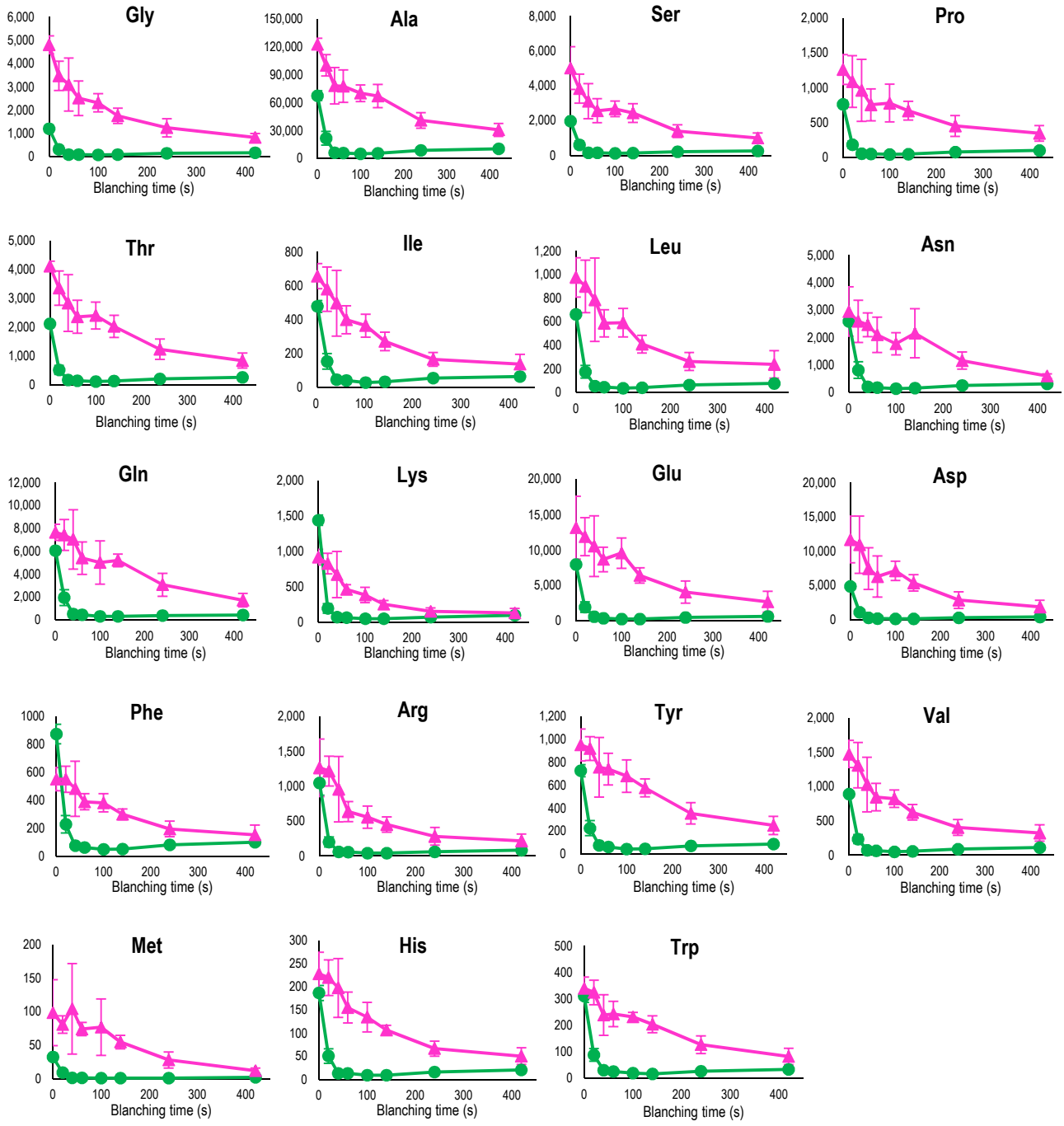

Figure 4.6a Metabolites changes during the blanching process in amino acids.

The green and pink lines indicate Wakame and Mekabu, respectively. The horizontal axis indicates the blanching time (s) and the vertical axis indicates the metabolite concentration (nmol g^{-1}). The values indicate average ± SD, *n=5*.

b) Sugars

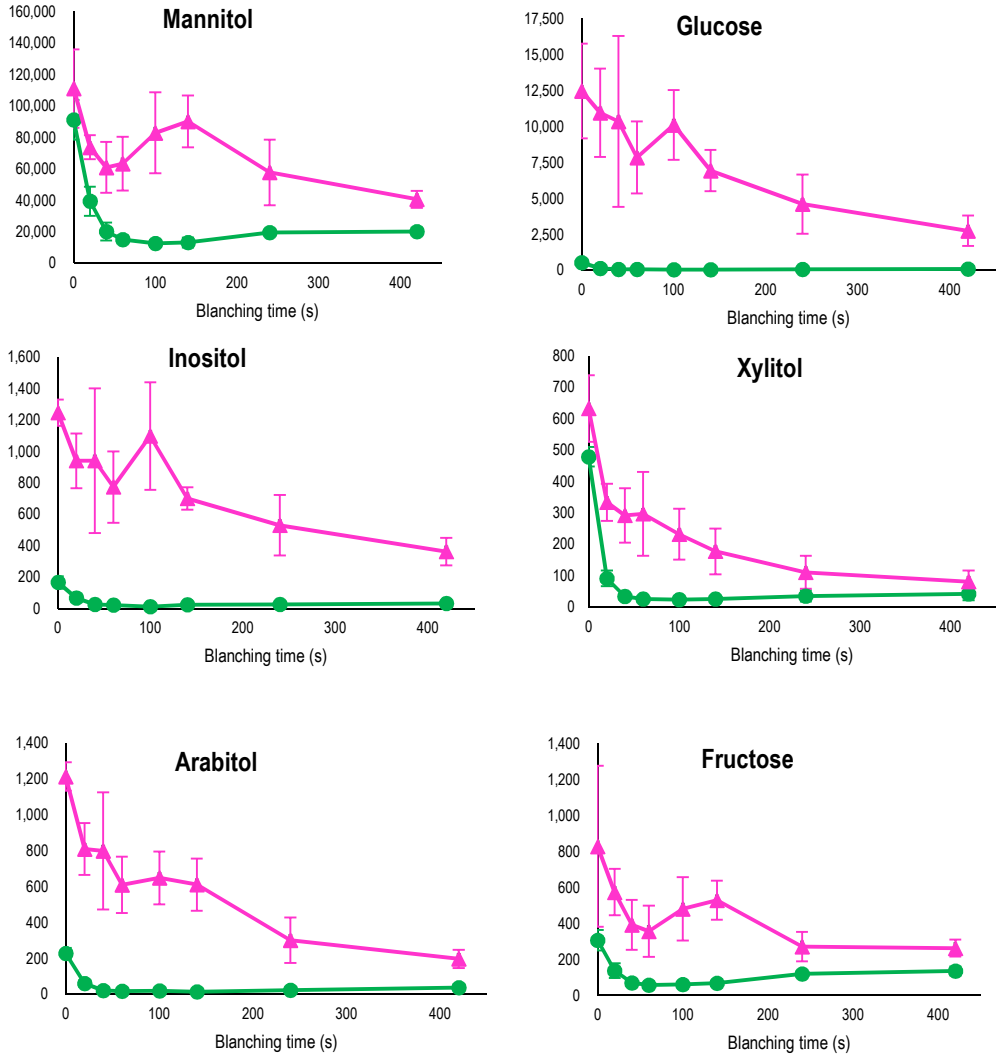

Figure 4.6b Metabolites changes during the blanching process in sugars.

The green and pink lines indicate Wakame and Mekabu, respectively. The horizontal axis indicates the blanching time (s) and the vertical axis indicates the metabolite concentration (nmol g^{-1}). The values indicate average ± SD, *n=5*.

c) Organic acids

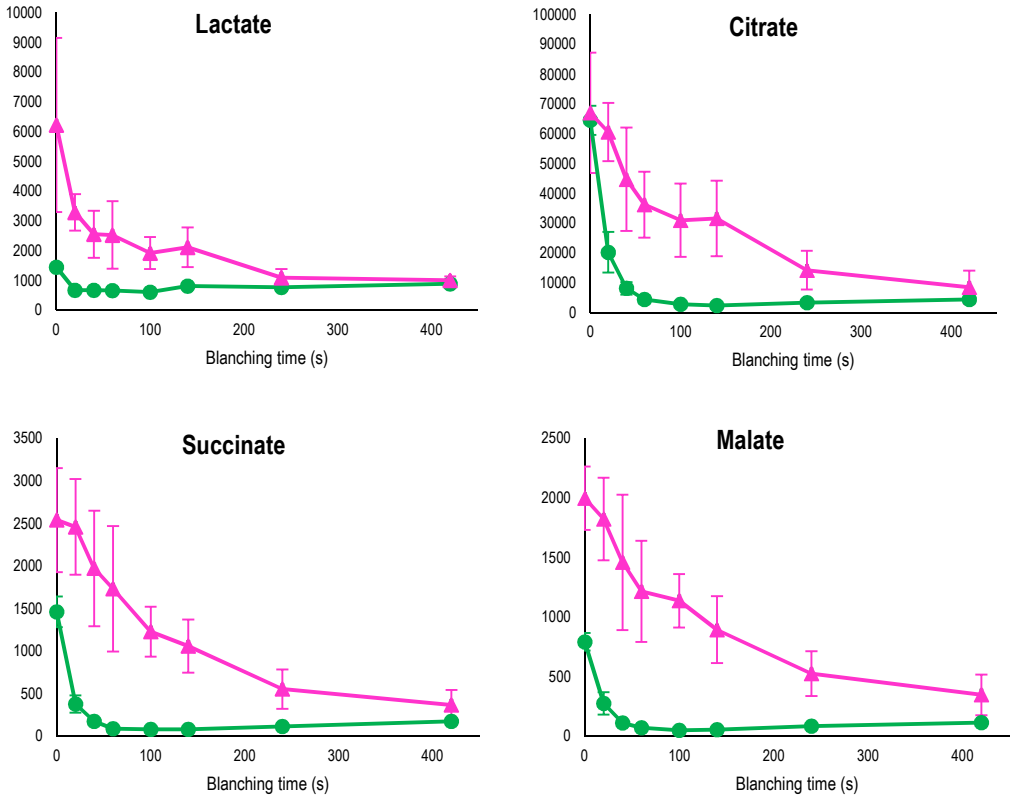

Figure 4.6c Metabolites changes during the blanching process in organic acids.

The green and pink lines indicate Wakame and Mekabu, respectively. The horizontal axis indicates the blanching time (s) and the vertical axis indicates the metabolite concentration (nmol g^{-1}). The values indicate average ± SD, *n=5*.

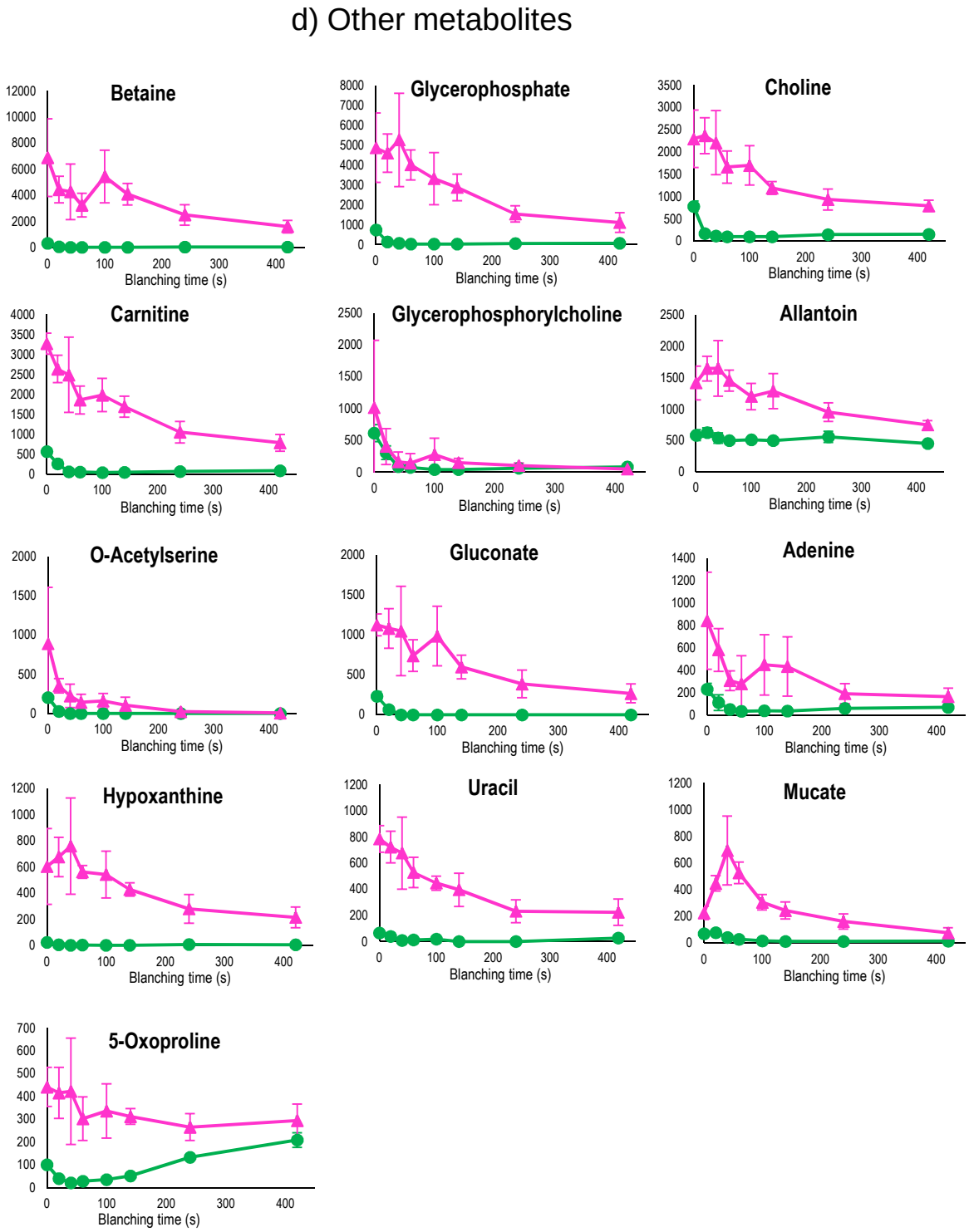

Figure 4.6d Metabolites changes during the blanching process in other metabolites.

The green and pink lines indicate Wakame and Mekabu, respectively. The horizontal axis indicates the blanching time (s) and the vertical axis indicates the metabolite concentration (nmol g^{-1}). The values indicate average ± SD, *n=5*.

4.4 Discussion

4.4.1 Characteristics of the early blanching process in Wakame and Mekabu

Among all the different blanching times for both Wakame and Mekabu assessed here, the clearest changes in color, drying ratio, sensory indices, and metabolite concentrations were seen between the raw sample and the samples 20 s after blanching (Figures 4.1-4.6, S4.1-S4.8). During this time, the color changed from brown to green, and the drying ratio and the concentration of most metabolites decreased. In previous studies, color changes from brown to green arising from the result of a short blanching process have been reported to be due to changes in chlorophyll related enzymes (Balbas *et al.*, 2015). Brown algae contain red color pigments (which are bound to fucoxanthin) and green pigments (which are bound to chlorophylls a and c) (Endo *et al.*, 2017; Yamanaka & Akiyama, 1993). Heating, as a result of the blanching process, results in denaturation of the red colored protein, but not the green. Thus, color changes from brown in the raw seaweed samples to green in the blanched samples can be seen in both the Wakame and Mekabu samples after a short blanching time.

A prolonged blanching process resulted in a slightly brownish coloration in Wakame and discoloration or fading in Mekabu (Figures 4.1, 4.2). Especially in Mekabu, the green pigment from chlorophyll a and c degraded into pheophytin a and c (Kaack, 1994; Rocha *et al.*,1993). Although numerous studies have been conducted to explore the nutrient composition in Wakame and Mekabu with respect to bioactive compounds (Fung *et al.*, 2013; Synytsya *et al.*, 2010; Wang *et al.*, 2018), use as food supplements (Kolb *et al.*, 2004; Prabhasankar *et al.*, 2009) and the effect of the environment (Skriptsova *et al.*, 2004; Zhou *et al.*, 2015), the effect on metabolite concentration and/or composition are limited number of studies. This study showed that the concentration of water-soluble metabolites in Wakame and Mekabu decreased early on in the blanching process, and that color degradation also occurred.

4.4.2 The best blanching time for Wakame and Mekabu

Color is an important factor in determining the market value of Wakame and Mekabu salad, especially in Japan. This is because a brighter green color is preferred as compared to discoloration or faded color (Endo *et al.*, 2017). Even though high metabolite concentrations were found in the raw samples (Figure 4.3–4.6), raw seaweed is not preferred by consumers. Shorter blanching times of 20 s and 40 s in Wakame and Mekabu, respectively (Figure 4.3–4.5) resulted in high concentration of metabolites. In Wakame, both greenness and crunchiness remained high early in the blanching process (20–40 s). In contrast, in Mekabu, stickiness was found to be high early in the blanching process, but crunchiness did increase after 60 s of blanching. Thus, if a consumer prefers sticky Mekabu; the shorter blanching times are better. A previous study exploring the relationship between blanching temperature and color changes in Wakame suggested that heating for 30–60 s at a temperature between 80 °C to 95 °C was the best in retaining the ideal color of processed Wakame (Yamanaka & Akiyama, 1993). In contrast, for Mekabu, blanching at 90–100 °C for 60 s resulted in a change from the brown color of raw Mekabu change to green (Endo *et al*., 2017). These studies therefore also suggest that blanching times of less than 60 s are sufficient to produce a bright green color.

4.4.3 Preserving metabolite concentrations

Since this study aimed to investigate the effect of heat on Wakame and Mekabu by quantifying the metabolite concentrations after different blanching times in boiling water, the blanched sample was not cooled down under running tap water, as it would be in any commercial process. This is because cooling down under running tap water will significantly increase the loss of quality vegetable nutrients as they leach out into the water, and also increase the sample weight due to water absorption (Fellows, 2000). This is why, in this study, we elected to stop the blanching process rapidly by quenching the sample in liquid nitrogen in order to retain as many metabolites as possible. A freeze-drying method was chosen to dry the blanched samples based on a previous study that showed that freeze-drying is the preferred method for preserving metabolite concentration (Hamid *et al.*, 2018). A moisture content graph showed that liquid in the sample leaches

out as the blanching time increased (Figure S4.2). Mekabu contained a higher percentage of moisture compared to Wakame, possibly because Mekabu has thicker cells than Wakame.

4.4.4 Metabolite changes in the blanching process

The blanching process inactivates enzymes present in vegetable tissues, resulting in changes in the sensory and nutritional qualities (Fellows, 2000). This is because, water-soluble vitamins, minerals, and other components are lost due to leaching into the blanching water. Several factors that influence the leaching process are the type of food, the blanching method, the time, and the temperature, as well as the method of cooling (Fellows, 2000; Reyes De Corcuera *et al.*, 2004). Some blanching studies demonstrated that metabolite concentrations vary depending on the species, blanching conditions, and analytical conditions, such as protein hydrolysate amino acids or free amino acids. For example, in broccoli, ascorbic acid concentration decrease during hot water blanching but remain stable during the microwave blanching process (Severini *et al.*, 2016). In soybean, sugars and chlorophyll concentrations were decreased during hot water blanching, but protein hydrolysate amino acids were maintained at a stable concentration (Song *et al.*, 2003). A similar trend for protein hydrolysate amino acids was also found in spinach (Lisiewska *et al.*, 2011). However, these reports did not reveal the adverse effect of blanching on metabolite profiles, especially in edible seaweed like Wakame and Mekabu, which are routinely blanched for an undetermined time before being consumed.

In this study of blanching Wakame and Mekabu, the concentration of most metabolites decreased at early blanching times. The patterns of behavior were however different between Wakame and Mekabu, even though they are derived from two parts of the same seaweed species. In Wakame (thin texture), the metabolite concentration decreased rapidly, but in Mekabu (thick texture), the metabolite concentration decreased more slowly. Especially in Mekabu, the concentration of some metabolites such as sugars were increased at 100–120 s (Figure 4.6b). Similarly, crunchiness (Figure S4.1) and the drying ratio (Figure S4.2) were also increased over these time periods. Thus, we propose that there is some relationship between Mekabu structure and metabolite changes during blanching.

The use of a metabolomics approach to investigate the blanching process has allowed us to understand the effect of heat on changes in metabolites comprehensively. Not only does heat affect the metabolite concentrations, but changes in seaweed color were also observed during the blanching process. Controlling the blanching time or checking color are both easy ways for quality control in cooking. This study also showed that there is relationship between metabolite concentrations and blanching-related characteristics, such as color and drying ratio. The research approach used here is also applicable to other food blanching processes. The output from these studies will contribute to other food-related industries and hopefully benefit consumers.

Chapter 5

CONCLUDING REMARKS

5.1 Main Conclusion

Seaweed is a metabolically unique organism as it is neither a plant nor an animal. Seaweed have neither leaves, roots, nor a specialized vascular system compared to terrestrial plants, yet, they nourish themselves by osmosis (Gupta & Abu-Ghannam, 2011). Seaweed are classified into three major groups; brown, red and green algae (West *et al*., 2017; Guiry & Guiry, 2018). The demand for seaweed is increasing every year due to their various usages, either for food or non-food applications (Pulidindi & Rajpathak, 2017). Japan is the one of the highest producers and consumers of seaweed in the world. The location of Japan which is surrounded by vast seas gives it advantage in having a rich diversity of seaweed. Most seaweed research done by phycologists in Japan had mainly focused on the study of seaweed taxonomy compared to other research in omics. Among all other omics studies, there are abundant seaweed data in proteomics and genomics compared to metabolomics. As mentioned in Chapter 1, the previous studies on seaweed metabolomics are very limited (~1109 metabolites) compared to terrestrial plants (> 50,000 metabolites) (Davis & Vasanthi, 2011; Nakamura *et al*., 2014). Due to this factor, this seaweed metabolites study is aimed to further understand the metabolites profiles of seaweed.

Previously, most of seaweed metabolome studies were carried out based on specific seaweed or targeted compound. NMR tools were used in most of the studies. The disadvantage of NMR method is that it needs large volume of samples compared to MS. The Institute for Advanced Bioscience (IAB) at Keio University in Tsuruoka Town Campus is fully equipped with the latest metabolome analysis instruments such CE-MS

and LC-MS. Thus, in order to comprehensively analyze the seaweed metabolites profiling, the CE-MS and LC-MS were used throughout the study.

The merit of using MS-based method is that only a small amount of sample is required (~5–10 mg). Nevertheless, the samples must be disrupted and extracted before quantitation. Pre-treatment (drying and extraction) methods are important steps for MS analysis and must be performed swiftly to stop any biochemical reactions. Thus, the evaluation of pre-treatment methods was important for a comprehensive analysis of various seaweed metabolites. Previously, there were no optimized methods of pre-treatment for simultaneous extraction all the metabolites in seaweed especially for the MS-based methods. Thus, in Chapter 2 of this thesis, water-soluble metabolite on 3 edible brown algae species; *Cladosiphon okamuranu*s (Mozuku), *Saccharina japonica* (Kombu), and *Undaria pinnatifida* (Wakame) were evaluated using different pre-treatment methods. The optimized drying method, which is freeze-drying, was able to retain higher metabolites concentration, as compared to oven drying at 40 °C and 80 °C. On the other hand, the evaluation of different extraction methods (methanol-water with and without chloroform) showed limited effects on metabolite concentrations and they were varied within individual species. These findings had been published (Hamid *et al*., 2018), and data from the paper were extracted and rewritten in Chapter 2 of this thesis.

The established freeze-drying method from Chapter 2 was used to investigate the metabolites profiles from the various seaweed groups. Seaweed samples used in this study were collected from two different sites in northern Japan; the Shonai coastal area in the Yamagata Prefecture (Sea of Japan) and Muroran in Hokkaido (Pacific Ocean). A total of eleven species of seaweed; 4 brown algae, 5 red algae, and 2 green algae were metabolically compared after extraction using 2 extraction methods; methanol-water with and without chloroform. As discussed in Chapter 3 and published in (Hamid *et al.*, 2019), the findings from this study showed that species is the principal factor affecting the metabolites profiles compared to the extraction methods. The results also revealed that the sugar profiling is able to discriminate between the different seaweed groups; (i.e., brown, red, and green algae) as compared to amino acids or other metabolites profiling. To the best of my knowledge, this study provides a novel way of categorizing the seaweed groups using the platform of metabolite profiling. The comprehensive results of the

analyzed of metabolite profiles in Chapter 2 and 3 of this thesis could have further usage in foods and health-related fields, such as in nutraceutical.

Instead of focusing on basic research, the last part of the study aimed to implement the understanding of the metabolites profiling into daily application. As described in chapter 4 of this thesis, the heating effect from the blanching process on the metabolites profiles was evaluated on two parts of *U. pinnatifida* (a brown algae species); Wakame (leaf) and Mekabu (sporophyll). This species was chosen as it is one of the most common seaweed eaten by Japanese and Korean after blanching. Although many studies were done on the nutrient content and the effect of blanching on the color of Wakame and Mekabu, there was very little study done on the effects of blanching at different periods on the concentration of metabolites. This study showed that most metabolites were at the highest concentration in raw samples. The metabolites concentration in Wakame was drastically decreased after blanching for 20 s, but the concentration in Mekabu was gradually decreased after 40 s. During this process, the color changed from brown to green. The blanching process did not significantly affect the amino acid and sugar component ratio. In general, metabolite concentrations decreased after prolonged blanching. Currently, the manuscript from the finding has been submitted to *Algal Research* and under-reviewed. The information generated can be utilized and act as a guidance in the food-related industry as well as for the ordinary consumers of Wakame and Mekabu.

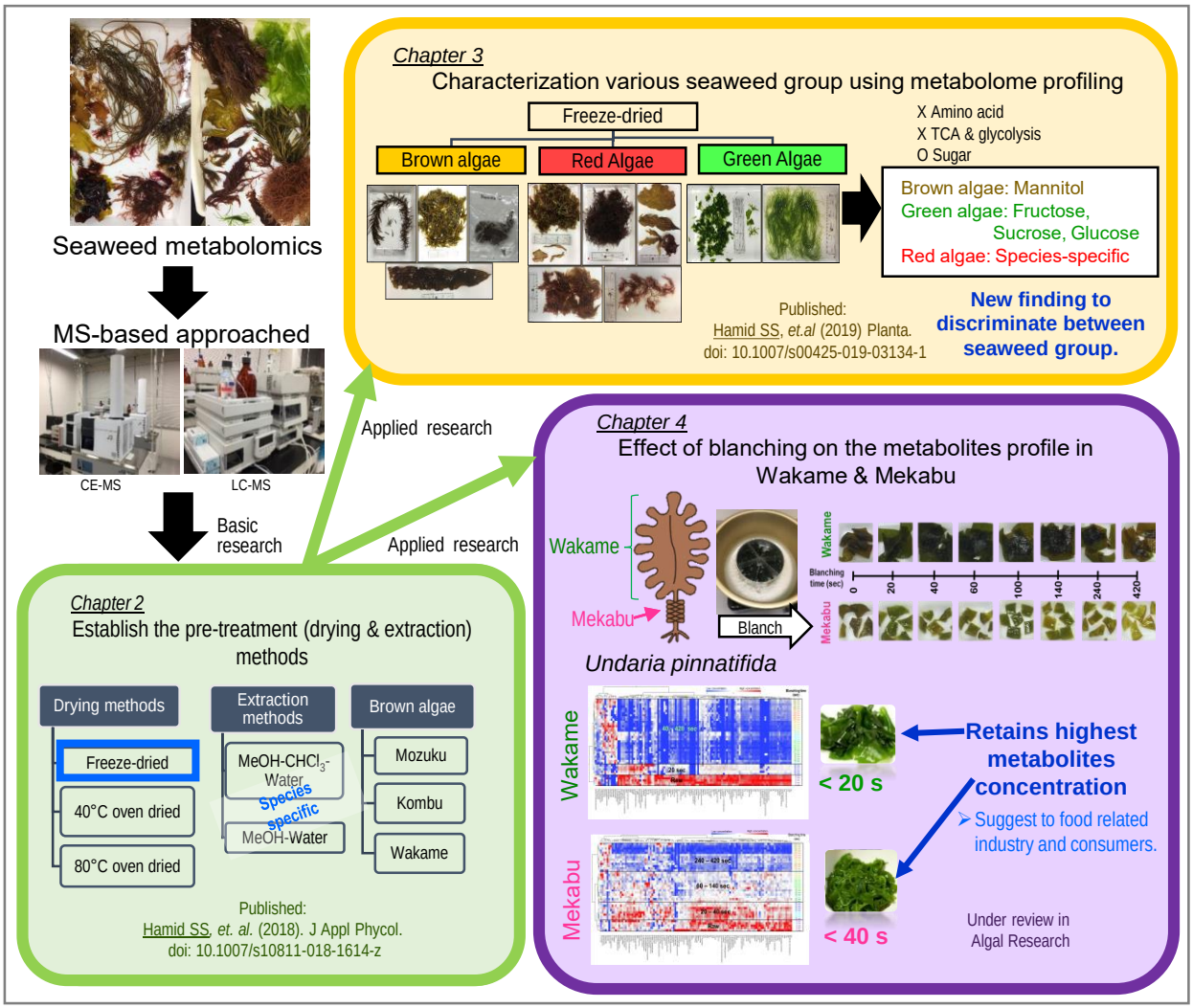

Figure 5.1 Overall conclusion of the study.

5.2 Future Direction

The continuation of metabolomics study is necessary. Besides that, the vast data generated can also contribute to the seaweed metabolites database which is currently quite scarce and mostly derived from one red algae species, *Laurencia sp.* (Davis & Vasanthi, 2011).

By conducting a seaweed metabolomics study, it has opened up the opportunity to further explore other fields such as pharmaceutical (drug) and nutraceutical (food). Additionally, the knowledge gained from this study can also be used to overcome the limitation in other research area such as seaweed tissue culture or defense system, mainly in the seaweed aquaculture industry.

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Abbreviations

	Amino acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Others

40	Oven-dry at 40 °C
80	Oven-dry at 80 °C
2,3-DPG	2,3-diphosphoglycerate
2AB	2-Aminobutyrate
2PG	2-Phosphoglycerate
3′ AMP	3’-adenylic acid
3PG	3-Phosphoglycerate
ADMA	Asymmetric dimethyl arginine
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
CE	Capillary electrophoresis
$CHCl_3$	Chloroform
Cm	Centimeter
CMP	Cytidine monophosphate
CSA	D-camphor-10-sulfonic acid
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
ESI	Electrospray ionization
F6P	Fructose-6-phosphate
FD	Freeze-dry
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GDP	Guanosine 5’-phosphate
H	Hour(s)
H_2O	Water
HCA	Hierarchical clustering analysis
HPLC	High-performance liquid chromatography
i.d.	Inner diameter
IDP	Inosine 5-diphosphate
IMP	Inosine monophosphate
IS	Internal standard
kV	Kilovolt
LC	Liquid chromatography
M	Molar
M&C&W	Methanol-chloroform-water extraction method
M&W	Methanol-water extraction method
MeOH	Methanol

MES	2-(*N*-morpholino)ethanesulfonic acid
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PCA	Principle component analysis
S	Second(s)
S7P	Sedoheptulose 7-phosphate
SAH	S-adenosyl homocysteine
SAM+	S-adenosylmethionine
SD	Standard deviation
SDMA	Symmetric dimethylarginine
TOF	Time-of-fight
UDP	Uridine diphosphate
UMP	Uridine-monophosphate

Appendixes (Supplementary Figures and Tables)

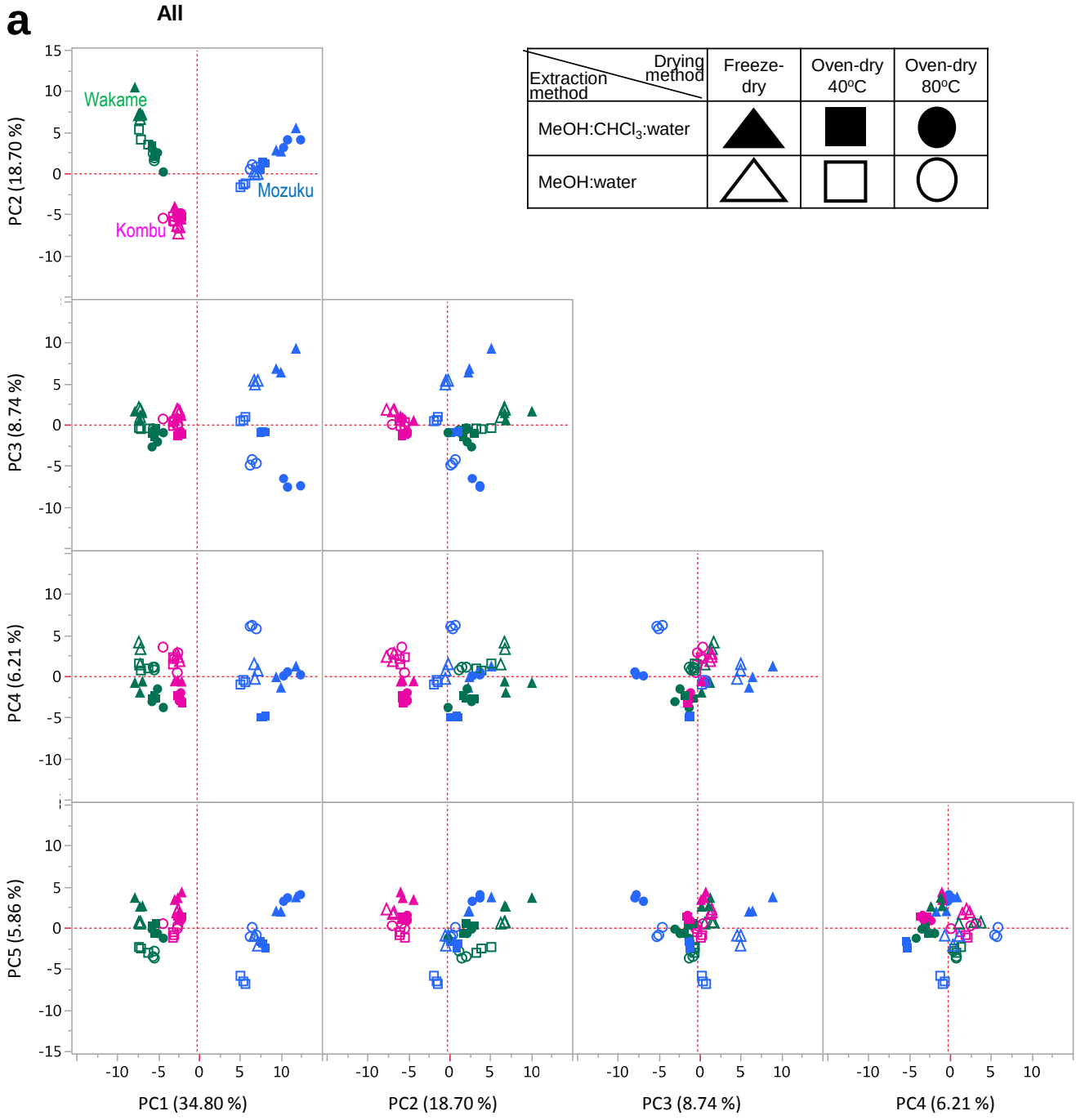


Figure S2.1a PCA score plots of metabolites concentrations in three brown algae after all drying and extractions methods.

The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-5 separation variance.

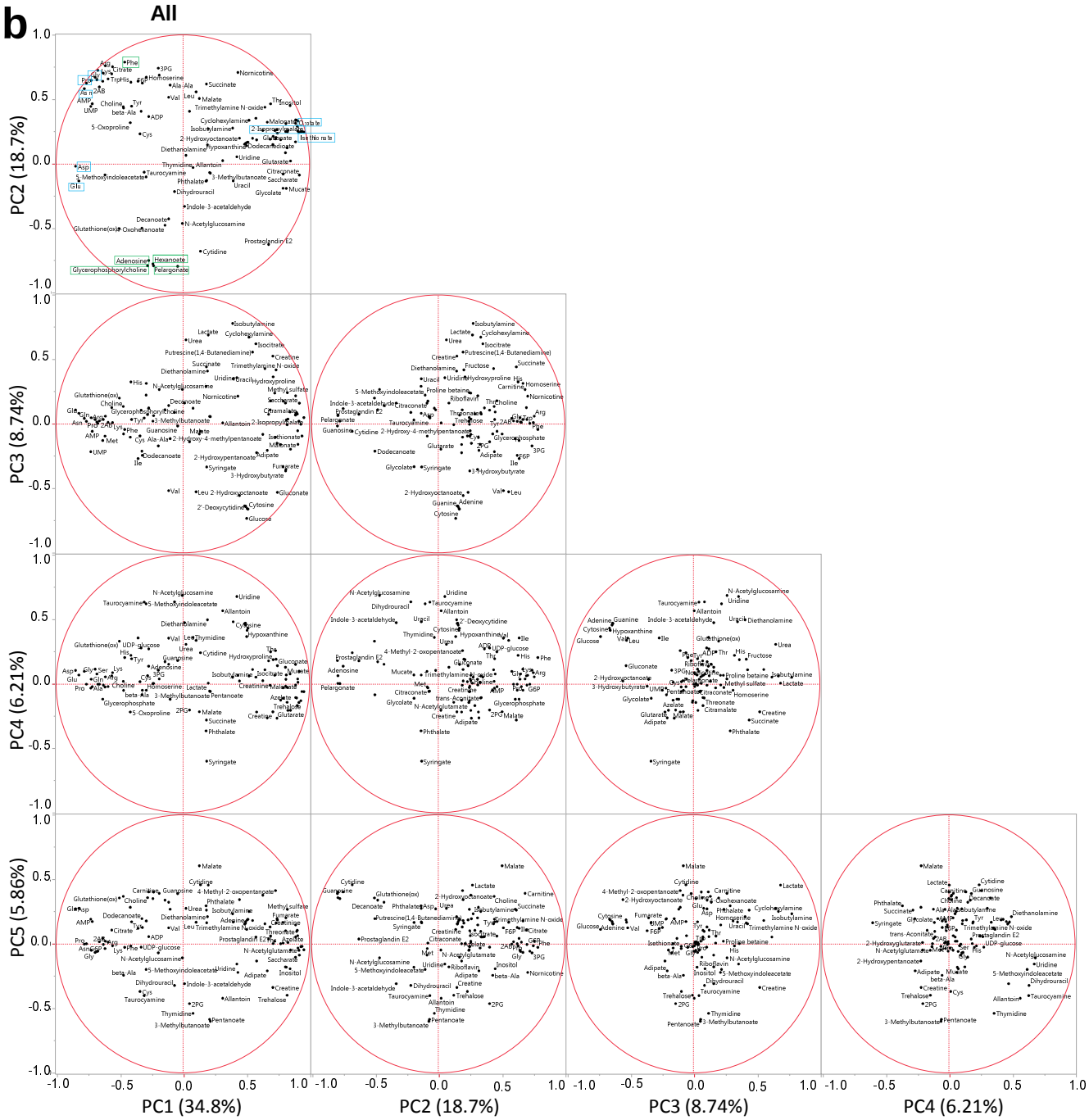


Figure S2.1b PCA loading plots of metabolites concentrations in three brown seaweed after all drying and extractions methods.

The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

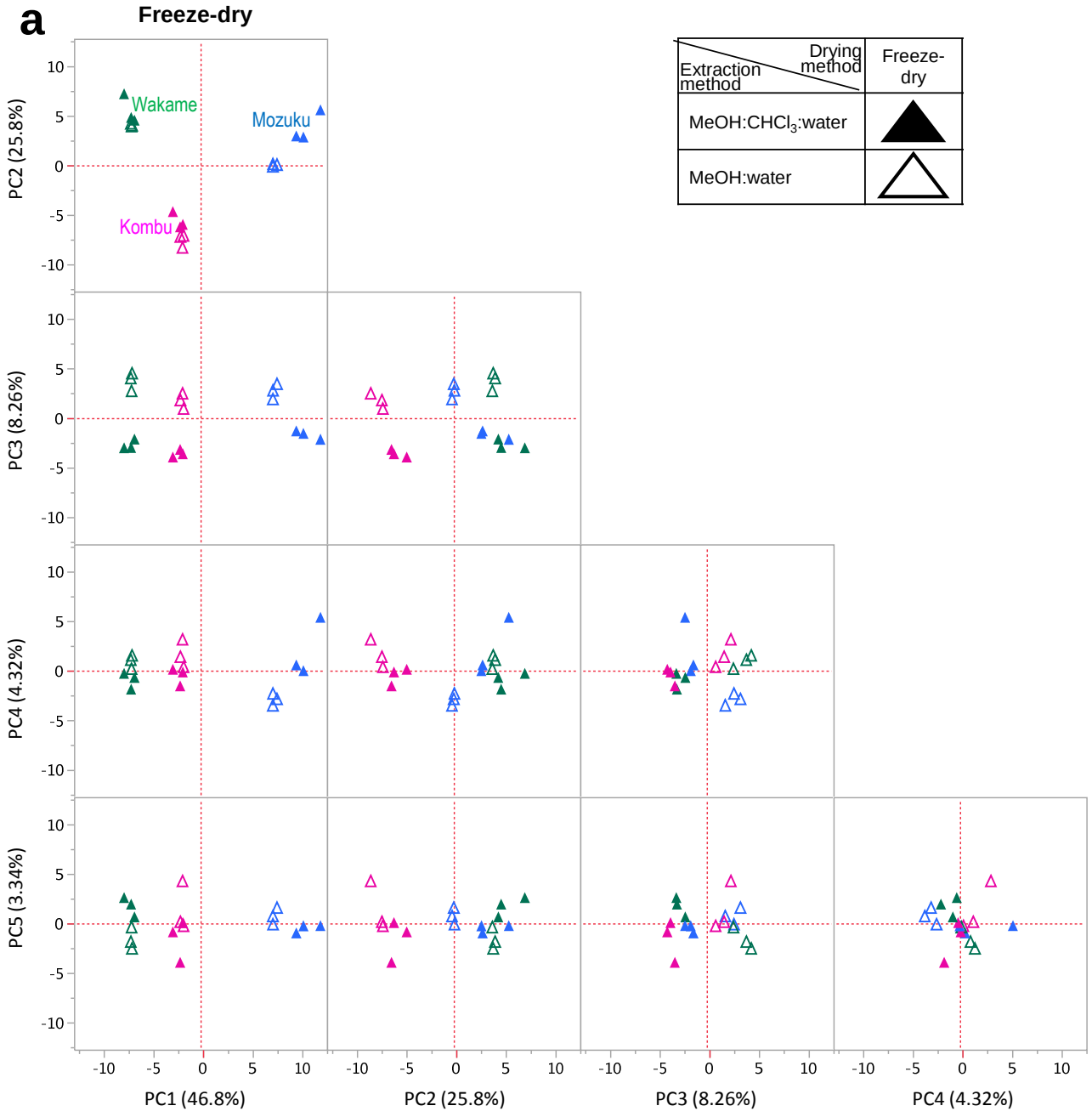


Figure S2.2a PCA score plots of metabolites concentrations in freeze-dry methods.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

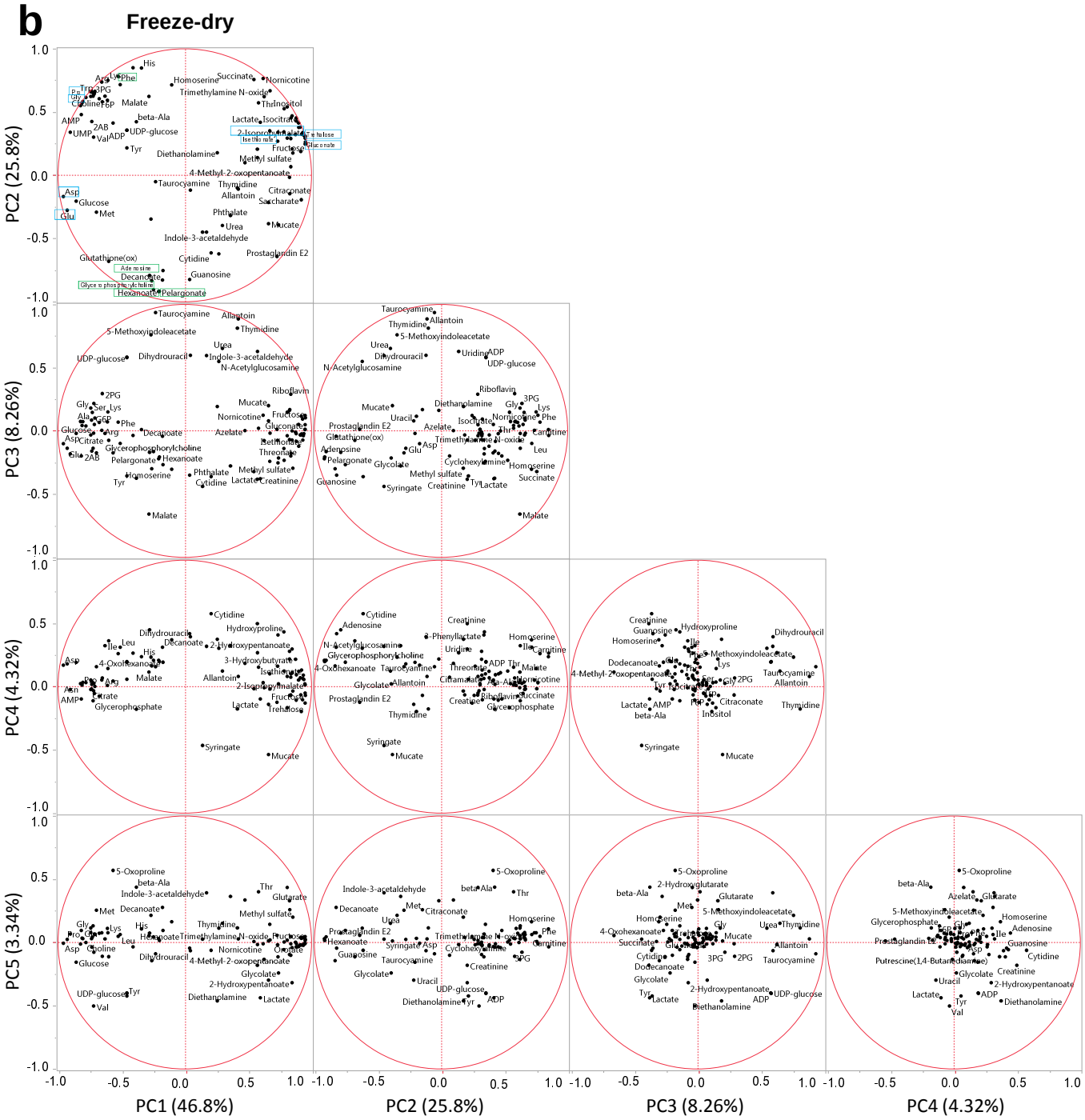


Figure S2.2b PCA loading plots of metabolites concentrations in freeze-dry methods.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

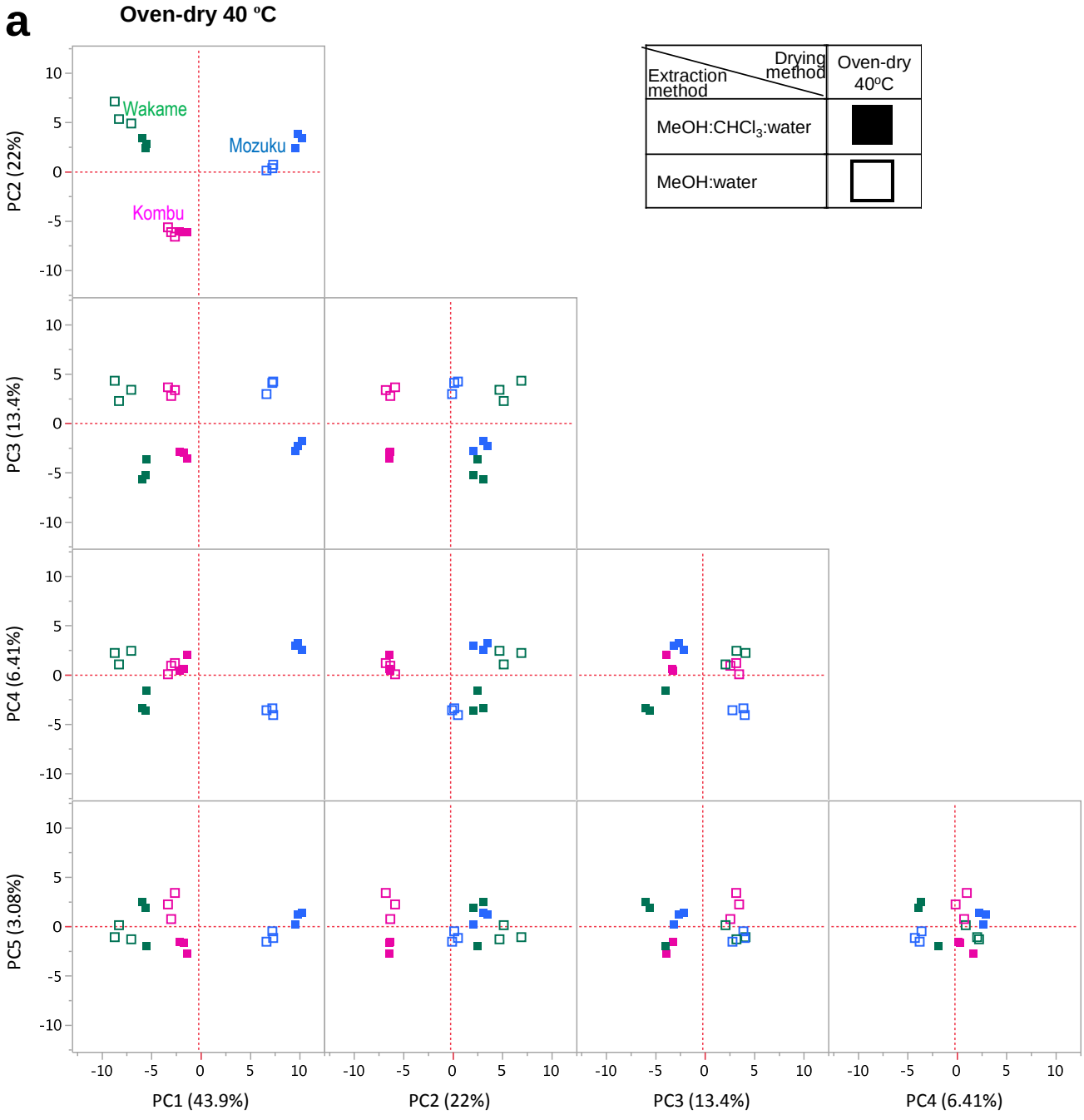


Figure S2.3a PCA score plots of metabolites concentrations in oven-dry at 40° C.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

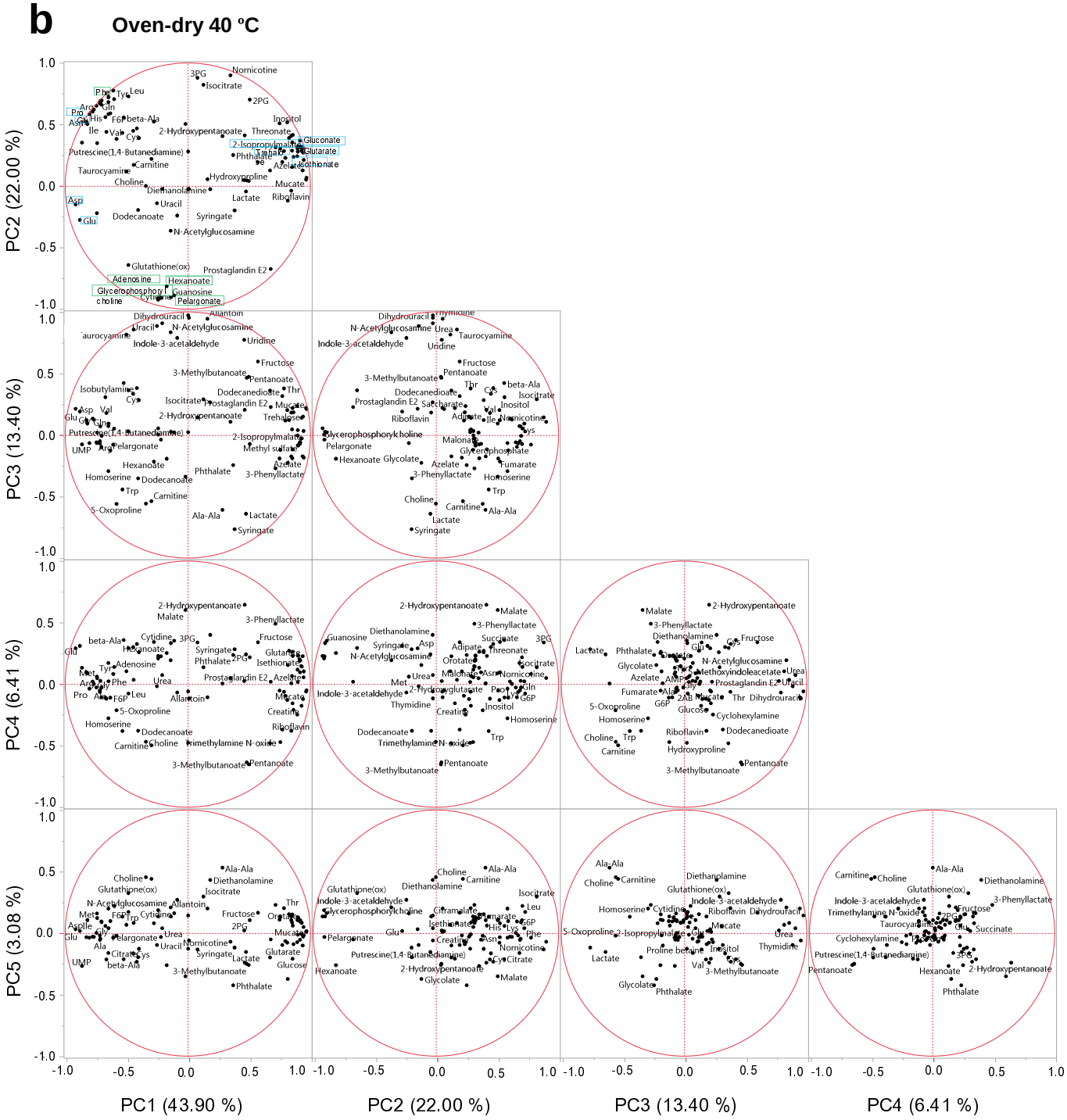


Figure S2.3b PCA loading plots of metabolites concentrations in oven-dry at 40° C.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

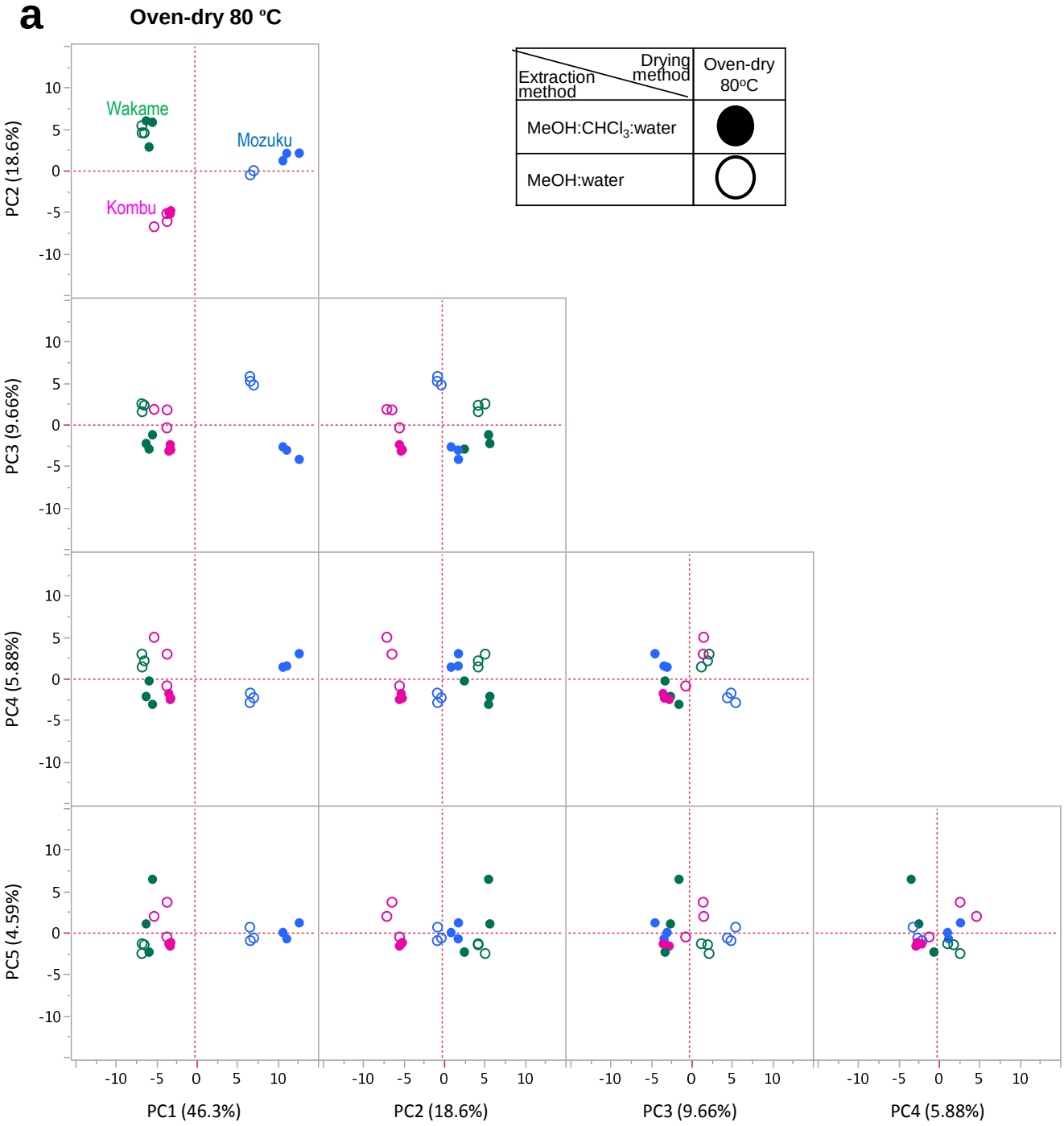


Figure S2.4a PCA score plots of metabolites concentrations in oven-dry at 80° C.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

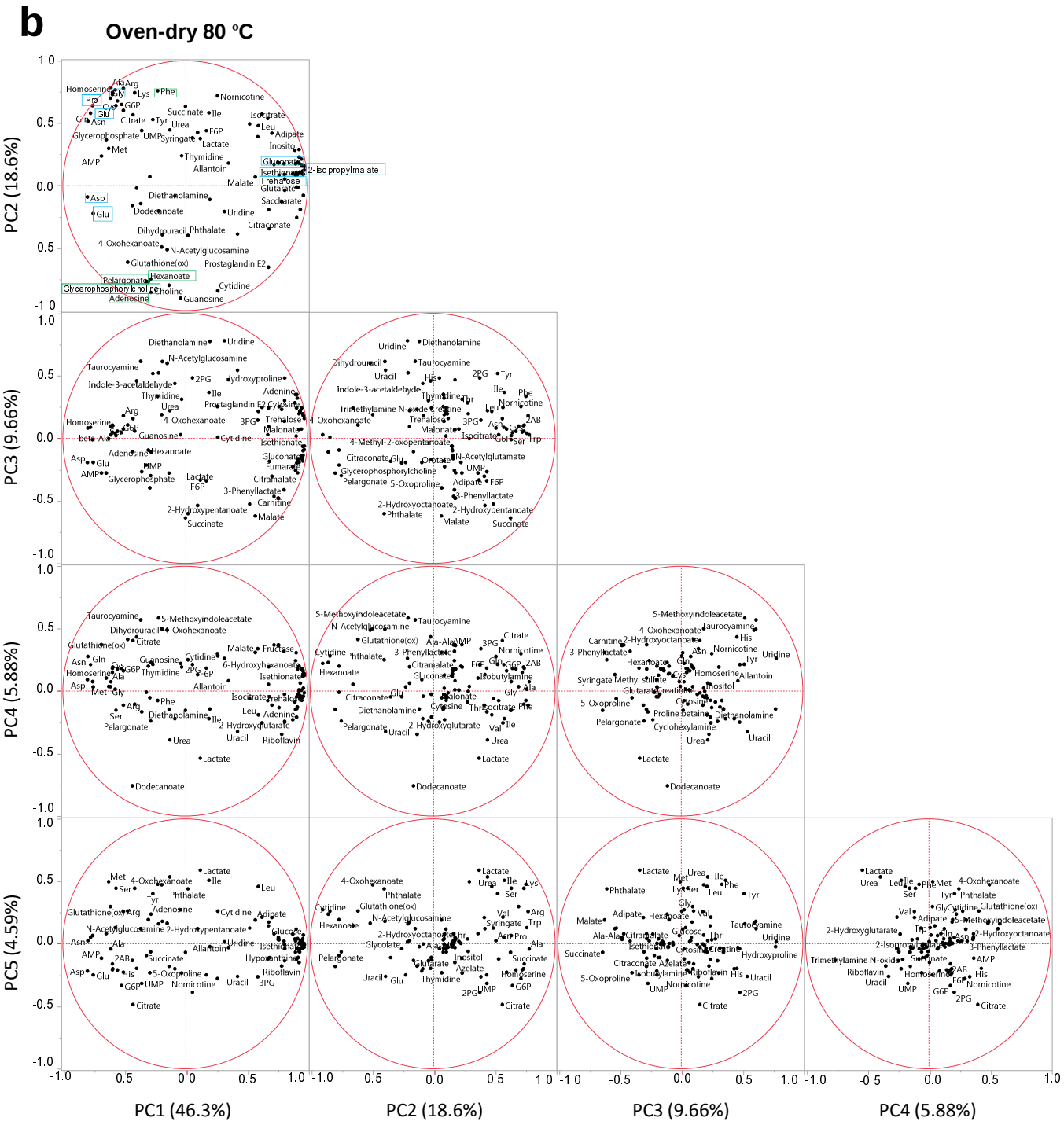


Figure S2.4b PCA loading plots of metabolites concentrations in oven-dry at 80° C.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

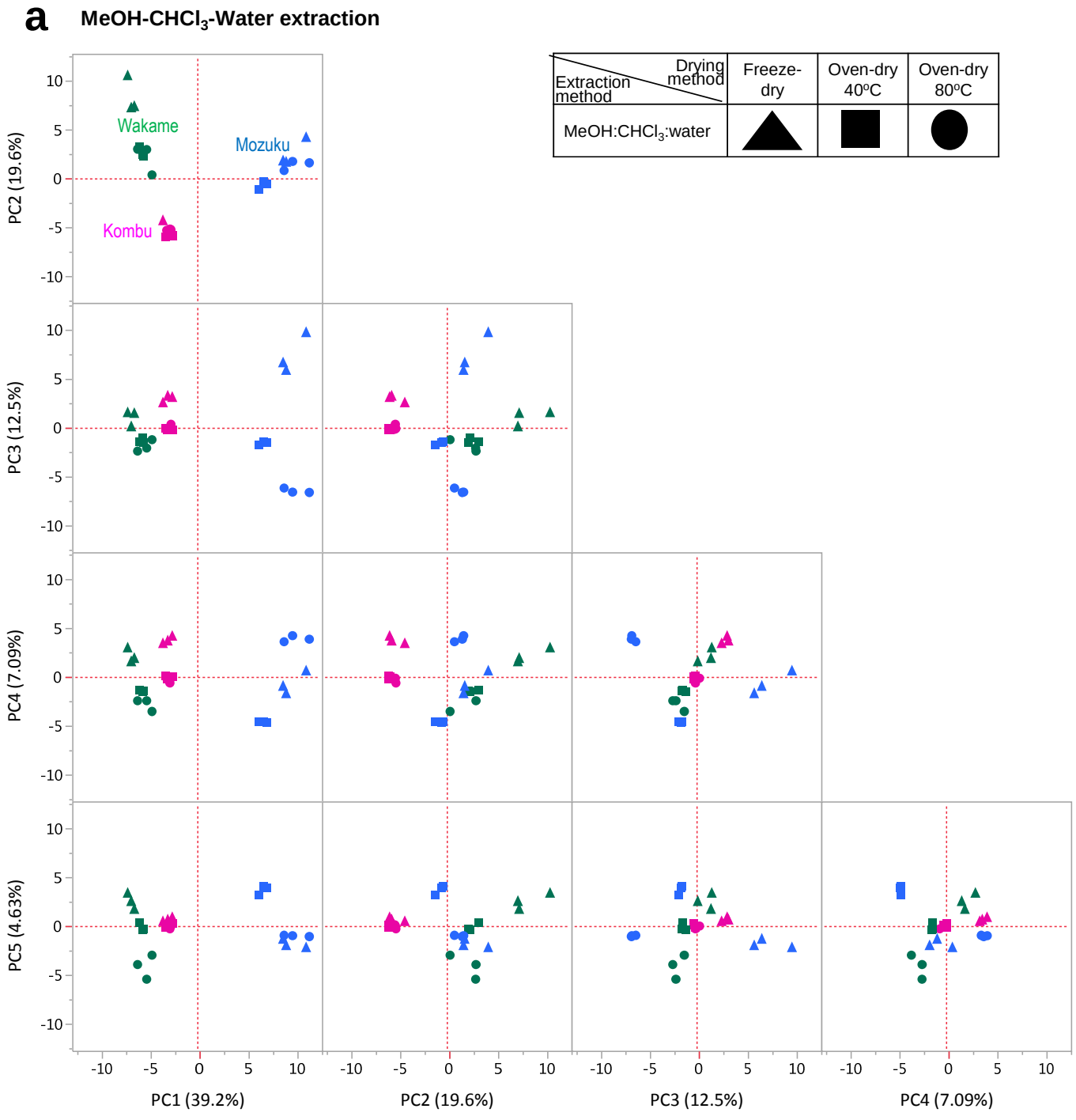


Figure S2.5a PCA score plots of metabolites concentrations in methanol-chloroform-water extraction methods.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

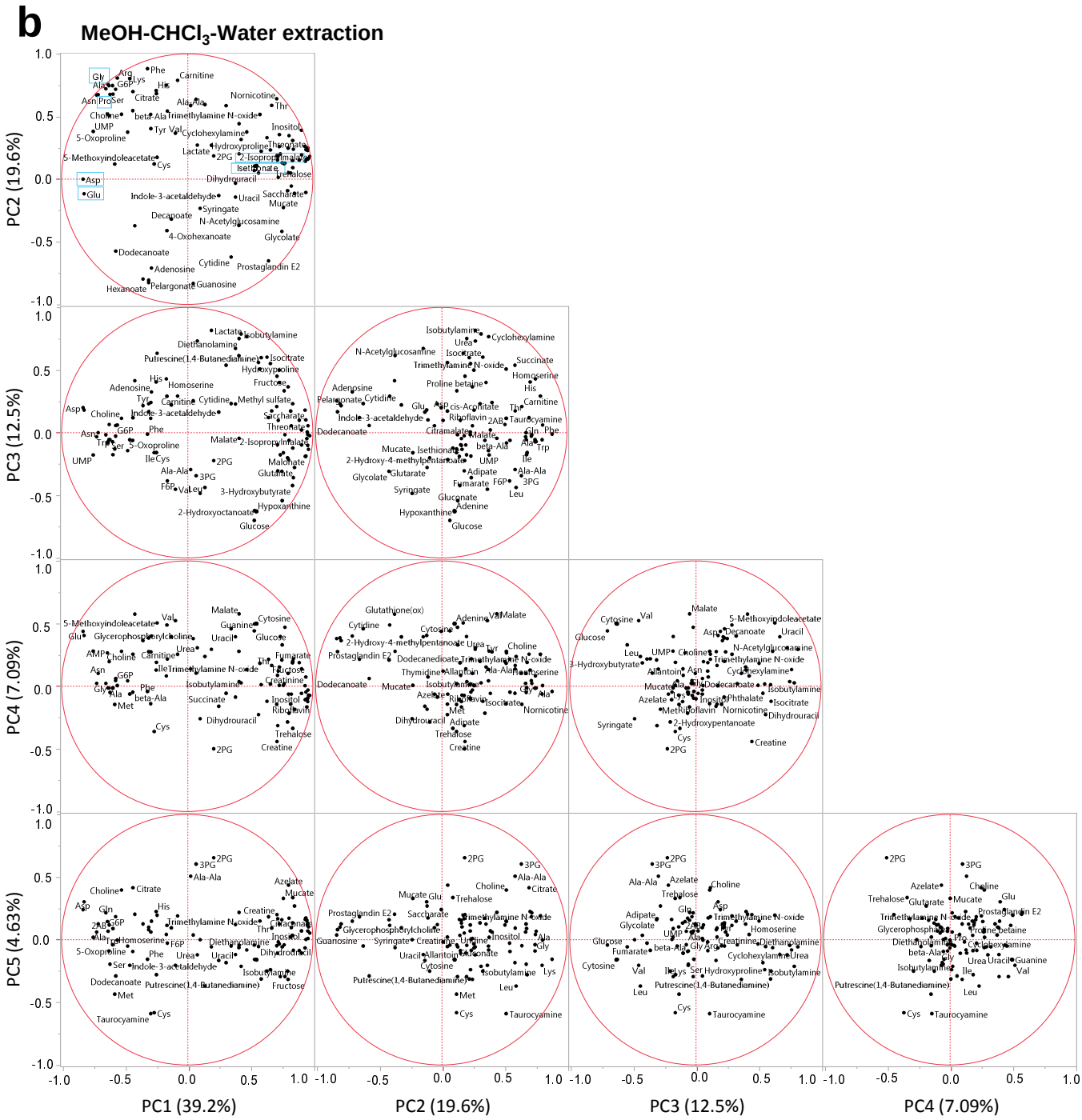


Figure S2.5b PCA loading plots of metabolites concentrations in methanol-chloroform-water extraction methods.

The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

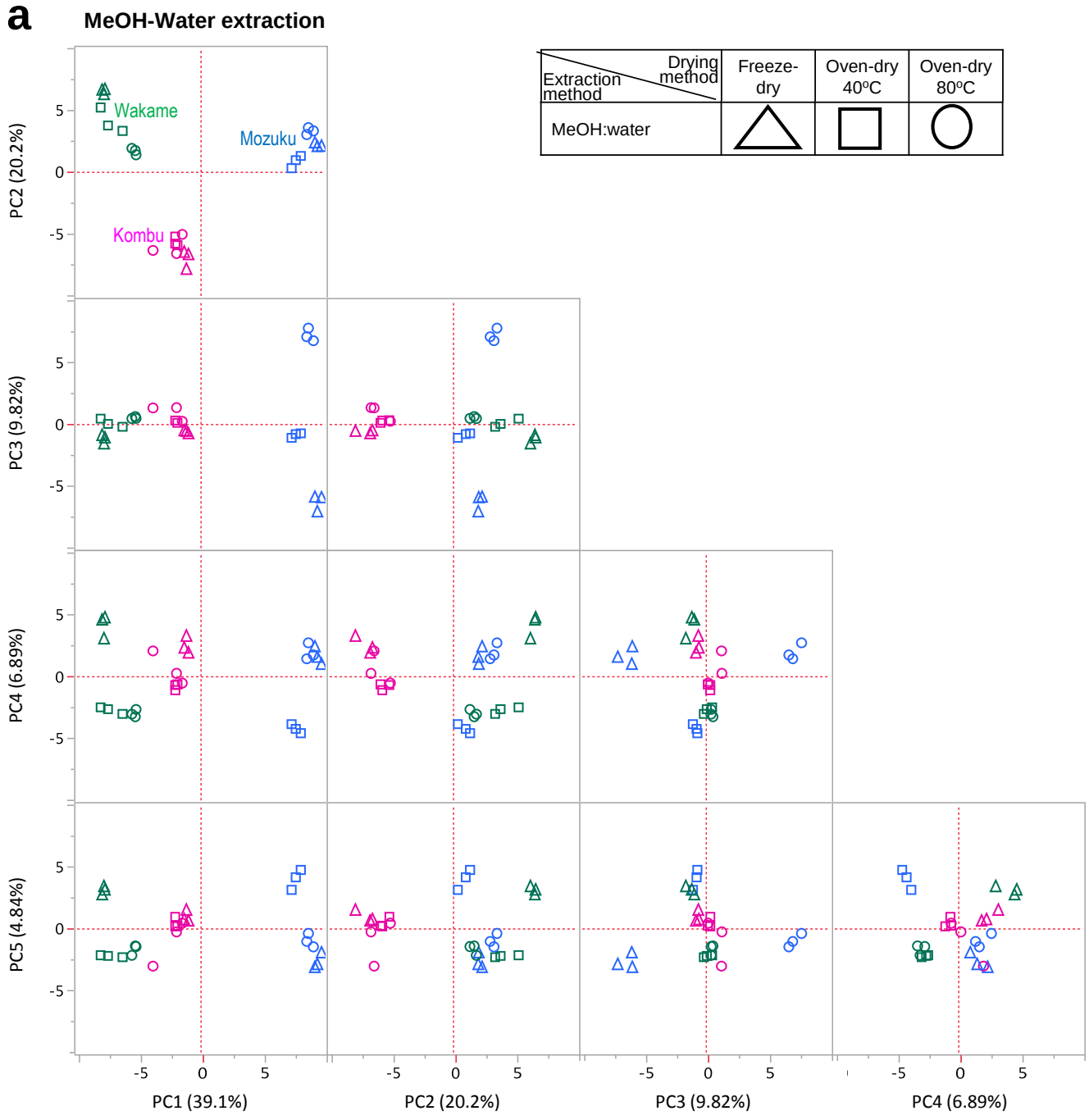


Figure S2.6a PCA score plots of metabolites concentrations in methanol-water extraction methods.

The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

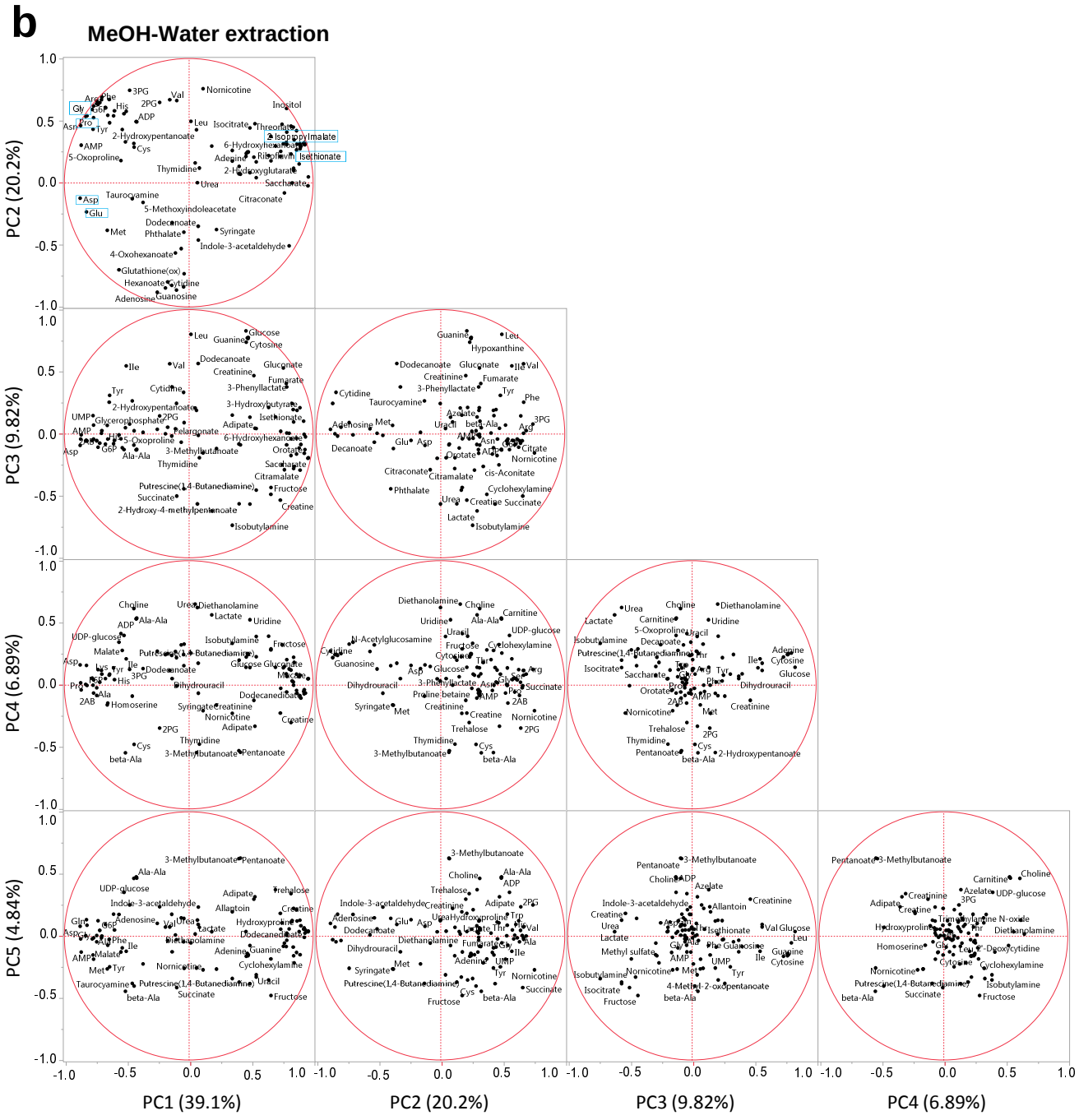


Figure S2.6b PCA loading plots of metabolites concentrations in methanol-water extraction methods.

The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

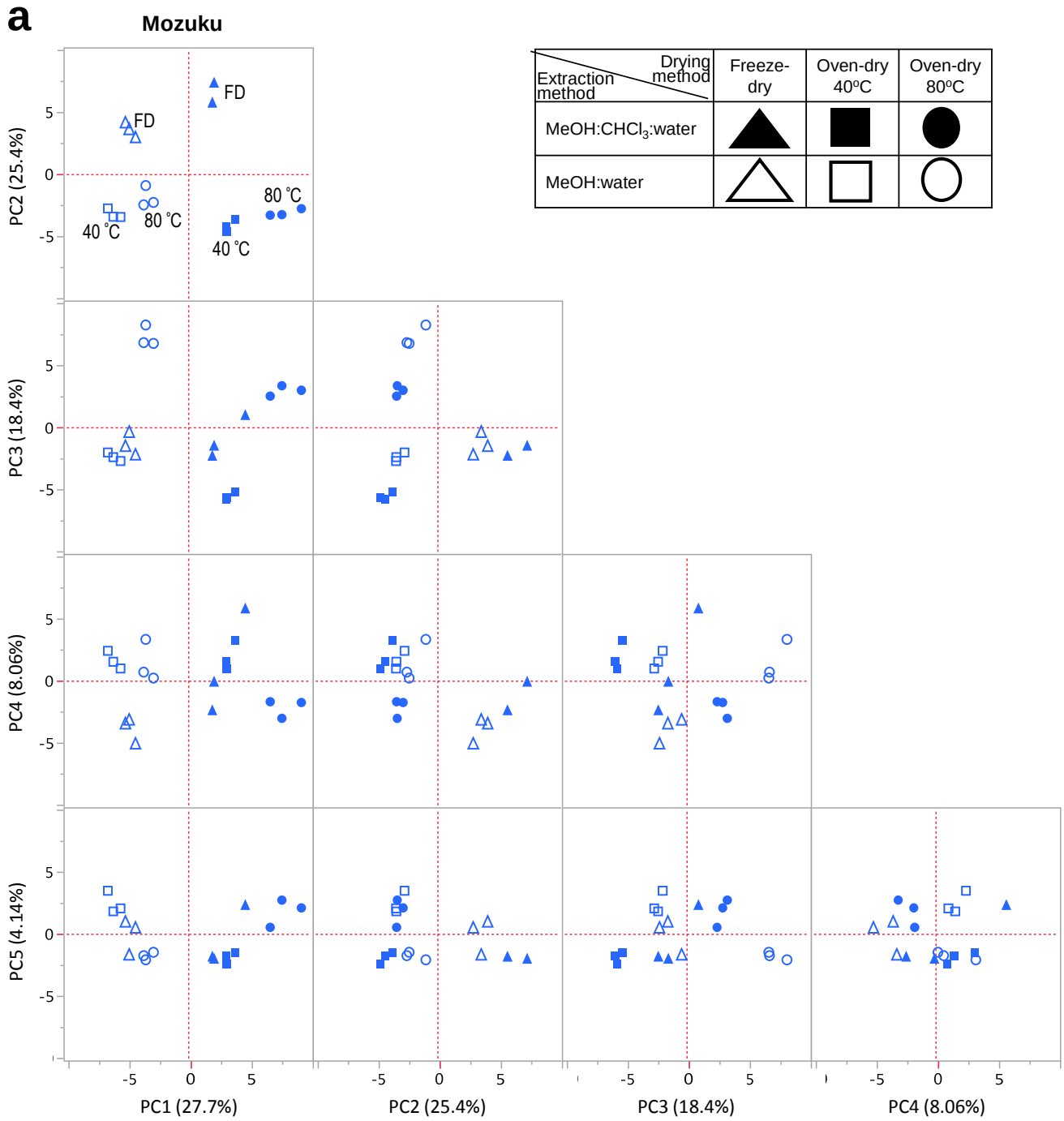


Figure S2.7a PCA score plots of metabolites concentrations in Mozuku samples.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

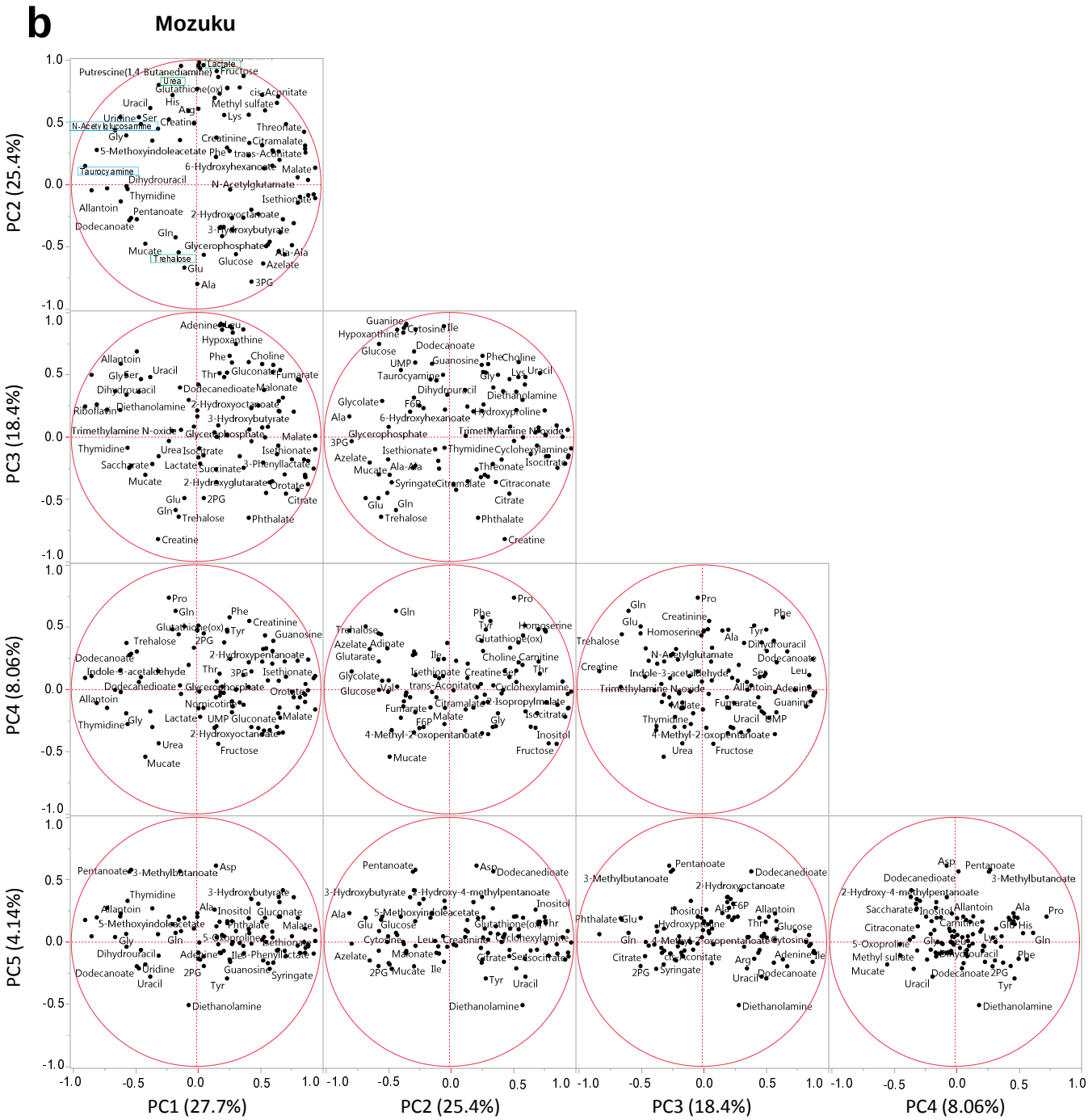


Figure S2.7b PCA loading plots of metabolites concentrations in Mozuku samples.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

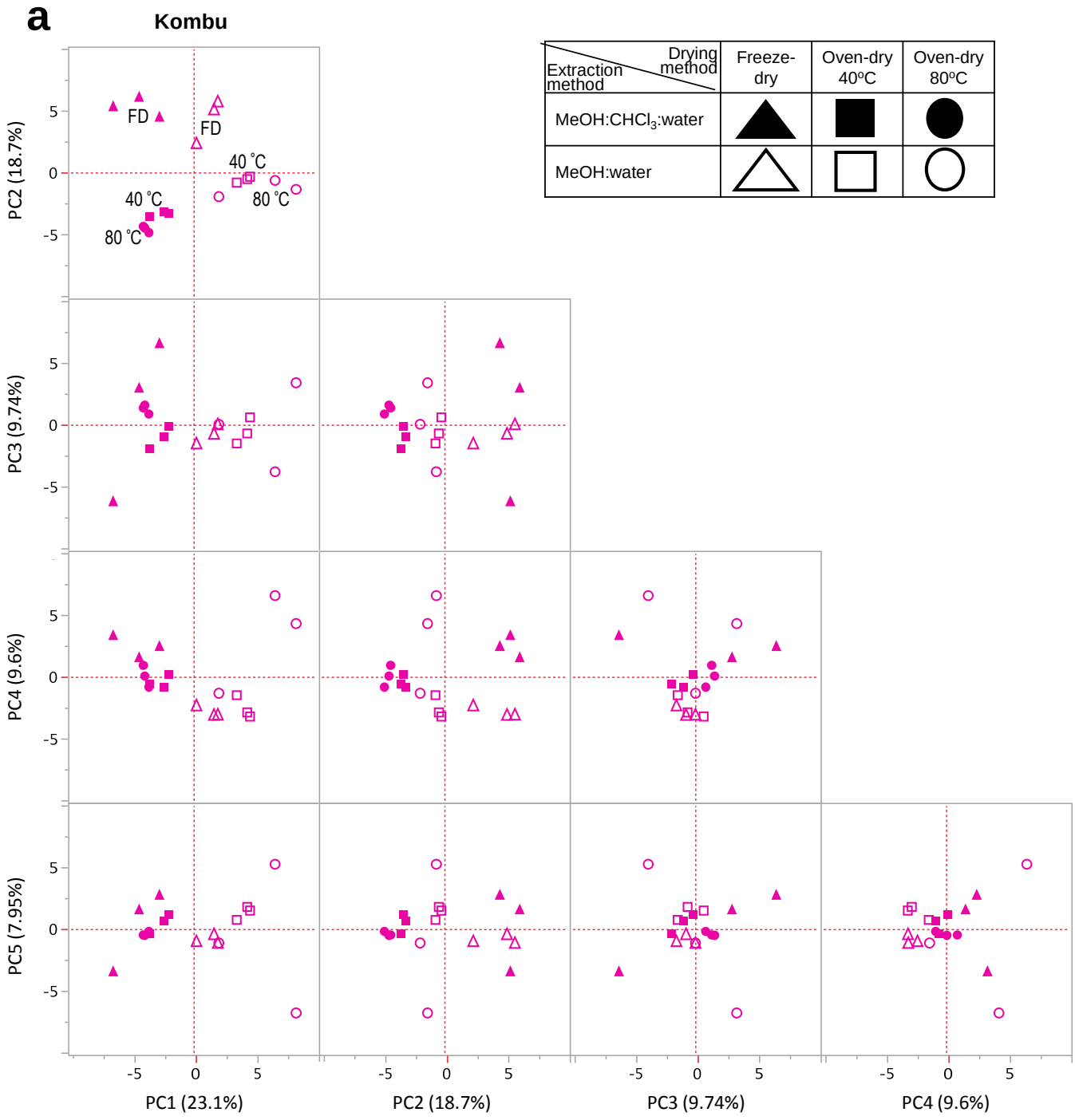


Figure S2.8a PCA score plots of metabolites concentrations in Kombu.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

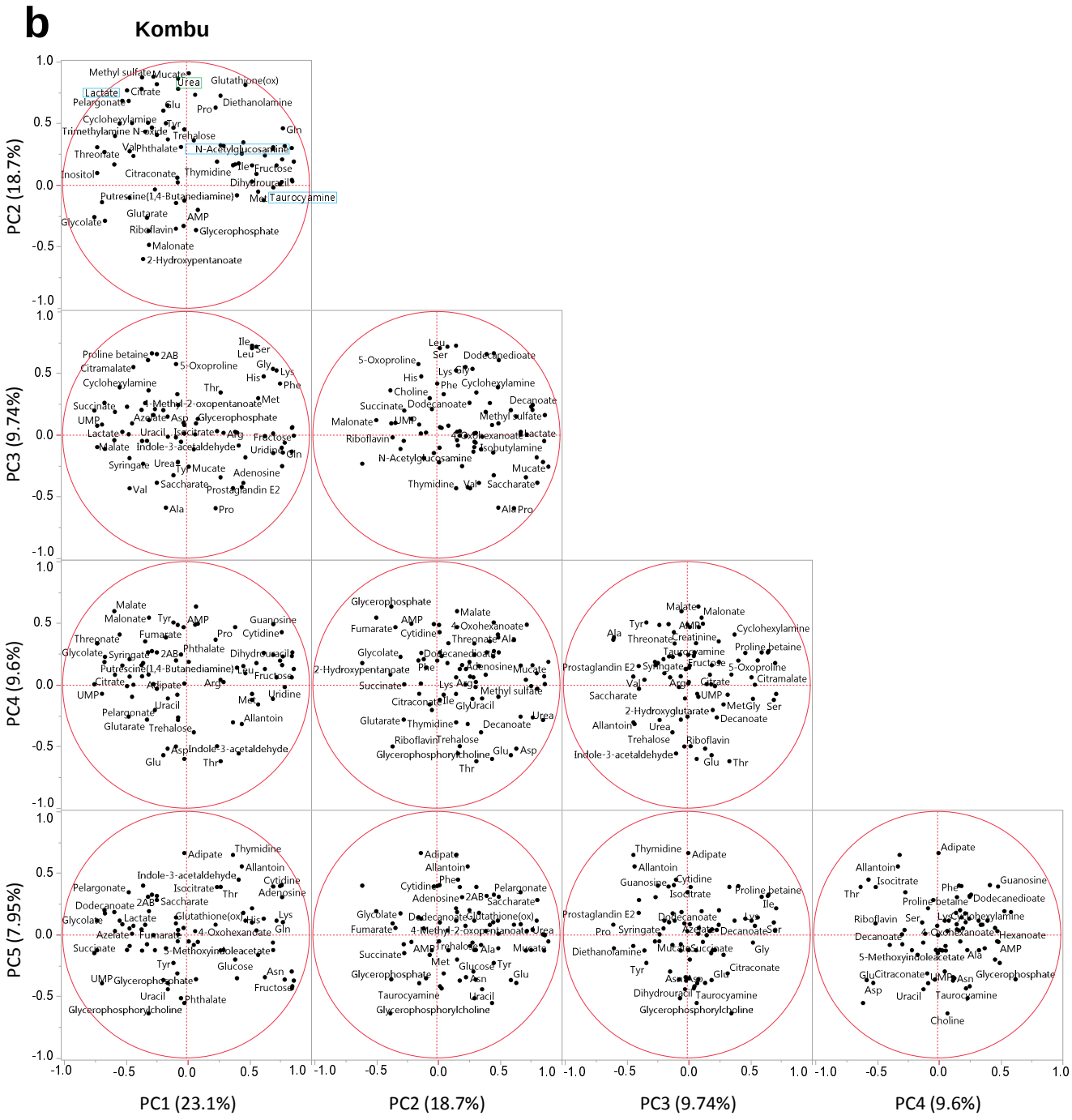


Figure S2.8b PCA loading plots of metabolites concentrations in Kombu.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

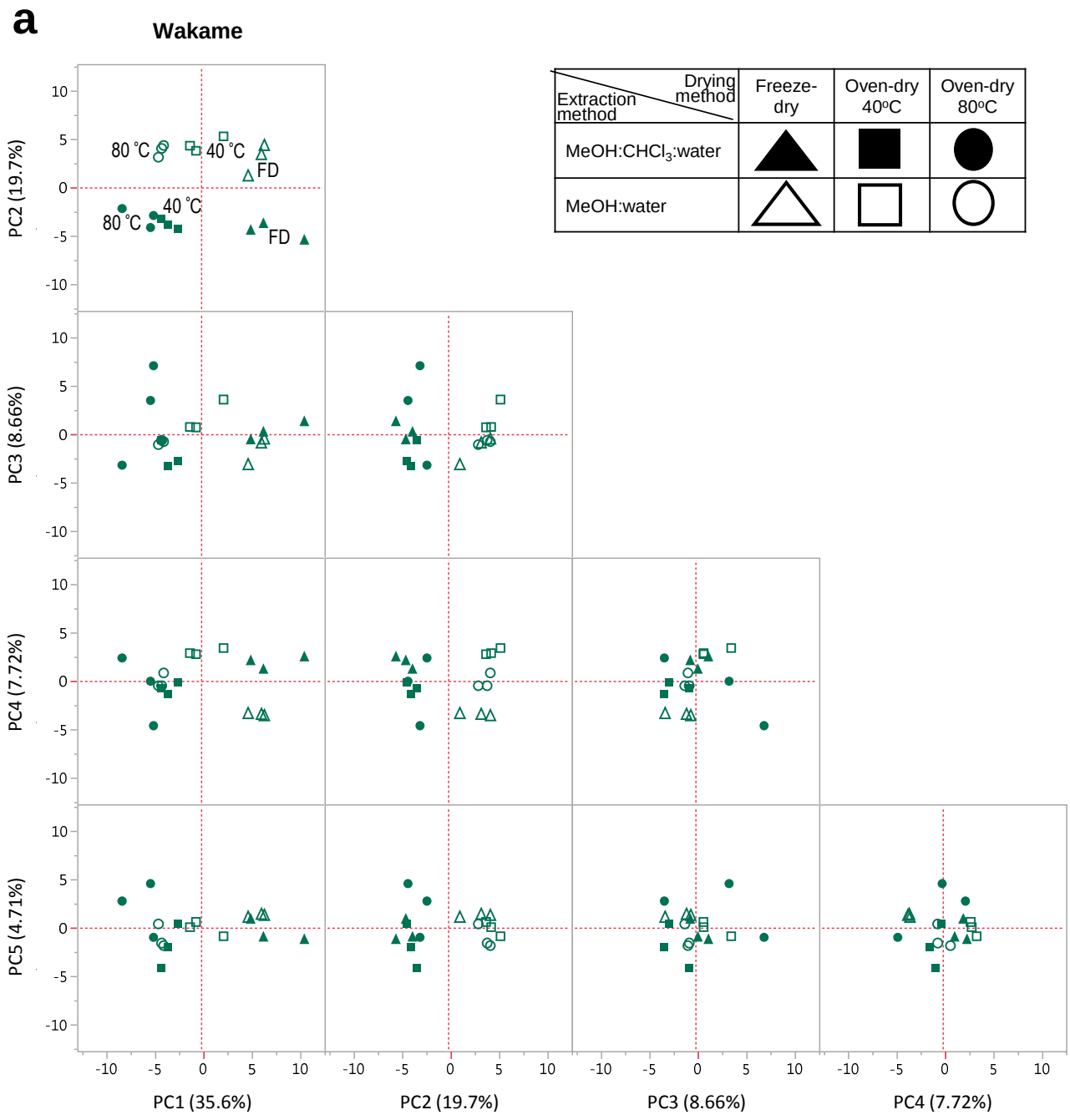


Figure S2.9a PCA score plots of metabolites concentrations in Wakame.
The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-PC5 separation variance.

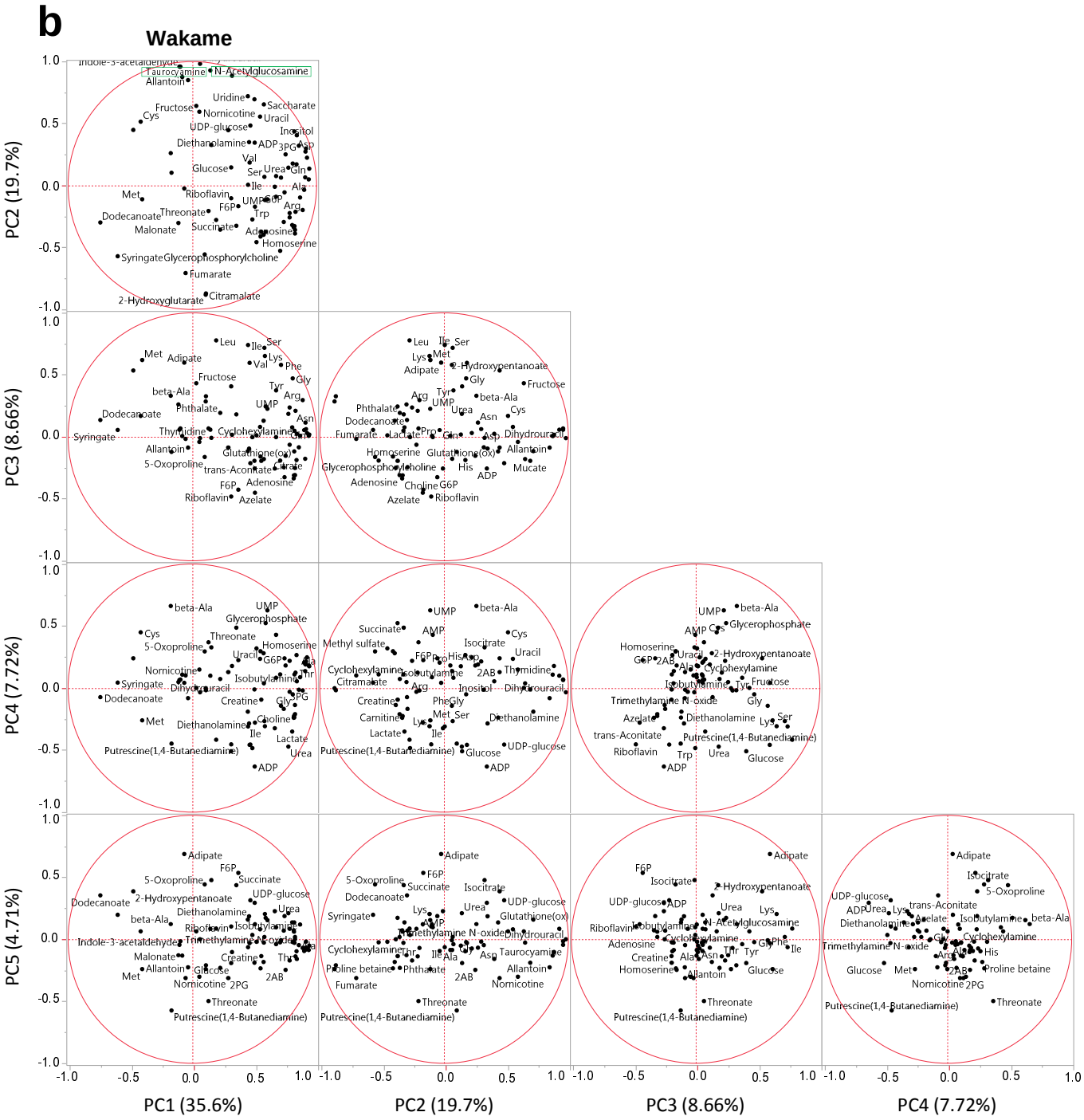


Figure S2.9b PCA loading plots of metabolites concentrations in Wakame.
The blue color box indicate the metabolites which contributed to PC1. The green color box indicate the metabolites which contributed to PC2.

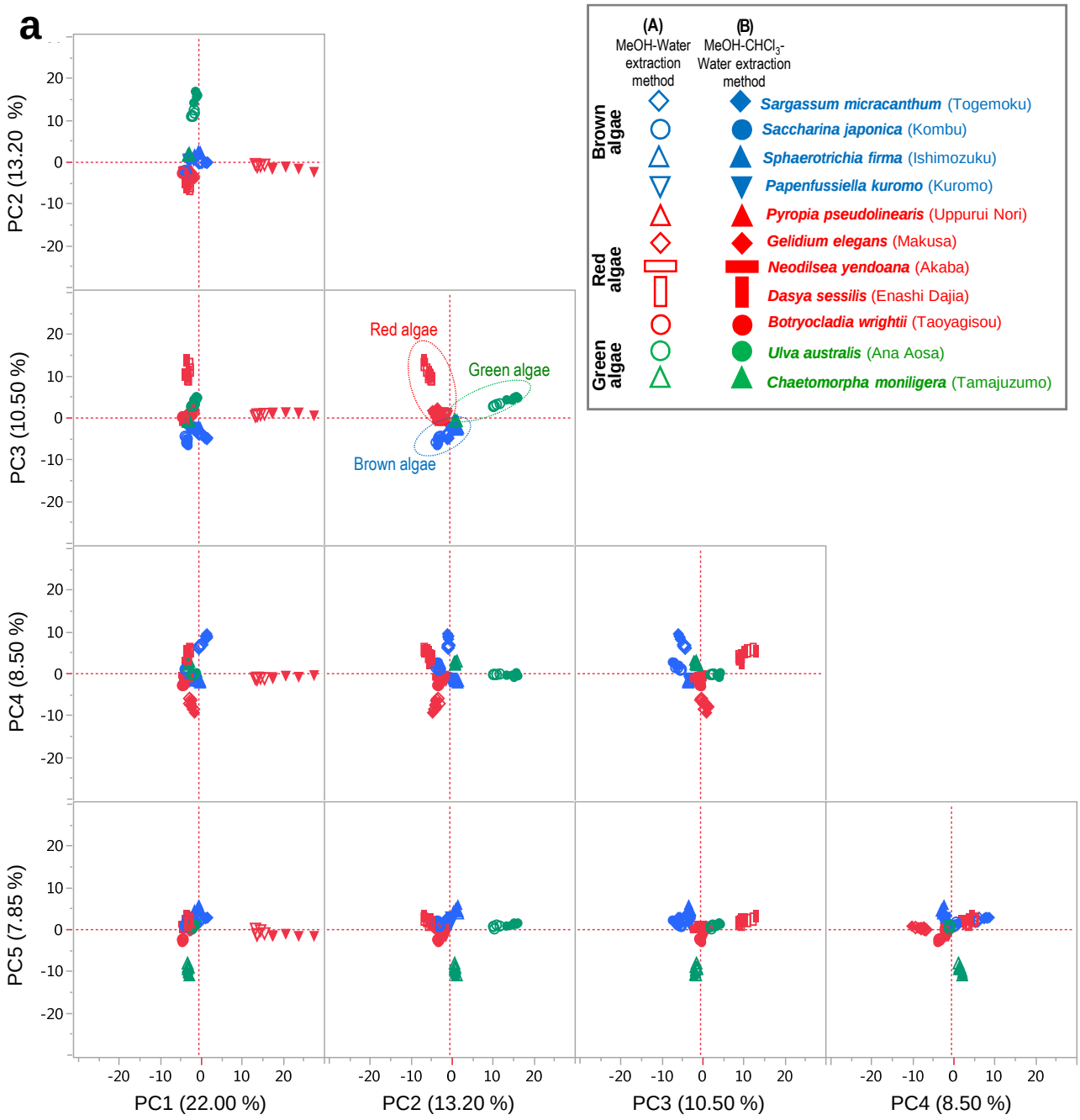
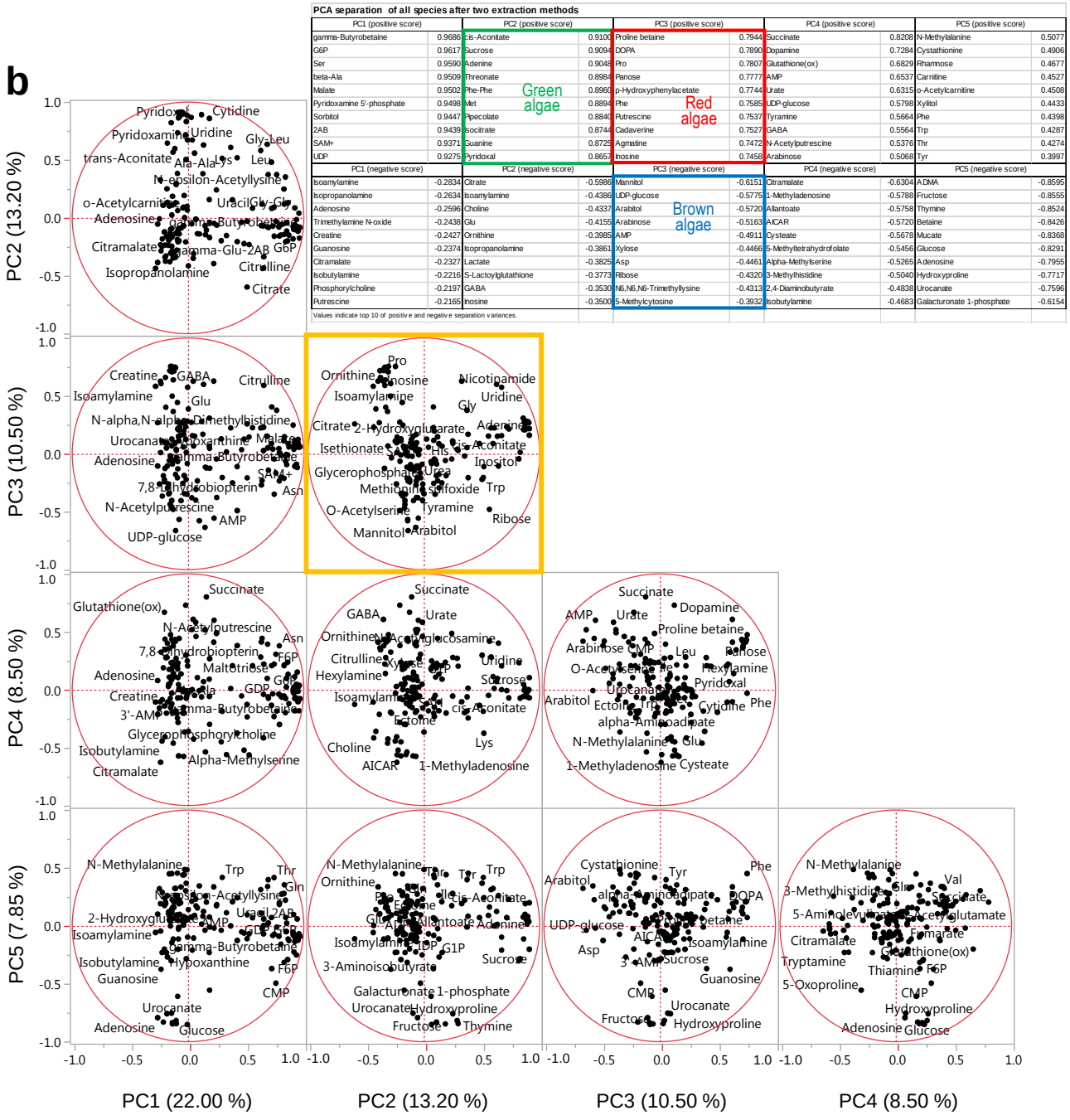


Figure S3.1a PCA score plot of dry weight based metabolites concentrations in three seaweed groups.
Four brown algae are indicated by blue color symbols; *S. micracanthum* (Togemoku), *S. japonica* (Kombu), *S. firma* (Ishimozuku) and *P. kuromo* (Kuromo); five red algae are indicated by red color symbols; *P. pseudolinearis* (Uppurui Nori), *G. elegans* (Makusa), *N. yendoana* (Akaba), *D. sessilis* (Enashi-Dajia) and *B. wrightii* (Taoyagisou) and two green algae are indicated by green color symbols; *U. australis* (Ana Aosa) and *C. moniligera* (Tamajuzumo). Filled and unfilled color indicate the methanol-water extractions with and without chloroform, respectively. The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-5 separation variance. Blue, red and green dotted circle indicate the species group; brown, red and green algae respectively.



PCA separation of all species after two extraction methods

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
gamma-Butyrobetaine	0.9686	cis-Aconitate	0.9100	Proline betaine	0.7944	Succinate	0.8208	N-Methylalanine	0.5077
G6P	0.9617	Sucrose	0.9094	DOPA	0.7890	Dopamine	0.7284	Cystathionine	0.4906
Ser	0.9590	Adenine	0.9048	Pro	0.7807	Glutathione(ox)	0.6829	Rhamnose	0.4677
beta-Ala	0.9509	Threonate	0.8984	Panose	0.7777	AMP	0.6537	Carnitine	0.4527
Malate	0.9502	Phe-Phe	0.8966	p-Hydroxyphenylacetate	0.7744	Urate	0.6315	o-Acetylcarnitine	0.4508
Pyridoxamine 5'-phosphate	0.9498	Met	0.8894	Phe	0.7585	UDP-glucose	0.5798	Xylitol	0.4433
Sorbitol	0.9447	Pipecolate	0.8840	Putrescine	0.7537	Tyramine	0.5664	Phe	0.4398
2AB	0.9439	Isocitrate	0.8744	Cadaverine	0.7527	GABA	0.5564	Trp	0.4287
SAM+	0.9371	Guanine	0.8725	Agmatine	0.7472	N-Acetylputrescine	0.5376	Thr	0.4274
UDP	0.9275	Pyridoxal	0.8657	Inosine	0.7458	Arabinose	0.5068	Tyr	0.3997
PC1 (negative score)		**PC2 (negative score)**		**PC3 (negative score)**		**PC4 (negative score)**		**PC5 (negative score)**	
Isoamylamine	-0.2834	Citrate	-0.5988	Mannitol	-0.6151	Citramalate	-0.6304	ADMA	-0.8595
Isopropanolamine	-0.2634	Isoamylamine	-0.4386	UDP-glucose	-0.5775	1-Methyladenosine	-0.5788	Fructose	-0.8555
Adenosine	-0.2596	Choline	-0.4337	Arabitol	-0.5720	Allantoate	-0.5758	Thymine	-0.8524
Trimethylamine N-oxide	-0.2438	Glu	-0.4155	Arabinose	-0.5163	AICAR	-0.5720	Betaine	-0.8426
Creatine	-0.2427	Ornithine	-0.3985	AMP	-0.4911	Cysteate	-0.5678	Mucate	-0.8368
Guanosine	-0.2374	Isopropanolamine	-0.3861	Xylose	-0.4466	5-Methyltetrahydrofolate	-0.5456	Glucose	-0.8291
Citramalate	-0.2327	Lactate	-0.3825	Asp	-0.4461	Alpha-Methylserine	-0.5265	Adenosine	-0.7955
Isobutylamine	-0.2216	S-Lactoylglutathione	-0.3773	Ribose	-0.4320	3-Methylhistidine	-0.5040	Hydroxyproline	-0.7717
Phosphorylcholine	-0.2197	GABA	-0.3530	N6,N6,N6-Trimethyllysine	-0.4313	2,4-Diaminobutyrate	-0.4838	Urocanate	-0.7596
Putrescine	-0.2165	Inosine	-0.3500	5-Methylcytosine	-0.3932	Isobutylamine	-0.4683	Galacturonate 1-phosphate	-0.6154

Values indicate top 10 of positive and negative separation variances.

Green algae

Red algae

Brown algae

Figure S3.1b PCA loading plot of dry weight based metabolites concentrations in three seaweed groups.
Four brown algae are indicated by blue color symbols; *S. micracanthum* (Togemoku), *S. japonica* (Kombu), *S. firma* (Ishimozuku) and *P. kuromo* (Kuromo); five red algae are indicated by red color symbols; *P. pseudolinearis* (Uppurui Nori), *G. elegans* (Makusa), *N. yendoana* (Akaba), *D. sessilis* (Enashi-Dajia) and *B. wrightii* (Taoyagisou) and two green algae are indicated by green color symbols; *U. australis* (Ana Aosa) and *C. moniligera* (Tamajuzumo). Filled and unfilled color indicate the methanol-water extractions with and without chloroform, respectively. The horizontal axis value indicates PC1-PC4 separation variance and the vertical axis value indicates PC2-5 separation variance. Blue, red and green dotted circle indicate the species group; brown, red and green algae respectively.

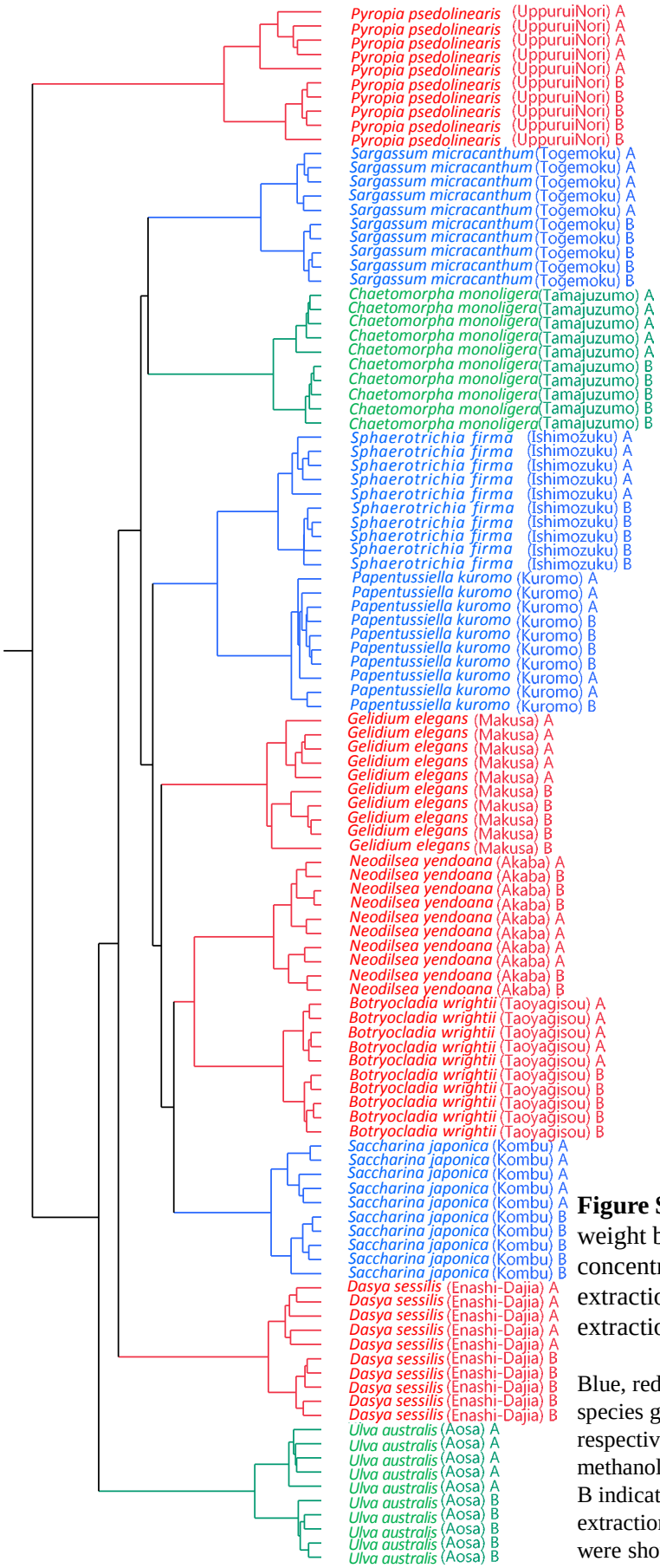


Figure S3.2 HCA clusterization of dry weight based of metabolites concentrations in all data set after both extraction methods; methanol-water extractions with and without chloroform.

Blue, red and green font indicate the seaweed species groups; brown, red and green algae respectively. Alphabet A indicates the methanol-water extraction method; alphabet B indicates the methanol-chloroform-water extraction method. This data set of heat map were shown are the same as in Figure 3.3.

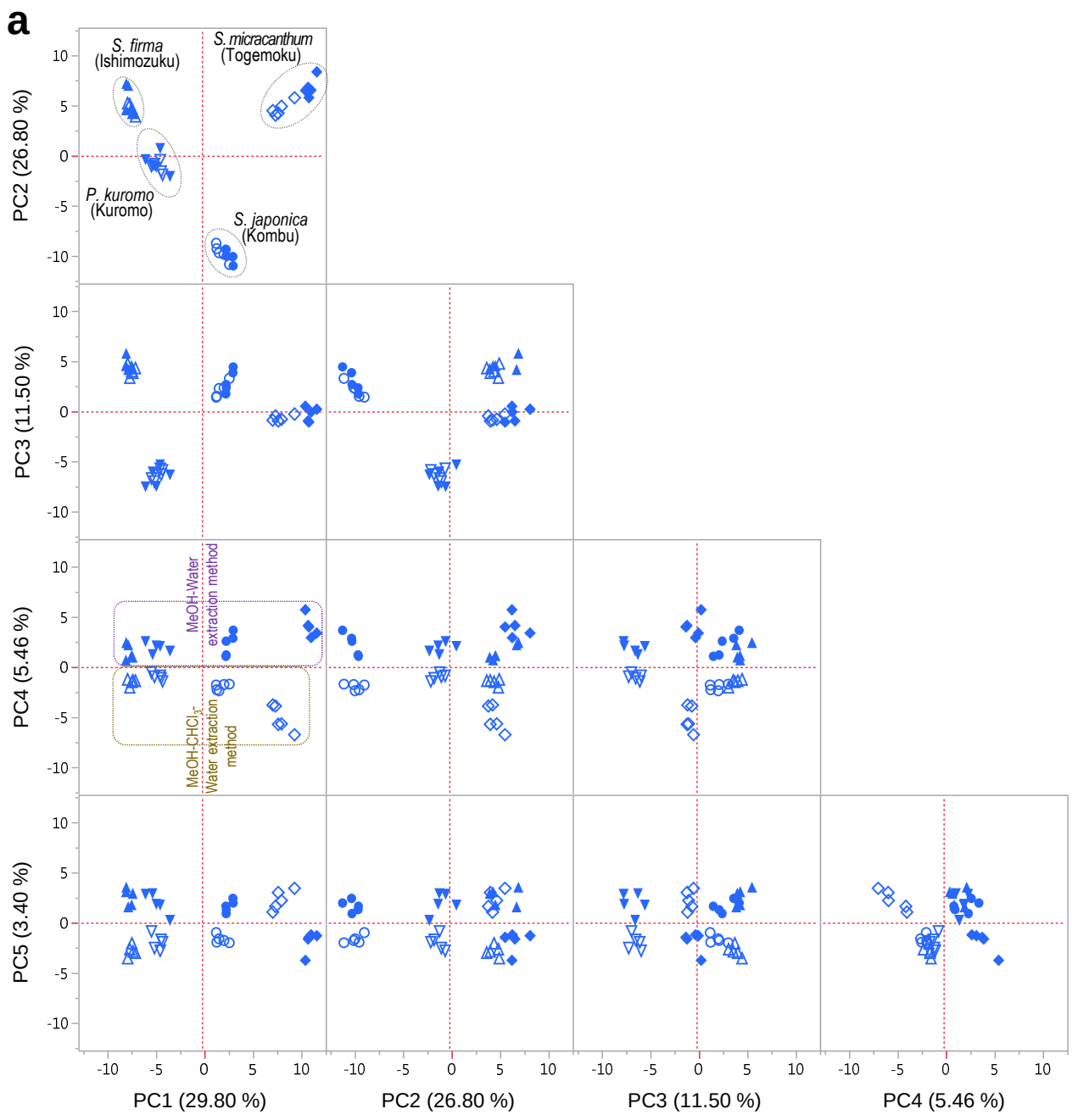


Figure S3.3a PCA score plots of dry weight based metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

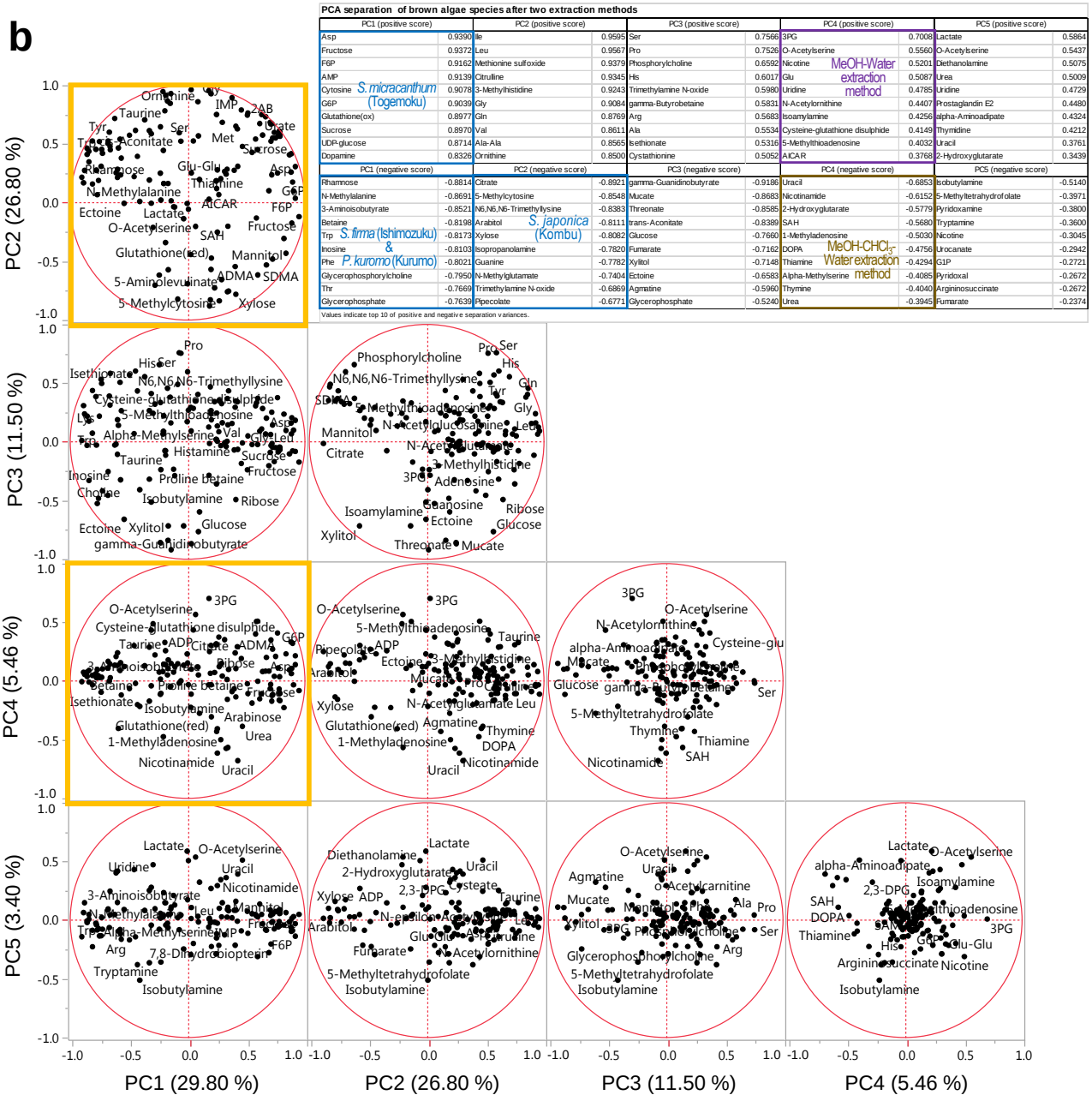


Figure S3.3b PCA loading plots of dry weight based metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

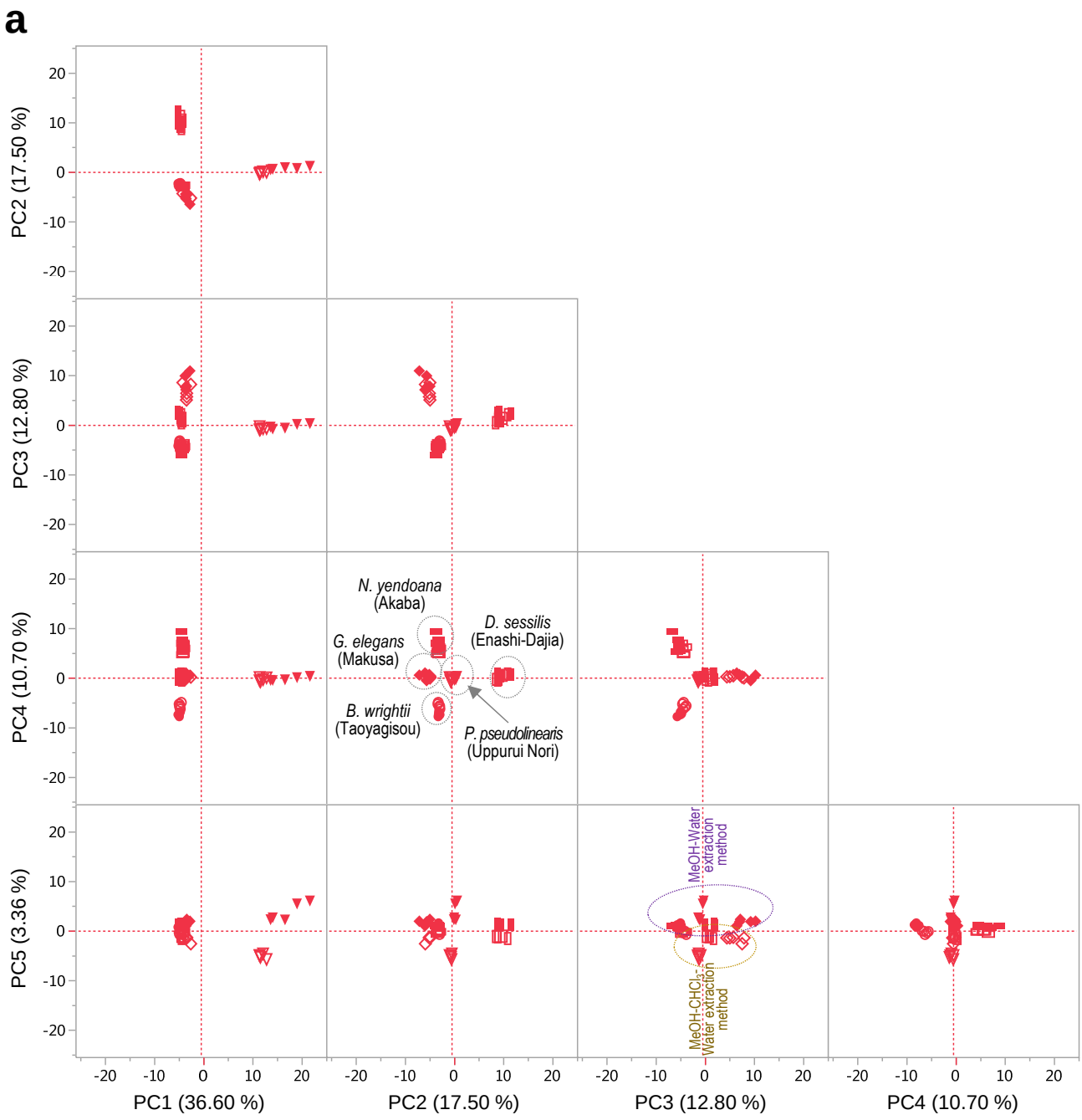


Figure S3.4a PCA score plots of dry weight based metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

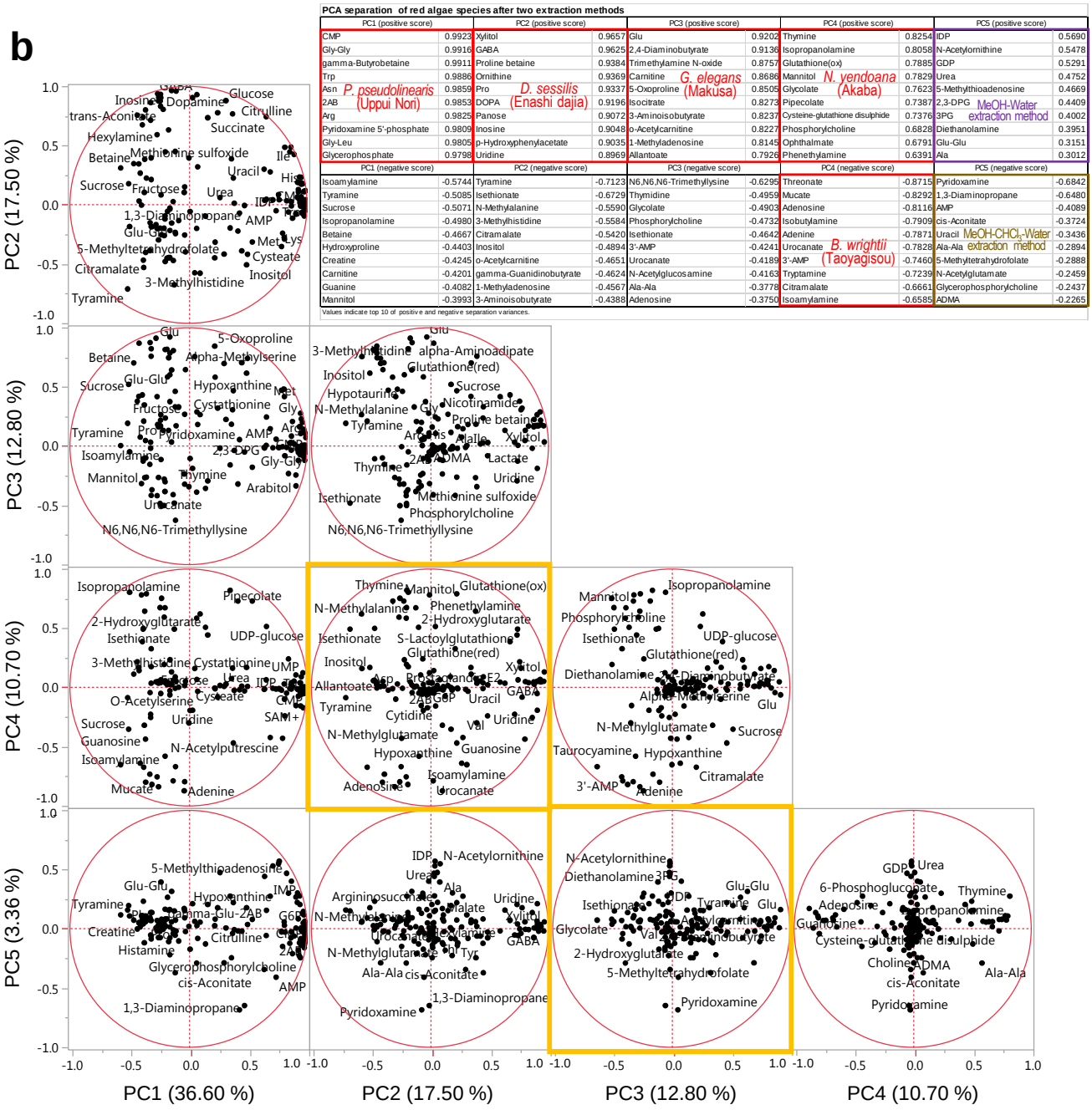


Figure S3.4b PCA loading plots of dry weight based metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

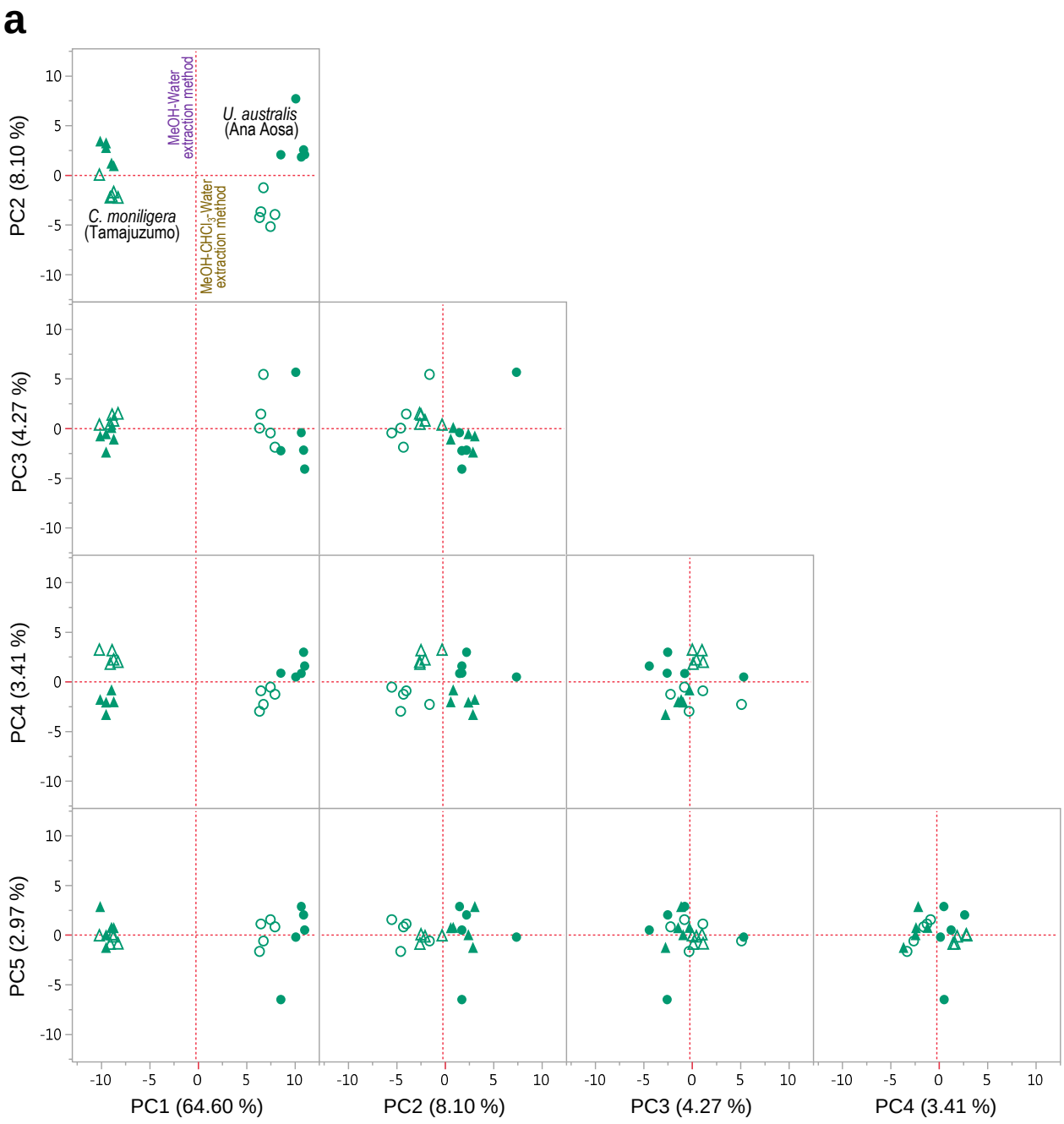
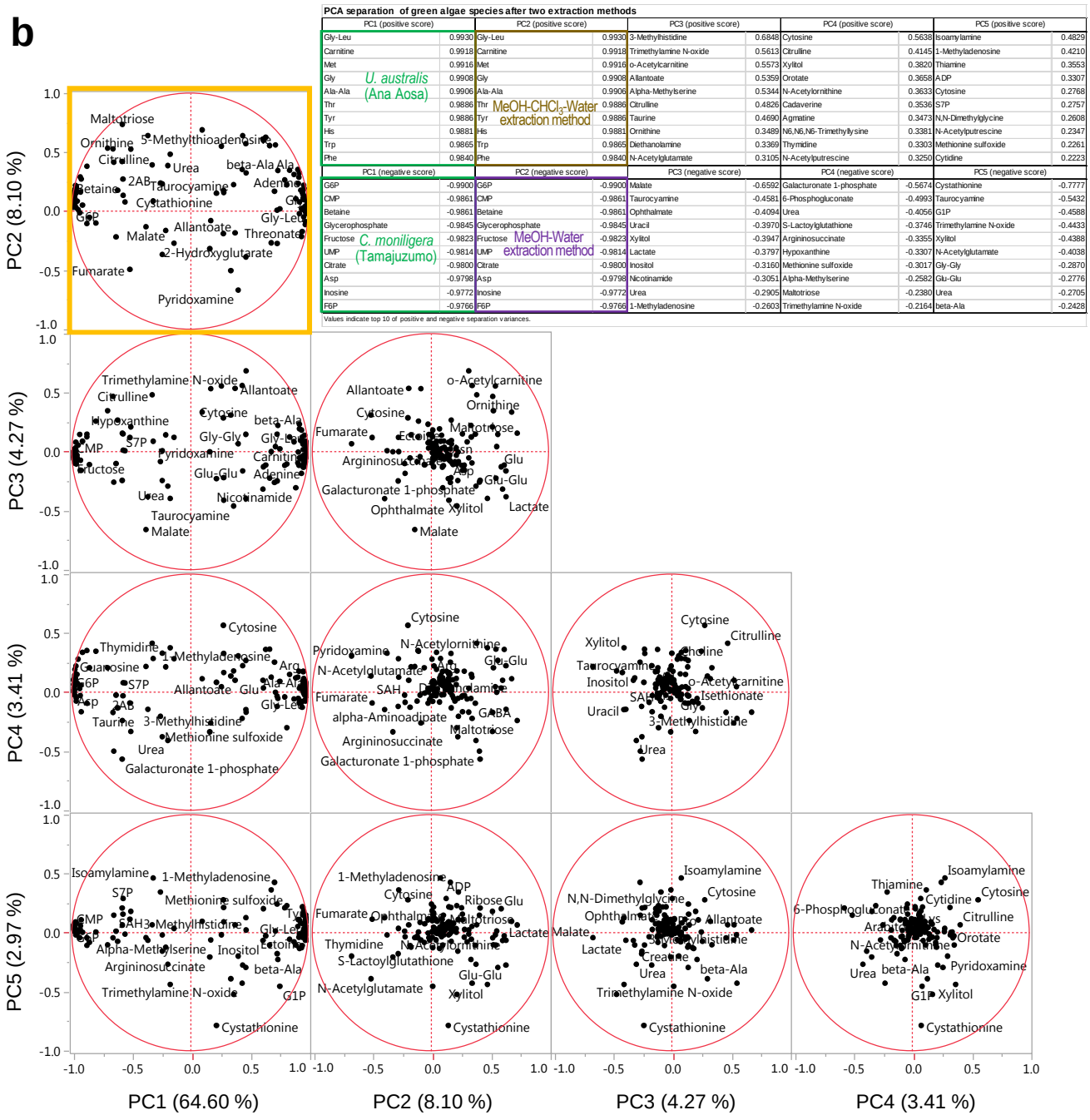


Figure S3.5a PCA score plots of dry weight based metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.



PCA separation of green algae species after two extraction methods

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Gly-Leu	0.9930	Gly-Leu	0.9930	3-Methylhistidine	0.6848	Cytosine	0.5638	Isoamylamine	0.4829
Carnitine	0.9918	Carnitine	0.9918	Trimethylamine N-oxide	0.5613	Citrulline	0.4145	1-Methyladenosine	0.4210
Met	0.9916	Met	0.9916	o-Acetylcarnitine	0.5573	Xylitol	0.3820	Thiamine	0.3553
Gly	0.9908	Gly	0.9908	Allantoate	0.5359	Orotate	0.3658	ADP	0.3307
Ala-Ala	0.9906	Ala-Ala	0.9906	Alpha-Methylserine	0.5344	N-Acetylornithine	0.3633	Cytosine	0.2768
Thr	0.9886	Thr	0.9886	Citrulline	0.4826	Cadaverine	0.3536	S7P	0.2757
Tyr	0.9886	Tyr	0.9886	Taurine	0.4690	Agmatine	0.3473	N,N-Dimethylglycine	0.2608
His	0.9881	His	0.9881	Ornithine	0.3489	N6,N6,N6-Trimethyllysine	0.3381	N-Acetylputrescine	0.2347
Trp	0.9865	Trp	0.9865	Diethanolamine	0.3369	Thymidine	0.3303	Methionine sulfoxide	0.2261
Phe	0.9840	Phe	0.9840	N-Acetylglutamate	0.3105	N-Acetylputrescine	0.3250	Cytidine	0.2223
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
G6P	-0.9900	G6P	-0.9900	Malate	-0.6592	Galacturonate 1-phosphate	-0.5674	Cystathionine	-0.7777
CMP	-0.9861	CMP	-0.9861	Taurocyamine	-0.4581	6-Phosphogluconate	-0.4993	Taurocyamine	-0.5432
Betaine	-0.9861	Betaine	-0.9861	Ophthalmate	-0.4094	Urea	-0.4056	G1P	-0.4588
Glycerophosphate	-0.9845	Glycerophosphate	-0.9845	Uracil	-0.3970	S-Lactoylglutathione	-0.3746	Trimethylamine N-oxide	-0.4433
Fructose	-0.9823	Fructose	-0.9823	Xylitol	-0.3947	Argininosuccinate	-0.3355	Xylitol	-0.4388
UMP	-0.9814	UMP	-0.9814	Lactate	-0.3797	Hypoxanthine	-0.3307	N-Acetylglutamate	-0.4038
Citrate	-0.9800	Citrate	-0.9800	Inositol	-0.3160	Methionine sulfoxide	-0.3017	Gly-Gly	-0.2870
Asp	-0.9798	Asp	-0.9798	Nicotinamide	-0.3051	Alpha-Methylserine	-0.2582	Glu-Glu	-0.2776
Inosine	-0.9772	Inosine	-0.9772	Urea	-0.2905	Maltotriose	-0.2380	Urea	-0.2705
F6P	-0.9766	F6P	-0.9766	1-Methyladenosine	-0.2603	Trimethylamine N-oxide	-0.2164	beta-Ala	-0.2428

U. australis (Ana Aosa) — MeOH-$CHCl_3$-Water extraction method

C. moniligera (Tamajuzumo) — MeOH-Water extraction method

Values indicate top 10 of positive and negative separation variances.

Figure S3.5b PCA loading plots of dry weight based metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.

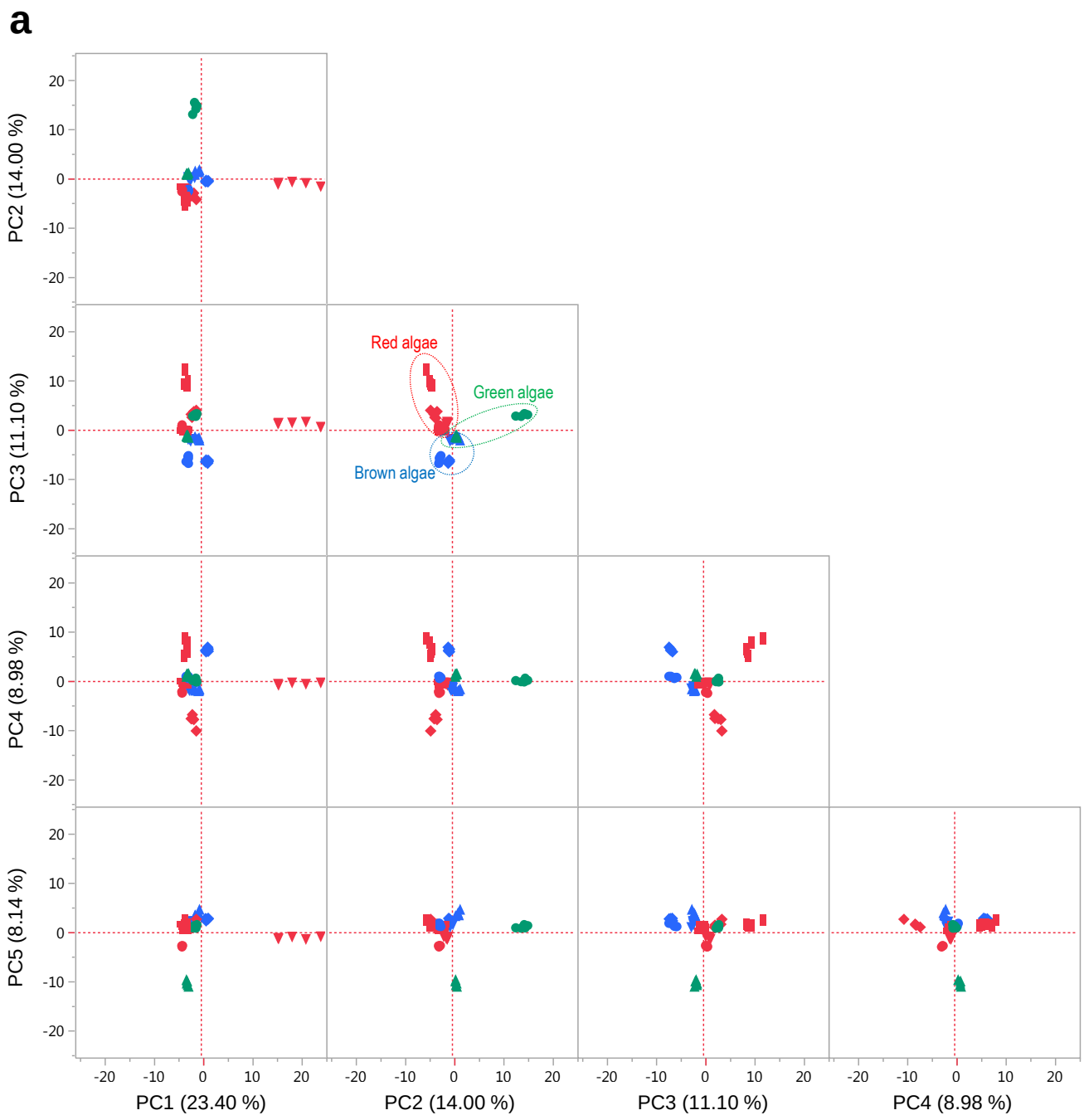
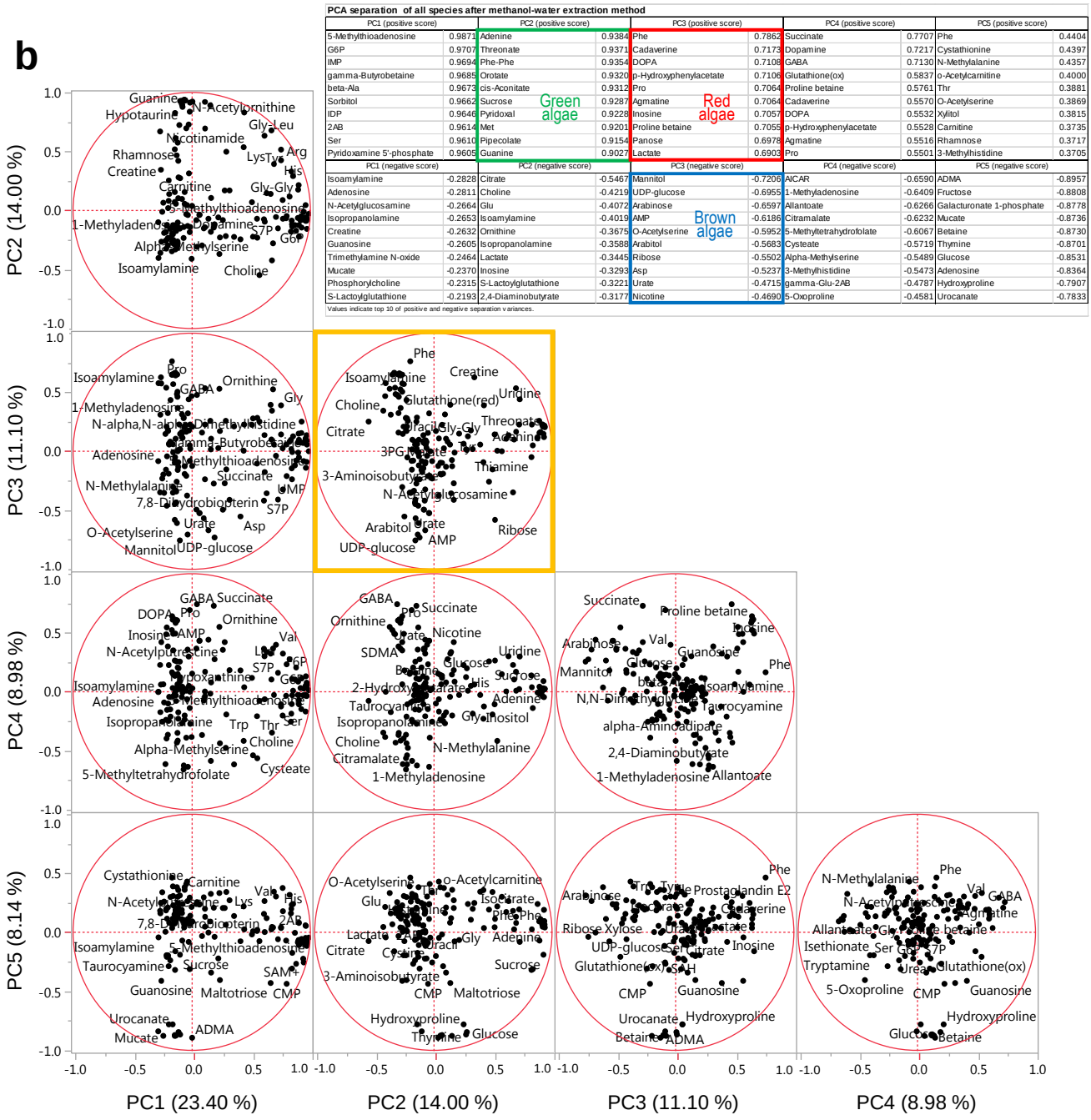


Figure S3.6a PCA score plots of dry weight based metabolites concentrations in all data set after extracted using methanol-water extraction methods.



PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
5-Methylthioadenosine	0.9871	Adenine	0.9384	Phe	0.7862	Succinate	0.7707	Phe	0.4404
G6P	0.9707	Threonate	0.9371	Cadaverine	0.7173	Dopamine	0.7217	Cystathionine	0.4397
IMP	0.9694	Phe-Phe	0.9354	DOPA	0.7108	GABA	0.7130	N-Methylalanine	0.4357
gamma-Butyrobetaine	0.9685	Orotate	0.9320	p-Hydroxyphenylacetate	0.7106	Glutathione(ox)	0.5837	o-Acetylcarnitine	0.4000
beta-Ala	0.9673	cis-Aconitate	0.9312	Pro	0.7064	Proline betaine	0.5761	Thr	0.3881
Sorbitol	0.9662	Sucrose	0.9287	Agmatine	0.7064	Cadaverine	0.5570	O-Acetylserine	0.3869
IDP	0.9646	Pyridoxal	0.9228	Inosine	0.7057	DOPA	0.5532	Xylitol	0.3815
2AB	0.9614	Met	0.9201	Proline betaine	0.7055	p-Hydroxyphenylacetate	0.5528	Carnitine	0.3735
Ser	0.9610	Pipecolate	0.9154	Panose	0.6978	Agmatine	0.5516	Rhamnose	0.3717
Pyridoxamine 5'-phosphate	0.9605	Guanine	0.9027	Lactate	0.6903	Pro	0.5501	3-Methylhistidine	0.3705
PC1 (negative score)		**PC2 (negative score)**		**PC3 (negative score)**		**PC4 (negative score)**		**PC5 (negative score)**	
Isoamylamine	-0.2828	Citrate	-0.5467	Mannitol	-0.7206	AICAR	-0.6590	ADMA	-0.8957
Adenosine	-0.2811	Choline	-0.4219	UDP-glucose	-0.6955	1-Methyladenosine	-0.6409	Fructose	-0.8808
N-Acetylglucosamine	-0.2664	Glu	-0.4072	Arabinose	-0.6597	Allantoate	-0.6266	Galacturonate 1-phosphate	-0.8778
Isopropanolamine	-0.2653	Isoamylamine	-0.4019	AMP	-0.6186	Citramalate	-0.6232	Mucate	-0.8736
Creatine	-0.2632	Ornithine	-0.3675	O-Acetylserine	-0.5952	5-Methyltetrahydrofolate	-0.6067	Betaine	-0.8730
Guanosine	-0.2605	Isopropanolamine	-0.3588	Arabitol	-0.5683	Cysteate	-0.5719	Thymine	-0.8701
Trimethylamine N-oxide	-0.2464	Lactate	-0.3445	Ribose	-0.5502	Alpha-Methylserine	-0.5489	Glucose	-0.8531
Mucate	-0.2370	Inosine	-0.3293	Asp	-0.5237	3-Methylhistidine	-0.5473	Adenosine	-0.8364
Phosphorylcholine	-0.2315	S-Lactoylglutathione	-0.3221	Urate	-0.4715	gamma-Glu-2AB	-0.4787	Hydroxyproline	-0.7907
S-Lactoylglutathione	-0.2193	2,4-Diaminobutyrate	-0.3177	Nicotine	-0.4690	5-Oxoproline	-0.4581	Urocanate	-0.7833

Values indicate top 10 of positive and negative separation variances.

Figure S3.6b PCA loading plots of dry weight based metabolites concentrations in all data set after extracted using methanol-water extraction methods.

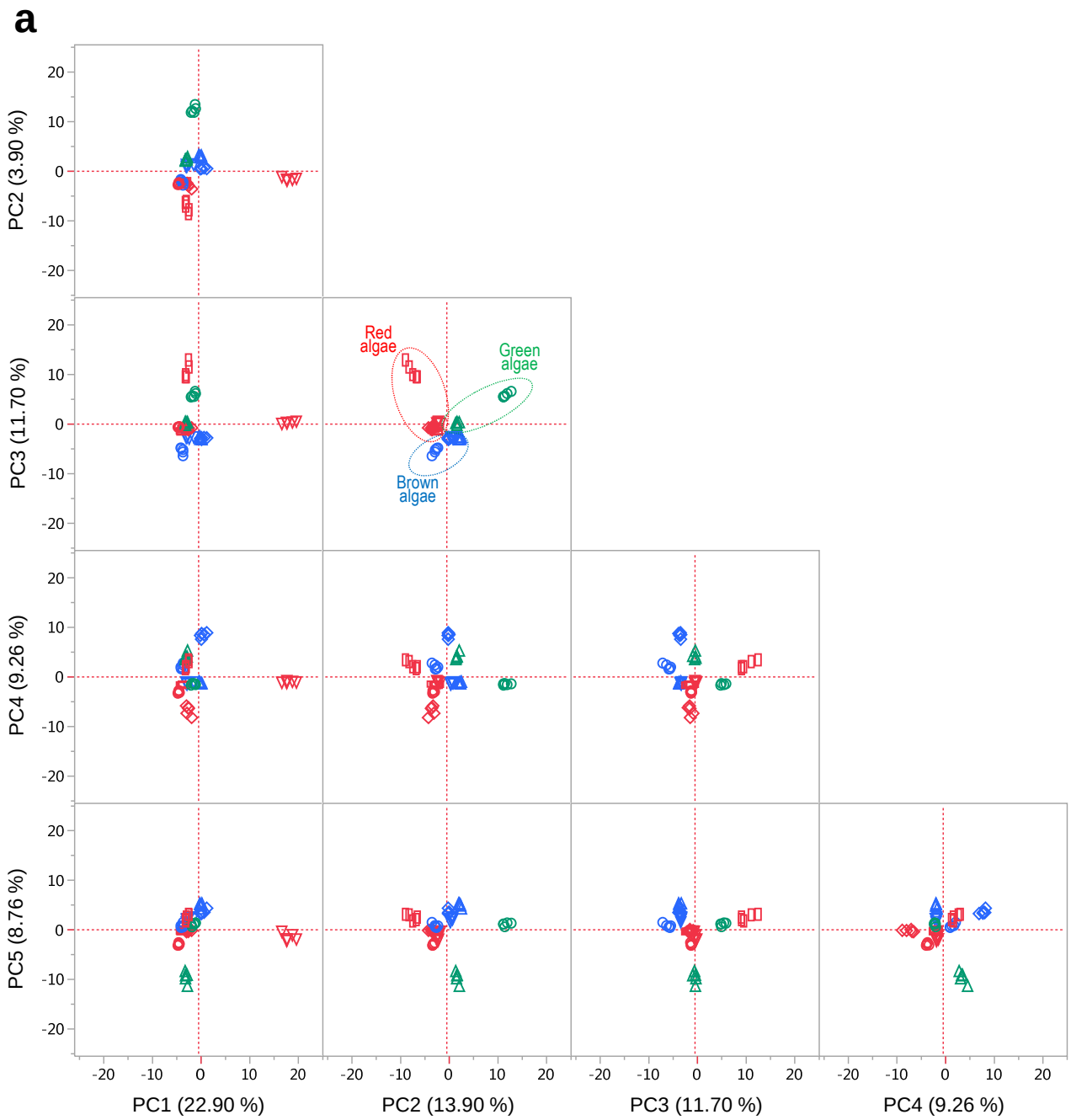


Figure S3.7a PCA score plots of dry weight based metabolites concentrations in all data set after extracted using methanol-chloroform-water extraction methods.

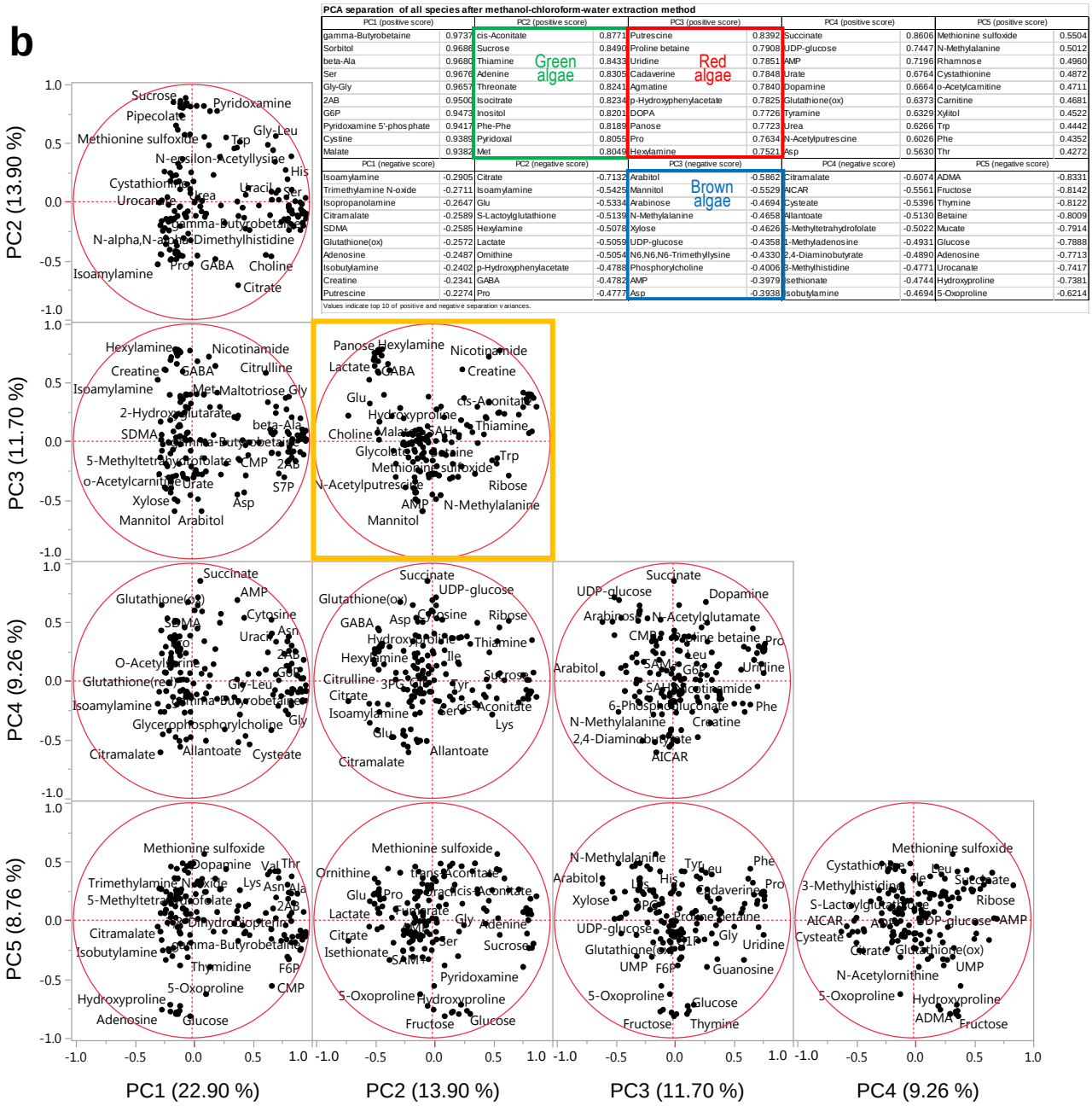


Figure S3.7b PCA loading plots of dry weight based metabolites concentrations in all data set after extracted using methanol-chloroform-water extraction methods.

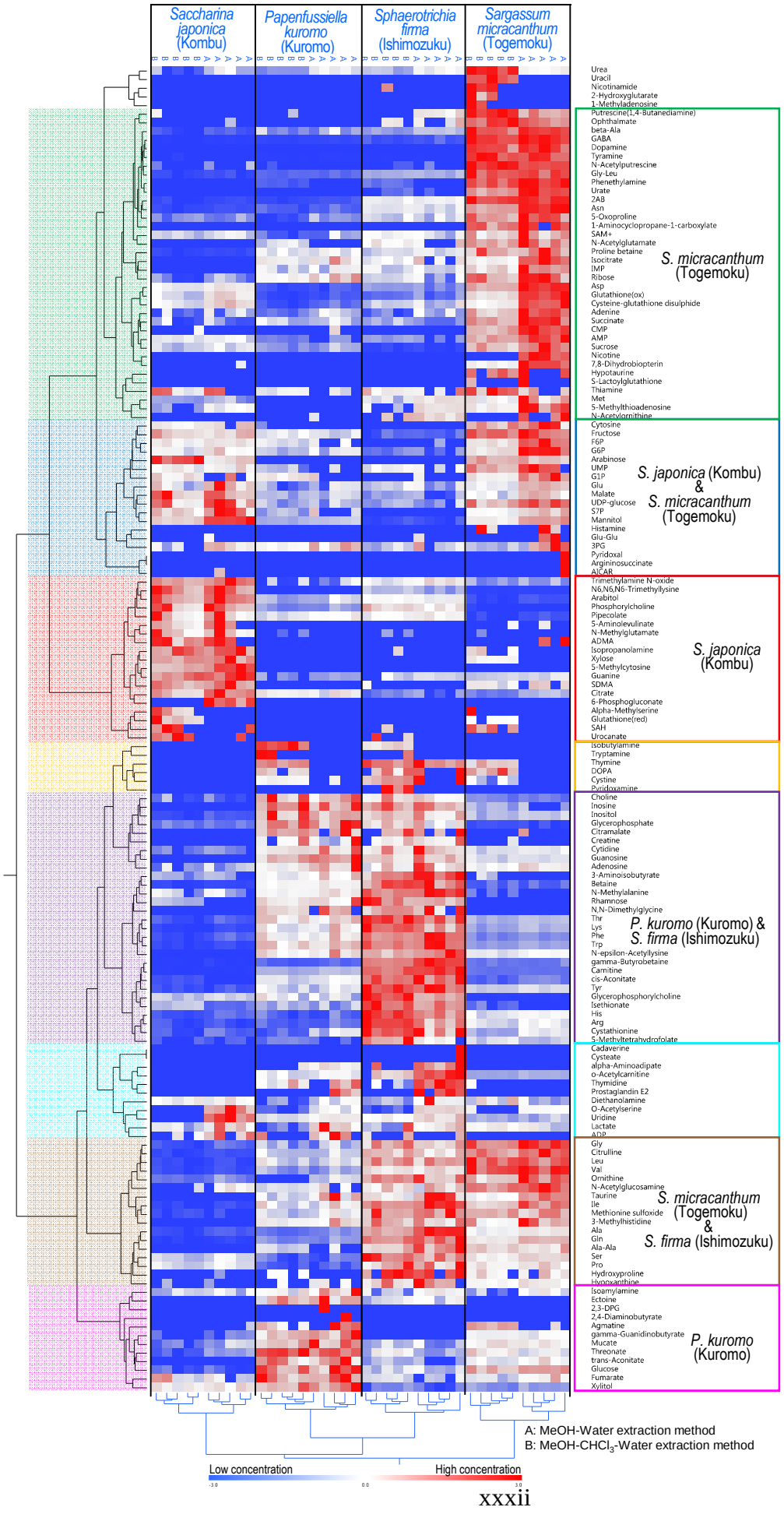


Figure S3.8 HCA and heat map of dry weight based of metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

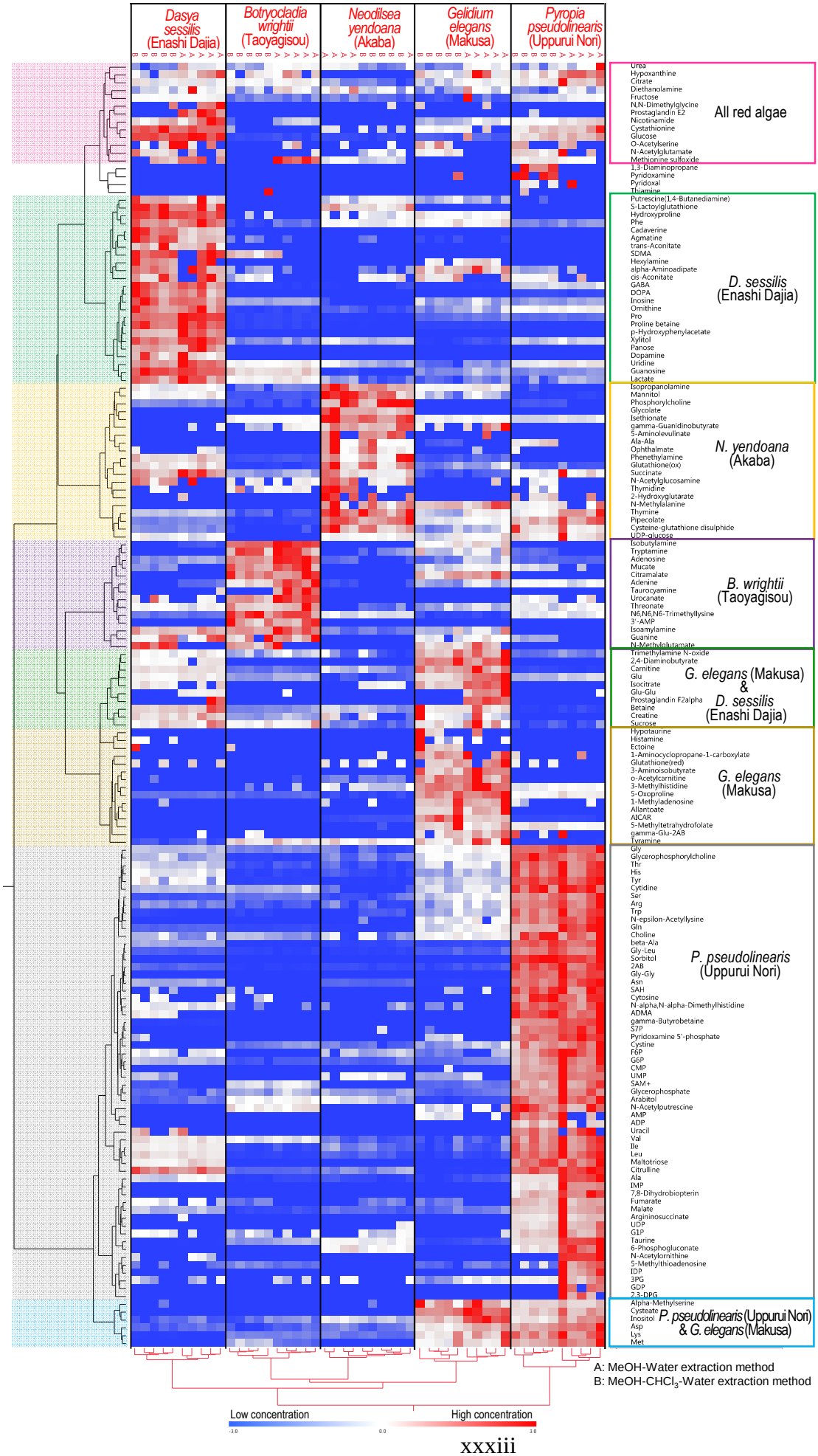


Figure S3.9 HCA and heat map of dry weight based of metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

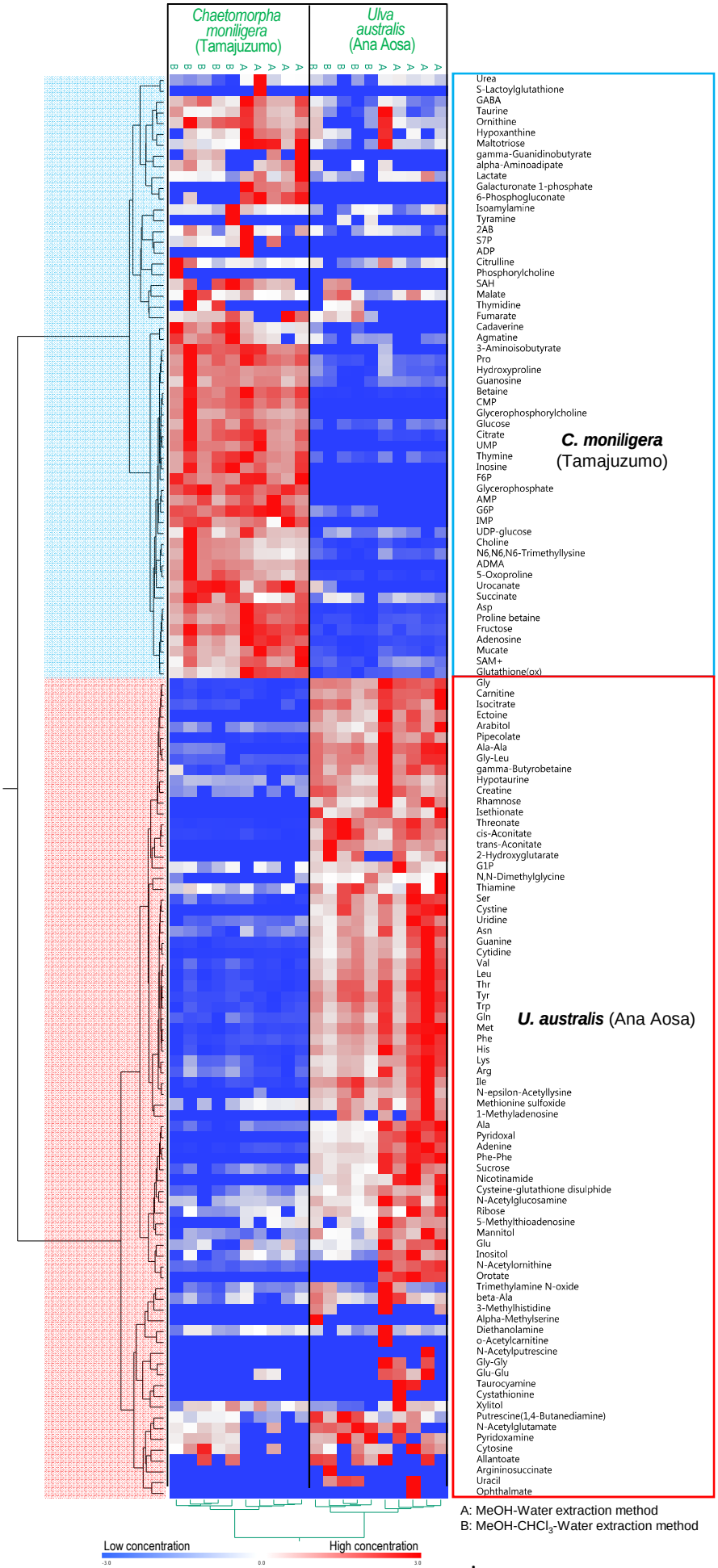


Figure S3.10 HCA and heat map of dry weight based of metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.

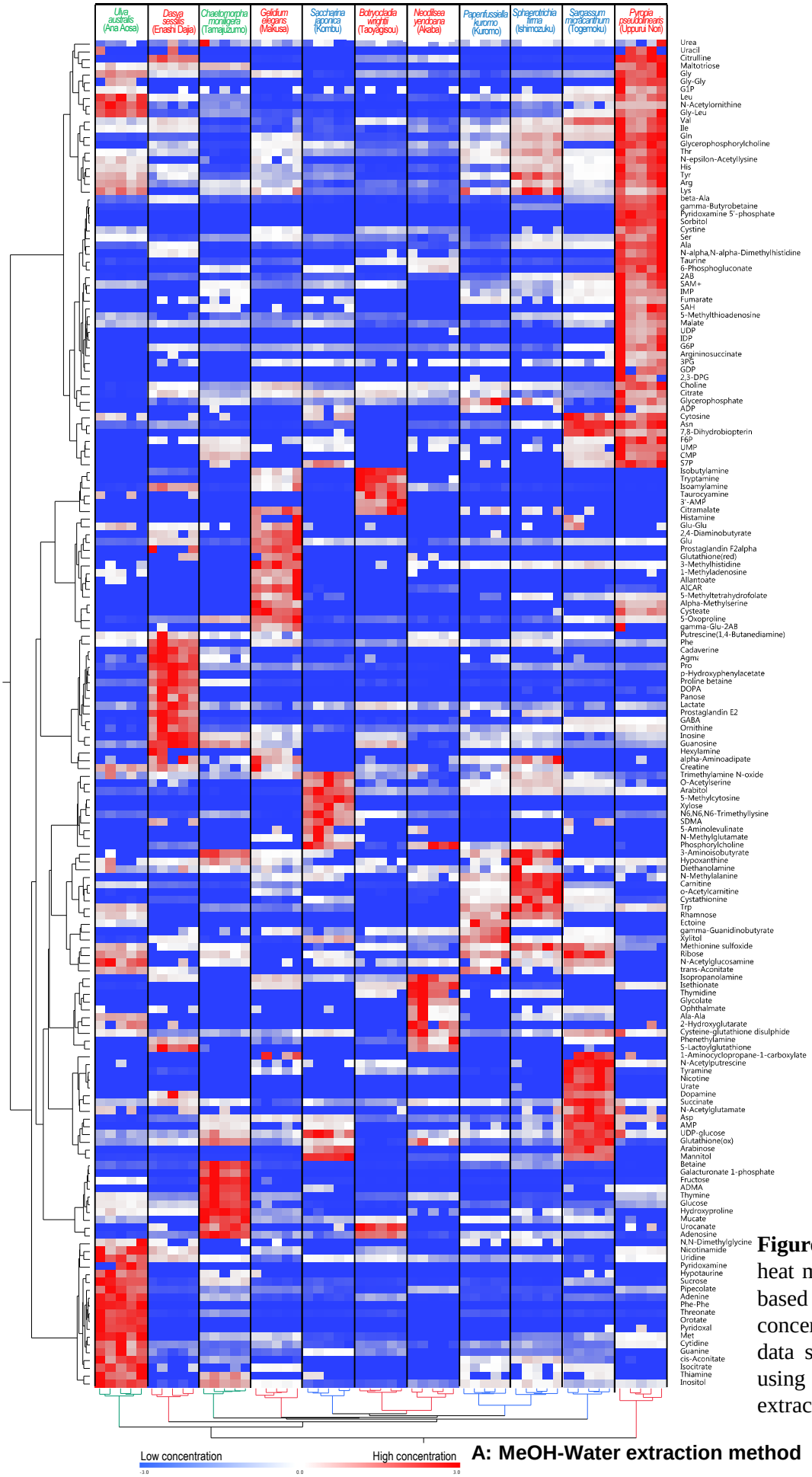


Figure S3.11 HCA and heat map of dry weight based of metabolites concentrations in all data set after extracted using methanol-water extraction methods.

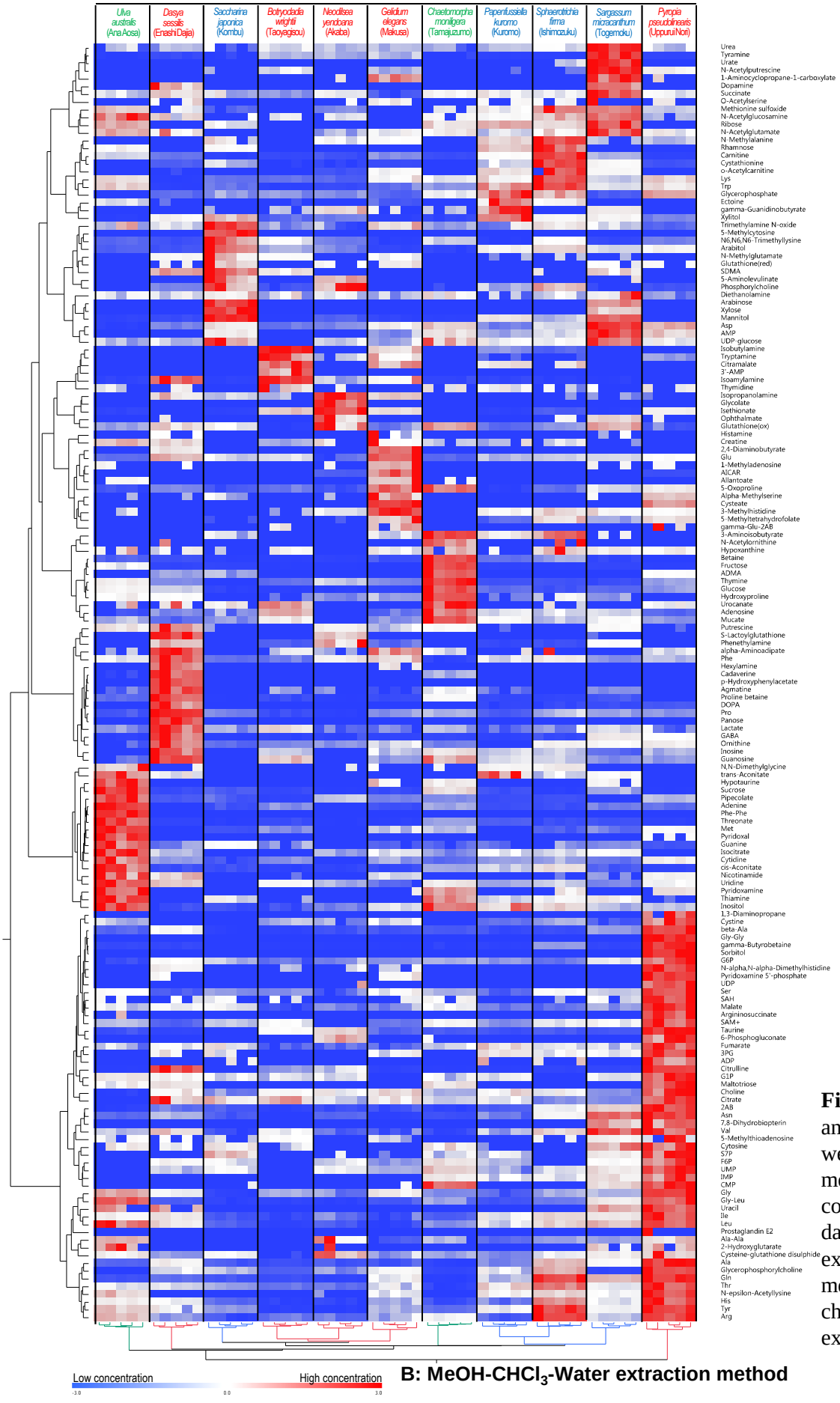


Figure S3.12 HCA and heat map of dry weight based of metabolites concentrations in all data set after extracted using methanol-chloroform-water extraction methods.

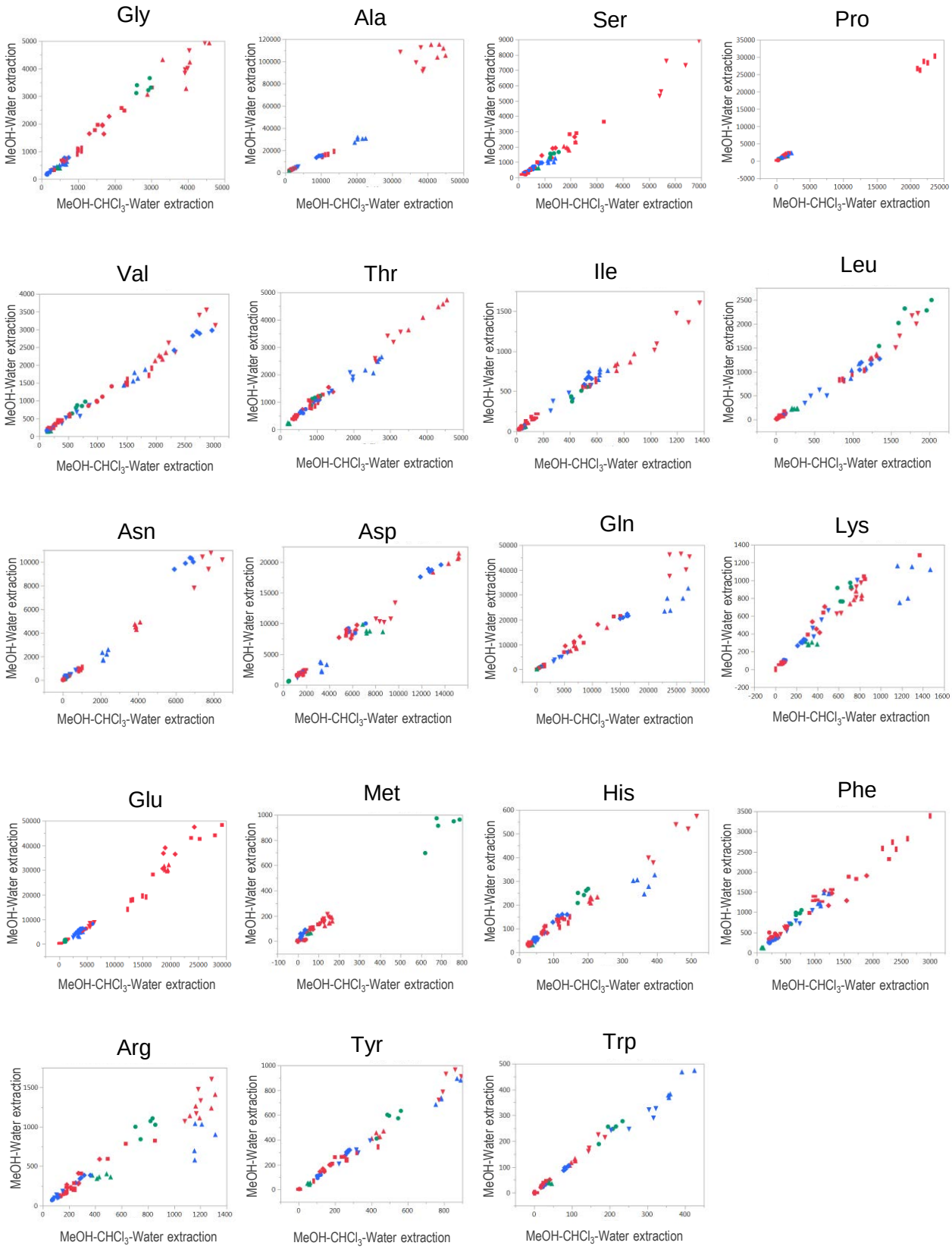


Figure S3.13a Comparison of dry weight based metabolite concentration methanol-water and methanol-chloroform-water extraction methods in amino acids.
X- and Y-axis indicate the methanol-chloroform-water and methanol-water extraction methods, respectively. The numbers of both axes indicate mean ± SD (nmol g^{-1}) of metabolites concentration of dry weight (n=5).

b) TCA and glycolysis organic acids.

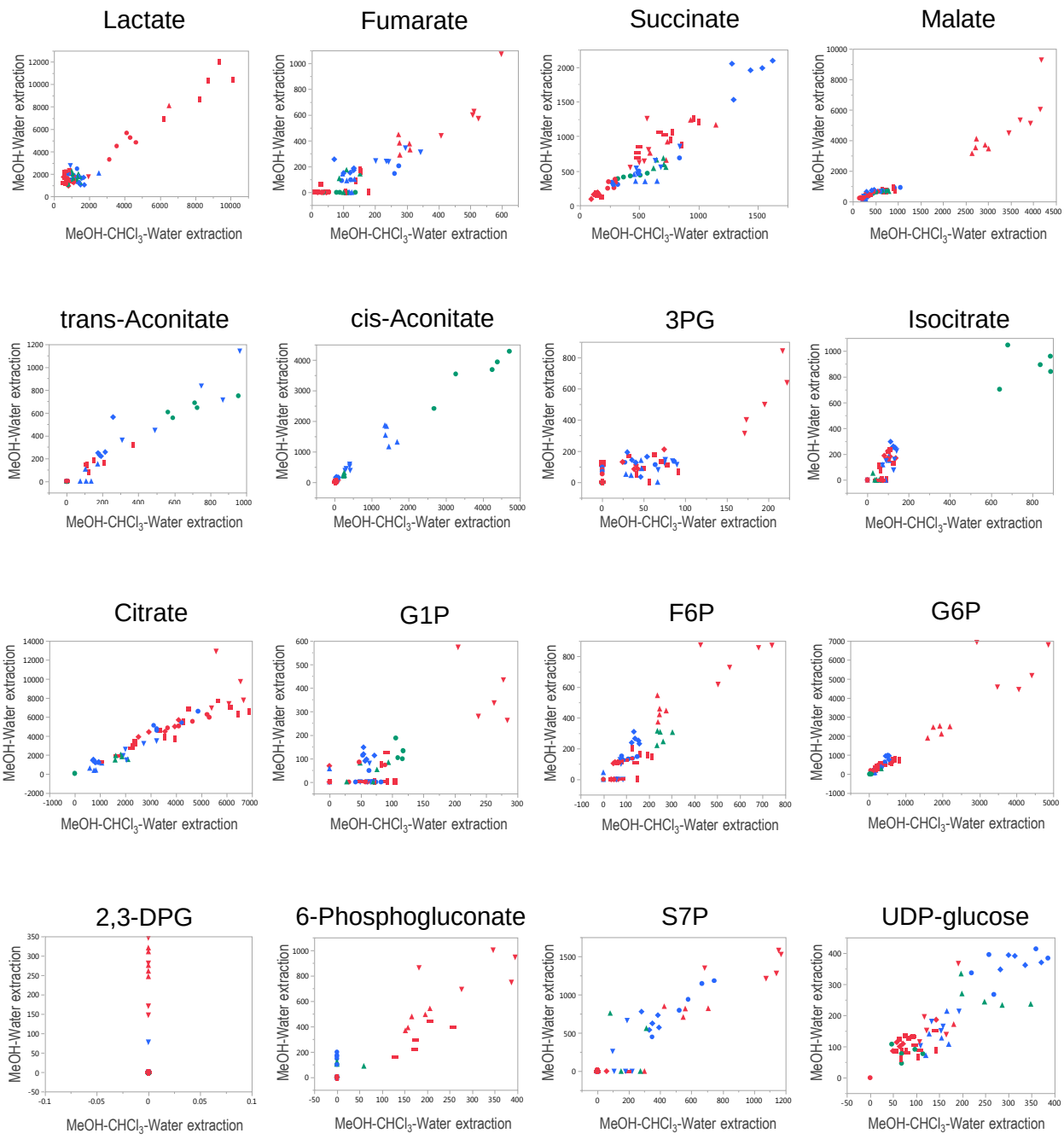


Figure S3.13b Comparison of dry weight based metabolite concentration methanol-water and methanol-chloroform-water extraction methods in TCA and glycolysis organic acids.
X- and Y-axis indicate the methanol-chloroform-water and methanol-water extraction methods, respectively. The numbers of both axes indicate mean ±SD (nmol g^{-1}) of metabolites concentration of dry weight (n=5).

c) Sugars

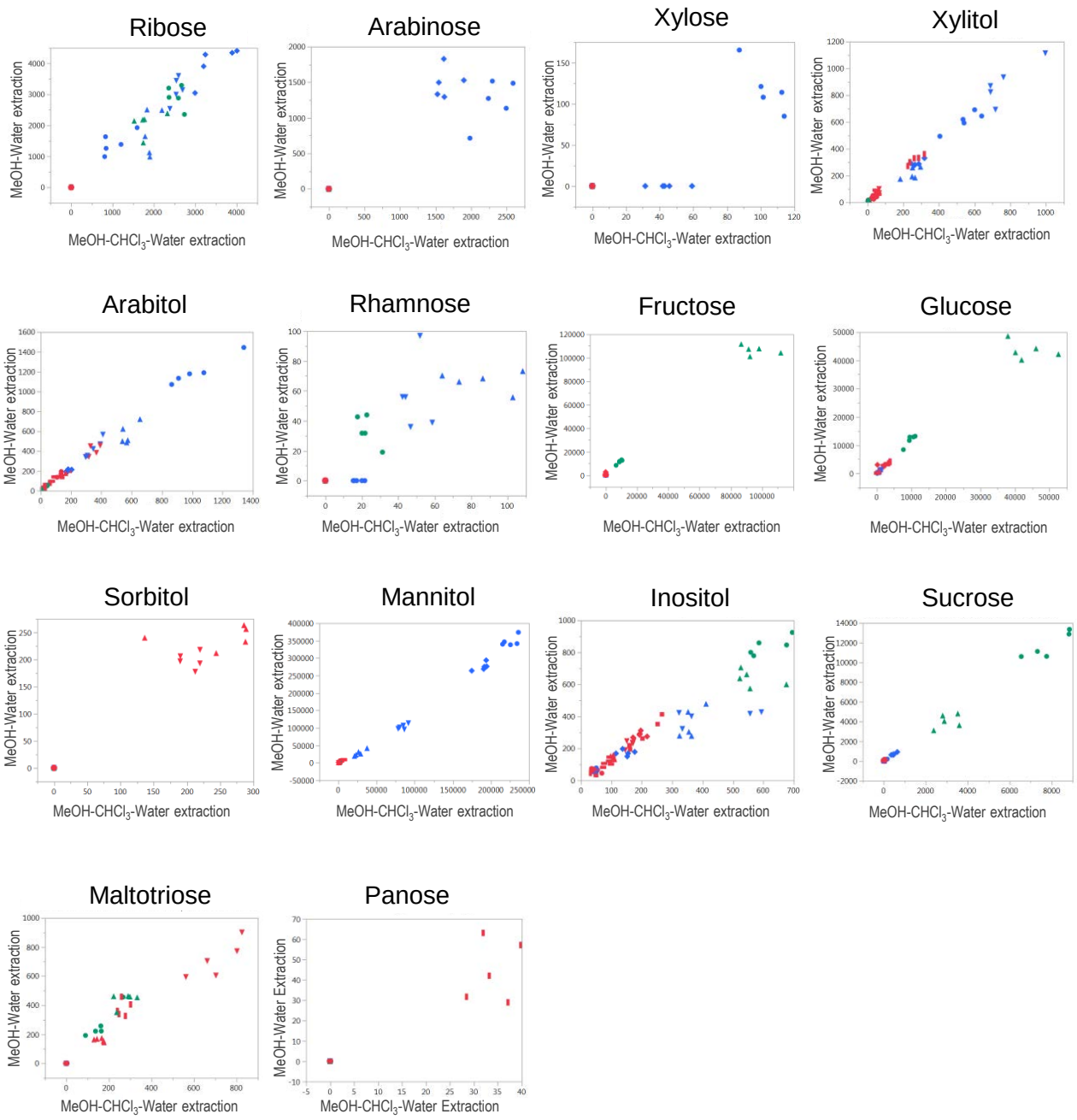


Figure S3.13c Comparison of dry weight based metabolite concentration methanol-water and methanol-chloroform-water extraction methods in sugars.
X- and Y-axis indicate the methanol-chloroform-water and methanol-water extraction methods, respectively. The numbers of both axes indicate mean ± SD (nmol g^{-1}) of metabolites concentration of dry weight (*n*=5).

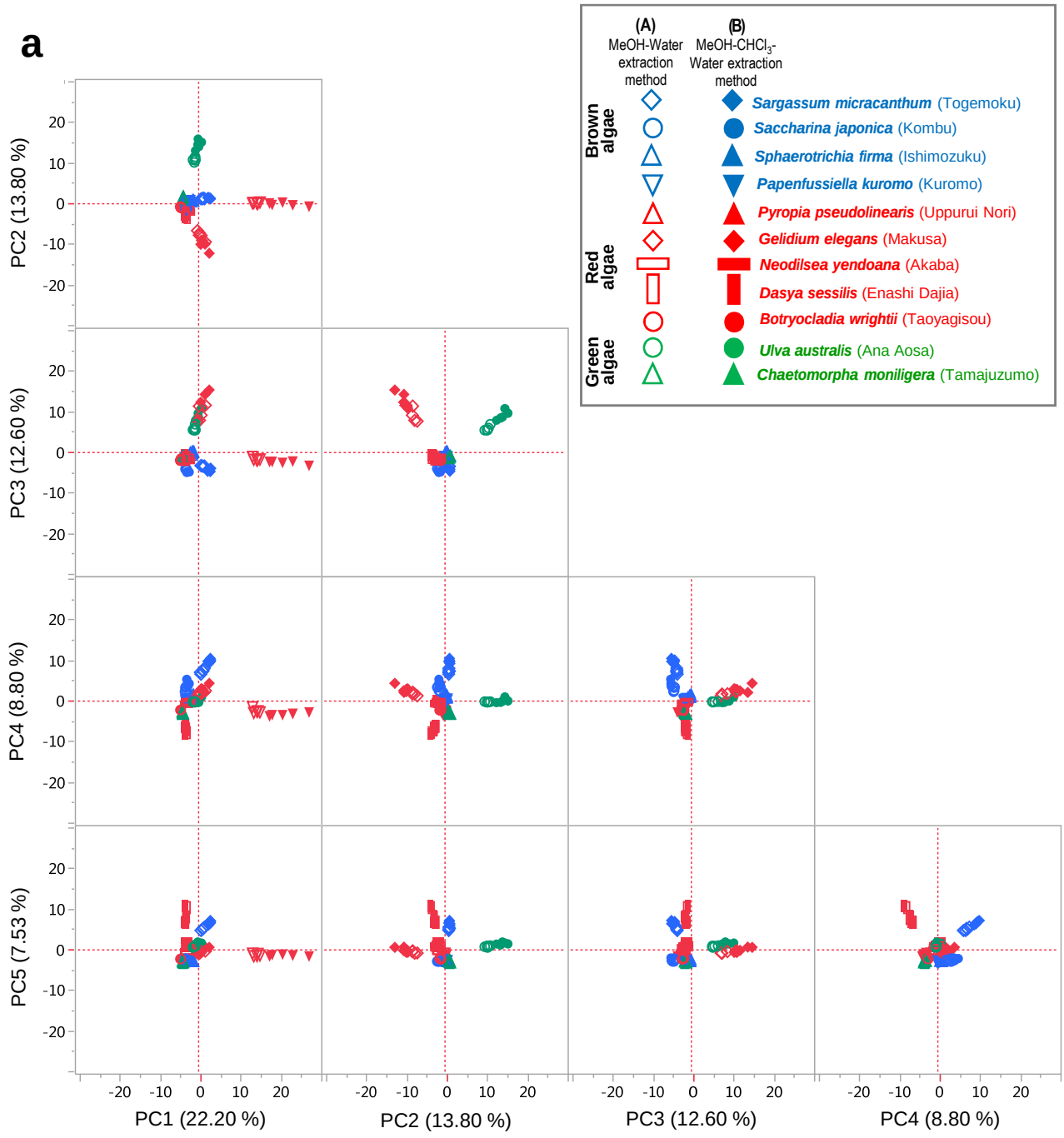


Figure S3.14a PCA score plots of fresh weight based metabolites concentrations in all data set after both extraction methods; methanol-water extractions with and without chloroform.
Blue, red and green color indicate the seaweed species groups; brown, red and green algae respectively.

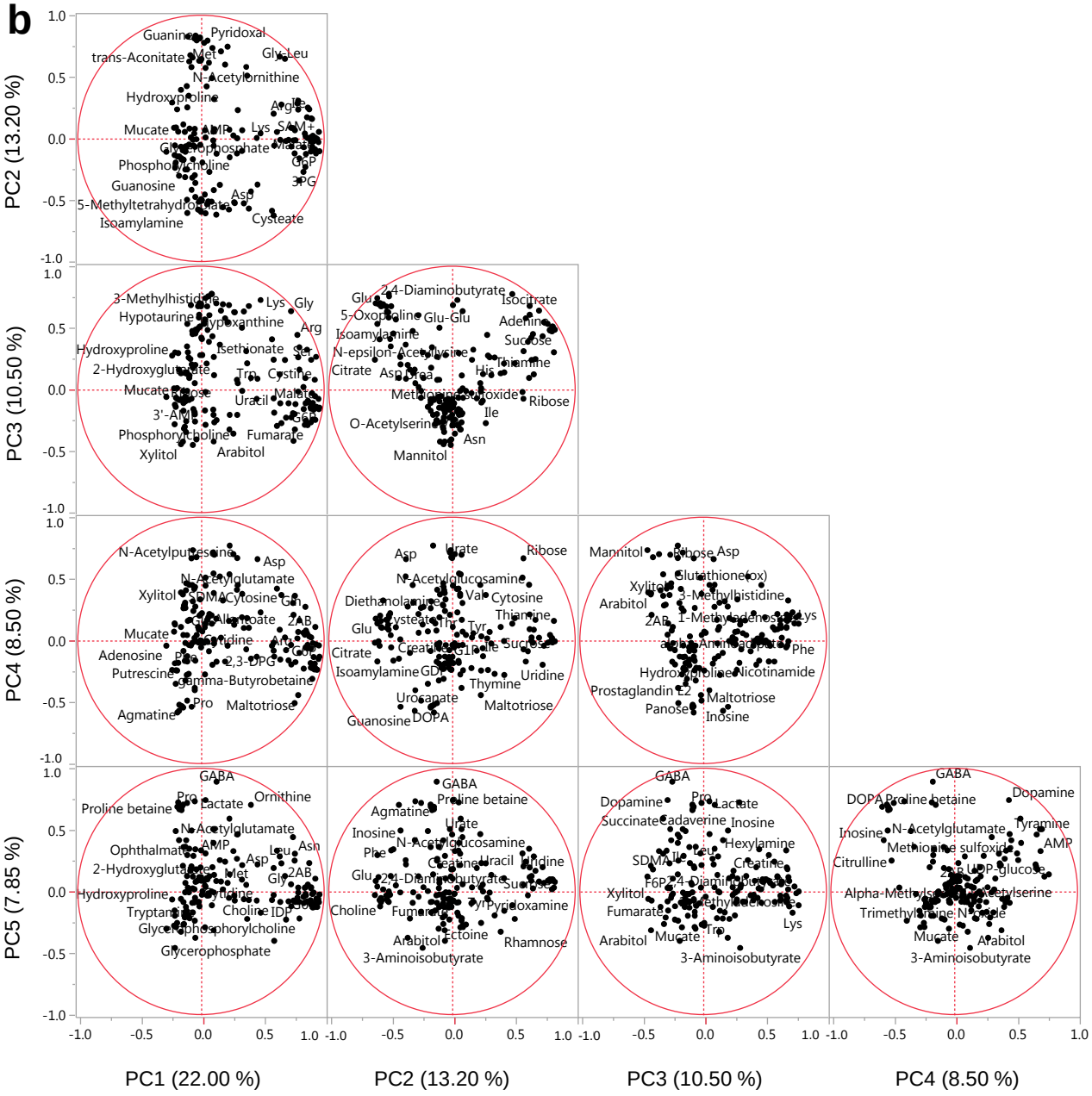


Figure S3.14b PCA loading plots of fresh weight based metabolites concentrations in all data set after both extraction methods; methanol-water extractions with and without chloroform.

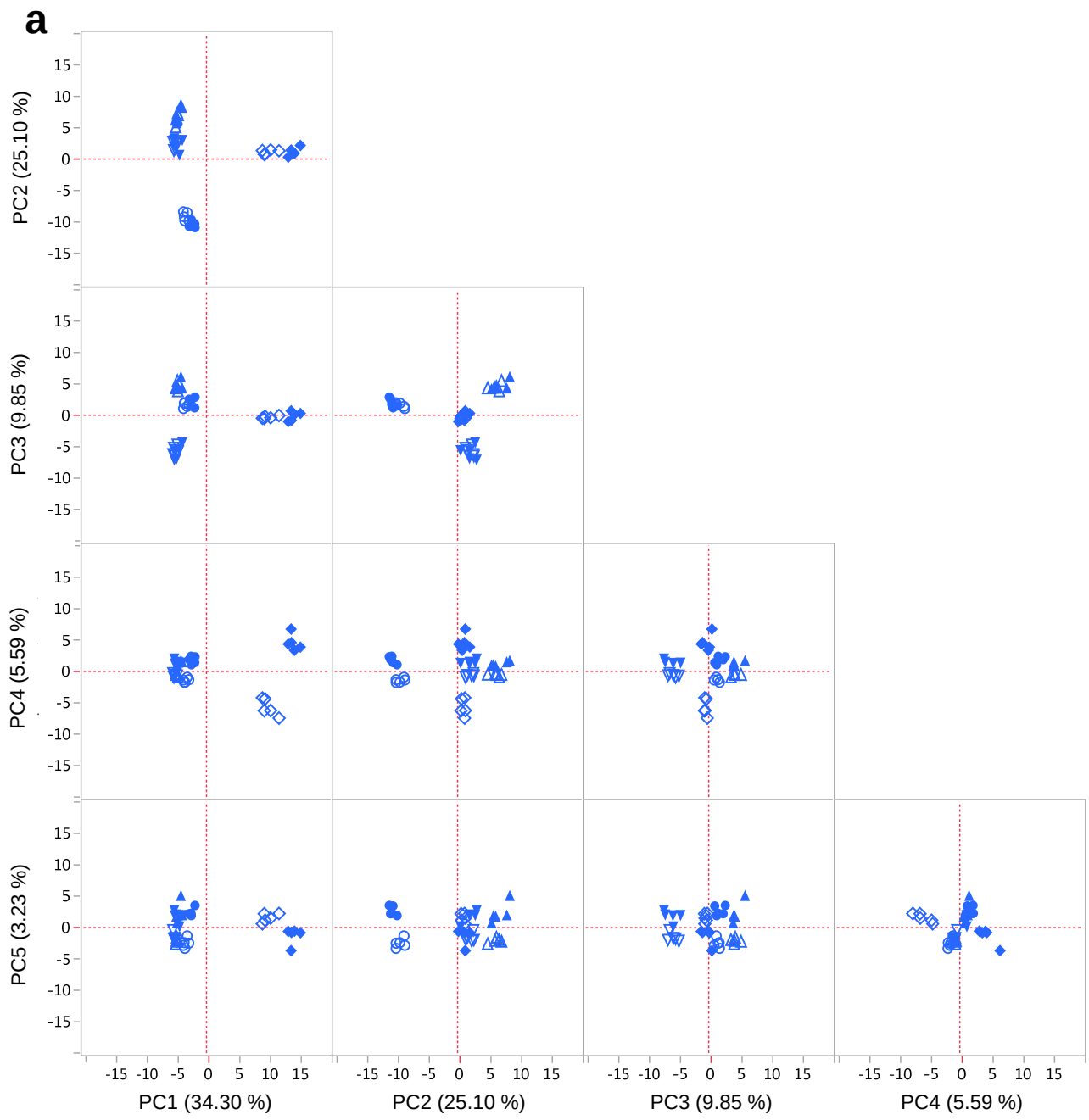


Figure S3.15a PCA score plots of fresh weight based metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

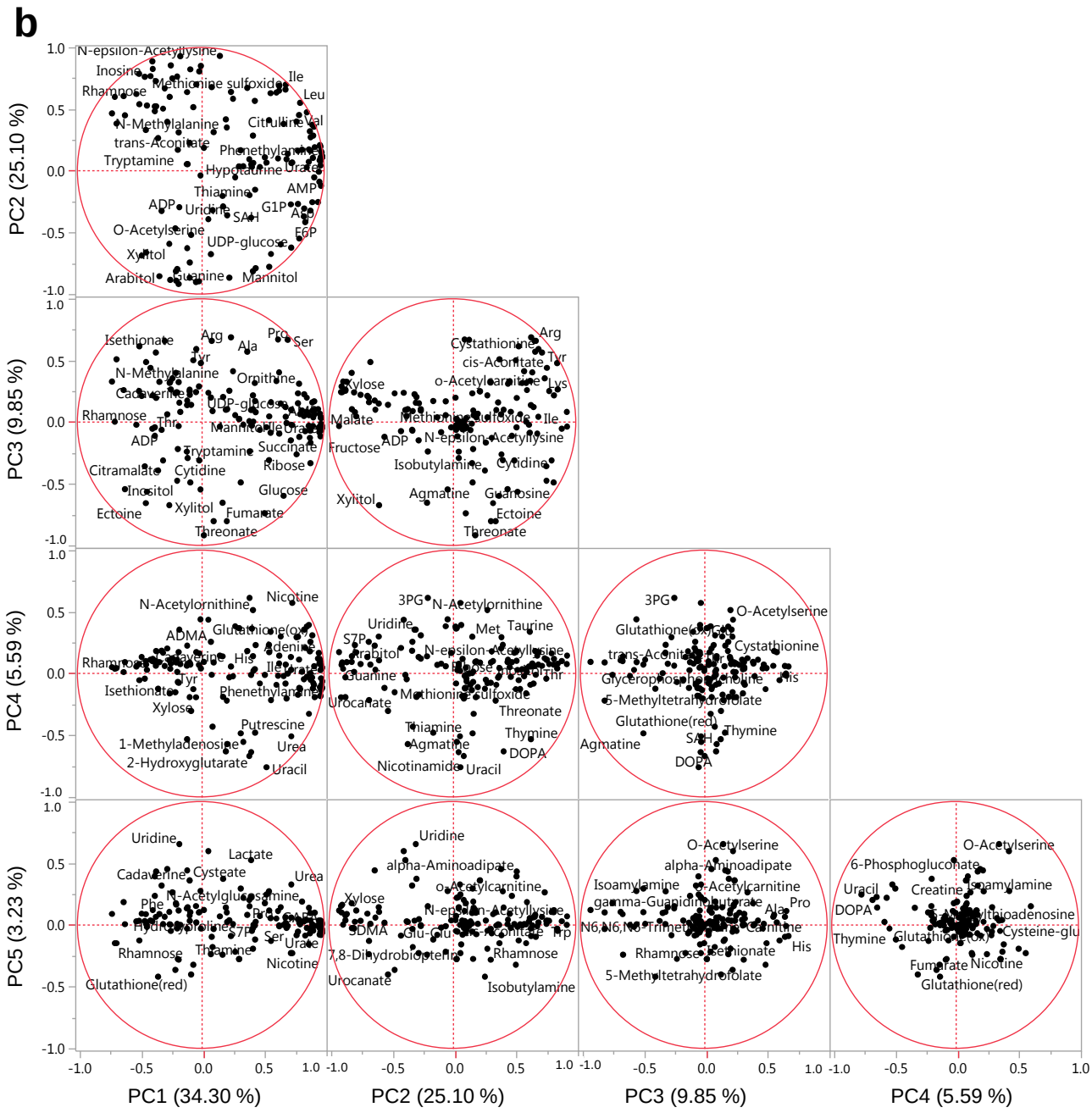


Figure S3.15b PCA loading plots of fresh weight based metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

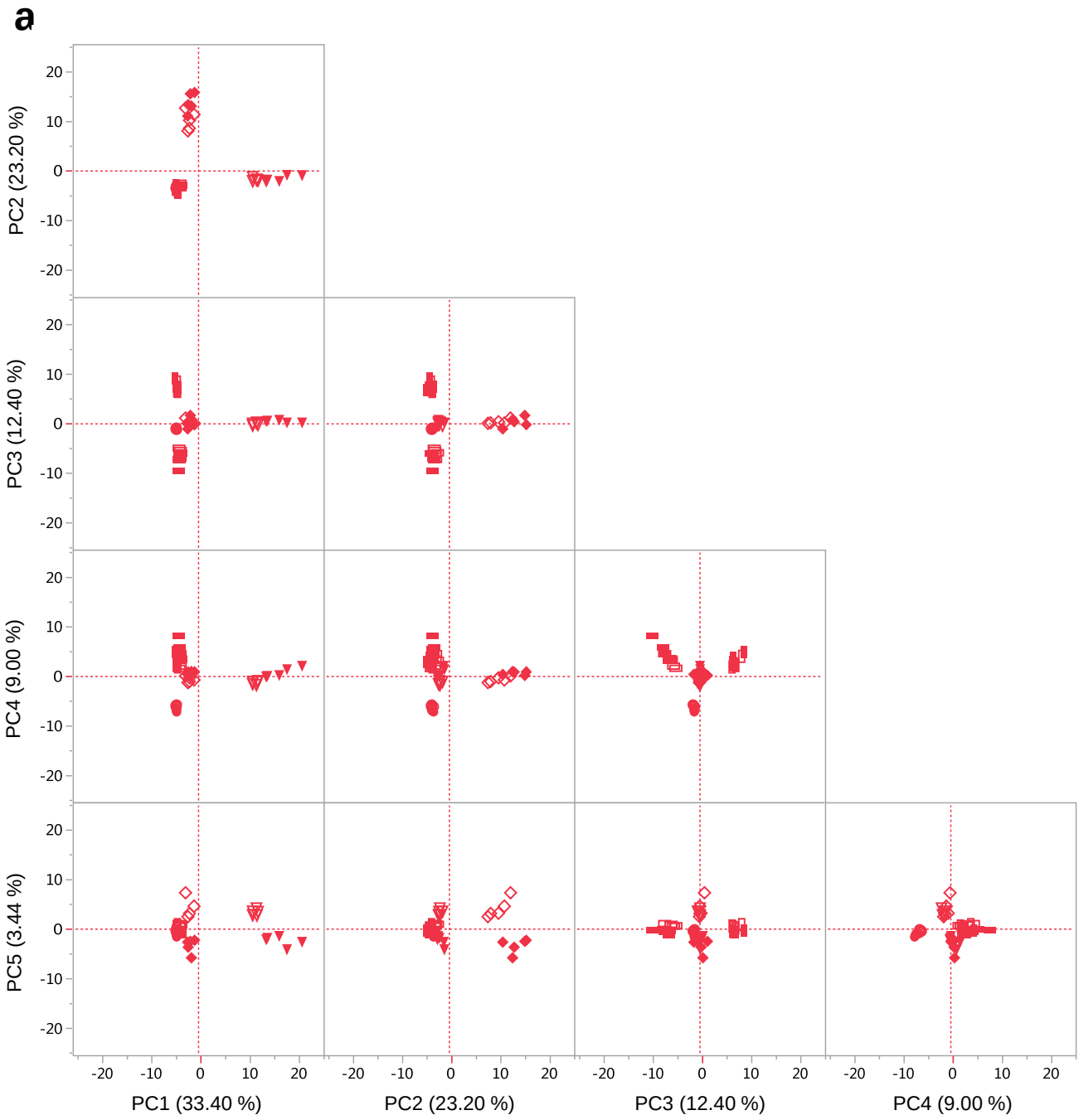


Figure S3.16a PCA score plots of fresh weight based metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

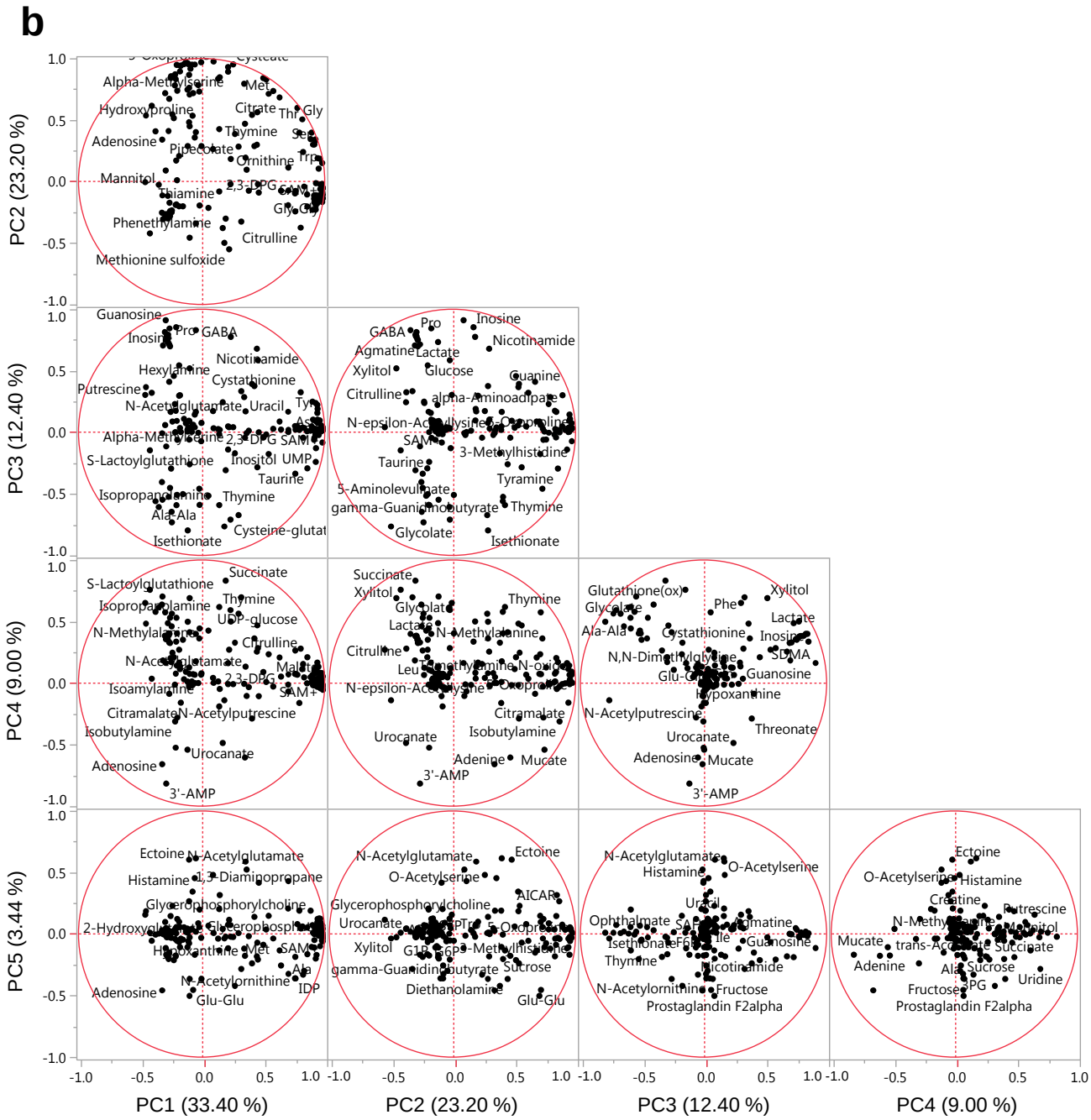


Figure S3.16b PCA loading plots of fresh weight based metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

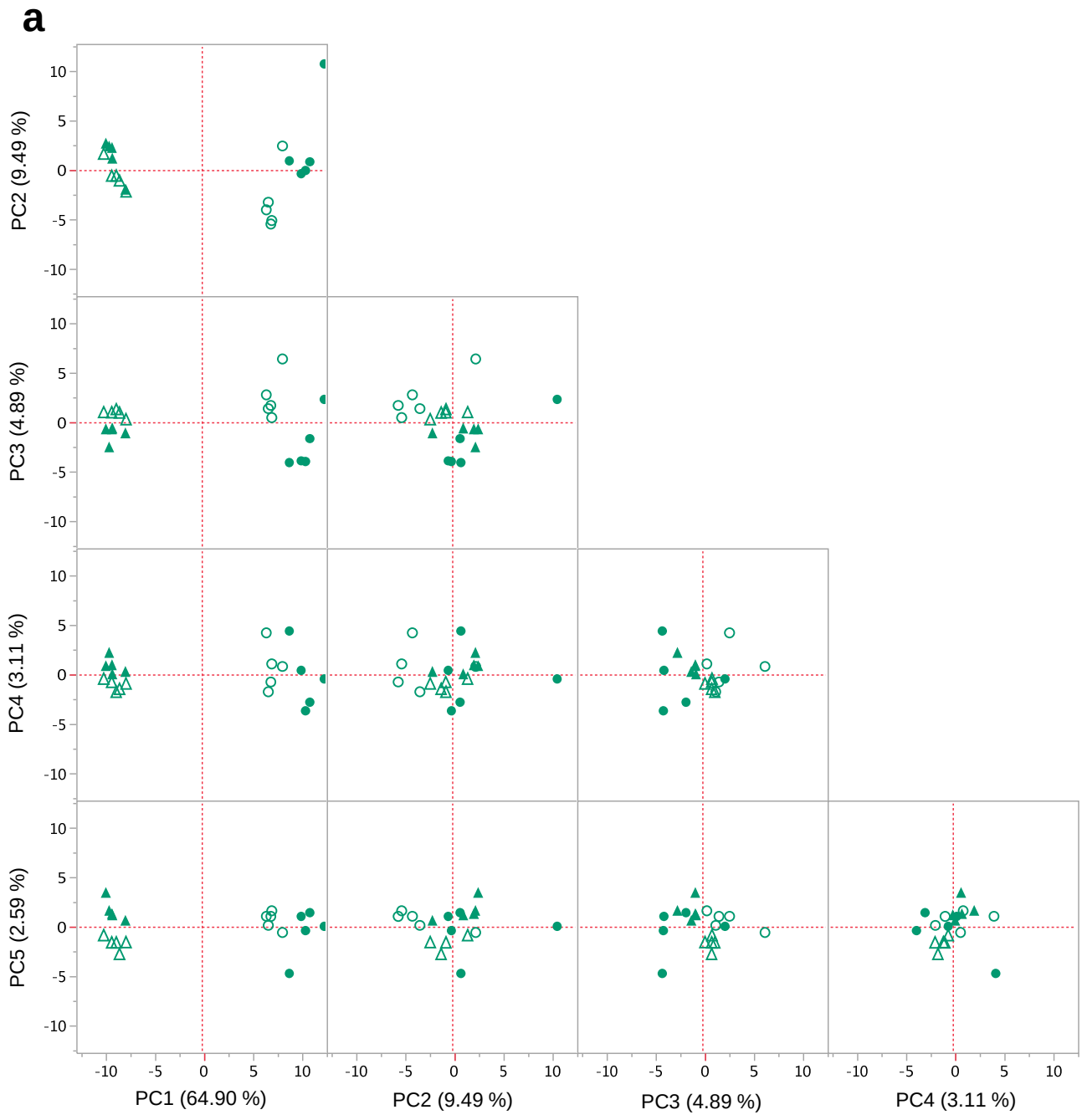


Figure S3.17a PCA score plots of fresh weight based metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.

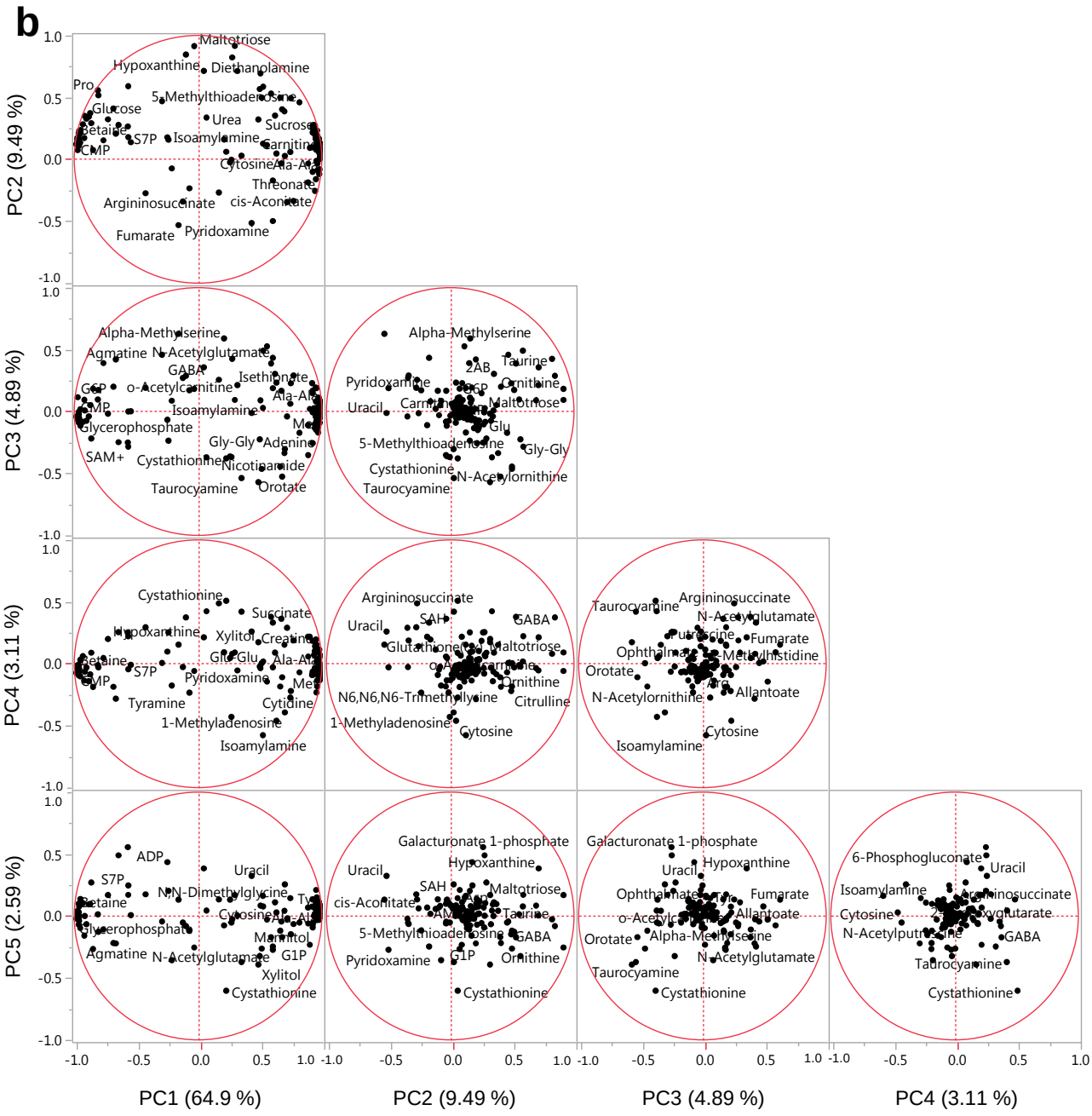


Figure S3.17b PCA loading plots of fresh weight based metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.

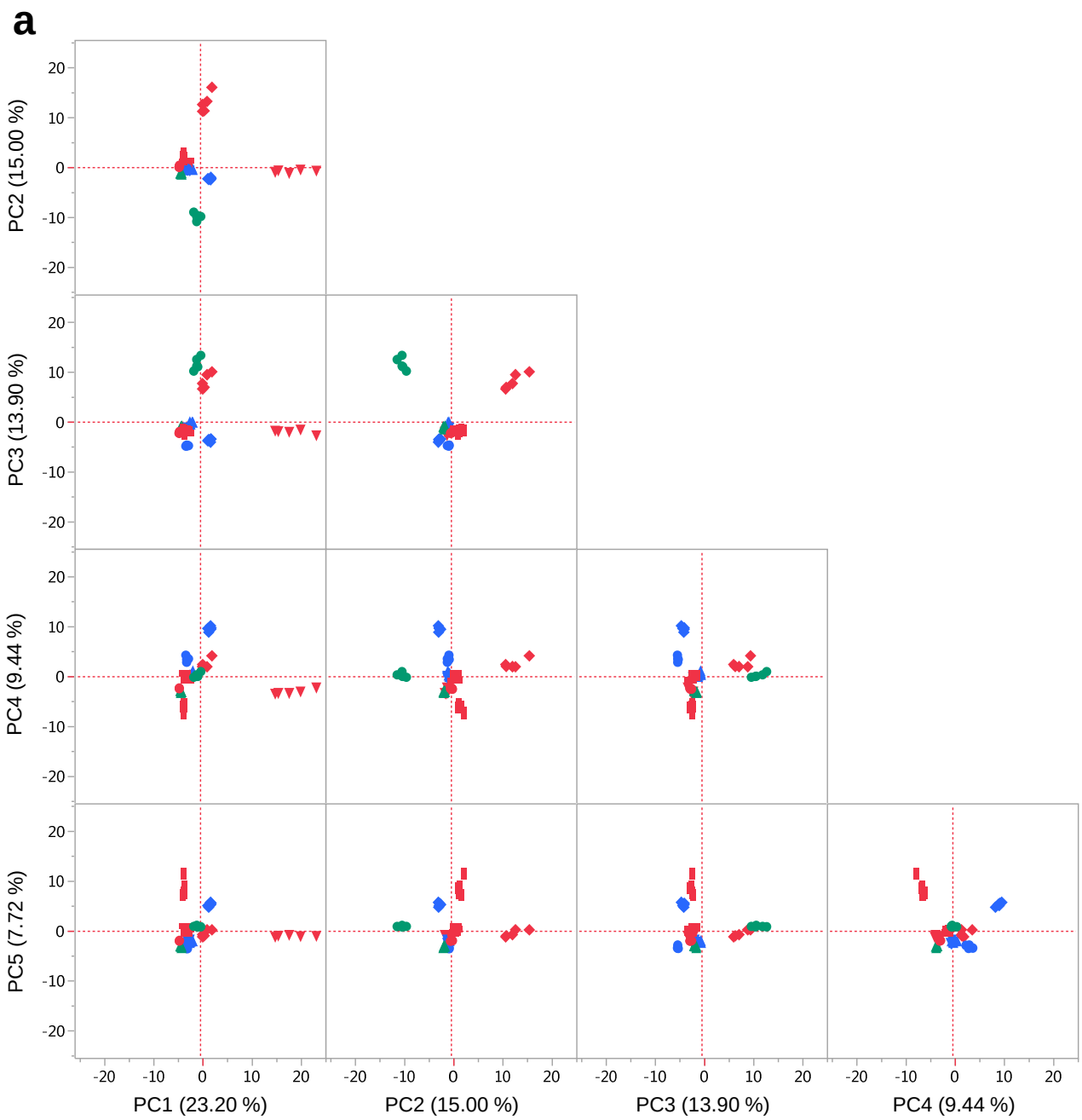


Figure S3.18a PCA score plots of fresh weight based metabolites concentrations in all data set after extracted using methanol-water extraction methods.

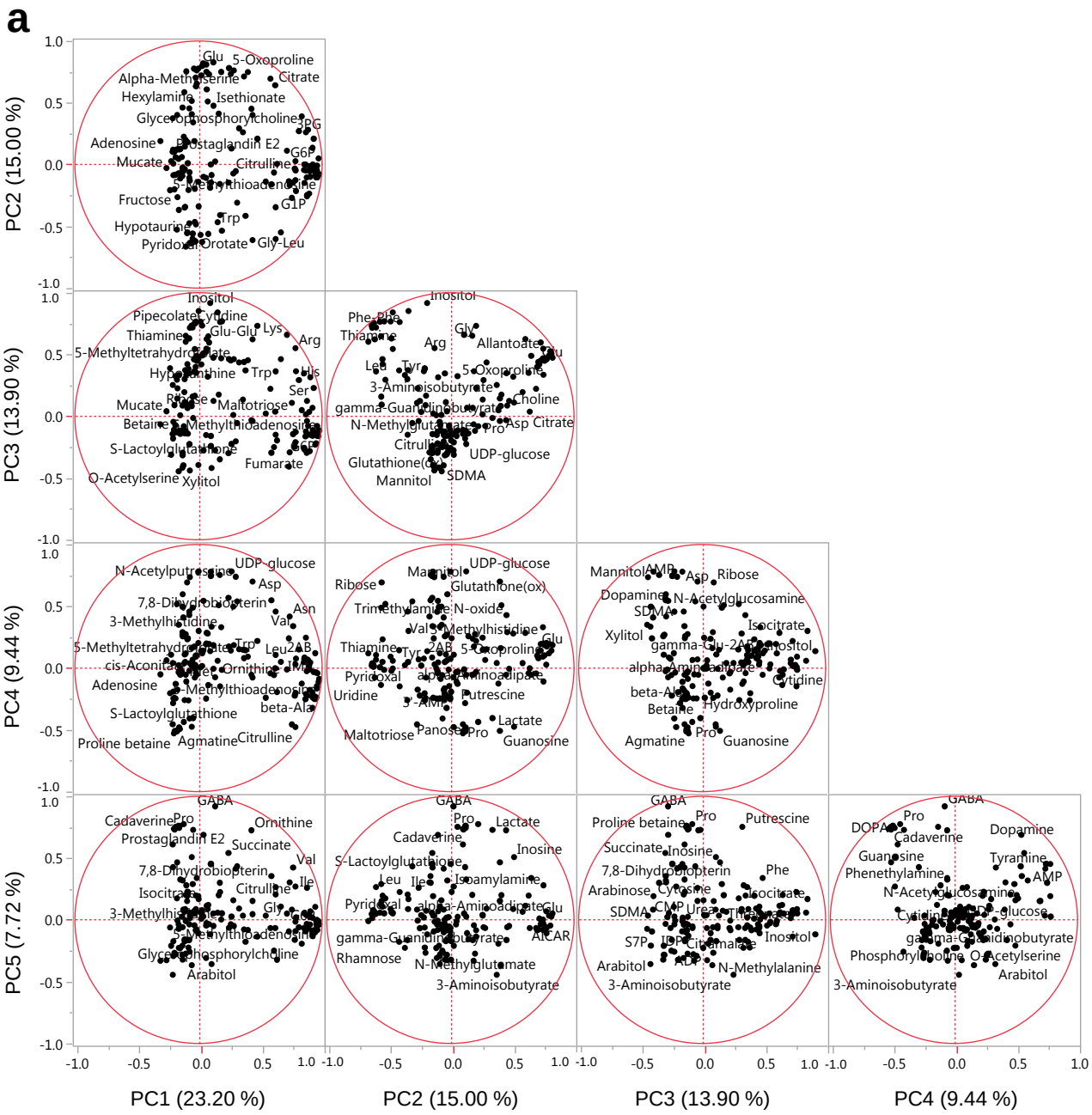


Figure S3.18b PCA loading plots of fresh weight based metabolites concentrations in all data set after extracted using methanol-water extraction methods.

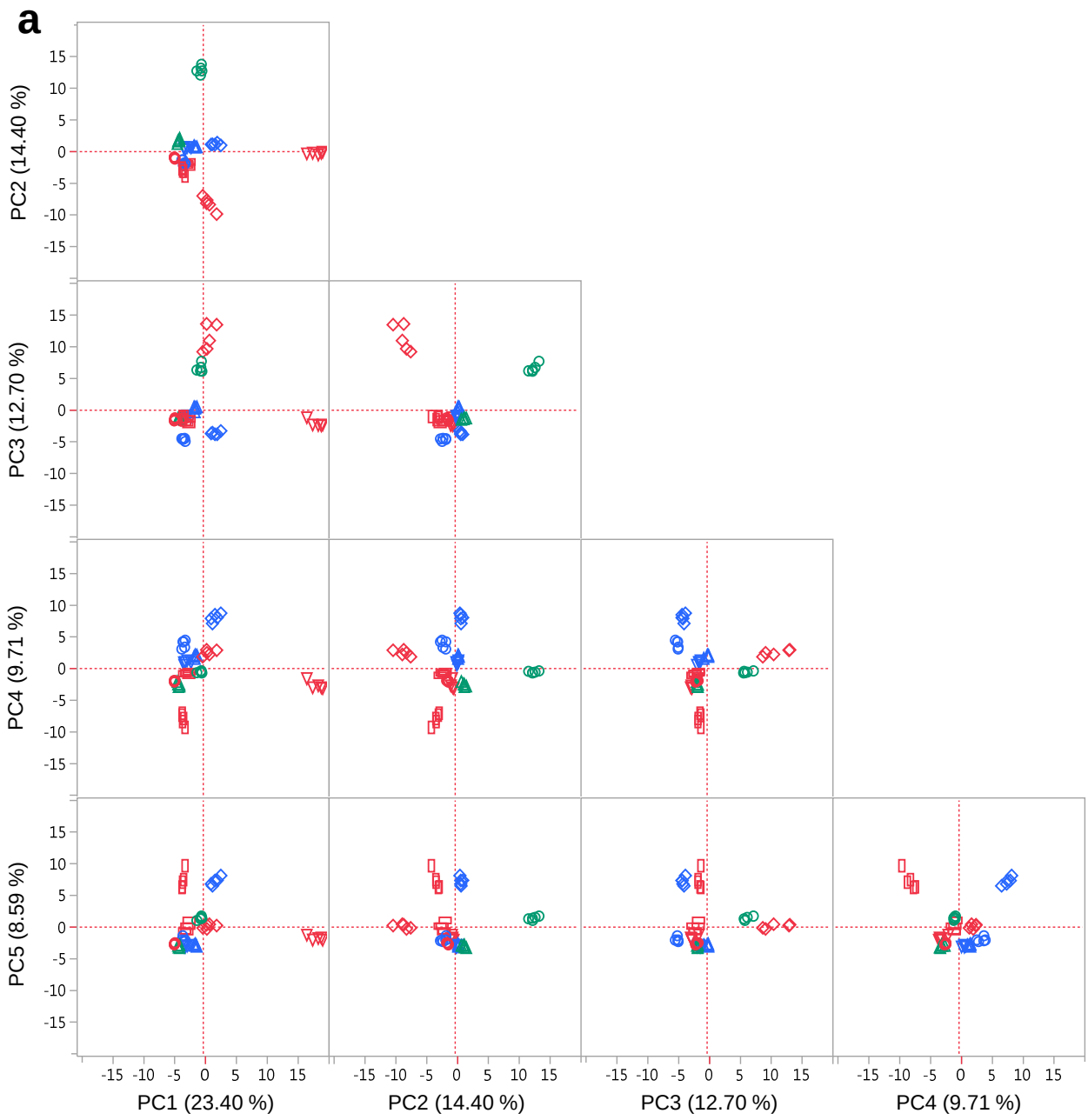


Figure S3.19a PCA score plots of fresh weight based metabolites concentrations in all data set after extracted using methanol-chloroform-water extraction methods.

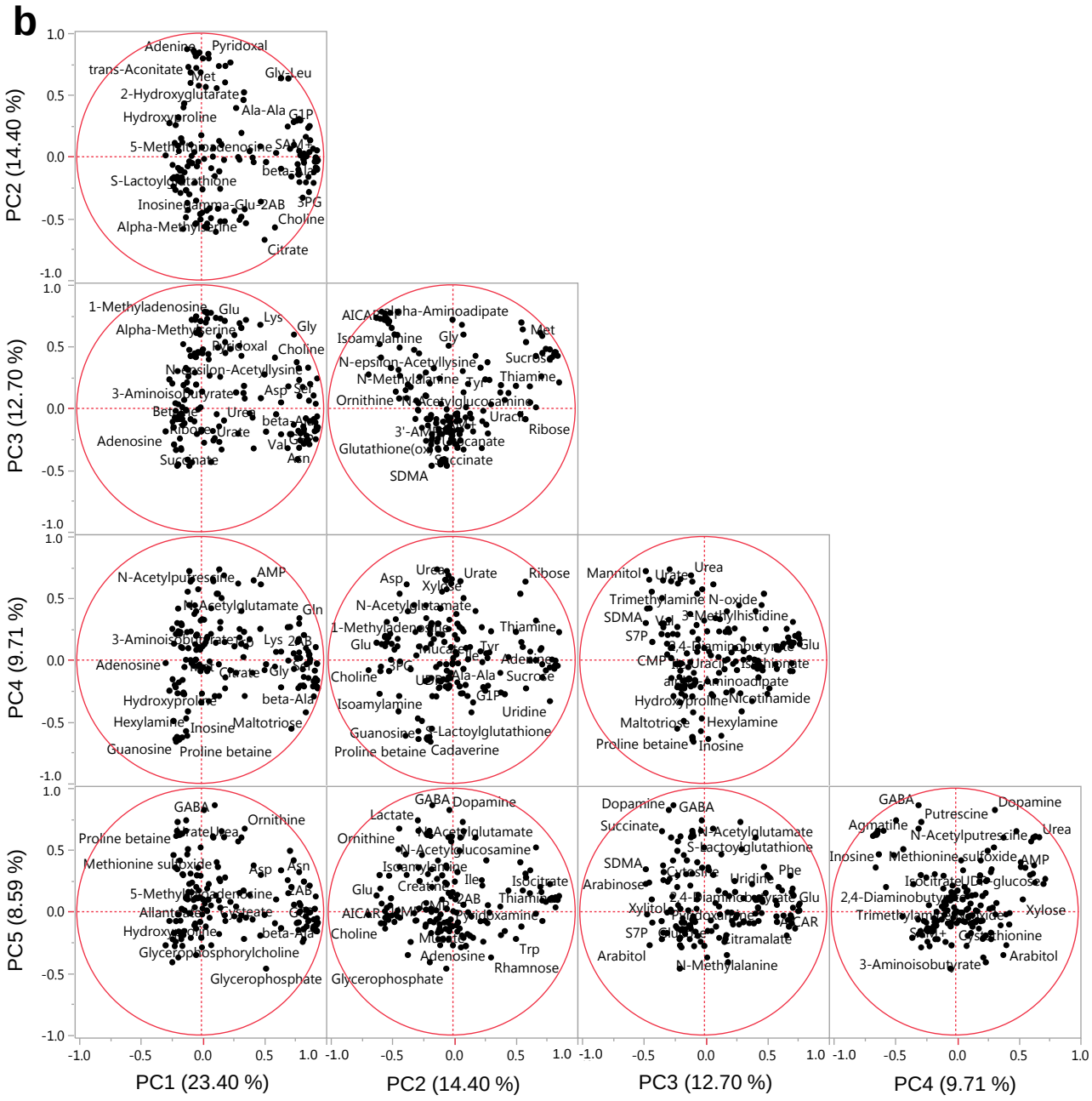


Figure S3.19b PCA loading plots of fresh weight based metabolites concentrations in all data set after extracted using methanol-chloroform-water extraction methods.

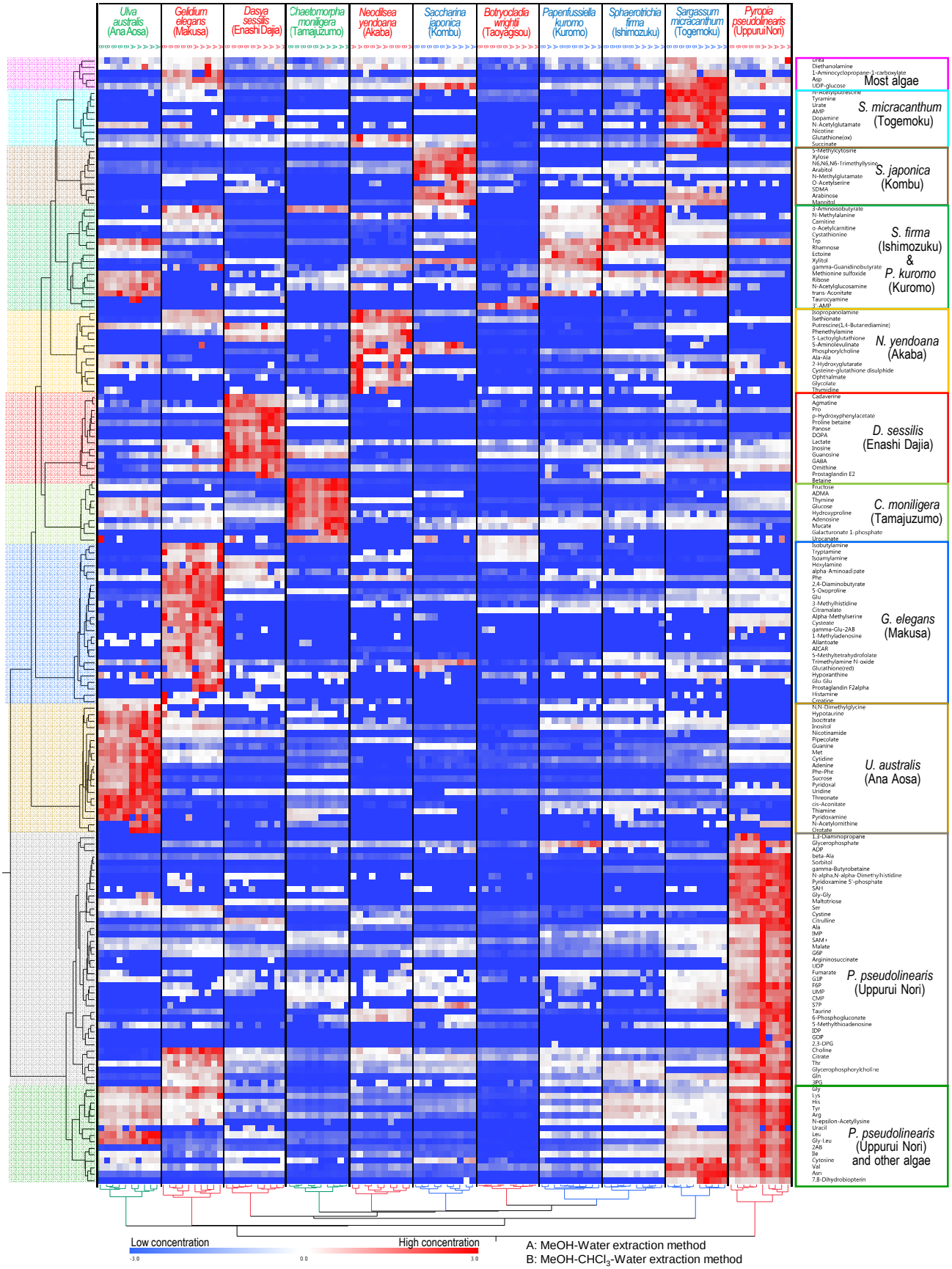


Figure S3.20 HCA and heat map of fresh weight based of metabolites concentrations in all data set after both extraction methods; methanol-water extractions with and without chloroform.

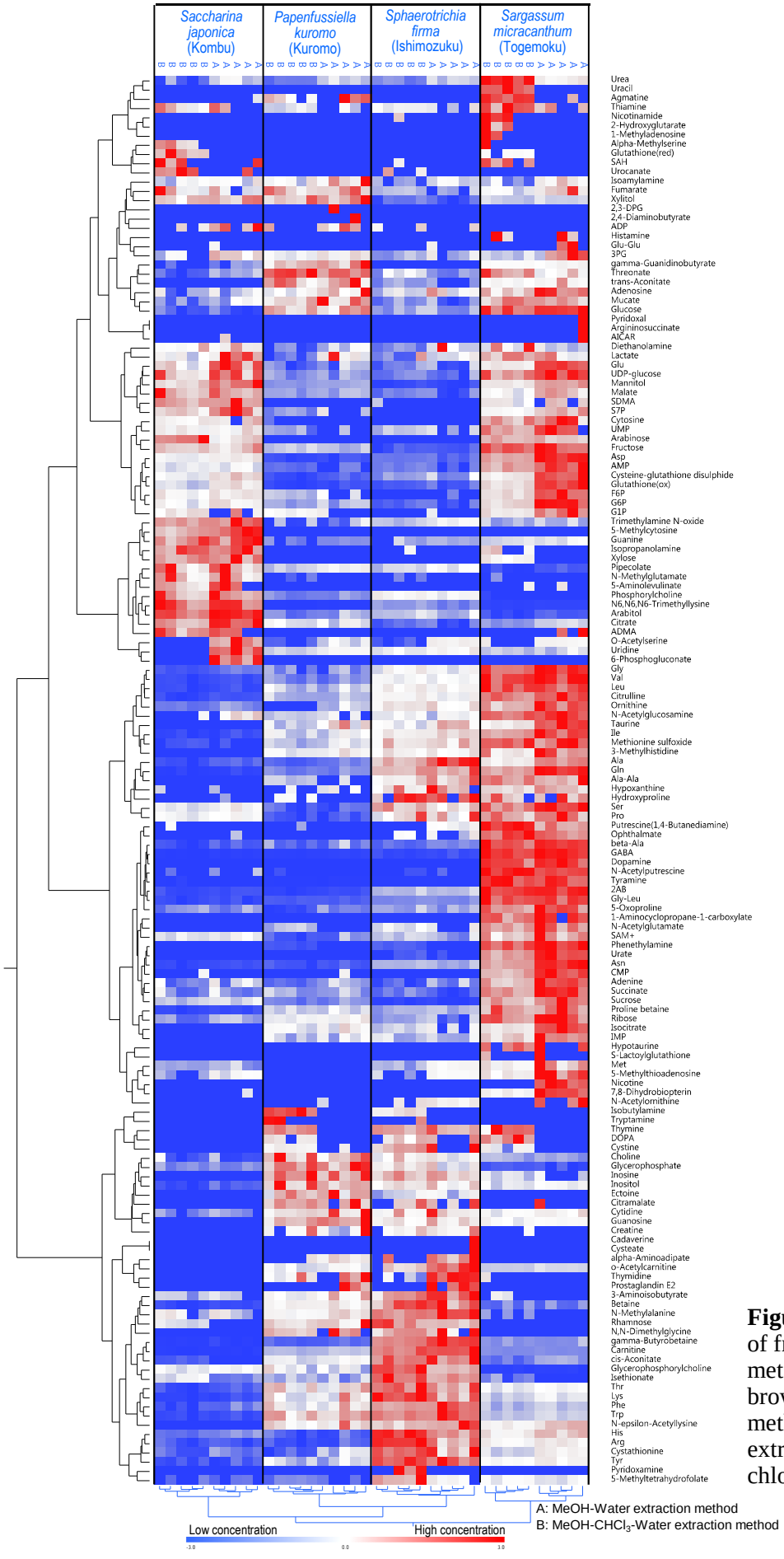


Figure S3.21 HCA and heat map of fresh weight based of metabolites concentrations in brown algae after both extraction methods; methanol-water extractions with and without chloroform.

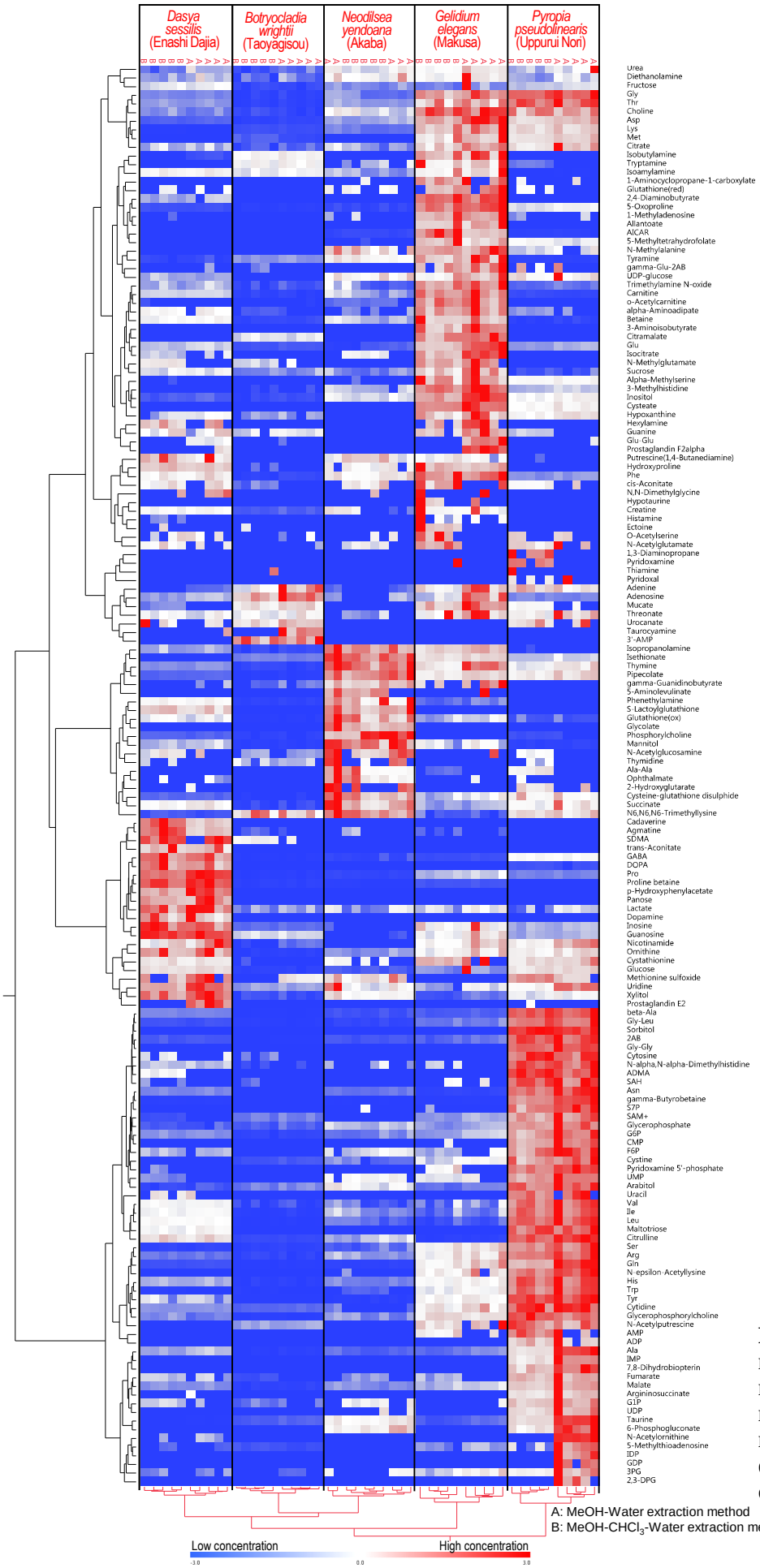


Figure S3.22 HCA and heat map of fresh weight based of metabolites concentrations in red algae after both extraction methods; methanol-water extractions with and without chloroform.

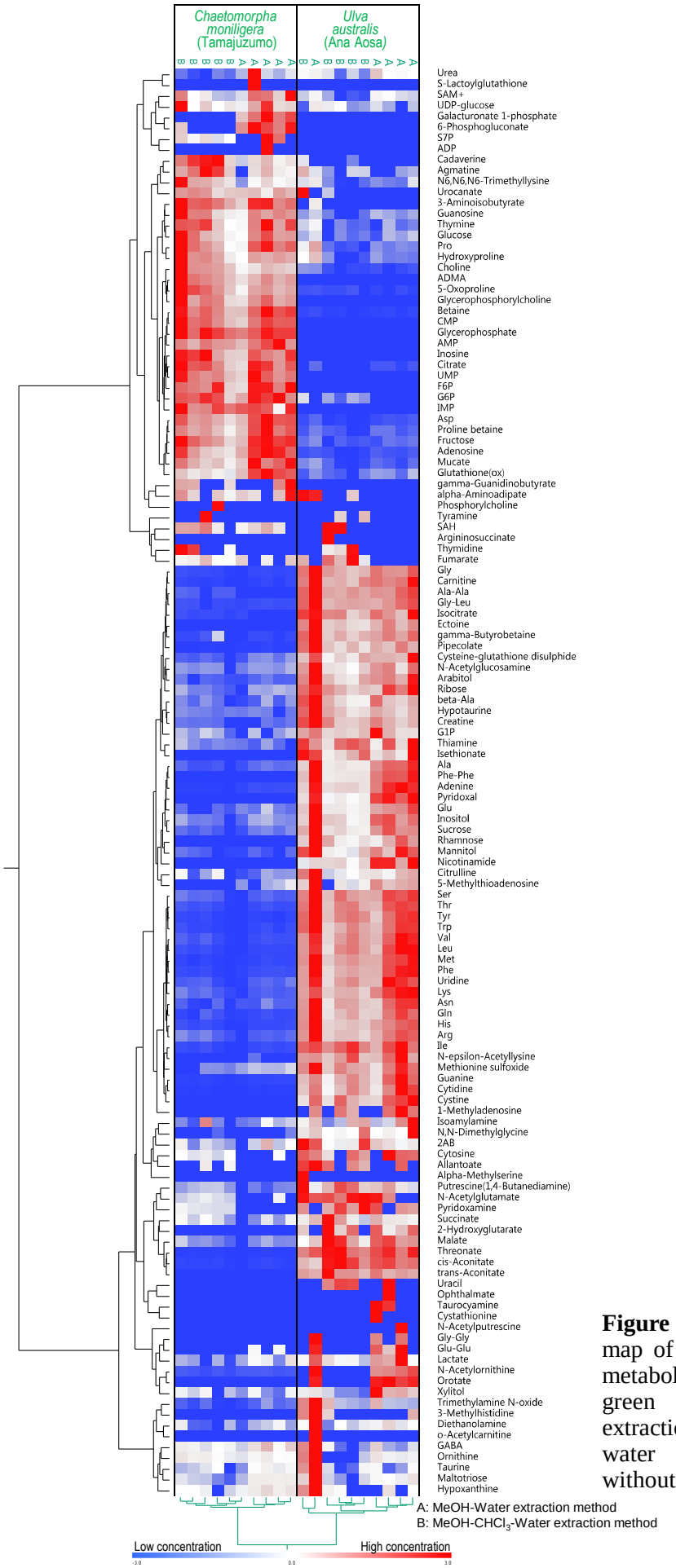
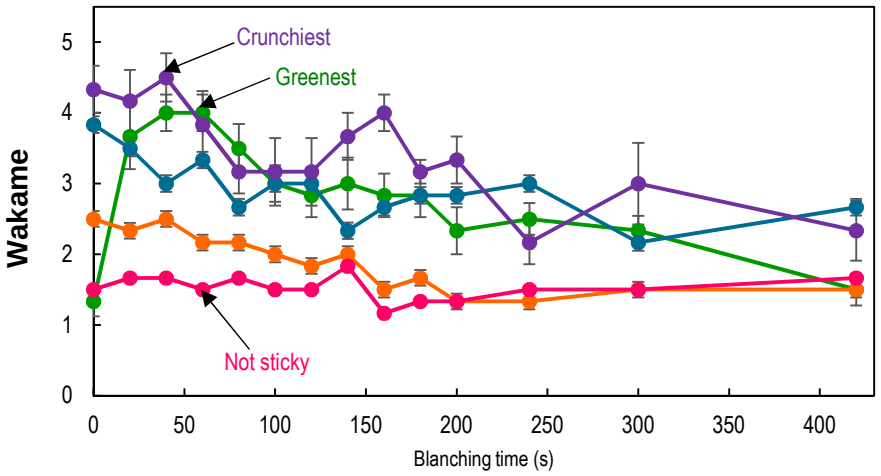
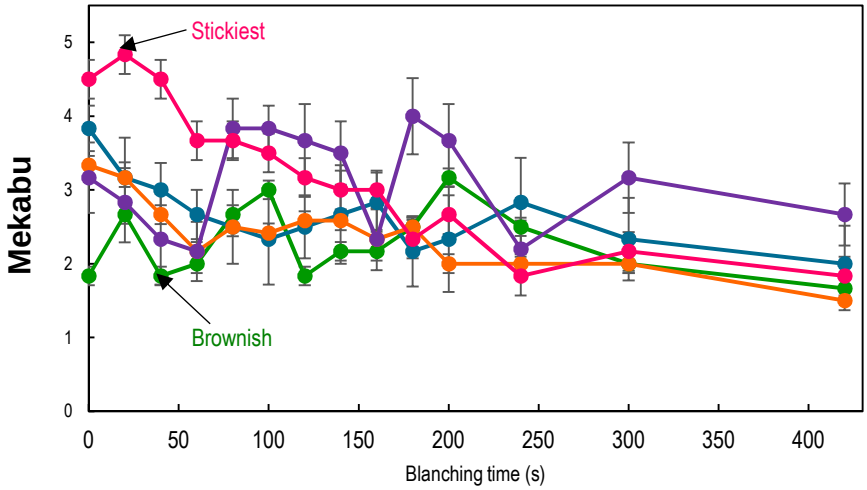


Figure S3.23 HCA and heat map of fresh weight based of metabolites concentrations in green algae after both extraction methods; methanol-water extractions with and without chloroform.





Characteristics	Hedonic scale 5	4	3	2	1
Greenness	Dark green		Brown		Light brown
Odour of seawater	Strong		Moderate		Weak
Umami strength	Strong		Moderate		Weak
Crunchiness	Strong		Moderate		Weak
Stickiness	Strong		Moderate		Weak

Figure S4.1 Sensory evaluation of the blanched Wakame and Mekabu were performed by 6 untrained panelist but familiar with the samples.
They graded the greenness color (green line), odor of seawater smell (blue line), umami strength (orange line) and texture; crunchiness (purple line) and stickiness (pink line), respectively after different blanching time. The grading of the odor, umami strength and texture were based on the five hedonic scale with scores ranging from five (strong) to one (weak). While, the greenness color was graded from five (dark green) to one (brown). The values indicate the average ±SD of 6 panelists score.

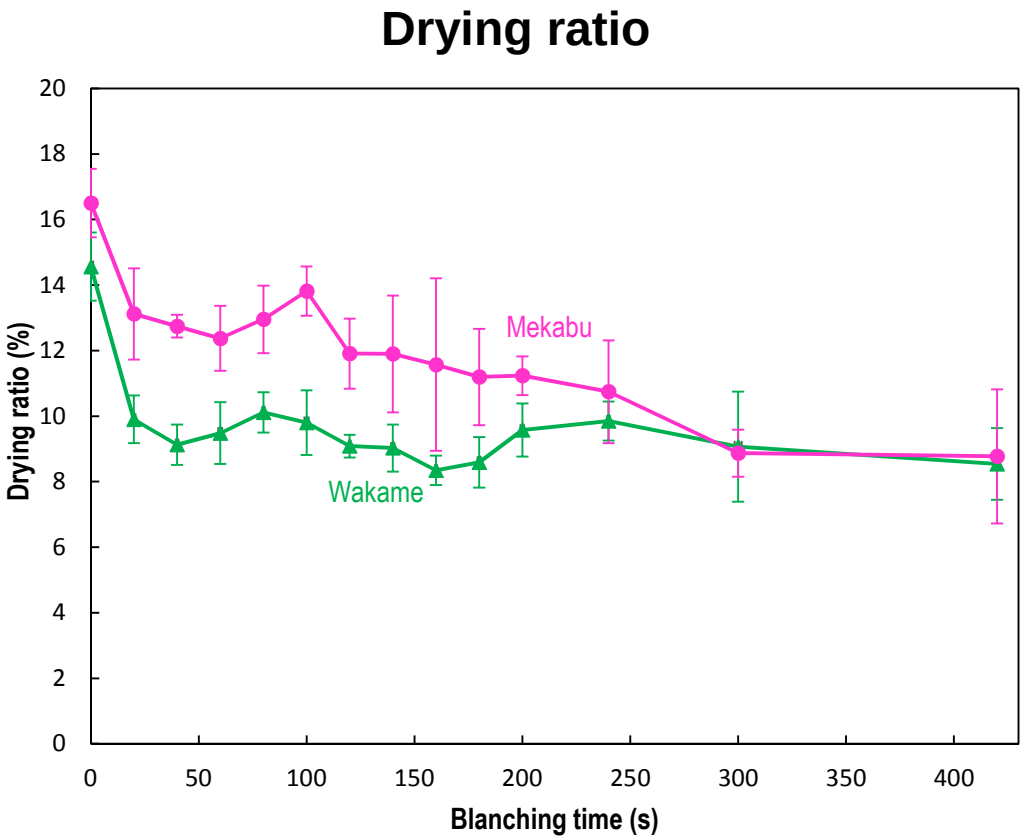


Figure S4.2 The drying ratio (%) of the Wakame and Mekabu after each blanching process.
The green line indicated blanched Wakame, while the pink line indicated blanched Mekabu. The values indicate average ± SD (*n*=5).

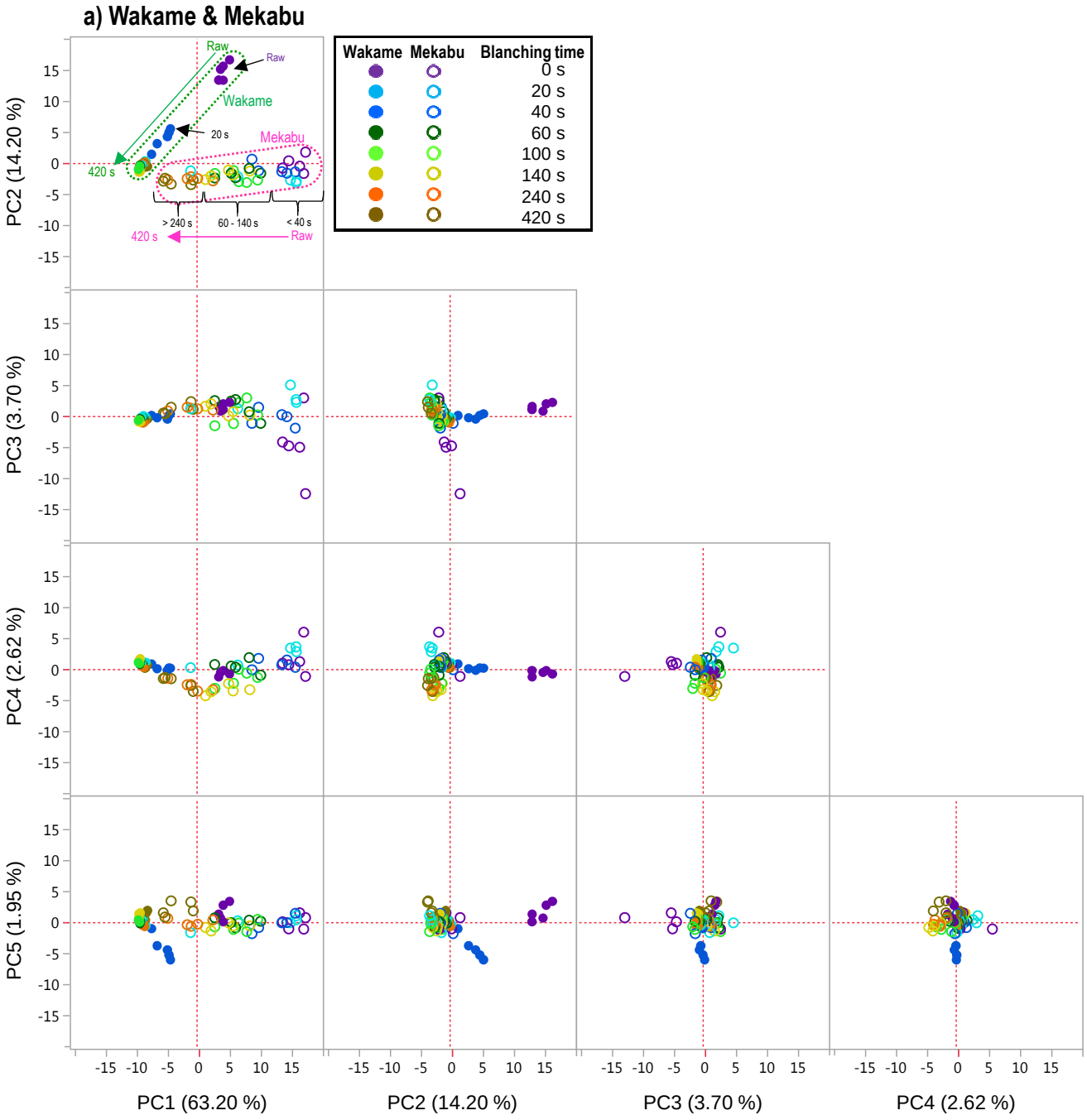
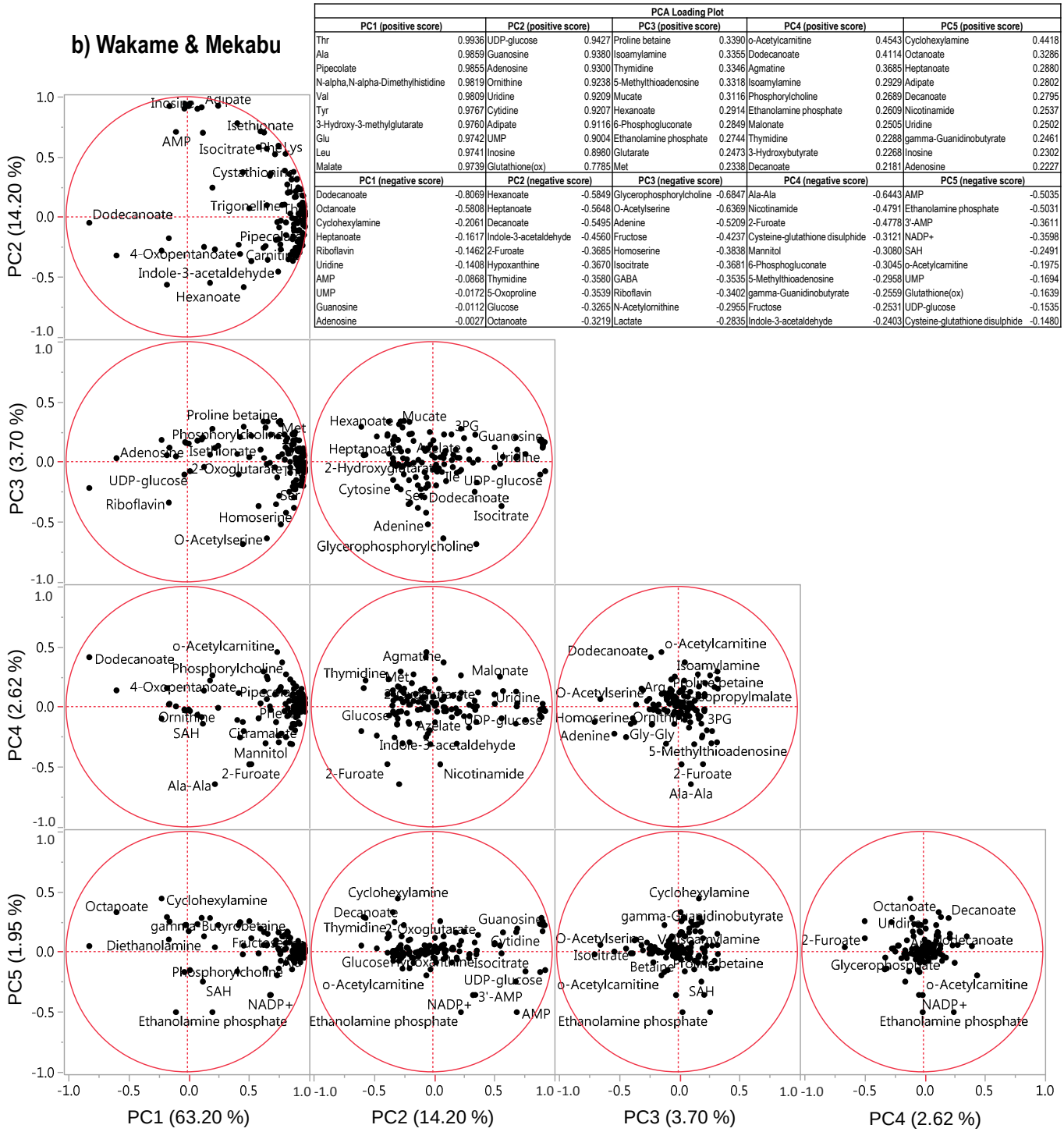


Figure S4.3a PCA score plots of dry weight based metabolites concentrations in entire data set (both Wakame and Mekabu) of different blanching time.
The arrow showed the tendency of the clusterization of the metabolites in each part, Wakame (green dotted eclipse) and Mekabu (pink dotted eclipse). The horizontal axis value indicates PC1-PC5 separation variance of blanching time and the vertical axis value indicates PC2-5 separation variance of 2 part of seaweed, Wakame and Mekabu.



PCA Loading Plot

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Thr	0.9936	UDP-glucose	0.9427	Proline betaine	0.3390	o-Acetylcarnitine	0.4543	Cyclohexylamine	0.4418
Ala	0.9859	Guanosine	0.9380	Isoamylamine	0.3355	Dodecanoate	0.4114	Octanoate	0.3286
Pipecolate	0.9855	Adenosine	0.9300	Thymidine	0.3346	Agmatine	0.3685	Heptanoate	0.2880
N-alpha,N-alpha-Dimethylhistidine	0.9819	Ornithine	0.9238	5-Methylthioadenosine	0.3318	Isoamylamine	0.2929	Adipate	0.2802
Val	0.9809	Uridine	0.9209	Mucate	0.3116	Phosphorylcholine	0.2689	Decanoate	0.2795
Tyr	0.9767	Cytidine	0.9207	Hexanoate	0.2914	Ethanolamine phosphate	0.2609	Nicotinamide	0.2537
3-Hydroxy-3-methylglutarate	0.9760	Adipate	0.9116	6-Phosphogluconate	0.2849	Malonate	0.2505	Uridine	0.2502
Glu	0.9742	UMP	0.9004	Ethanolamine phosphate	0.2744	Thymidine	0.2288	gamma-Guanidinobutyrate	0.2461
Leu	0.9741	Inosine	0.8980	Glutarate	0.2473	3-Hydroxybutyrate	0.2268	Inosine	0.2302
Malate	0.9739	Glutathione(ox)	0.7785	Met	0.2338	Decanoate	0.2181	Adenosine	0.2227
PC1 (negative score)		**PC2 (negative score)**		**PC3 (negative score)**		**PC4 (negative score)**		**PC5 (negative score)**	
Dodecanoate	-0.8069	Hexanoate	-0.5849	Glycerophosphorylcholine	-0.6847	Ala-Ala	-0.6443	AMP	-0.5035
Octanoate	-0.5808	Heptanoate	-0.5648	O-Acetylserine	-0.6369	Nicotinamide	-0.4791	Ethanolamine phosphate	-0.5031
Cyclohexylamine	-0.2061	Decanoate	-0.5495	Adenine	-0.5209	2-Furoate	-0.4778	3'-AMP	-0.3611
Heptanoate	-0.1617	Indole-3-acetaldehyde	-0.4560	Fructose	-0.4237	Cysteine-glutathione disulphide	-0.3121	NADP+	-0.3598
Riboflavin	-0.1462	2-Furoate	-0.3685	Homoserine	-0.3838	Mannitol	-0.3080	SAH	-0.2491
Uridine	-0.1408	Hypoxanthine	-0.3670	Isocitrate	-0.3681	6-Phosphogluconate	-0.3045	o-Acetylcarnitine	-0.1975
AMP	-0.0868	Thymidine	-0.3580	GABA	-0.3535	5-Methylthioadenosine	-0.2958	UMP	-0.1694
UMP	-0.0172	5-Oxoproline	-0.3539	Riboflavin	-0.3402	gamma-Guanidinobutyrate	-0.2559	Glutathione(ox)	-0.1639
Guanosine	-0.0112	Glucose	-0.3265	N-Acetylornithine	-0.2955	Fructose	-0.2531	UDP-glucose	-0.1535
Adenosine	-0.0027	Octanoate	-0.3219	Lactate	-0.2835	Indole-3-acetaldehyde	-0.2403	Cysteine-glutathione disulphide	-0.1480

Figure S4.3b PCA loading plots of dry weight based metabolites concentrations in entire data set (both Wakame and Mekabu) of different blanching time.

The horizontal axis value indicates PC1-PC5 separation variance of blanching time and the vertical axis value indicates PC2-5 separation variance of 2 part of seaweed, Wakame and Mekabu. Top 10 metabolites that detected in the positive and negative loading plot of PC1-PC5 were indicated in the table.

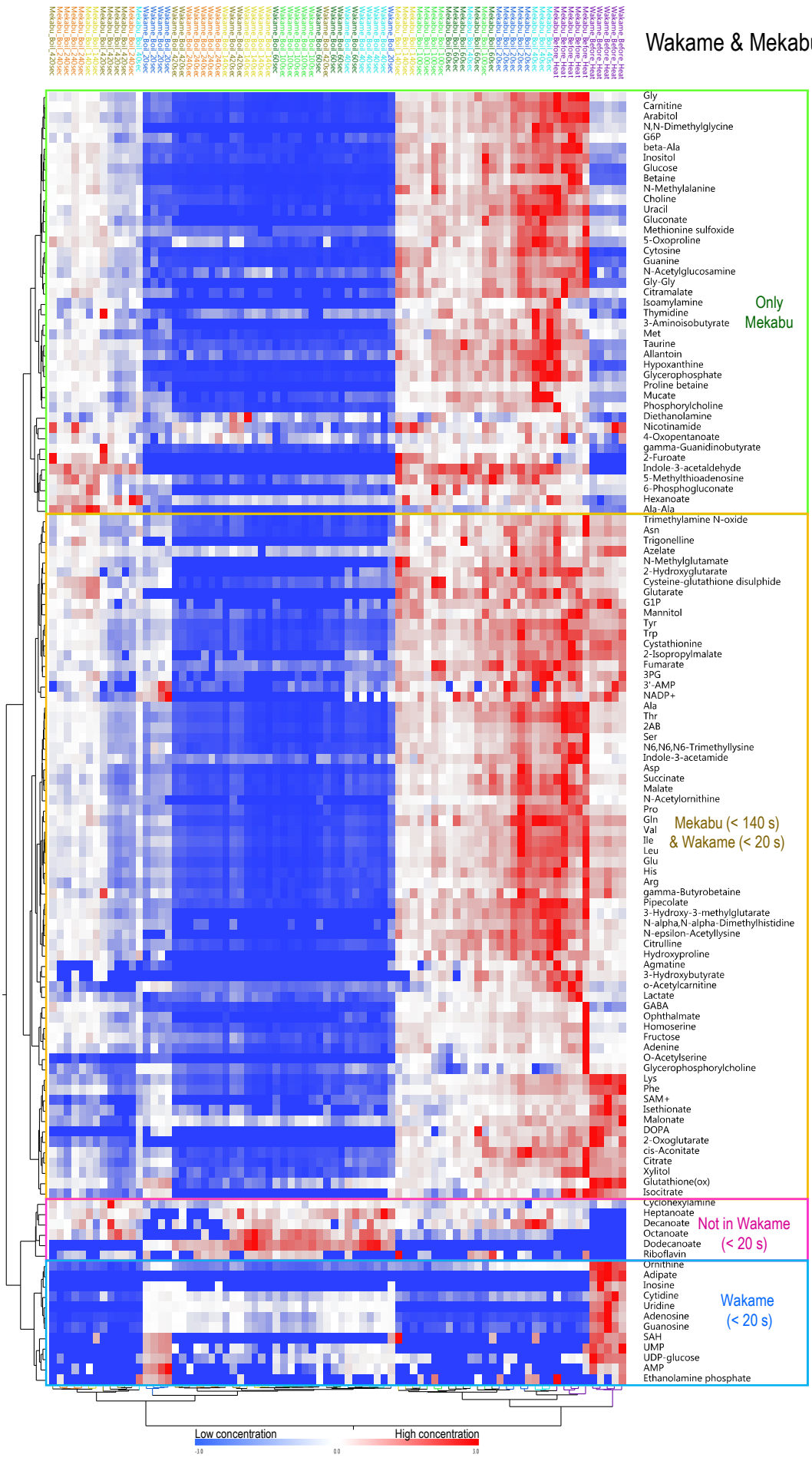


Figure S4.4 HCA and heat map of dry weight based on all metabolites concentrations in all data set (both Wakame and Mekabu) of blanching time.

Metabolites detection were clustered in several clades indicate by color font; green: metabolites detects in Mekabu only, brown: metabolites detect in Mekabu (<140 s) and Wakame (<20 s), pink: metabolites does not detect in Wakame (<20 s) and blue: metabolites detects in Wakame (<20 s).

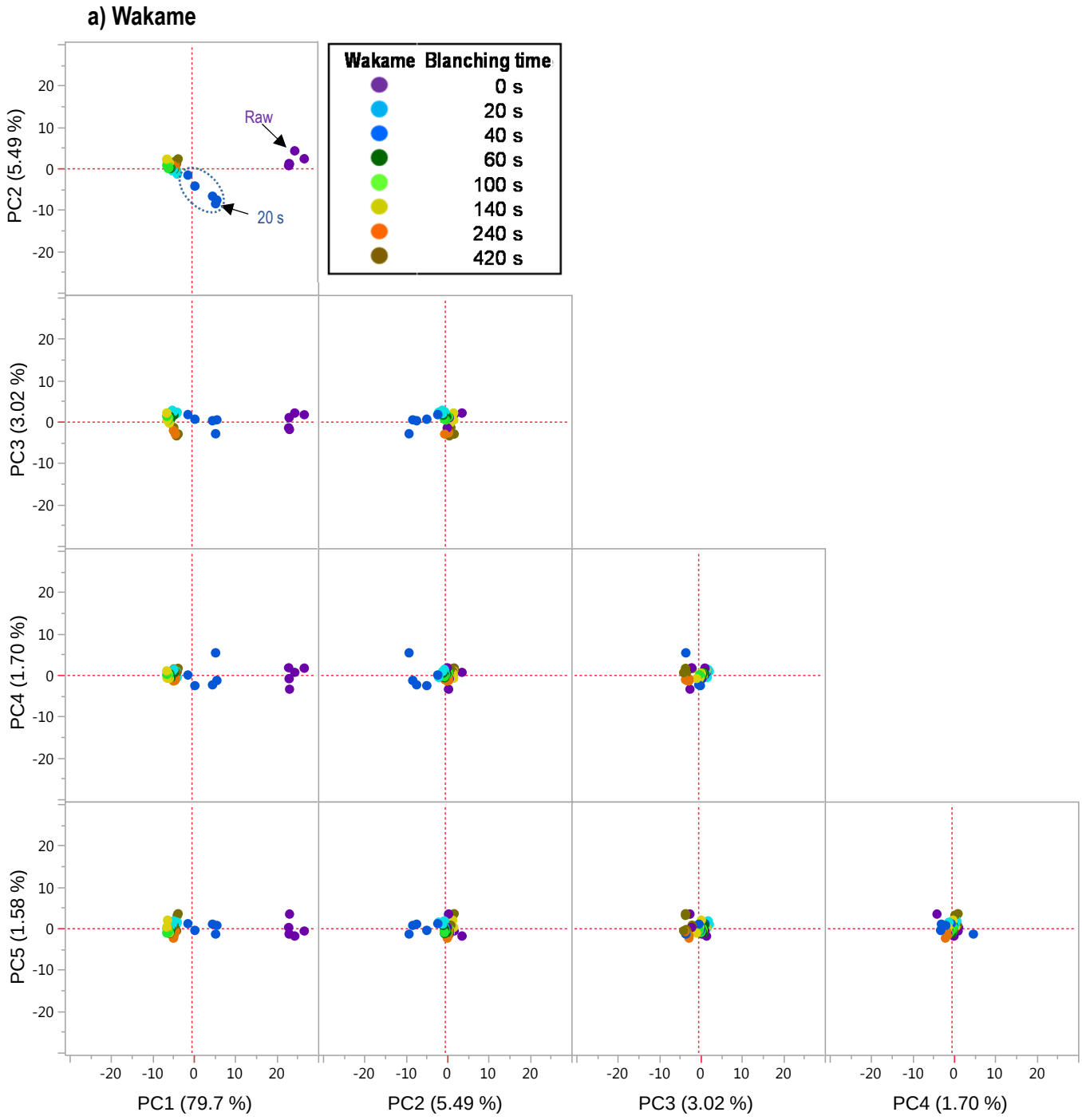
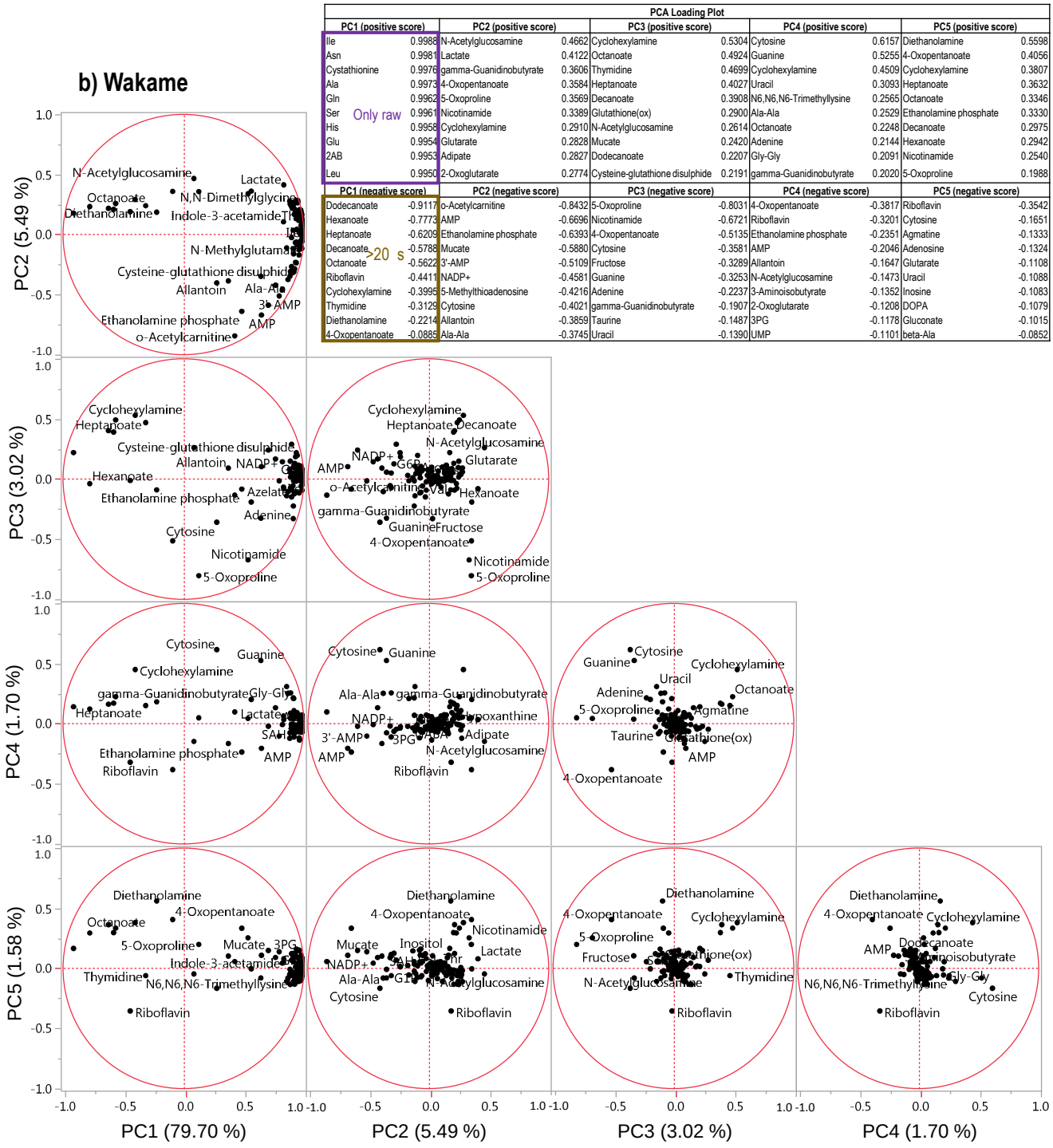


Figure S4.5a PCA score plots of dry weight based metabolites concentrations in Wakame after different blanching time.



PCA Loading Plot

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Ile	0.9988	N-Acetylglucosamine	0.4662	Cyclohexylamine	0.5304	Cytosine	0.6157	Diethanolamine	0.5598
Asn	0.9981	Lactate	0.4122	Octanoate	0.4924	Guanine	0.5255	4-Oxopentanoate	0.4056
Cystathionine	0.9976	gamma-Guanidinobutyrate	0.3606	Thymidine	0.4699	Cyclohexylamine	0.4509	Cyclohexylamine	0.3807
Ala	0.9973	4-Oxopentanoate	0.3584	Heptanoate	0.4027	Uracil	0.3093	Heptanoate	0.3632
Gln	0.9962	5-Oxoproline	0.3569	Decanoate	0.3908	N6,N6,N6-Trimethyllysine	0.2565	Octanoate	0.3346
Ser	0.9961	Nicotinamide	0.3389	Glutathione(ox)	0.2900	Ala-Ala	0.2529	Ethanolamine phosphate	0.3330
His	0.9958	Cyclohexylamine	0.2910	N-Acetylglucosamine	0.2614	Octanoate	0.2248	Decanoate	0.2975
Glu	0.9954	Glutarate	0.2828	Mucate	0.2420	Adenine	0.2144	Hexanoate	0.2942
2AB	0.9953	Adipate	0.2827	Dodecanoate	0.2207	Gly-Gly	0.2091	Nicotinamide	0.2540
Leu	0.9950	2-Oxoglutarate	0.2774	Cysteine-glutathione disulphide	0.2191	gamma-Guanidinobutyrate	0.2020	5-Oxoproline	0.1988
PC1 (negative score)		**PC2 (negative score)**		**PC3 (negative score)**		**PC4 (negative score)**		**PC5 (negative score)**	
Dodecanoate	-0.9117	o-Acetylcarnitine	-0.8432	5-Oxoproline	-0.8031	4-Oxopentanoate	-0.3817	Riboflavin	-0.3542
Hexanoate	-0.7773	AMP	-0.6696	Nicotinamide	-0.6721	Riboflavin	-0.3201	Cytosine	-0.1651
Heptanoate	-0.6209	Ethanolamine phosphate	-0.6393	4-Oxopentanoate	-0.5135	Ethanolamine phosphate	-0.2351	Agmatine	-0.1333
Decanoate	-0.5788	Mucate	-0.5880	Cytosine	-0.3581	AMP	-0.2046	Adenosine	-0.1324
Octanoate	-0.5622	3'-AMP	-0.5109	Fructose	-0.3289	Allantoin	-0.1647	Glutarate	-0.1108
Riboflavin	-0.4411	NADP+	-0.4581	Guanine	-0.3253	N-Acetylglucosamine	-0.1473	Uracil	-0.1088
Cyclohexylamine	-0.3995	5-Methylthioadenosine	-0.4216	Adenine	-0.2237	3-Aminoisobutyrate	-0.1352	Inosine	-0.1083
Thymidine	-0.3129	Cytosine	-0.4021	gamma-Guanidinobutyrate	-0.1907	2-Oxoglutarate	-0.1208	DOPA	-0.1079
Diethanolamine	-0.2214	Allantoin	-0.3859	Taurine	-0.1487	3PG	-0.1178	Gluconate	-0.1015
4-Oxopentanoate	-0.0885	Ala-Ala	-0.3745	Uracil	-0.1390	UMP	-0.1101	beta-Ala	-0.0852

Figure S4.5b PCA loading plots of dry weight based metabolites concentrations in Wakame after different blanching time.

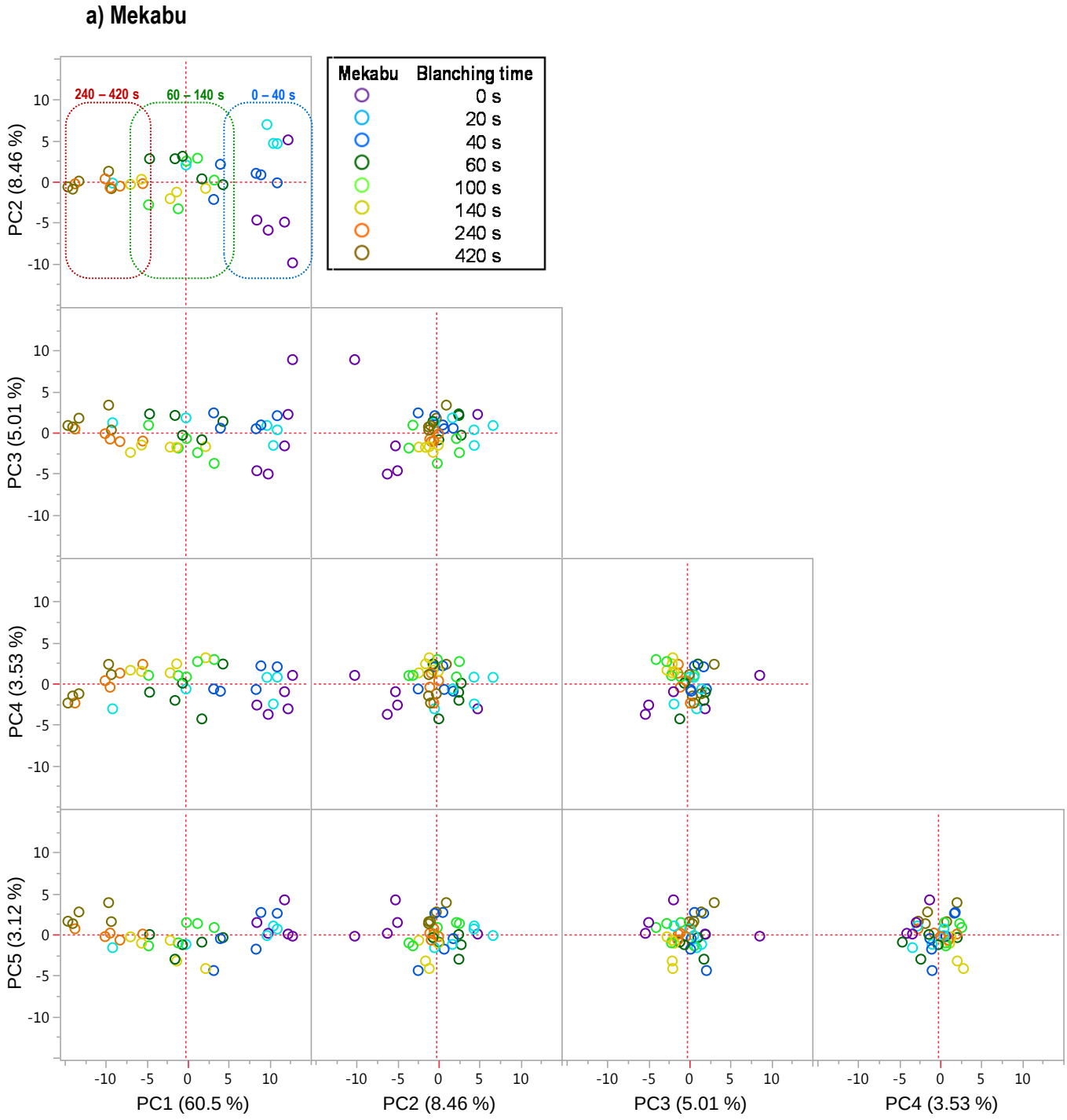


Figure S4.6a PCA score plots of dry weight based metabolites concentrations in Mekabu after different blanching time.

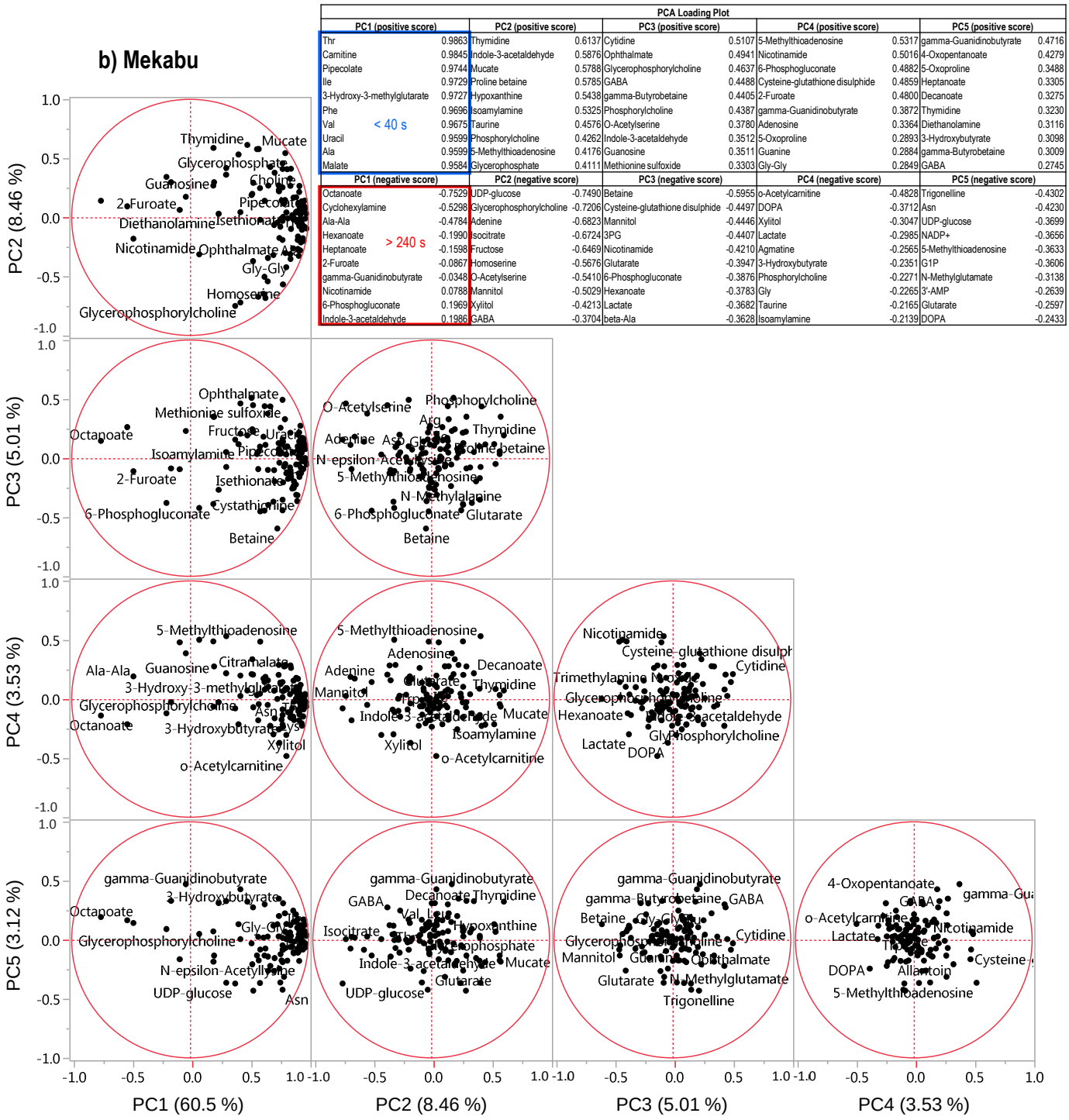


Figure S4.6b PCA loading plots of dry weight based metabolites concentrations in Mekabu after different blanching time.

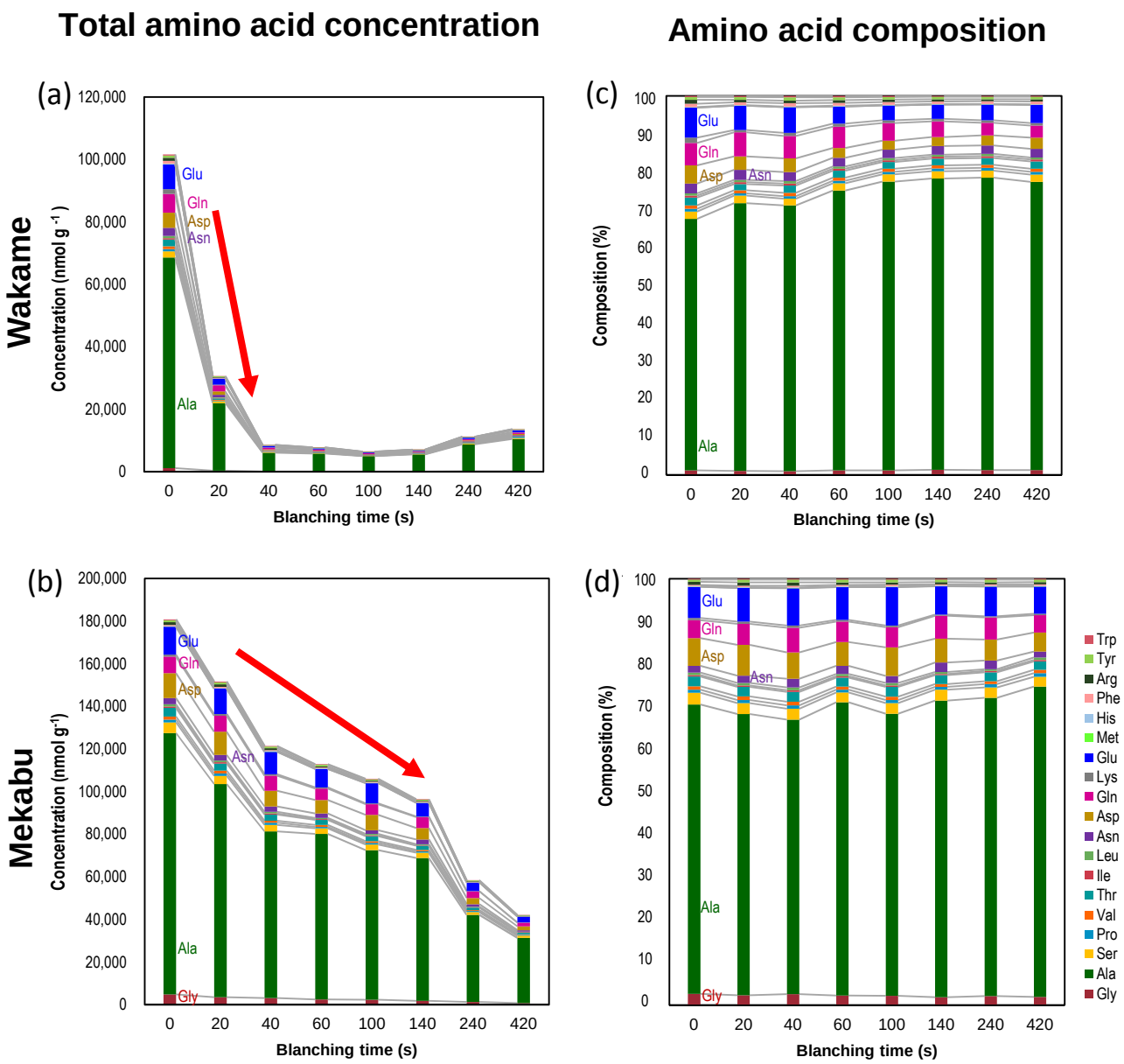


Figure S4.7 Total of 19 amino acids concentrations (a, b) and composition (c, d) in Wakame and Mekabu after blanching.

The concentrations are shown in (nmol g^{-1}), and the composition in percentage.

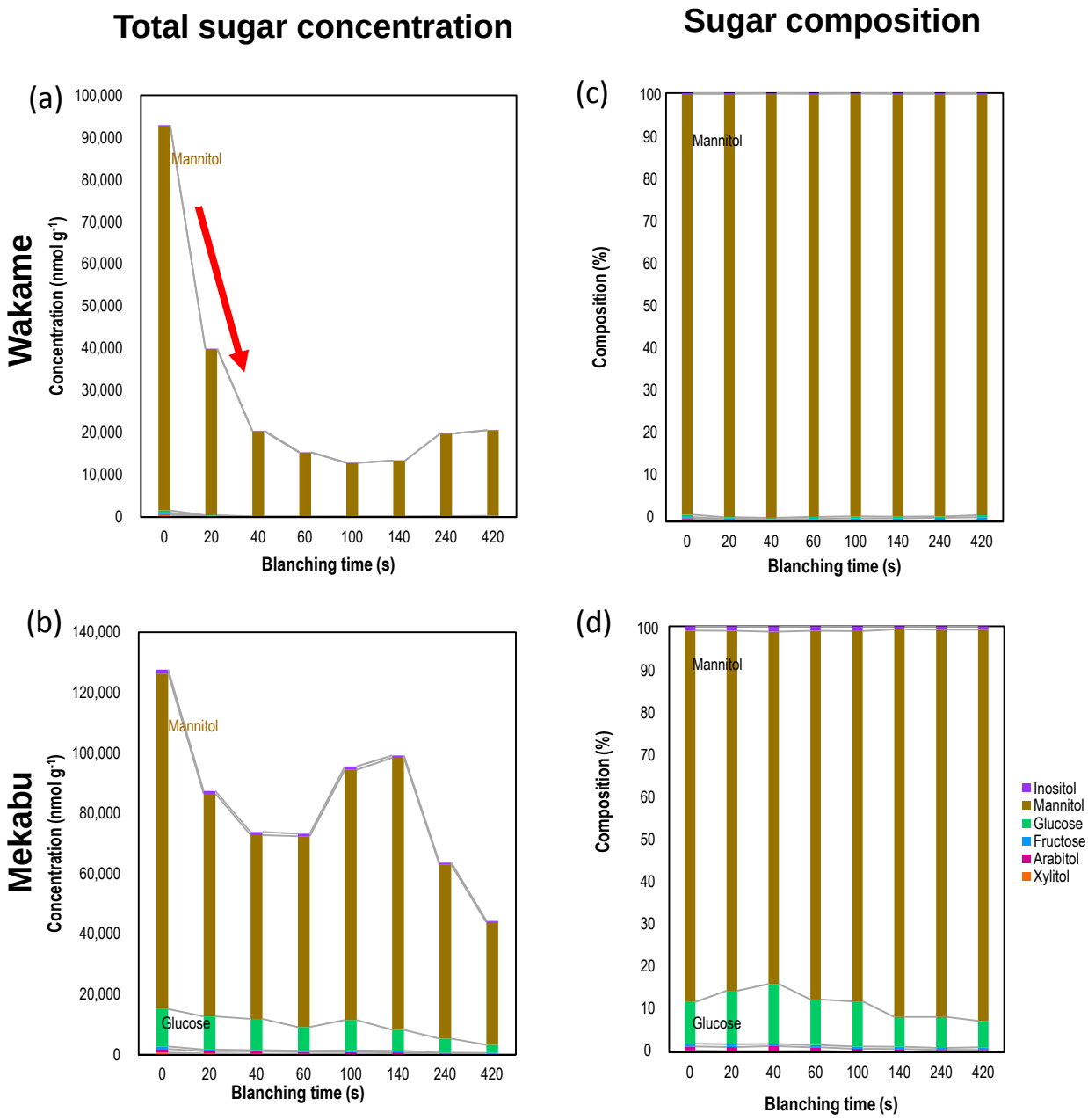


Figure S4.8 Total of 6 sugars concentration (a, b) and composition distributions (c, d) in Wakame and Mekabu after blanching.
Sugars concentrations are shown in (nmol g^{-1}), and the composition in percentage.

Table S2.1. PCA separation variances of all samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
2-Isopropylmalate	0.9565	Phe	0.7885	Isobutylamine	0.7784	N-Acetylglucosamine	0.6855	Malate	0.6054
Isethionate	0.9416	Arg	0.7597	Lactate	0.6890	Uridine	0.6769	Cytidine	0.4579
Orotate	0.9324	Lys	0.7523	Cyclohexylamine	0.6721	Dihydrouracil	0.6356	Lactate	0.4552
Saccharate	0.9209	3PG	0.7419	Urea	0.6507	Taurocyamine	0.6333	4-Methyl-2-oxopentanoate	0.4130
N-Acetylglutamate	0.9174	Ala	0.7247	Isocitrate	0.6206	5-Methoxyindoleacetate	0.6209	Adenosine	0.4029
Malonate	0.9094	Nornicotine	0.7083	Putrescine	0.5560	Allantoin	0.5667	Pelargonate	0.4020
6-Hydroxyhexanoate	0.9079	G6P	0.7024	Creatine	0.5245	Diethanolamine	0.4968	Carnitine	0.3917
Citramalate	0.9040	Citrate	0.6975	Succinate	0.4413	Uracil	0.4810	Guanosine	0.3898
Threonate	0.8991	Carnitine	0.6876	Trimethylamine N-oxide	0.4284	Indole-3-acetaldehyde	0.4745	2-Hydroxy-4-methylpentanoate	0.3777
cis-Aconitate	0.8913	Homoserine	0.6709	Fructose	0.4182	Adenine	0.4712	Choline	0.3680
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.8473	Guanosine	-0.7952	Glucose	-0.7331	Syringate	-0.5997	3-Methylbutanoate	-0.6004
Glu	-0.8211	Pelargonate	-0.7912	Cytosine	-0.6615	Phthalate	-0.3647	Pentanoate	-0.5854
Asn	-0.7785	Glycerophosphorylcholine	-0.7910	Hypoxanthine	-0.6563	Succinate	-0.2810	Thymidine	-0.5392
Pro	-0.7615	Hexanoate	-0.7760	2'-Deoxycytidine	-0.6417	3-Phenyllactate	-0.2663	2PG	-0.4637
UMP	-0.7269	Adenosine	-0.7497	Adenine	-0.6379	Adipate	-0.2639	Allantoin	-0.4221
Gln	-0.7236	Cytidine	-0.6778	Guanine	-0.6360	2-Hydroxypentanoate	-0.2189	Taurocyamine	-0.4013
AMP	-0.7153	Prostaglandin E2	-0.6256	2-Hydroxyoctanoate	-0.5562	5-Oxoproline	-0.2185	Trehalose	-0.3989
Gly	-0.6926	Glutathione(ox)	-0.5026	Gluconate	-0.5315	Creatine	-0.2169	Cys	-0.3692
Ser	-0.6790	Dodecanoate	-0.4993	Leu	-0.5272	Malate	-0.2161	Creatine	-0.3383
Ala	-0.6712	4-Oxohexanoate	-0.4767	Val	-0.5183	2-Hydroxyglutarate	-0.2059	Dihydrouracil	-0.3223

Values indicate top 10 of positive and negative separation variances.

Table S2.2. PCA separation variances of freeze-dry samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Fructose	0.9526	Carnitine	0.8489	Taurocyamine	0.9363	Cytidine	0.5772	5-Oxoproline	0.5697
Trehalose	0.9498	His	0.8480	Allantoin	0.8854	Creatinine	0.4992	beta-Ala	0.4379
Gluconate	0.9493	Phe	0.7814	Thymidine	0.8129	Adenosine	0.4497	2-Hydroxyglutarate	0.4343
2-Isopropylmalate	0.9491	Nornicotine	0.7652	5-Methoxyindoleacetate	0.7592	Hydroxyproline	0.4329	Thr	0.4001
Orotate	0.9481	Succinate	0.7557	Urea	0.6517	Guanosine	0.4193	Indole-3-acetaldehyde	0.3935
6-Hydroxyhexanoate	0.9401	Lys	0.7500	Uridine	0.6280	3-Phenyllactate	0.4099	Phthalate	0.3634
Isethionate	0.9361	Arg	0.7362	Dihydrouracil	0.5986	Dihydrouracil	0.3942	Azelate	0.3360
Citramalate	0.9314	Leu	0.7163	Indole-3-acetaldehyde	0.5961	Diethanolamine	0.3757	Glutarate	0.3299
Creatine	0.9299	Homoserine	0.7141	ADP	0.5832	Homoserine	0.3705	Decanoate	0.2764
N-Acetylglutamate	0.9220	Trimethylamine N-oxide	0.6666	UDP-glucose	0.5806	Glutathione(ox)	0.3635	Citraconate	0.2590
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.9572	Pelargonate	-0.9195	Malate	-0.6575	Mucate	-0.5369	Val	-0.5024
Glu	-0.9267	Hexanoate	-0.9173	Syringate	-0.4389	Syringate	-0.4642	Diethanolamine	-0.4615
UMP	-0.9032	Glycerophosphorylcholine	-0.9060	Creatinine	-0.3824	Saccharate	-0.1955	Lactate	-0.4364
Glucose	-0.8574	Dodecanoate	-0.8348	Lactate	-0.3767	beta-Ala	-0.1781	Tyr	-0.4224
Asn	-0.8228	Decanoate	-0.8280	beta-Ala	-0.3739	Thymidine	-0.1750	ADP	-0.4033
AMP	-0.8166	Guanosine	-0.8241	Cytidine	-0.3605	Inositol	-0.1656	UDP-glucose	-0.3983
Pro	-0.8099	Adenosine	-0.7914	Tyr	-0.3553	Uracil	-0.1385	2-Hydroxypentanoate	-0.3182
Gln	-0.8039	4-Oxohexanoate	-0.7541	Guanosine	-0.3495	Trehalose	-0.1290	Uracil	-0.2974
Choline	-0.7806	Glutathione(ox)	-0.6802	Succinate	-0.3212	Prostaglandin E2	-0.1235	Glycolate	-0.2409
Gly	-0.7418	Prostaglandin E2	-0.6420	Homoserine	-0.3030	Glycerophosphate	-0.1106	Creatinine	-0.1801

Values indicate top 10 of positive and negative separation variances.

Table S2.3. PCA separation variances of oven-dry at 40ºC samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Mucate	0.9684	Nornicotine	0.8991	Dihydrouracil	0.9728	2-Hydroxypentanoate	0.6443	Ala-Ala	0.5322
Saccharate	0.9649	3PG	0.8792	Thymidine	0.9519	Malate	0.6014	Choline	0.4540
Azelate	0.9458	Isocitrate	0.8222	Allantoin	0.9444	3-Phenyllactate	0.4899	Carnitine	0.4411
2-Isopropylmalate	0.9447	Glycerophosphate	0.7758	Urea	0.9084	Diethanolamine	0.3999	Diethanolamine	0.4321
Trehalose	0.9404	Leu	0.7278	Uracil	0.8860	beta-Ala	0.3578	Glutathione(ox)	0.3253
Glutarate	0.9370	Phe	0.7210	Taurocyamine	0.8568	Cytidine	0.3530	Isocitrate	0.2973
Isethionate	0.9293	Tyr	0.7049	N-Acetylglucosamine	0.8343	Succinate	0.3417	Indole-3-acetaldehyde	0.2709
Creatine	0.9280	2PG	0.7021	5-Methoxyindoleacetate	0.8161	Fructose	0.3396	3-Phenyllactate	0.2301
Gluconate	0.9248	Lys	0.6945	Indole-3-acetaldehyde	0.7891	3PG	0.3386	Tyr	0.2206
N-Acetylglutamate	0.9170	Gln	0.6908	Uridine	0.7736	Guanosine	0.3328	N-Acetylglucosamine	0.2094
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.9148	Glycerophosphorylcholine	-0.9240	Syringate	-0.7654	3-Methylbutanoate	-0.6520	Phthalate	-0.4239
Glu	-0.8802	Adenosine	-0.9121	Lactate	-0.6397	Pentanoate	-0.6359	Glycolate	-0.3716
UMP	-0.8626	Pelargonate	-0.9090	Ala-Ala	-0.6077	Carnitine	-0.4966	Malate	-0.3504
Asn	-0.8258	Guanosine	-0.9037	5-Oxoproline	-0.5585	Hydroxyproline	-0.4802	UMP	-0.2666
Ile	-0.8182	Cytidine	-0.8900	Choline	-0.5565	Putrescine	-0.4776	Hexanoate	-0.2601
Pro	-0.8023	Hexanoate	-0.8151	Carnitine	-0.5374	Trimethylamine N-oxide	-0.4717	3-Methylbutanoate	-0.2578
Ser	-0.7751	Prostaglandin E2	-0.6749	Trp	-0.4426	Choline	-0.4685	Pentanoate	-0.2493
Gly	-0.7657	Glutathione(ox)	-0.6425	Dodecanoate	-0.3513	Trp	-0.3818	2-Hydroxypentanoate	-0.2397
Arg	-0.7447	N-Acetylglucosamine	-0.3640	Malate	-0.3378	Riboflavin	-0.3792	Isobutylamine	-0.2326
Met	-0.7406	Glu	-0.2751	Homoserine	-0.2937	Dodecanoate	-0.3787	Val	-0.2134

Values indicate top 10 of positive and negative separation variances.

Table S2.4. PCA separation variances of oven-dry at 80ºC samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Isethionate	0.9731	Ala	0.7871	Uridine	0.7812	5-Methoxyindoleacetate	0.5849	Lactate	0.5891
Trehalose	0.9726	Arg	0.7782	Diethanolamine	0.7763	Taurocyamine	0.5697	Urea	0.5353
2-Isopropylmalate	0.9723	Trp	0.7669	Taurocyamine	0.6171	4-Oxohexanoate	0.4996	Ile	0.5084
Saccharate	0.9650	Phe	0.7599	Dihydrouracil	0.6152	Dihydrouracil	0.4951	Met	0.4977
Glucose	0.9648	2AB	0.7461	N-Acetylglucosamine	0.6008	N-Acetylglucosamine	0.4888	Phe	0.4764
Gluconate	0.9621	Lys	0.7436	Uracil	0.5448	His	0.4340	4-Oxohexanoate	0.4723
Methyl sulfate	0.9620	Homoserine	0.7408	5-Methoxyindoleacetate	0.5243	Glutathione(ox)	0.4143	Leu	0.4586
cis-Aconitate	0.9607	beta-Ala	0.7297	Tyr	0.5194	Citrate	0.4055	Ser	0.4454
Fumarate	0.9604	Nornicotine	0.7197	2PG	0.4846	AMP	0.3718	Lys	0.4449
Dodecanedioate	0.9603	Gly	0.7001	Hydroxyproline	0.4833	Carnitine	0.3698	Phthalate	0.4395
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.7984	Guanosine	-0.8995	Succinate	-0.6364	Dodecanoate	-0.7582	Citrate	-0.4856
Asn	-0.7948	Adenosine	-0.8544	Malate	-0.6204	Lactate	-0.5372	2PG	-0.3878
Gln	-0.7728	Cytidine	-0.8409	Phthalate	-0.6018	Urea	-0.3902	G6P	-0.3341
Pro	-0.7558	Choline	-0.7966	Syringate	-0.5363	Riboflavin	-0.3462	UMP	-0.3169
Glu	-0.7509	Hexanoate	-0.7671	2-Hydroxypentanoate	-0.5249	Uracil	-0.3235	Nornicotine	-0.2782
AMP	-0.6835	Glycerophosphorylcholine	-0.7668	2-Hydroxyoctanoate	-0.4797	Val	-0.2547	3PG	-0.2638
Glycerophosphate	-0.6447	Pelargonate	-0.7478	2-Hydroxy-4-methylpentanoate	-0.4789	Hydroxyproline	-0.2481	Uracil	-0.2608
Met	-0.6247	Prostaglandin E2	-0.6522	Ala-Ala	-0.4776	Cyclohexylamine	-0.2418	Glu	-0.2491
Gly	-0.6098	Glutathione(ox)	-0.6117	Carnitine	-0.4638	Pelargonate	-0.2390	F6P	-0.2487
Ala	-0.6070	N-Acetylglucosamine	-0.5119	3-Phenyllactate	-0.4114	2-Hydroxyglutarate	-0.2287	Thymidine	-0.2341

Values indicate top 10 of positive and negative separation variances.

Table S2.5. PCA separation variances of Methanol-Chloroform-Water extraction methods.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
2-Isopropylmalate	0.9761	Phe	0.8820	Lactate	0.8161	Malate	0.5770	2PG	0.6533
Isethionate	0.9661	Arg	0.8072	Isobutylamine	0.7879	Glutathione(ox)	0.5752	3PG	0.6037
Threonate	0.9610	Lys	0.8023	Cyclohexylamine	0.7670	Val	0.5227	Ala-Ala	0.5065
Citramalate	0.9603	Carnitine	0.7894	Diethanolamine	0.7515	5-Methoxyindoleacetate	0.5050	Azelate	0.4349
Orotate	0.9598	Ala	0.7560	Urea	0.7312	2'-Deoxycytidine	0.5012	Citrate	0.4146
trans-Aconitate	0.9552	Homoserine	0.7503	Uracil	0.6730	Cytosine	0.5010	Choline	0.3959
N-Acetylglutamate	0.9526	Trp	0.7468	5-Methoxyindoleacetate	0.6331	Hypoxanthine	0.5008	Trehalose	0.3364
Malonate	0.9452	Gly	0.7466	N-Acetylglucosamine	0.6154	Adenine	0.4998	Mucate	0.3295
Saccharate	0.9444	Gln	0.7213	Isocitrate	0.6033	2-Hydroxyoctanoate	0.4968	Glu	0.3021
3-Phenyllactate	0.9405	G6P	0.7171	Putrescine	0.5990	Guanine	0.4966	Glutarate	0.2689
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.8289	Guanosine	-0.8321	Glucose	-0.6998	2PG	-0.4991	Taurocyamine	-0.5897
Glu	-0.8204	Hexanoate	-0.8254	Cytosine	-0.6294	Creatine	-0.4415	Cys	-0.5821
UMP	-0.7479	Pelargonate	-0.8056	Adenine	-0.6283	Cys	-0.3621	Met	-0.4345
Pro	-0.7211	Glycerophosphorylcholine	-0.7969	2'-Deoxycytidine	-0.6283	Trehalose	-0.3362	Leu	-0.3703
Asn	-0.7078	Adenosine	-0.7085	Hypoxanthine	-0.6282	Adipate	-0.3147	Fructose	-0.3172
Gln	-0.6476	Prostaglandin E2	-0.6507	2-Hydroxyoctanoate	-0.6249	2-Hydroxypentanoate	-0.2850	Putrescine	-0.3133
AMP	-0.6347	Cytidine	-0.6212	Guanine	-0.6203	Syringate	-0.2594	4-Methyl-2-oxopentanoate	-0.2945
Ala	-0.6296	Dodecanoate	-0.5736	Gluconate	-0.5417	Dihydrouracil	-0.2267	2-Hydroxy-4-methylpentanoate	-0.2943
Gly	-0.6223	Glycolate	-0.4158	Syringate	-0.4836	2-Hydroxyglutarate	-0.2153	Dodecanoate	-0.2894
2AB	-0.6211	4-Oxohexanoate	-0.4101	Val	-0.4508	Glutarate	-0.1842	Ile	-0.2790

Values indicate top 10 of positive and negative separation variances.

Table S2.6. PCA separation variances of Methanol-Water extraction methods.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Saccharate	0.9608	Nornicotine	0.7564	Glucose	0.8291	Diethanolamine	0.6509	3-Methylbutanoate	0.6269
Mucate	0.9580	3PG	0.7439	Leu	0.8009	Urea	0.6223	Pentanoate	0.6225
6-Hydroxyhexanoate	0.9375	Arg	0.6900	Adenine	0.7769	Choline	0.6143	Ala-Ala	0.4745
2-Isopropylmalate	0.9333	Phe	0.6694	2'-Deoxycytidine	0.7724	Lactate	0.5645	ADP	0.4742
Orotate	0.9285	Val	0.6690	Guanine	0.7708	Carnitine	0.5379	Carnitine	0.4668
Dodecanedioate	0.9281	Lys	0.6689	Cytosine	0.7684	Ala-Ala	0.5320	Choline	0.4630
Isethionate	0.9238	Succinate	0.6625	Hypoxanthine	0.7371	ADP	0.5320	Azelate	0.3724
N-Acetylglutamate	0.9010	Ala	0.6584	Dodecanoate	0.5654	Uridine	0.5244	UDP-glucose	0.3513
Citramalate	0.8947	2PG	0.6468	Val	0.5644	5-Oxoproline	0.4128	Trehalose	0.3407
3-Hydroxybutyrate	0.8942	G6P	0.6446	Ile	0.5475	UDP-glucose	0.3975	Adipate	0.3148
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Asp	-0.8746	Adenosine	-0.8831	Isobutylamine	-0.7371	beta-Ala	-0.5474	Fructose	-0.4790
Asn	-0.8700	Guanosine	-0.8657	Lactate	-0.6215	2-Hydroxypentanoate	-0.5450	beta-Ala	-0.4460
AMP	-0.8661	Glycerophosphorylcholine	-0.8484	Isocitrate	-0.5656	3-Methylbutanoate	-0.5432	Succinate	-0.4168
Pro	-0.8289	Cytidine	-0.8389	Urea	-0.5652	Pentanoate	-0.5301	Cys	-0.4028
Glu	-0.8229	Pelargonate	-0.8263	2-Hydroxy-4-methylpentanoate	-0.5645	Cys	-0.4795	Isocitrate	-0.3836
Gln	-0.8190	Hexanoate	-0.7986	Creatine	-0.5344	Thymidine	-0.4783	Taurocyamine	-0.3815
Gly	-0.7770	N-Acetylglucosamine	-0.7327	Succinate	-0.5016	2PG	-0.3486	2-Hydroxy-4-methylpentanoate	-0.3708
UMP	-0.7710	Glutathione(ox)	-0.7026	Cyclohexylamine	-0.4870	Adipate	-0.3334	4-Methyl-2-oxopentanoate	-0.3540
Ser	-0.7668	4-Oxohexanoate	-0.5661	Putrescine	-0.4531	Trehalose	-0.3031	Isobutylamine	-0.3431
Glycerophosphate	-0.7665	Decanoate	-0.5311	Phthalate	-0.4439	Allantoin	-0.2298	Putrescine	-0.3313

Values indicate top 10 of positive and negative separation variances.

Table S2.7. PCA separation variances of Mozuku samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Malate	0.9543	Cyclohexylamine	0.9791	Adenine	0.9078	Pro	0.7361	Asp	0.6106
Isethionate	0.9538	Isocitrate	0.9581	2'-Deoxycytidine	0.8974	Gln	0.6295	Pentanoate	0.5776
3-Phenyllactate	0.9420	Isobutylamine	0.9536	Guanine	0.8945	Phe	0.5783	Dodecanedioate	0.5649
2-Hydroxypentanoate	0.8981	Trimethylamine N-oxide	0.9514	Ile	0.8881	Creatinine	0.5487	3-Methylbutanoate	0.5619
Orotate	0.8964	Lactate	0.9310	Cytosine	0.8736	Arg	0.5124	2-Hydroxyoctanoate	0.4134
trans-Aconitate	0.8766	Putrescine	0.9098	Leu	0.8616	Glu	0.5044	2-Hydroxy-4-methylpentanoate	0.3600
Citramalate	0.8738	Succinate	0.8699	Val	0.8598	His	0.4817	3-Hydroxybutyrate	0.3577
2-Isopropylmalate	0.8727	Fructose	0.8628	Hypoxanthine	0.8364	Tyr	0.4792	F6P	0.3433
Threonate	0.8647	Urea	0.7988	Glucose	0.7464	Hydroxyproline	0.4787	Thymidine	0.3283
Malonate	0.8324	Nornicotine	0.7781	Dodecanoate	0.6850	Homoserine	0.4725	6-Hydroxyhexanoate	0.3189
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Indole-3-acetaldehyde	-0.8897	Ala	-0.7986	Creatine	-0.8207	Mucate	-0.5407	Diethanolamine	-0.5087
Taurocyamine	-0.8383	3PG	-0.7803	Phthalate	-0.6501	Fructose	-0.4374	Tyr	-0.2945
5-Methoxyindoleacetate	-0.7966	Glu	-0.6682	Trehalose	-0.6412	Urea	-0.4332	Uracil	-0.2798
Prostaglandin E2	-0.7136	Azelate	-0.6353	Gln	-0.5877	4-Methyl-2-oxopentanoate	-0.3599	Syringate	-0.2175
N-Acetylglucosamine	-0.6490	2PG	-0.5652	2PG	-0.4910	2-Hydroxy-4-methylpentanoate	-0.3515	Uridine	-0.2141
Riboflavin	-0.6090	Glutarate	-0.5619	Glu	-0.4909	2-Hydroxyoctanoate	-0.3470	Dodecanoate	-0.1973
Allantoin	-0.6044	Glucose	-0.5602	Citrate	-0.4555	5-Oxoproline	-0.3311	2PG	-0.1949
Gly	-0.5610	Trehalose	-0.5447	Adipate	-0.4496	Syringate	-0.3279	Mucate	-0.1815
Dihydrouracil	-0.5588	Glycolate	-0.5355	N-Acetylglutamate	-0.4253	AMP	-0.3106	Glycerophosphate	-0.1712
Thymidine	-0.5493	Adipate	-0.4949	Orotate	-0.3802	Inositol	-0.3081	Guanosine	-0.1680

Values indicate top 10 of positive and negative separation variances.

Table S2.8. PCA separation variances of Kombu samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Fructose	0.8726	Mucate	0.9058	Ile	0.7233	Glycerophosphate	0.6344	Asp	0.6106
Dihydrouracil	0.8605	Methyl sulfate	0.8778	Leu	0.7147	Malate	0.5967	Pentanoate	0.5776
Taurocyamine	0.8566	Isobutylamine	0.8722	Ser	0.7037	Malonate	0.5438	Dodecanedioate	0.5649
N-Acetylglucosamine	0.8557	Urea	0.8624	Proline betaine	0.6610	Tyr	0.5055	3-Methylbutanoate	0.5619
Uridine	0.7992	Saccharate	0.8170	2AB	0.6551	AMP	0.4952	2-Hydroxyoctanoate	0.4134
Gln	0.7834	Glutathione(ox)	0.8114	Dodecanedioate	0.6060	Guanosine	0.4924	2-Hydroxy-4-methylpentanoate	0.3600
Adenosine	0.7778	Decanoate	0.7794	5-Oxoproline	0.5742	4-Oxohexanoate	0.4875	3-Hydroxybutyrate	0.3577
Cytidine	0.7744	4-Methyl-2-oxopentanoate	0.7776	Citramalate	0.5502	Hexanoate	0.4851	F6P	0.3433
Phe	0.7608	Citrate	0.7667	Gly	0.5350	Glucose	0.4679	Thymidine	0.3283
Lys	0.7324	4-Oxohexanoate	0.7313	Lys	0.5230	Fumarate	0.4674	6-Hydroxyhexanoate	0.3189
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Succinate	-0.7423	2-Hydroxypentanoate	-0.6003	Pro	-0.5946	Thr	-0.6200	Diethanolamine	-0.5087
Inositol	-0.7194	Malonate	-0.4865	Ala	-0.5902	Glycerophosphorylcholine	-0.6009	Tyr	-0.2945
Threonate	-0.7185	Choline	-0.3729	Val	-0.4332	Glu	-0.5711	Uracil	-0.2798
UMP	-0.6800	Glycerophosphate	-0.3658	Thymidine	-0.4326	Indole-3-acetaldehyde	-0.5566	Syringate	-0.2175
Dodecanoate	-0.6597	Riboflavin	-0.3547	Allantoin	-0.4235	Asp	-0.5178	Uridine	-0.2141
Glycolate	-0.6567	Fumarate	-0.3321	Prostaglandin E2	-0.3896	Riboflavin	-0.4999	Dodecanoate	-0.1973
Malate	-0.5809	Glycolate	-0.2907	Saccharate	-0.3878	Isocitrate	-0.4978	2PG	-0.1949
Trimethylamine N-oxide	-0.5755	Glutarate	-0.2651	Diethanolamine	-0.3438	Trehalose	-0.3849	Mucate	-0.1815
Cyclohexylamine	-0.5373	Succinate	-0.2609	Tyr	-0.3275	Allantoin	-0.3180	Glycerophosphate	-0.1712
Lactate	-0.5158	AMP	-0.2026	Mucate	-0.2583	Thymidine	-0.3043	Guanosine	-0.1680

Values indicate top 10 of positive and negative separation variances.

Table S2.9. PCA separation variances of Wakame samples.

PC1 (positive score)		PC2 (positive score)		PC3 (positive score)		PC4 (positive score)		PC5 (positive score)	
Gln	0.9409	Dihydrouracil	0.9809	Leu	0.7773	beta-Ala	0.6617	Adipate	0.6886
Ala	0.9365	Taurocyamine	0.9594	Ile	0.7398	UMP	0.6268	F6P	0.5364
Glu	0.9232	Indole-3-acetaldehyde	0.9584	Ser	0.7172	Glycerophosphate	0.5239	Isocitrate	0.4772
Asp	0.9156	N-Acetylglucosamine	0.9285	Lys	0.6513	Succinate	0.4875	5-Oxoproline	0.4432
Asn	0.9114	5-Methoxyindoleacetate	0.8853	Met	0.6195	Cys	0.4491	Succinate	0.4369
Citrate	0.9084	Thymidine	0.8755	Adipate	0.5974	AMP	0.4298	2-Hydroxypentanoate	0.3867
Pro	0.9001	Allantoin	0.8500	Val	0.5960	Threonate	0.3690	Dodecanoate	0.3545
Arg	0.8888	Uridine	0.7193	Phe	0.5798	Isocitrate	0.3288	UDP-glucose	0.3146
Isobutylamine	0.8672	Mucate	0.6949	2-Hydroxypentanoate	0.5349	Methyl sulfate	0.3193	ADP	0.2939
3PG	0.8582	Saccharate	0.6533	Gly	0.4716	Homoserine	0.2963	trans-Aconitate	0.2279
PC1 (negative score)		PC2 (negative score)		PC3 (negative score)		PC4 (negative score)		PC5 (negative score)	
Dodecanoate	-0.7412	Citramalate	-0.8810	Riboflavin	-0.4819	ADP	-0.6332	Putrescine	-0.5730
Syringate	-0.6010	2-Hydroxyglutarate	-0.8706	Azelate	-0.4515	Glucose	-0.5103	Threonate	-0.4984
2-Hydroxypentanoate	-0.4748	Fumarate	-0.7070	F6P	-0.4272	Trp	-0.4840	2PG	-0.3127
Cys	-0.4161	Syringate	-0.5710	Choline	-0.3357	Urea	-0.4719	Fumarate	-0.3122
Met	-0.4038	5-Oxoproline	-0.5560	G6P	-0.3264	Val	-0.4589	Nornicotine	-0.3013
beta-Ala	-0.1740	Glycerophosphorylcholine	-0.5269	Carnitine	-0.3103	UDP-glucose	-0.4560	2AB	-0.2460
Putrescine	-0.1681	Methyl sulfate	-0.4560	Adenosine	-0.3088	Riboflavin	-0.4547	Citramalate	-0.2395
Malonate	-0.1122	Homoserine	-0.4108	trans-Aconitate	-0.2564	Putrescine	-0.4484	Met	-0.2393
Taurocyamine	-0.1037	Proline betaine	-0.3978	ADP	-0.2558	Leu	-0.4171	Homoserine	-0.2345
Indole-3-acetaldehyde	-0.0964	Trimethylamine N-oxide	-0.3880	Trimethylamine N-oxide	-0.2510	Lactate	-0.3508	Phthalate	-0.2310

Values indicate top 10 of positive and negative separation variances.

Table S2.10: Metabolites concentrations in Mozuku samples after various drying and extractions methods.

		MOZUKU					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Sugars	UDP-glucose	ND	ND	ND	ND	ND	ND
	Fructose	539.13±28.36^{a}	113.21±4.77^{e}	305.17±70.72^{c}	464.86±35.81^{b}	102.23±12.08^{e}	208.94±18.64^{d}
	Glucose	ND	43.59±3.35^{bc}	187.89±28.13^{a}	ND	52.72±4.87^{b}	173.69±14.26^{a}
	Inositol	132.62±10.32^{a}	108.25±12.77^{c}	121.37±13.94^{abc}	127.61±1.75^{ab}	113.92±2.20^{bc}	112.19±2.47^{bc}
	Trehalose	127.99±4.58^{b}	263.57±25.06^{a}	138.86±21.33^{b}	123.93±2.48^{b}	269.84±8.25^{a}	126.84±6.59^{b}
Organic acids	5-Methoxyindoleacetate	90.58±10.18^{a}	66.65±6.64^{a}	70.16±7.57^{a}	122.51±25.51^{a}	109.71±6.06^{a}	114.44±12.43^{a}
	Glycolate	457.11±158.83^{bc}	775.18±143.26^{ab}	1118.67±122.93^{a}	404.13±17.82^{bc}	584.68±160.59^{bc}	699.19±76.25^{b}
	Lactate	8149.65±503.77^{a}	1731.09±412.19^{d}	1463.72±68.43^{d}	5869.97±486.73^{b}	1130.21±122.27^{d}	1165.77±199.74^{d}
	Pentanoate	ND	ND	ND	ND	68.22±24.31^{a}	ND
	3-Methylbutanoate	ND	ND	ND	ND	132.58±15.62^{a}	ND
	Malonate	369.34±10.85^{bc}	367.69±1.66^{bc}	458.77±41.38^{a}	295.00±16.27^{cd}	260.12±15.54^{d}	385.18±24.48^{ab}
	3-Hydroxybutyrate	203.95±108.56^{bc}	320.52±114.72^{b}	654.74±183.47^{a}	127.21±38.92^{cd}	184.06±35.63^{bc}	203.79±1.78^{bc}
	Methyl sulfate	68.56±4.62^{a}	42.81±4.47^{bc}	54.34±5.01^{abc}	48.85±0.54^{b}	20.86±2.09^{d}	27.56±6.50^{cd}
	Fumarate	118.21±6.81^{b}	99.31±5.68^{bc}	215.11±8.10^{a}	68.89±9.52^{cd}	68.47±7.89^{cd}	118.05±25.05^{b}
	Hexanoate	ND	ND	ND	ND	ND	ND
	Succinate	371.00±25.30^{a}	158.49±6.23^{bcdef}	162.76±16.94^{bcde}	222.18±11.79^{bc}	72.90±5.52^{efg}	77.27±8.51^{efg}
	2-Hydroxypentanoate	203.78±22.20^{ab}	236.84±26.27^{a}	239.59±50.94^{a}	85.78±5.42^{de}	115.79±19.41^{bcd}	118.72±23.90^{bcd}
	Isethionate	298.02±21.56^{b}	333.14±27.69^{ab}	369.92±27.61^{a}	166.43±8.57^{c}	185.23±7.01^{c}	205.92±37.83^{c}
	Citraconate	13.62±3.30^{ab}	12.87±3.78^{ab}	12.21±1.77^{ab}	15.88±5.51^{a}	9.98±3.61^{abc}	8.25±0.40^{abc}
	4-Methyl-2-oxopentanoate	62.45±7.51^{ab}	ND	81.84±18.98^{a}	49.47±11.81^{cd}	ND	46.33±12.89^{bc}
	4-Oxohexanoate	ND	ND	ND	ND	ND	ND
	Glutarate	95.53±5.93^{c}	188.83±2.60^{a}	160.69±3.64^{ab}	67.30±10.89^{c}	102.87±2.69^{bc}	92.65±13.98^{c}
	2-Hydroxy-4-methylpentanoate	81.66±1.59^{b}	ND	97.91±18.07^{a}	45.24±5.38^{bc}	ND	ND
	6-Hydroxyhexanoate	412.92±38.02^{a}	348.31±28.18^{b}	521.12±96.20^{a}	246.44±22.48^{b}	244.33±23.30^{b}	234.82±7.61^{b}
	Malate	302.85±14.61^{ab}	258.30±16.78^{bcd}	384.44±46.08^{a}	180.44±12.27^{cdef}	129.59±17.21^{ef}	159.51±13.71^{def}
	Threonate	1647.58±110.86^{a}	1233.97±122.67^{b}	1481.77±112.01^{a}	883.29±86.65^{c}	631.83±33.86^{d}	670.96±40.72^{d}
	Adipate	56.51±10.46^{bcd}	117.57±8.98^{a}	84.84±10.90^{ab}	43.13±9.63^{cdefg}	68.76±1.55^{bc}	50.06±18.27^{bcdef}
	Citramalate	287.15±17.36^{a}	262.88±16.97^{a}	276.37±9.78^{a}	172.34±8.26^{b}	87.62±2.93^{c}	91.57±4.58^{c}
	2-Hydroxyglutarate	56.31±15.18^{a}	66.85±16.87^{a}	54.65±9.76^{a}	46.51±7.40^{ab}	38.47±8.69^{abc}	38.01±13.01^{abc}
	Orotate	734.81±54.19^{b}	860.62±21.81^{a}	834.37±84.98^{a}	495.46±41.00^{c}	402.20±51.04^{d}	353.30±6.73^{d}
	Pelargonate	ND	ND	ND	ND	ND	ND
	2-Hydroxyoctanoate	ND	ND	74.78±12.38^{a}	ND	ND	ND
	Phthalate	15.62±3.44^{a}	15.17±8.00^{a}	11.75±5.03^{ab}	10.37±0.86^{ab}	10.50±4.22^{ab}	ND
	3-Phenyllactate	446.43±30.69^{b}	618.92±49.15^{a}	624.61±56.07^{a}	22.60±9.34^{c}	35.42±9.78^{c}	51.75±5.40^{c}
	Glycerophosphate	ND	51.48±3.66^{ef}	79.24±46.65^{ef}	ND	ND	27.74±6.60^{f}
	Decanoate	ND	ND	ND	ND	ND	ND
	trans-Aconitate	107.62±6.33^{a}	101.34±10.15^{a}	99.70±10.92^{a}	56.40±7.70^{b}	43.00±6.99^{b}	42.16±5.59^{b}
	cis-Aconitate	82.95±4.53^{a}	41.29±3.11^{c}	59.28±4.22^{b}	47.37±0.96^{c}	17.71±1.13^{e}	27.14±2.20^{d}
	2-Isopropylmalate	159.64±9.39^{a}	141.77±13.75^{a}	158.99±17.46^{a}	108.41±2.11^{b}	97.94±4.88^{bc}	83.12±2.82^{c}
	2PG	ND	16.09±5.65^{a}	2.41±0.39^{b}	ND	7.01±2.54^{b}	5.59±0.59^{b}
	3PG	ND	19.95±0.39^{bcd}	17.41±2.34^{bcd}	ND	9.35±1.77^{de}	16.50±4.72^{cde}
	Azelate	58.82±9.04^{bcde}	118.09±5.84^{a}	93.75±4.04^{ab}	48.77±4.64^{cdef}	71.22±9.04^{bcd}	73.85±19.37^{bc}
	N-Acetylglutamate	45.31±6.36^{a}	53.44±2.54^{a}	45.50±4.99^{a}	25.21±8.83^{b}	26.18±0.53^{b}	20.61±5.85^{b}
	Isocitrate	137.23±14.39^{a}	27.27±4.44^{cd}	28.22±5.04^{cd}	92.19±9.86^{b}	20.25±3.77^{cdef}	25.70±5.23^{cd}
	Citrate	150.83±16.82^{e}	127.50±2.06^{e}	103.23±10.72^{e}	80.01±8.26^{e}	52.08±4.85^{e}	43.07±0.86^{e}
	Gluconate	389.10±14.23^{d}	558.60±42.87^{c}	2106.59±166.97^{a}	296.56±39.02^{d}	309.91±63.60^{d}	971.30±71.54^{b}
	Syringate	249.22±192.81^{abc}	491.17±36.25^{a}	437.06±140.83^{abc}	252.00±22.51^{abc}	211.60±51.52^{abc}	199.44±76.62^{abc}
	Dodecanoate	ND	ND	ND	ND	107.44±25.46^{def}	303.00±38.91^{abcde}
	Saccharate	809.51±157.80^{b}	815.87±62.12^{b}	791.55±57.77^{b}	1092.21±79.90^{a}	851.66±96.63^{b}	773.87±49.67^{b}
	Mucate	618.59±228.51^{bc}	746.92±38.12^{a}	637.14±49.26^{ab}	887.56±56.38^{a}	753.38±85.48^{a}	619.74±76.53^{ab}
	Dodecanedioate	15.31±6.12^{a}	11.37±1.38^{bc}	15.02±1.34^{ab}	14.72±2.46^{ab}	15.46±1.76^{a}	14.74±2.87^{ab}

		MOZUKU					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Others	Isobutylamine	203.51±35.77^{a}	ND	ND	119.90±9.19^{b}	ND	ND
	Putrescine	19.98±8.60^{a}	1.69±0.29^{c}	4.37±2.02^{bc}	8.98±4.85^{b}	2.30±0.53^{bc}	3.21±0.61^{bc}
	Cyclohexylamine	25.99±6.25^{a}	2.69±0.63^{d}	2.97±0.12^{d}	14.68±2.08^{b}	3.71±0.31^{cd}	4.65±1.79^{cd}
	Choline	21.57±8.97^{c}	4.09±2.30^{c}	16.29±0.35^{c}	8.70±0.75^{c}	4.64±0.63^{c}	15.50±2.41^{c}
	Diethanolamine	565.29±184.44^{a}	173.84±81.87^{ab}	87.63±25.09^{b}	406.81±73.56^{ab}	133.09±3.92^{b}	451.74±226.91^{ab}
	Cytosine	ND	ND	23.23±0.88^{a}	ND	ND	25.17±2.92^{a}
	Creatinine	10.21±7.50^{a}	5.63±1.14^{ab}	5.58±2.19^{ab}	ND	5.64±0.44^{ab}	6.76±1.39^{ab}
	Homoserine	13.13±7.04^{ab}	ND	ND	ND	ND	ND
	Hydroxyproline	8.94±5.48^{a}	1.87±0.14^{b}	2.25±0.42^{b}	2.83±1.16^{b}	3.92±1.56^{b}	4.29±0.81^{b}
	Creatine	84.59±6.33^{a}	73.33±6.58^{b}	7.61±1.18^{c}	77.23±3.82^{ab}	79.34±5.99^{ab}	7.68±2.29^{c}
	Adenine	ND	ND	12.86±1.21^{b}	ND	ND	17.54±0.44^{a}
	Hypoxanthine	ND	ND	30.27±1.76^{a}	ND	ND	31.44±10.01^{a}
	Proline betaine	8.60±2.66^{a}	4.66±0.79^{ab}	4.77±0.29^{ab}	8.73±2.69^{ab}	5.87±2.50^{ab}	8.53±2.55^{ab}
	Nornicotine	36.07±4.37^{a}	21.18±2.63^{bcd}	22.03±3.36^{bcd}	25.53±2.92^{abc}	16.20±3.84^{cd}	17.99±9.28^{bcd}
	Guanine	ND	ND	15.83±3.00^{b}	ND	ND	21.25±2.53^{a}
	Carnitine	10.72±5.28^{bc}	ND	4.46±1.97^{cd}	ND	ND	ND
	Taurocyamine	304.19±17.21^{de}	231.68±10.67^{e}	257.30±11.13^{de}	468.80±14.24^{bcde}	530.55±53.46^{bcde}	618.48±86.50^{abcde}
	N-Acetylglucosamine	50.32±13.23^{cdef}	30.24±8.43^{efg}	31.24±4.65^{defg}	63.32±9.49^{abcde}	50.59±7.17^{cdef}	63.91±9.10^{abcde}
	2'-Deoxycytidine	ND	ND	6.30±0.41^{b}	ND	ND	7.86±1.49^{a}
	Thymidine	183.11±14.68^{a}	156.49±12.26^{a}	174.69±23.36^{a}	273.01±20.04^{a}	300.21±17.73^{a}	317.45±6.45^{a}
	Cytidine	16.01±2.69^{bc}	5.55±2.03^{cd}	11.08±2.45^{bc}	ND	ND	9.04±2.03^{bcd}
	Uridine	173.14±15.84^{ab}	96.87±14.42^{bcdef}	93.77±38.26^{cdef}	167.35±52.37^{abc}	132.37±25.89^{abcde}	181.87±25.22^{a}
	Glycerophosphorylcholine	ND	ND	ND	ND	ND	ND
	Adenosine	10.84±5.07^{d}	3.10±1.30^{d}	3.44±1.12^{d}	ND	ND	ND
	Guanosine	7.71±3.13^{cd}	3.61±2.13^{d}	6.76±0.92^{d}	ND	ND	7.46±1.39^{cd}
	5-Oxoproline	107.29±35.00^{ab}	93.06±20.38^{ab}	153.68±26.92^{ab}	116.66±7.32^{ab}	64.77±33.41^{ab}	107.13±23.58^{ab}
	Urea	823.28±151.52^{abc}	211.26±13.13^{f}	208.61±32.67^{f}	997.45±111.22^{abc}	313.94±27.79^{ef}	303.19±46.60^{efg}
	Trimethylamine N-oxide	23.70±6.23^{a}	9.60±0.78^{cdef}	11.16±1.23^{cde}	20.30±2.04^{ab}	11.93±2.43^{bcd}	13.82±1.43^{bc}
	2AB	ND	ND	ND	ND	ND	ND
	Uracil	689.23±62.71^{ab}	84.62±21.96^{c}	393.84±35.15^{abc}	759.96±334.09^{a}	241.93±52.15^{abc}	744.16±49.25^{a}
	Allantoin	810.46±56.27^{abc}	708.00±28.20^{abc}	944.34±220.79^{abc}	1043.50±40.72^{abc}	1124.81±33.30^{ab}	1199.02±38.59^{a}
	Indole-3-acetaldehyde	337.07±52.29^{bc}	264.22±16.06^{c}	249.05±39.13^{c}	444.20±30.01^{abc}	474.12±42.04^{abc}	451.75±38.65^{abc}
	Glutathione(ox)	4.95±2.32^{ef}	ND	ND	ND	ND	ND
	Riboflavin	34.43±6.09^{a}	23.12±2.91^{abc}	23.23±3.87^{abc}	33.70±7.00^{ab}	33.52±1.56^{ab}	33.96±0.69^{a}
	G6P	ND	ND	ND	ND	ND	ND
	UMP	ND	ND	78.77±69.54^{bcd}	ND	ND	42.22±7.75^{bcd}
	AMP	ND	ND	26.71±3.17^{cd}	ND	ND	ND
	ADP	ND	ND	ND	ND	ND	ND

The values indicate as mean ±SD (nmolg^{-1}) of dry weight (n=3). ND, not detected. Different character indicates the significant differences (P<0.05) between each type of sample by Tukey-HSD analysis. Statistical analysis were compared between all data set (species and treatments (Table S2.10-S2.12)).

Table S2.11: Metabolites concentrations in Kombu samples after various drying and extractions methods

		KOMBU					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Sugars	UDP-glucose	ND	ND	ND	ND	ND	ND
	Fructose	69.36±5.34^{e}	62.89±3.01^{e}	58.72±3.03^{e}	99.89±2.24^{e}	97.53±9.26^{e}	127.23±31.29^{e}
	Glucose	24.30±9.25^{bcd}	22.50±1.57^{bcd}	15.82±4.28^{cd}	21.25±6.97^{bcd}	18.45±3.85^{cd}	34.94±3.83^{bc}
	Inositol	7.07±2.72^{gh}	ND	6.65±1.46^{gh}	ND	ND	ND
	Trehalose	ND	2.54±1.22^{c}	ND	8.71±5.46^{c}	ND	ND
Organic acids	5-Methoxyindoleacetate	98.54±9.25^{a}	76.52±1.60^{a}	77.81±2.11^{a}	153.78±19.20^{a}	140.16±18.32^{a}	224.87±33.37^{a}
	Glycolate	403.28±54.95^{bc}	614.21±221.05^{bc}	566.07±78.40^{bc}	448.90±75.73^{cd}	ND	427.20±100.47^{cd}
	Lactate	5862.45±739.38^{b}	1460.22±307.20^{d}	1211.49±218.85^{d}	4301.71±299.90^{cd}	1033.74±55.49^{d}	1018.25±10.35^{d}
	Pentanoate	ND	ND	ND	ND	ND	ND
	3-Methylbutanoate	ND	ND	ND	ND	ND	ND
	Malonate	79.10±12.82^{ef}	102.79±12.83^{ef}	83.80±11.91^{ef}	51.46±1.38^{f}	57.82±4.93^{f}	74.67±28.64^{ef}
	3-Hydroxybutyrate	ND	ND	ND	ND	ND	ND
	Methyl sulfate	24.55±7.25^{d}	ND	ND	27.31±0.26^{d}	ND	ND
	Fumarate	30.87±8.62^{e}	37.47±0.13^{e}	49.19±5.19^{de}	23.77±5.63^{de}	24.53±2.02^{e}	42.39±9.99^{de}
	Hexanoate	67.61±16.23^{a}	82.08±6.53^{a}	66.39±27.87^{a}	69.98±16.73^{a}	60.01±7.56^{ab}	101.49±11.46^{a}
	Succinate	120.40±15.97^{cdefg}	136.74±25.28^{cdefg}	116.26±8.90^{cdefg}	83.06±0.01^{fg}	69.47±3.79^{efg}	72.48±5.38^{g}
	2-Hydroxypentanoate	55.14±3.22^{de}	104.84±10.34^{cd}	102.63±5.58^{cd}	50.67±0.58^{de}	54.30±46.94^{de}	117.50±6.35^{de}
	Isethionate	ND	ND	ND	ND	ND	ND
	Citraconate	5.03±0.30^{bc}	ND	11.88±7.43^{abc}	12.67±1.45^{abc}	ND	7.73±1.04^{c}
	4-Methyl-2-oxopentanoate	20.25±11.30^{cde}	ND	ND	10.88±6.30^{de}	ND	ND
	4-Oxohexanoate	18.61±2.84^{a}	ND	ND	17.60±13.55^{ab}	ND	16.95±7.12^{ab}
	Glutarate	ND	65.19±11.69^{c}	80.05±32.44^{cd}	70.75±59.15^{cd}	ND	ND
	2-Hydroxy-4-methylpentanoate	ND	ND	ND	ND	ND	ND
	6-Hydroxyhexanoate	ND	ND	ND	ND	ND	ND
	Malate	275.05±30.20^{abc}	205.46±59.88^{bcdef}	237.62±22.30^{bcde}	178.11±19.36^{cdef}	124.45±15.97^{f}	193.57±80.06^{bcdef}
	Threonate	220.16±21.97^{e}	189.85±21.16^{e}	145.50±41.14^{e}	135.76±15.62^{e}	120.70±8.09^{e}	121.21±20.97^{e}
	Adipate	24.16±12.64^{defg}	16.90±10.20^{fg}	25.75±2.78^{defg}	17.35±0.73^{g}	30.56±13.43^{defg}	31.62±8.26^{efg}
	Citramalate	16.79±0.26^{de}	ND	13.93±4.61^{de}	13.64±8.54^{de}	ND	ND
	2-Hydroxyglutarate	4.29±1.24^{d}	ND	24.60±3.25^{bcd}	25.54±22.78^{cd}	ND	ND
	Orotate	ND	ND	ND	ND	ND	ND
	Pelargonate	143.34±30.42^{a}	94.29±15.05^{b}	90.69±2.94^{b}	149.45±9.62^{a}	78.69±18.46^{b}	83.49±7.57^{b}
	2-Hydroxyoctanoate	ND	ND	ND	ND	ND	ND
	Phthalate	12.31±2.90^{ab}	11.59±3.07^{ab}	10.16±2.39^{ab}	14.98±6.95^{ab}	8.32±2.05^{ab}	13.08±4.65^{ab}
	3-Phenyllactate	ND	ND	ND	ND	ND	ND
	Glycerophosphate	44.45±14.52^{f}	ND	93.75±19.48^{def}	ND	ND	95.98±38.91^{def}
	Decanoate	43.36±11.49^{b}	ND	ND	54.34±2.93^{a}	ND	ND
	trans-Aconitate	ND	ND	ND	ND	ND	ND
	cis-Aconitate	ND	ND	ND	ND	ND	ND
	2-Isopropylmalate	ND	ND	ND	ND	ND	ND
	2PG	ND	ND	ND	ND	ND	ND
	3PG	ND	ND	ND	ND	ND	ND
	Azelate	39.65±14.76^{cdefg}	28.68±12.03^{efg}	17.73±12.75^{fg}	53.67±18.65^{cdefg}	4.13±0.87^{g}	17.92±12.63^{fg}
	N-Acetylglutamate	ND	ND	ND	ND	ND	ND
	Isocitrate	5.14±2.50^{ef}	3.30±0.47^{f}	4.67±0.81^{ef}	5.50±3.84^{ef}	12.05±2.60^{def}	4.42±1.60^{f}
	Citrate	206.42±32.25^{de}	78.66±7.69^{e}	72.55±7.74^{e}	120.32±9.53^{e}	88.85±4.15^{e}	74.49±2.85^{e}
	Gluconate	ND	ND	ND	ND	ND	ND
	Syringate	337.26±27.68^{abc}	496.91±91.93^{a}	393.43±2.57^{abc}	269.69±63.43^{abc}	192.33±68.52^{abc}	237.19±52.54^{abc}
	Dodecanoate	552.05±81.30^{a}	408.86±162.32^{bcdef}	485.53±38.24^{ab}	357.29±70.98^{abcd}	134.54±14.04^{def}	253.94±28.59^{cdef}
	Saccharate	455.85±132.14^{c}	214.96±23.72^{def}	169.04±9.54^{ef}	429.20±19.08^{cd}	267.21±9.05^{cde}	296.64±122.39^{def}
	Mucate	403.25±116.89^{bc}	176.68±2.73^{cd}	149.54±8.44^{cd}	379.67±16.88^{bc}	236.38±8.01^{cd}	262.07±76.56^{cd}
	Dodecanedioate	5.77±0.59^{c}	ND	ND	ND	ND	ND

		KOMBU					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Others	Isobutylamine	21.98±5.11^{cde}	ND	ND	17.03±3.56^{cde}	ND	ND
	Putrescine	1.35±0.43^{c}	0.54±0.04^{c}	1.25±0.35^{bc}	1.15±0.22^{bc}	1.72±0.40^{bc}	3.15±1.33^{c}
	Cyclohexylamine	4.99±1.20^{cd}	ND	1.62±0.97^{d}	ND	ND	ND
	Choline	48.16±10.53^{bc}	34.77±2.82^{c}	89.23±2.44^{bc}	34.18±2.02^{c}	31.52±3.48^{c}	81.00±11.17^{bc}
	Diethanolamine	253.16±107.63^{ab}	60.23±20.73^{b}	74.33±20.31^{b}	322.19±48.42^{ab}	180.00±107.73^{ab}	237.84±43.22^{ab}
	Cytosine	ND	ND	ND	ND	ND	ND
	Creatinine	4.33±1.79^{b}	ND	ND	ND	ND	ND
	Homoserine	ND	ND	ND	ND	ND	ND
	Hydroxyproline	ND	ND	ND	ND	ND	ND
	Creatine	ND	ND	ND	ND	ND	ND
	Adenine	ND	ND	ND	ND	ND	ND
	Hypoxanthine	ND	ND	ND	ND	ND	ND
	Proline betaine	3.97±1.42^{b}	ND	ND	ND	ND	ND
	Nornicotine	ND	ND	ND	ND	ND	ND
	Guanine	ND	ND	ND	ND	ND	ND
	Carnitine	ND	ND	ND	ND	ND	ND
	Taurocyamine	270.11±50.39^{de}	236.41±28.01^{def}	234.42±10.50^{de}	524.82±81.94^{bcde}	648.73±40.29^{abcd}	1023.52±545.13^{a}
	N-Acetylglucosamine	44.94±10.27^{cdefg}	37.02±7.05^{defg}	36.83±3.40^{defg}	88.15±7.18^{abc}	75.81±10.34^{abc}	95.99±32.29^{a}
	2'-Deoxycytidine	ND	ND	ND	ND	ND	ND
	Thymidine	144.72±16.90^{a}	148.07±29.52^{a}	128.13±19.17^{a}	207.41±29.01^{a}	277.72±19.94^{a}	277.45±67.42^{a}
	Cytidine	13.49±2.43^{ab}	13.89±1.51^{ab}	11.40±1.02^{abc}	13.98±1.07^{ab}	17.44±1.62^{ab}	20.43±6.49^{a}
	Uridine	66.70±5.25^{def}	59.37±7.75^{ef}	52.55±16.69^{f}	141.27±20.14^{abcd}	104.09±17.16^{abcdef}	144.14±39.51^{abcd}
	Glycerophosphorylcholine	153.70±46.09^{ab}	136.05±2.35^{ab}	118.20±8.07^{b}	199.27±11.40^{a}	162.26±22.31^{ab}	163.86±24.29^{b}
	Adenosine	25.05±2.29^{c}	26.70±2.20^{bc}	24.11±1.88^{c}	37.28±2.25^{ab}	33.19±2.24^{abc}	39.92±12.49^{a}
	Guanosine	17.48±2.59^{b}	17.96±2.17^{b}	15.34±0.73^{bc}	17.69±1.18^{b}	21.94±3.74^{ab}	26.23±9.03^{a}
	5-Oxoproline	196.31±67.68^{ab}	120.56±35.30^{ab}	207.24±12.54^{ab}	138.42±59.52^{ab}	75.48±24.97^{b}	161.86±20.91^{ab}
	Urea	694.03±172.93^{bcd}	216.02±13.23^{f}	174.52±9.55^{f}	1077.52±220.45^{a}	349.34±71.43^{def}	492.30±242.54^{f}
	Trimethylamine N-oxide	5.76±1.28^{cdefg}	2.56±0.98^{fg}	4.77±1.75^{efg}	5.32±0.58^{defg}	ND	ND
	2AB	16.29±11.75^{a}	ND	ND	ND	ND	ND
	Uracil	486.51±203.35^{abc}	159.81±34.34^{bc}	319.95±30.46^{abc}	778.98±50.02^{abc}	248.74±23.33^{abc}	530.23±90.35^{abc}
	Allantoin	538.62±40.02^{bc}	458.13±15.20^{c}	465.92±32.21^{c}	937.74±59.35^{abc}	1061.51±102.63^{abc}	1586.40±703.91^{abc}
	Indole-3-acetaldehyde	294.04±108.54^{c}	348.76±2.21^{c}	371.12±2.62^{abc}	696.81±41.45^{a}	658.87±162.52^{ab}	548.47±73.15^{abc}
	Glutathione(ox)	28.87±3.09^{ab}	10.07±0.97^{d}	8.04±0.93^{de}	32.41±0.68^{a}	25.58±0.78^{bc}	28.28±7.04^{ab}
	Riboflavin	ND	8.09±1.84^{cd}	22.51±12.78^{bcd}	13.11±0.16^{cd}	17.45±1.15^{cd}	ND
	G6P	ND	ND	ND	ND	ND	ND
	UMP	67.59±7.09^{bcd}	76.00±11.37^{bcd}	69.39±19.39^{bcd}	53.08±13.19^{bcd}	50.66±28.03^{cd}	53.91±1.69^{cd}
	AMP	20.83±1.74^{bcd}	27.30±8.34^{cd}	23.20±1.58^{bcd}	22.16±0.64^{cd}	22.94±2.53^{cd}	27.99±6.17^{bcd}
	ADP	ND	ND	ND	ND	ND	ND

The values indicate as mean ±SD (nmolg^{-1}) of dry weight (n=3). ND, not detected. Different character indicates the significant differences (P<0.05) between each type of sample by Tukey-HSD analysis. Statistical analysis were compared between all data set (species and treatments (Table S2.10-S2.12)).

Table S2.12: Metabolites concentrations in Wakame samples after various drying and extractions methods

		WAKAME					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Sugars	UDP-glucose	ND	ND	ND	50.61±0.47^{a}	4.24±2.95^{b}	ND
	Fructose	68.72±4.69^{e}	55.23±10.00^{e}	82.83±8.66^{e}	87.25±15.90^{e}	91.59±20.19^{e}	89.98±26.98^{e}
	Glucose	20.54±7.80^{bcd}	16.38±3.44^{cd}	26.32±18.89^{cd}	27.39±2.86^{bcd}	26.29±4.09^{cd}	21.47±11.69^{bcd}
	Inositol	41.88±2.36^{d}	22.20±4.34^{efg}	17.55±3.83^{fgh}	44.16±2.20^{d}	38.88±4.25^{de}	30.33±7.37^{def}
	Trehalose	ND	ND	ND	50.61±0.47^{c}	4.24±2.95^{cd}	ND
Organic acids	5-Methoxyindoleacetate	97.42±5.40^{a}	80.24±14.79^{a}	68.48±5.07^{a}	140.29±4.77^{a}	160.95±25.33^{a}	137.88±11.97^{a}
	Glycolate	ND	ND	ND	ND	ND	ND
	Lactate	5016.21±235.33^{bc}	1353.56±300.26^{d}	2157.65±1287.71^{d}	4710.01±263.19^{bc}	898.70±138.97^{d}	676.82±30.78^{d}
	Pentanoate	ND	ND	ND	ND	ND	ND
	3-Methylbutanoate	ND	ND	ND	ND	ND	ND
	Malonate	111.98±7.46^{ef}	103.31±30.19^{efg}	139.12±19.79^{e}	94.34±5.66^{ef}	93.59±15.74^{ef}	128.44±12.83^{ef}
	3-Hydroxybutyrate	ND	ND	ND	ND	ND	ND
	Methyl sulfate	28.80±13.17^{cd}	ND	ND	ND	ND	ND
	Fumarate	43.66±12.29^{de}	55.39±10.71^{de}	40.48±1.31^{e}	30.66±4.49^{e}	34.26±5.22^{e}	26.35±6.61^{e}
	Hexanoate	ND	ND	ND	ND	ND	ND
	Succinate	264.00±20.15^{b}	203.37±7.72^{cdefg}	212.86±29.19^{bcd}	151.89±8.80^{cdefg}	218.59±23.06^{bcd}	114.40±5.12^{defg}
	2-Hydroxypentanoate	ND	ND	188.17±11.96^{abc}	70.11±1.52^{de}	185.77±41.39^{abc}	104.05±15.45^{cd}
	Isethionate	ND	ND	ND	ND	ND	ND
	Citraconate	ND	ND	ND	ND	ND	ND
	4-Methyl-2-oxopentanoate	ND	ND	ND	ND	ND	ND
	4-Oxohexanoate	ND	ND	ND	ND	ND	ND
	Glutarate	ND	ND	ND	ND	ND	ND
	2-Hydroxy-4-methylpentanoate	ND	ND	ND	ND	ND	ND
	6-Hydroxyhexanoate	ND	ND	ND	ND	ND	ND
	Malate	375.79±14.05^{a}	198.50±65.94^{bcdef}	204.06±35.21^{bcdef}	258.75±10.18^{bcd}	243.74±45.17^{bcd}	185.59±7.37^{cdef}
	Threonate	292.91±82.04^{e}	257.84±67.12^{e}	207.82±30.58^{e}	179.00±12.57^{e}	263.90±119.85^{e}	211.51±41.14^{e}
	Adipate	30.30±1.39^{defg}	16.15±9.26^{fg}	55.04±18.94^{bcde}	33.87±5.74^{cdefg}	39.17±12.58^{cdefg}	24.12±6.77^{defg}
	Citramalate	26.98±4.62^{d}	22.99±8.93^{de}	25.05±0.39^{de}	ND	ND	ND
	2-Hydroxyglutarate	23.22±4.07^{bcd}	18.83±8.35^{bcd}	22.53±3.03^{d}	ND	ND	ND
	Orotate	ND	ND	ND	ND	ND	ND
	Pelargonate	ND	ND	ND	ND	ND	ND
	2-Hydroxyoctanoate	ND	ND	ND	ND	ND	ND
	Phthalate	11.45±1.60^{ab}	9.23±4.21^{ab}	14.44±1.48^{ab}	7.35±1.35^{ab}	9.82±2.28^{ab}	ND
	3-Phenyllactate	ND	ND	ND	ND	ND	ND
	Glycerophosphate	318.44±18.64^{a}	175.19±27.69^{bcd}	195.15±95.74^{bc}	186.29±11.75^{bcd}	233.92±37.01^{ab}	136.18±7.23^{cde}
	Decanoate	ND	ND	ND	ND	ND	ND
	trans-Aconitate	12.33±7.20^{cd}	ND	ND	20.25±13.07^{c}	ND	ND
	cis-Aconitate	5.99±1.38^{f}	ND	ND	5.80±3.01^{f}	ND	ND
	2-Isopropylmalate	ND	ND	ND	ND	ND	ND
	2PG	3.78±1.73^{b}	8.46±0.87^{b}	ND	6.79±2.35^{b}	7.48±4.61^{b}	8.78±0.66^{b}
	3PG	31.29±2.45^{ab}	14.41±4.47^{de}	ND	39.51±5.34^{a}	23.91±6.95^{bc}	17.89±2.63^{cde}
	Azelate	31.75±16.45^{defg}	16.32±3.49^{fg}	17.68±2.08^{fg}	38.42±18.47^{cdefg}	ND^{g}	24.91±4.62^{efg}
	N-Acetylglutamate	ND	ND	ND	ND	ND	ND
	Isocitrate	16.33±2.19^{cdef}	16.26±7.93^{cdefg}	18.58±5.94^{cdef}	22.73±1.59^{cde}	31.83±5.08^{c}	19.73±3.35^{cdef}
	Citrate	1319.65±119.95^{a}	391.21±175.50^{d}	222.32±48.37^{e}	1070.79±35.73^{b}	631.67±151.94^{c}	617.59±58.63^{c}
	Gluconate	ND	ND	ND	ND	ND	ND
	Syringate	245.35±65.72^{abc}	327.99±59.11^{abc}	462.68±75.23^{ab}	120.03±27.36^{c}	243.95±7.46^{bc}	272.71±90.62^{abc}
	Dodecanoate	ND	259.26±66.36^{bcdef}	422.69±127.27^{abc}	92.93±49.82^{f}	153.11±16.98^{def}	149.40±30.64^{cdef}
	Saccharate	34.05±4.82^{ef}	41.27±8.13^{f}	ND	66.68±3.21^{ef}	64.90±15.23^{ef}	37.28±4.20^{ef}
	Mucate	26.30±3.70^{d}	27.97±11.93^{d}	ND	55.94±6.83^{d}	53.65±15.59^{d}	33.62±4.62^{d}
	Dodecanedioate	ND	ND	ND	ND	ND	ND

		WAKAME					
		MeOH: $CHCl_3$: H_2O			MeOH: H_2O		
		Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC	Freeze-Dry	Oven-Dry 40 ºC	Oven-Dry 80 ºC
Others	Isobutylamine	44.90±6.59^{c}	ND	6.28±2.38^{de}	29.45±4.38^{cd}	15.98±3.65^{de}	11.96±7.88^{de}
	Putrescine	0.37±0.12^{c}	4.12±0.00^{bc}	0.86±0.95^{c}	1.94±0.74^{c}	1.19±0.96^{c}	1.36±0.62^{bc}
	Cyclohexylamine	9.25±3.43^{bc}	ND	ND	5.38±1.03^{cd}	ND	ND
	Choline	286.45±23.28^{a}	204.35±16.95^{b}	ND	267.93±11.42^{a}	ND	ND
	Diethanolamine	204.10±16.48^{ab}	99.01±50.51^{a}	109.70±57.37^{a}	677.24±71.91^{ab}	124.94±53.56^{b}	177.14±110.27^{ab}
	Cytosine	ND	ND	ND	ND	ND	ND
	Creatinine	ND	ND	ND	ND	ND	ND
	Homoserine	12.15±5.78^{a}	7.86±2.93^{ab}	3.04±0.42^{b}	8.00±1.61^{ab}	5.70±2.15^{ab}	4.98±0.74^{ab}
	Hydroxyproline	ND	ND	ND	ND	ND	ND
	Creatine	7.86±5.05^{c}	6.64±1.07^{c}	ND	5.29±0.98^{c}	ND	ND
	Adenine	ND	ND	ND	ND	ND	ND
	Hypoxanthine	ND	ND	ND	ND	ND	ND
	Proline betaine	4.73±3.31^{b}	ND	ND	ND	ND	ND
	Nornicotine	16.76±2.67^{bcd}	11.48±2.54^{de}	10.76±0.40^{de}	18.18±3.74^{bcd}	19.30±1.51^{bcd}	30.32±14.15^{ab}
	Guanine	ND	ND	ND	ND	ND	ND
	Carnitine	19.52±2.73^{a}	13.65±1.43^{c}	ND	17.66±2.73^{ab}	ND	ND
	Taurocyamine	298.91±28.16^{de}	311.72±29.78^{de}	381.48±14.08^{cdef}	631.70±78.59^{abcde}	788.63±39.03^{abc}	873.11±44.08^{ab}
	N-Acetylglucosamine	25.75±0.97^{fg}	16.57±3.61^{g}	25.17±1.91^{fg}	50.45±8.87^{cdefg}	55.49±1.04^{bcdef}	54.03±14.71^{cdef}
	2'-Deoxycytidine	ND	ND	ND	ND	ND	ND
	Thymidine	129.84±13.79^{a}	134.64±23.11^{a}	130.09±15.42^{a}	214.16±34.58^{a}	262.27±46.49^{a}	310.50±47.23^{a}
	Cytidine	ND	ND	ND	ND	ND	ND
	Uridine	79.00±30.99^{def}	41.45±5.76^{f}	55.76±11.33^{ef}	143.15±40.77^{abcd}	98.27±20.83^{bcdef}	106.43±2.79^{abcdef}
	Glycerophosphorylcholine	15.74±2.37^{c}	5.99±4.78^{c}	ND	8.30±4.31^{c}	ND	ND
	Adenosine	7.99±2.24^{d}	5.34±0.80^{d}	ND	7.01±0.28^{d}	ND	ND
	Guanosine	ND	ND	ND	ND	ND	ND
	5-Oxoproline	294.97±46.53^{a}	237.73±37.33^{ab}	397.68±137.56^{ab}	188.11±53.81^{ab}	147.91±29.53^{ab}	150.46±21.64^{ab}
	Urea	602.45±73.65^{cdef}	179.23±23.51^{f}	371.23±220.14^{def}	854.60±125.58^{abc}	356.62±17.54^{def}	295.55±54.19^{ef}
	Trimethylamine N-oxide	13.79±5.61^{bc}	8.76±1.44^{cdefg}	ND	10.45±1.04^{cde}	ND	ND
	2AB	18.02±6.46^{a}	13.76±0.27^{a}	8.66±0.24^{ab}	17.82±1.22^{a}	17.27±2.22^{a}	14.32±0.99^{a}
	Uracil	194.23±15.33^{bc}	75.78±7.78^{c}	74.43±16.13^{c}	353.41±32.16^{abc}	268.79±30.41^{abc}	153.72±16.16^{bc}
	Allantoin	501.77±13.18^{bc}	506.68±24.38^{bc}	820.26±0.50^{bc}	918.35±68.46^{abc}	948.82±28.22^{abc}	1079.41±76.12^{abc}
	Indole-3-acetaldehyde	198.19±35.30^{c}	207.08±15.28^{c}	255.38±20.97^{c}	364.08±16.81^{abc}	435.43±6.64^{abc}	446.54±25.69^{abc}
	Glutathione(ox)	11.48±2.42^{d}	2.58±0.77^{ef}	2.44±0.58^{ef}	19.10±1.40^{c}	8.89±0.88^{de}	7.82±1.76^{de}
	Riboflavin	4.40±0.41^{d}	11.26±1.74^{cd}	ND	17.06±2.40^{cd}	ND	ND
	G6P	96.80±13.85^{a}	55.11±20.29^{bcd}	47.24±17.32^{de}	83.30±20.26^{ab}	66.88±7.96^{abc}	46.08±19.61^{cd}
	UMP	166.84±29.05^{a}	105.98±6.61^{abc}	105.47±45.58^{abc}	116.58±11.10^{ab}	161.86±26.56^{a}	81.92±11.05^{bc}
	AMP	85.90±5.98^{a}	49.76±1.90^{bcd}	35.90±13.25^{bcd}	50.42±3.61^{abc}	57.12±23.78^{ab}	40.01±11.79^{bc}
	ADP	ND	ND	ND	31.61^{a}	ND	ND

The values indicate as mean ±SD ($nmolg^{-1}$) of dry weight (n=3). ND, not detected. Different character indicates the significant differences (P<0.05) between each type of sample by Tukey-HSD analysis. Statistical analysis were compared between all data set (species and treatments (Table S2.10-S2.12)).

Table S3.1 Amino acids, sugars and major organic acids in 11 seaweed after methanol-water extraction method.

	Methanol-Water extraction methods										
	Brown algae				Red algae					Green algae	
Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Urea	196.64 ± 24.00^{b}	176.52 ± 29.42^{b}	153.40 ± 24.29^{b}	138.00 ± 57.33^{b}	287.26 ± 350.34^{ab}	160.41 ± 48.85^{b}	176.64 ± 38.72^{b}	203.72 ± 182.72^{b}	153.11 ± 36.53^{b}	139.65 ± 61.97^{b}	292.91 ± 363.23^{ab}
Isobutylamine	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	295.77 ± 132.90^{c}	ND^{d}	16.23 ± 6.00^{d}	738.88 ± 44.47^{a}	ND^{d}	ND^{d}
1,3-Diaminopropane	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Trimethylamine N-oxide	18.34 ± 5.17^{def}	47.96 ± 6.39^{bc}	19.44 ± 14.05^{def}	78.00 ± 10.13^{a}	6.39 ± 1.45^{f}	45.01 ± 12.35^{bc}	6.68 ± 6.01^{f}	21.14 ± 4.96^{def}	12.58 ± 2.93^{ef}	30.24 ± 27.54^{cde}	12.82 ± 2.69^{ef}
Isopropanolamine	ND^{d}	ND^{d}	ND^{d}	3.44 ± 0.82^{d}	ND^{d}	16.07 ± 1.78^{b}	29.76 ± 7.15^{a}	10.71 ± 2.07^{c}	2.81 ± 0.33^{d}	ND^{d}	ND^{d}
Isoamylamine	7.04 ± 1.92^{e}	7.72 ± 1.90^{e}	13.59 ± 6.16^{e}	5.72 ± 1.41^{e}	2.54 ± 0.26^{e}	485.86 ± 348.45^{c}	118.65 ± 49.36^{de}	885.69 ± 122.11^{b}	1190.76 ± 147.81^{a}	7.11 ± 2.65^{e}	8.38 ± 1.50^{e}
Putrescine	2.61 ± 0.41^{bcde}	1.65 ± 0.32^{cde}	ND^{e}	ND^{e}	ND^{e}	1.59 ± 0.68^{cde}	2.56 ± 0.79^{bcde}	10.88 ± 6.57^{a}	ND^{e}	4.27 ± 1.59^{bcd}	2.17 ± 0.61^{cde}
beta-Ala	110.56 ± 3.94^{bcd}	26.68 ± 1.68^{d}	24.57 ± 11.30^{d}	11.99 ± 1.87^{d}	2288.17 ± 314.59^{a}	32.02 ± 6.92^{d}	28.81 ± 10.13^{d}	224.05 ± 10.87^{bc}	11.07 ± 1.07^{d}	49.72 ± 6.62^{bcd}	33.87 ± 5.81^{cd}
1-Aminocyclopropane-1-carboxylate	2.39 ± 1.36^{a}	ND^{c}	ND^{c}	ND^{c}	0.40 ± 0.89^{bc}	1.53 ± 1.48^{ab}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Hexylamine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.76 ± 0.49^{b}	ND^{c}	0.97 ± 1.33^{b}	ND^{c}	ND^{c}	ND^{c}
Cadaverine	ND^{c}	0.56 ± 1.26^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	89.73 ± 13.57^{b}	ND^{c}	ND^{c}	4.38 ± 1.24^{c}
GABA	1091.65 ± 62.57^{b}	32.81 ± 16.71^{cd}	14.22 ± 2.47^{d}	7.50 ± 0.91^{d}	504.31 ± 79.25^{c}	16.45 ± 2.88^{d}	20.79 ± 21.48^{cd}	3009.88 ± 632.80^{a}	13.04 ± 5.10^{d}	9.28 ± 3.55^{d}	19.66 ± 2.29^{cd}
3-Aminoisobutyrate	ND^{f}	8.73 ± 1.00^{a}	4.47 ± 1.47^{c}	1.02 ± 0.95^{ef}	ND^{f}	2.45 ± 0.47^{de}	ND^{f}	ND^{f}	ND^{f}	0.45 ± 1.02^{f}	7.75 ± 0.85^{ab}
2AB	193.43 ± 9.78^{b}	61.97 ± 7.19^{c}	12.74 ± 1.54^{e}	10.46 ± 0.85^{e}	435.33 ± 76.31^{a}	10.49 ± 3.34^{e}	7.32 ± 0.99^{e}	15.17 ± 2.15^{de}	3.72 ± 0.41^{e}	2.89 ± 0.32^{e}	4.03 ± 1.27^{e}
N-Methylalanine	ND^{e}	18.11 ± 1.62^{a}	9.20 ± 1.70^{b}	4.00 ± 3.27^{d}	1.88 ± 1.99^{de}	3.96 ± 1.14^{d}	3.33 ± 2.23^{d}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
N,N-Dimethylglycine	ND^{c}	5.74 ± 4.25^{bc}	4.16 ± 3.96^{c}	ND^{c}	ND^{c}	0.57 ± 1.28^{c}	ND^{c}	3.52 ± 3.36^{c}	ND^{c}	16.55 ± 12.19^{a}	0.78 ± 1.74^{c}
Choline	186.46 ± 15.77^{f}	487.96 ± 96.41^{cde}	652.93 ± 169.38^{bc}	154.73 ± 43.09^{f}	1485.67 ± 188.19^{a}	905.17 ± 109.60^{b}	576.06 ± 181.88^{c}	569.95 ± 112.65^{cd}	550.63 ± 38.48^{cd}	23.20 ± 5.66^{f}	210.94 ± 17.92^{ef}
Diethanolamine	63.90 ± 26.83^{ab}	173.44 ± 153.34^{a}	92.05 ± 27.69^{ab}	125.96 ± 43.71^{ab}	70.76 ± 20.70^{ab}	97.52 ± 59.42^{ab}	120.67 ± 58.70^{ab}	121.50 ± 93.17^{ab}	100.53 ± 44.48^{ab}	137.77 ± 102.55^{ab}	103.68 ± 21.27^{ab}
Hypotaurine	8.37 ± 11.54^{bc}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	6.16 ± 13.78^{bc}	ND^{c}	ND^{c}	ND^{c}	76.10 ± 14.88^{a}	16.66 ± 9.65^{bc}
Cytosine	6.70 ± 2.36^{a}	ND^{e}	ND^{e}	3.58 ± 2.09^{bc}	6.62 ± 1.27^{a}	ND^{e}	ND^{e}	0.66 ± 1.47^{de}	ND^{e}	2.98 ± 1.75^{bcd}	0.68 ± 1.53^{de}
Histamine	0.43 ± 0.69^{ab}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	0.78 ± 1.12^{ab}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Uracil	ND^{c}	ND^{c}	ND^{c}	ND^{c}	74.09 ± 69.41^{ab}	ND^{c}	ND^{c}	21.40 ± 47.85^{bc}	ND^{c}	19.56 ± 43.73^{bc}	ND^{c}
Betaine	128.00 ± 59.24^{b}	1721.40 ± 340.83^{b}	794.00 ± 132.57^{b}	52.60 ± 5.61^{b}	14.10 ± 3.18^{b}	276.20 ± 99.36^{b}	86.40 ± 25.44^{b}	338.40 ± 94.87^{b}	56.20 ± 10.29^{b}	660.10 ± 139.18^{b}	41013.50 ± 3314.01^{a}
2,4-Diaminobutyrate	ND^{c}	ND^{c}	0.45 ± 1.00^{c}	ND^{c}	ND^{c}	6.85 ± 1.40^{a}	ND^{c}	2.46 ± 1.41^{b}	ND^{c}	ND^{c}	ND^{c}

Metabolites	Methanol-Water extraction methods										
	Brown algae				Red algae					Green algae	
	Sargassum micracanthum (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Alpha-Methylserine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	11.86 ± 2.19^{ab}	16.52 ± 5.49^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Phenethylamine	114.81 ± 7.64^{bc}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	27.25 ± 13.09^{b}	506.08 ± 322.83^{a}	406.74 ± 86.48^{a}	16.23 ± 3.60^{b}	ND^{b}	ND^{b}
Nicotinamide	ND^{e}	ND^{e}	ND^{e}	ND^{e}	11.32 ± 1.59^{cd}	4.67 ± 0.97^{de}	ND^{e}	18.24 ± 7.15^{bc}	3.61 ± 0.71^{de}	41.31 ± 12.33^{a}	ND^{e}
Taurine	267.40 ± 21.20^{f}	286.60 ± 72.63^{f}	224.60 ± 92.38^{f}	ND^{f}	24007.40 ± 1662.03^{a}	490.60 ± 131.22^{f}	6673.80 ± 770.14^{c}	57.50 ± 6.66^{f}	2619.70 ± 253.75^{e}	119.80 ± 66.02^{f}	196.90 ± 22.02^{f}
5-Methylcytosine	ND^{c}	ND^{c}	ND^{c}	4.88 ± 0.77^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Thymine	ND^{e}	76.93 ± 12.84^{e}	ND^{e}	ND^{e}	655.37 ± 81.91^{cd}	483.10 ± 30.25^{cde}	733.66 ± 70.36^{c}	330.17 ± 19.37^{cde}	169.69 ± 15.37^{de}	2074.82 ± 375.88^{b}	7447.73 ± 702.95^{a}
Pipecolate	2.87 ± 0.36^{c}	16.92 ± 2.03^{c}	14.67 ± 2.59^{c}	24.68 ± 11.09^{c}	111.30 ± 16.86^{c}	55.53 ± 4.38^{c}	120.91 ± 12.50^{c}	21.04 ± 1.45^{c}	6.53 ± 0.92^{c}	1763.29 ± 315.51^{a}	66.61 ± 9.97^{c}
N-Acetylputrescine	12.44 ± 0.25^{b}	0.21 ± 0.46^{e}	ND^{e}	0.65 ± 0.92^{de}	2.90 ± 0.73^{c}	0.92 ± 0.96^{de}	ND^{e}	ND^{e}	1.60 ± 0.30^{d}	0.24 ± 0.54^{e}	ND^{e}
Agmatine	0.55 ± 1.23^{d}	ND^{d}	3.60 ± 3.37^{cd}	0.17 ± 0.37^{d}	ND^{d}	0.19 ± 0.42^{d}	ND^{d}	61.39 ± 9.91^{b}	0.64 ± 1.44^{d}	1.00 ± 0.96^{cd}	6.08 ± 3.58^{cd}
Hydroxyproline	2.84 ± 1.82^{de}	5.85 ± 3.31^{cd}	ND^{e}	0.18 ± 0.40^{e}	ND^{e}	3.32 ± 0.60^{de}	2.01 ± 1.86^{de}	9.80 ± 0.64^{bc}	ND^{e}	14.41 ± 5.15^{b}	51.22 ± 1.63^{a}
5-Aminolevulinate	0.31 ± 0.69^{cd}	ND^{d}	ND^{d}	3.00 ± 2.89^{a}	ND^{d}	0.46 ± 1.02^{cd}	0.88 ± 1.27^{bcd}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Creatine	ND^{d}	2.80 ± 1.77^{abcd}	2.18 ± 2.92^{bcd}	ND^{d}	ND^{d}	4.42 ± 5.02^{abcd}	2.87 ± 3.54^{abcd}	7.22 ± 1.99^{ab}	1.45 ± 0.38^{cd}	7.91 ± 1.36^{a}	0.96 ± 0.90^{cd}
Gly-Gly	ND^{c}	ND^{c}	ND^{c}	ND^{c}	22.41 ± 2.53^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	7.43 ± 6.93^{b}	ND^{c}
Ornithine	138.17 ± 17.60^{bc}	116.97 ± 26.53^{bcd}	50.53 ± 18.06^{efg}	20.65 ± 9.37^{fg}	169.33 ± 23.99^{b}	83.93 ± 27.17^{cdef}	37.30 ± 4.95^{efg}	392.07 ± 81.87^{a}	25.36 ± 2.07^{fg}	12.28 ± 6.33^{g}	19.53 ± 1.96^{g}
Adenine	22.94 ± 2.29^{c}	6.43 ± 2.54^{c}	6.50 ± 1.77^{c}	6.50 ± 3.25^{c}	8.50 ± 2.16^{c}	6.62 ± 1.84^{c}	0.77 ± 0.29^{c}	1.68 ± 0.45^{c}	44.51 ± 8.26^{c}	950.65 ± 104.96^{a}	56.13 ± 6.72^{c}
Hypoxanthine	18.23 ± 3.46^{abcde}	29.78 ± 18.68^{ab}	8.62 ± 8.48^{cdef}	1.02 ± 2.28^{ef}	20.43 ± 1.26^{abcd}	16.62 ± 6.18^{abcdef}	ND^{f}	17.80 ± 3.60^{abcdef}	12.75 ± 7.76^{bcdef}	14.24 ± 15.65^{bcdef}	33.47 ± 4.24^{a}
Tyramine	1200.51 ± 136.83^{a}	0.34 ± 0.76^{c}	2.30 ± 1.49^{c}	ND^{c}	3.01 ± 0.30^{c}	115.07 ± 47.01^{b}	62.19 ± 18.44^{bc}	7.82 ± 4.53^{c}	78.80 ± 16.34^{bc}	ND^{c}	ND^{c}
Urocanate	ND^{b}	ND^{b}	ND^{b}	1.87 ± 4.18^{b}	3.62 ± 5.54^{b}	ND^{b}	ND^{b}	3.07 ± 4.22^{b}	23.70 ± 2.28^{a}	ND^{b}	20.75 ± 3.72^{a}
Ectoine	ND^{b}	29.09 ± 13.45^{b}	96.52 ± 50.99^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	9.48 ± 1.58^{b}	ND^{b}
Proline betaine	57.10 ± 14.28^{c}	17.96 ± 2.81^{c}	21.00 ± 6.01^{c}	1.56 ± 0.34^{c}	0.53 ± 0.24^{c}	29.22 ± 5.26^{c}	3.03 ± 2.44^{c}	5498.71 ± 684.93^{a}	33.94 ± 15.45^{c}	119.25 ± 11.91^{c}	1001.20 ± 76.26^{b}
gamma-Guanidinobutyrate	3.66 ± 0.68^{cde}	ND^{f}	13.47 ± 4.82^{a}	0.90 ± 0.56^{ef}	ND^{f}	4.47 ± 2.98^{cd}	5.41 ± 1.21^{c}	0.84 ± 1.24^{ef}	2.02 ± 1.18^{cdef}	ND^{f}	0.74 ± 1.04^{ef}
gamma-Butyrobetaine	44.40 ± 15.75^{c}	791.70 ± 46.77^{c}	29.00 ± 8.91^{c}	1.80 ± 2.74^{c}	23408.90 ± 2123.73^{a}	11.50 ± 2.23^{c}	5.60 ± 1.98^{c}	59.40 ± 10.68^{c}	9.10 ± 1.91^{c}	73.00 ± 7.37^{c}	6.80 ± 1.13^{c}
O-Acetylserine	31.05 ± 2.65^{c}	57.93 ± 3.04^{b}	25.19 ± 7.46^{cd}	90.33 ± 27.53^{a}	3.24 ± 4.45^{e}	ND^{e}	0.83 ± 1.85^{e}	6.03 ± 6.52^{e}	2.15 ± 3.74^{e}	ND^{e}	ND^{e}
Guanine	11.88 ± 1.85^{c}	11.21 ± 1.16^{c}	6.55 ± 0.65^{c}	43.13 ± 4.90^{c}	ND^{c}	6.69 ± 1.70^{c}	ND^{c}	9.35 ± 6.67^{c}	13.35 ± 3.60^{c}	343.72 ± 91.50^{a}	7.54 ± 1.58^{c}
Dopamine	8.76 ± 1.06^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	5.68 ± 4.14^{b}	ND^{c}	ND^{c}	ND^{c}
Ala-Ala	7.22 ± 1.76^{c}	11.33 ± 2.61^{bc}	0.85 ± 1.91^{c}	ND^{c}	ND^{c}	ND^{c}	64.53 ± 61.86^{a}	ND^{c}	ND^{c}	81.92 ± 6.83^{a}	ND^{c}

Metabolites	Methanol-Water extraction methods										
	Brown algae				Red algae					Green algae	
	Sargassum micracanthum (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Tryptamine	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	15.19 ± 11.07^{b}	1.15 ± 2.57^{d}	ND^{d}	45.54 ± 13.43^{a}	ND^{d}	ND^{d}
alpha-Aminoadipate	ND^{c}	16.73 ± 4.84^{a}	6.82 ± 1.89^{bc}	ND^{c}	0.72 ± 1.61^{c}	15.46 ± 4.70^{a}	0.61 ± 1.35^{c}	14.40 ± 9.16^{ab}	0.42 ± 0.93^{c}	0.53 ± 1.18^{c}	3.23 ± 2.07^{c}
N-Methylglutamate	ND^{b}	ND^{b}	ND^{b}	23.29 ± 16.87^{a}	ND^{b}	3.86 ± 2.40^{b}	ND^{b}	ND^{b}	1.53 ± 3.43^{b}	ND^{b}	ND^{b}
Carnitine	56.41 ± 3.04^{efg}	1039.58 ± 73.36^{a}	156.84 ± 15.63^{c}	1.38 ± 0.86^{h}	1.50 ± 0.09^{h}	41.44 ± 10.53^{fgh}	8.85 ± 3.77^{gh}	24.91 ± 1.80^{fgh}	4.02 ± 0.92^{gh}	97.10 ± 7.35^{de}	6.01 ± 1.26^{gh}
Nicotine	3.04 ± 0.43^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Methionine sulfoxide	96.42 ± 14.89^{ab}	112.00 ± 15.26^{a}	56.35 ± 15.06^{cde}	1.55 ± 3.46^{i}	12.49 ± 7.10^{hi}	ND^{i}	10.99 ± 6.42^{hi}	32.10 ± 18.27^{efgh}	44.82 ± 5.36^{def}	76.84 ± 14.08^{bc}	47.59 ± 6.69^{de}
Taurocyamine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	10.23 ± 22.87^{bc}	124.81 ± 34.68^{a}	28.89 ± 39.59^{b}	ND^{c}
Pyridoxal	0.29 ± 0.65^{c}	ND^{c}	ND^{c}	ND^{c}	0.70 ± 1.57^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	9.56 ± 0.75^{a}	ND^{c}
Pyridoxamine	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	1.10 ± 1.02^{bc}	ND^{e}
3-Methylhistidine	5.16 ± 1.01^{c}	4.43 ± 0.92^{cd}	3.77 ± 0.95^{cde}	ND^{i}	3.73 ± 0.52^{cde}	12.15 ± 1.72^{a}	2.43 ± 1.40^{efg}	ND^{i}	0.40 ± 0.90^{hi}	1.01 ± 1.47^{fghi}	ND^{i}
N-Acetylornithine	5.14 ± 4.86^{c}	3.88 ± 0.94^{c}	0.22 ± 0.50^{c}	ND^{c}	46.83 ± 2.66^{b}	ND^{c}	ND^{c}	2.37 ± 2.32^{c}	ND^{c}	83.91 ± 9.56^{a}	4.32 ± 0.66^{c}
Citrulline	79.80 ± 6.99^{e}	53.50 ± 9.64^{e}	26.70 ± 6.66^{e}	5.40 ± 0.65^{e}	13569.90 ± 791.24^{a}	63.30 ± 23.46^{e}	1988.40 ± 675.72^{c}	10875.60 ± 1088.47^{b}	858.90 ± 94.42^{de}	15.80 ± 3.21^{e}	15.40 ± 1.17^{e}
Phosphorylcholine	ND^{c}	195.01 ± 36.87^{b}	53.39 ± 17.43^{c}	332.73 ± 91.06^{a}	1.67 ± 3.74^{c}	ND^{c}	345.30 ± 121.95^{a}	22.21 ± 3.90^{c}	5.36 ± 5.02^{c}	ND^{c}	ND^{c}
N-alpha,N-alpha-Dimethylhistidine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	14.24 ± 2.78^{a}	ND^{c}	0.45 ± 0.64^{c}	3.50 ± 0.95^{b}	0.27 ± 0.61^{c}	ND^{c}	ND^{c}
Gly-Leu	65.89 ± 6.01^{c}	10.13 ± 1.33^{d}	3.86 ± 0.71^{d}	1.58 ± 0.79^{d}	178.00 ± 36.69^{ab}	4.89 ± 2.00^{d}	0.71 ± 0.28^{d}	4.41 ± 0.91^{d}	0.88 ± 0.50^{d}	194.73 ± 20.95^{a}	16.74 ± 1.24^{d}
N-epsilon-Acetyllysine	3.33 ± 0.28^{fg}	6.69 ± 0.95^{c}	4.41 ± 1.29^{def}	ND^{h}	13.24 ± 1.59^{a}	3.01 ± 2.02^{fg}	ND^{h}	ND^{h}	ND^{h}	6.18 ± 1.47^{cd}	0.22 ± 0.50^{h}
N6,N6,N6-Trimethyllysine	7.42 ± 0.35^{b}	112.14 ± 30.83^{b}	3.70 ± 0.79^{b}	946.69 ± 251.38^{a}	33.47 ± 5.40^{b}	3.12 ± 0.97^{b}	31.35 ± 4.86^{b}	9.19 ± 1.11^{b}	117.24 ± 18.64^{b}	24.14 ± 3.87^{b}	59.03 ± 6.15^{b}
SAH	ND^{b}	ND^{b}	ND^{b}	0.38 ± 0.84^{b}	12.69 ± 3.24^{a}	0.49 ± 1.09^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	1.96 ± 1.14^{b}
DOPA	ND^{b}	1.34 ± 1.85^{b}	ND^{b}	ND^{b}	3.23 ± 1.87^{b}	ND^{b}	ND^{b}	453.31 ± 131.32^{a}	ND^{b}	ND^{b}	ND^{b}
SAM+	15.18 ± 4.09^{cd}	6.94 ± 1.80^{cdefgh}	4.81 ± 1.75^{efgh}	6.82 ± 1.27^{cdefgh}	63.87 ± 17.45^{a}	3.13 ± 0.42^{fgh}	ND^{h}	ND^{h}	11.02 ± 1.99^{cdefg}	6.92 ± 0.61^{cdefgh}	16.92 ± 1.74^{c}
ADMA	2.19 ± 3.03^{e}	ND^{e}	ND^{e}	3.30 ± 3.03^{de}	10.49 ± 1.61^{cd}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	71.72 ± 5.59^{b}
SDMA	4.06 ± 3.73bcd	ND^{e}	ND^{e}	9.93 ± 2.46^{a}	ND^{e}	ND^{e}	ND^{e}	4.24 ± 3.89^{bc}	0.89 ± 1.99^{cde}	ND^{e}	ND^{e}
o-Acetylcarnitine	33.03 ± 2.24^{cde}	258.41 ± 27.95^{a}	65.90 ± 15.67^{c}	ND^{e}	ND^{e}	26.78 ± 8.91^{de}	0.89 ± 0.83^{e}	ND^{e}	0.11 ± 0.25^{e}	0.19 ± 0.43^{e}	ND^{e}
Cysteine-glutathione disulphide	41.63 ± 3.32^{ab}	14.96 ± 7.12^{efghi}	6.17 ± 1.14^{hijk}	26.34 ± 2.84^{cdef}	30.15 ± 13.68^{bcd}	11.10 ± 4.83^{ghijk}	44.95 ± 13.11^{a}	5.74 ± 1.09^{ijk}	0.45 ± 1.01^{jk}	27.15 ± 4.74^{cde}	10.05 ± 2.23^{ghijk}
N-Acetylglucosamine	44.66 ± 3.22^{ab}	31.06 ± 9.23^{bcdef}	16.67 ± 15.77^{efghijk}	23.61 ± 13.31^{cdefghi}	ND^{k}	4.13 ± 9.23^{ijk}	30.24 ± 3.72^{bcdefg}	7.93 ± 17.74^{hijk}	10.73 ± 6.19^{ghijk}	58.70 ± 10.65^{a}	21.66 ± 4.31^{defghij}
Cystathionine	292.91 ± 26.33^{b}	805.00 ± 290.78^{a}	270.03 ± 79.02^{b}	103.22 ± 31.16^{cd}	11.13 ± 2.73^{d}	3.35 ± 4.64^{d}	1.97 ± 2.70^{d}	13.35 ± 0.64^{cd}	ND^{d}	0.81 ± 1.81^{d}	ND^{d}

Metabolites	Methanol-Water extraction methods										
	Brown algae				Red algae					Green algae	
	Sargassum micracanthum (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
gamma-Glu-2AB	ND^{b}	ND^{b}	ND^{b}	ND^{b}	1.32 ± 2.95^{ab}	3.42 ± 1.27^{a}	ND^{b}	0.19 ± 0.44^{b}	ND^{b}	ND^{b}	ND^{b}
7,8-Dihydrobiopterin	44.08 ± 5.11^{a}	ND^{e}	ND^{e}	1.19 ± 2.65^{e}	28.41 ± 5.57^{b}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
Thymidine	ND^{c}	70.73 ± 8.67^{bc}	31.98 ± 30.73^{bc}	ND^{c}	ND^{c}	ND^{c}	202.58 ± 118.11^{a}	ND^{c}	54.56 ± 32.62^{bc}	ND^{c}	ND^{c}
Cytidine	5.80 ± 0.55^{e}	11.69 ± 3.21^{e}	11.22 ± 3.30^{e}	1.84 ± 1.21^{e}	49.53 ± 5.18^{c}	13.32 ± 3.76^{de}	1.64 ± 1.66^{e}	8.96 ± 0.66^{e}	8.93 ± 2.02^{e}	197.58 ± 55.69^{a}	4.61 ± 0.45^{e}
Uridine	42.80 ± 11.57^{klm}	122.06 ± 18.61^{ghi}	98.03 ± 13.15^{hijk}	194.67 ± 28.15^{ef}	189.98 ± 11.83^{ef}	51.10 ± 11.48^{jklm}	114.46 ± 31.52^{ghij}	321.82 ± 32.42^{c}	166.86 ± 18.98^{efg}	574.56 ± 85.66^{a}	219.26 ± 24.46^{de}
Pyridoxamine 5'-phosphate	ND^{d}	ND^{d}	ND^{d}	ND^{d}	35.85 ± 2.36^{a}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Glycerophosphorylcholine	29.16 ± 4.69^{ij}	2158.40 ± 183.58^{d}	831.34 ± 368.14^{efghi}	719.06 ± 100.58^{fghi}	4075.28 ± 329.62^{b}	1159.80 ± 120.01^{ef}	398.91 ± 161.32^{ij}	624.91 ± 146.75^{ghi}	116.95 ± 9.85^{ij}	ND^{j}	31.07 ± 3.39^{ij}
Thiamine	1.62 ± 1.50^{defg}	1.41 ± 1.45^{defg}	0.68 ± 0.99^{efg}	1.42 ± 1.94^{defg}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	ND^{g}	8.67 ± 1.74^{ab}	5.88 ± 1.38^{c}
Adenosine	16.52 ± 1.39^{de}	19.41 ± 4.72^{d}	19.85 ± 4.13^{d}	8.61 ± 3.02^{defgh}	2.64 ± 0.36^{fgh}	12.39 ± 3.28^{defg}	3.21 ± 0.97^{fgh}	9.04 ± 1.27^{defgh}	89.86 ± 9.46^{b}	2.53 ± 0.56^{fgh}	112.24 ± 8.07^{a}
Inosine	3.94 ± 5.66^{d}	33.88 ± 5.38^{cd}	35.12 ± 8.00^{cd}	ND^{d}	34.16 ± 5.70^{cd}	52.31 ± 30.33^{c}	1.72 ± 3.84^{d}	275.45 ± 44.90^{a}	38.79 ± 2.54^{cd}	ND^{d}	118.61 ± 13.09^{b}
Glu-Glu	3.17 ± 4.43^{bc}	0.49 ± 1.10^{c}	ND^{c}	0.22 ± 0.50^{c}	0.32 ± 0.72^{c}	8.50 ± 2.99^{a}	ND^{c}	2.63 ± 0.36^{bc}	0.24 ± 0.54^{c}	4.27 ± 2.71^{b}	0.98 ± 1.37^{c}
1-Methyladenosine	ND^{b}	ND^{b}	ND^{b}	ND^{b}	0.25 ± 0.56^{b}	4.34 ± 1.52^{a}	0.12 ± 0.27^{b}	ND^{b}	ND^{b}	0.92 ± 0.56^{b}	ND^{b}
Guanosine	6.11 ± 0.31^{fg}	13.30 ± 2.59^{ef}	16.34 ± 2.63^{e}	ND^{g}	7.94 ± 1.25^{fg}	12.11 ± 2.81^{ef}	0.45 ± 0.66^{g}	64.84 ± 3.70^{a}	37.85 ± 4.20^{cd}	6.84 ± 1.46^{fg}	45.78 ± 4.59^{bc}
Ophthalmate	3.95 ± 0.47^{abc}	1.24 ± 1.39^{bc}	ND^{c}	0.15 ± 0.34^{c}	ND^{c}	0.18 ± 0.41^{c}	6.47 ± 5.99^{a}	1.09 ± 1.35^{bc}	ND^{c}	0.31 ± 0.69^{c}	ND^{c}
5-Methylthioadenosine	8.68 ± 1.82^{b}	4.32 ± 0.36^{bc}	ND^{c}	2.62 ± 0.57^{bc}	53.09 ± 14.04^{a}	1.58 ± 0.48^{bc}	ND^{c}	0.58 ± 0.80^{bc}	1.29 ± 0.19^{bc}	3.62 ± 0.67^{bc}	1.25 ± 0.82^{bc}
Glutathione(ox)	306.04 ± 18.02^{a}	31.09 ± 15.33^{fg}	17.41 ± 4.49^{fg}	147.22 ± 27.05^{cd}	9.89 ± 9.85^{fg}	34.81 ± 18.07^{fg}	190.29 ± 77.82^{bc}	107.88 ± 31.41^{de}	1.67 ± 1.63^{g}	39.68 ± 6.24^{fg}	247.87 ± 14.71^{ab}
Glutathione(red)	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	10.40 ± 6.57^{ab}	2.12 ± 2.97^{c}	3.17 ± 4.34^{bc}	ND^{c}	ND^{c}	ND^{c}
Phe-Phe	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	10.37 ± 0.76^{a}	ND^{c}
AICAR	1.80 ± 4.02^{c}	ND^{c}	ND^{c}	0.75 ± 1.67^{c}	9.72 ± 5.75^{c}	1092.89 ± 148.24^{b}	15.15 ± 6.15^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
S-Lactoylglutathione	0.60 ± 1.34^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	31.01 ± 7.23^{b}	41.56 ± 9.03^{a}	ND^{d}	ND^{d}	0.29 ± 0.64^{d}
5-Methyltetrahydrofolate	39.18 ± 6.63^{de}	92.12 ± 62.99^{de}	5.93 ± 8.13^{e}	5.91 ± 5.67^{e}	177.04 ± 27.04^{cd}	352.27 ± 163.00^{ab}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
Glycolate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	3622.86 ± 1168.28^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Isethionate	ND^{d}	1284.50 ± 178.50^{d}	222.30 ± 123.30^{d}	83.60 ± 24.90^{d}	29841.50 ± 8384.50^{bc}	33428.50 ± 3016.70^{b}	80676.50 ± 11830.80^{a}	ND^{d}	34461.40 ± 1697.40^{b}	359.20 ± 115.90^{d}	ND^{d}
5-Oxoproline	1077.25 ± 195.71^{e}	264.04 ± 77.05^{fg}	163.40 ± 53.32^{fg}	133.20 ± 48.00^{fg}	2463.25 ± 409.20^{d}	5470.99 ± 871.19^{a}	121.52 ± 45.98^{g}	379.25 ± 38.99^{efg}	321.88 ± 32.61^{fg}	94.37 ± 27.91^{g}	3497.00 ± 382.44^{c}
Threonate	1074.00 ± 270.10^{b}	580.00 ± 168.30^{b}	2476.00 ± 217.00^{b}	553.00 ± 84.30^{b}	678.00 ± 188.40^{b}	377.00 ± 101.60^{b}	ND^{b}	628.00 ± 197.00^{b}	1547.00 ± 468.50^{b}	240069.00 ± 23575.20^{a}	8418.00 ± 838.80^{b}
Citramalate	9.80 ± 21.90^{de}	32.69 ± 32.20^{cde}	39.69 ± 26.72^{cd}	ND^{e}	ND^{e}	97.88 ± 15.62^{a}	ND^{e}	ND^{e}	108.96 ± 23.11^{a}	ND^{e}	ND^{e}

Metabolites	Methanol-Water extraction methods										
	Brown algae				Red algae					Green algae	
	Sargassum micracanthum (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	*Dasya sessilis* (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
2-Hydroxyglutarate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	24.06 ± 53.80^{bc}	ND^{c}	121.28 ± 71.60^{a}	16.53 ± 36.96^{bc}	ND^{c}	80.40 ± 52.74^{ab}	ND^{c}
p-Hydroxyphenylacetate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	2432.44 ± 513.31^{a}	ND^{c}	ND^{c}	ND^{c}
Orotate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	11682.40 ± 916.38^{a}	ND^{b}
Urate	4008.28 ± 362.90^{a}	28.62 ± 64.00^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Cysteate	ND^{d}	11.60 ± 25.93^{d}	ND^{d}	ND^{d}	374.60 ± 81.43^{b}	502.19 ± 63.62^{a}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Glycerophosphate	59.45 ± 71.87^{d}	1604.76 ± 854.56^{c}	3141.69 ± 922.82^{a}	304.66 ± 76.64^{d}	2932.19 ± 599.80^{ab}	183.60 ± 34.58^{d}	340.75 ± 70.25^{d}	149.74 ± 97.75^{d}	413.28 ± 123.09^{d}	ND^{d}	716.43 ± 42.56^{d}
Allantoate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	1158.41 ± 585.07^{a}	ND^{b}	ND^{b}	ND^{b}	104.17 ± 142.75^{b}	ND^{b}
N-Acetylglutamate	68.46 ± 17.96^{a}	16.10 ± 23.68^{cde}	ND^{e}	ND^{e}	21.98 ± 32.25^{cde}	ND^{e}	ND^{e}	15.35 ± 21.05^{cde}	4.57 ± 10.22^{de}	24.07 ± 22.22^{cde}	7.19 ± 16.08^{de}
Mucate	185.99 ± 21.39^{ef}	95.05 ± 38.90^{fgh}	282.27 ± 65.38^{cde}	108.65 ± 25.88^{fgh}	11.51 ± 25.74^{gh}	96.91 ± 18.03^{fgh}	ND^{h}	ND^{h}	381.54 ± 85.79^{c}	116.21 ± 28.38^{fg}	1190.18 ± 130.58^{a}
Galacturonate 1-phosphate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	191.94 ± 36.37^{a}
Argininosuccinate	19.66 ± 43.97^{c}	ND^{c}	ND^{c}	ND^{c}	293.12 ± 154.07^{a}	ND^{c}	ND^{c}	15.58 ± 34.84^{c}	ND^{c}	ND^{c}	ND^{c}
CMP	65.23 ± 5.74^{c}	ND^{e}	5.97 ± 13.36^{e}	ND^{e}	127.50 ± 27.25^{a}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	78.25 ± 6.35^{bc}
UMP	224.50 ± 126.61^{cd}	66.52 ± 63.82^{fgh}	18.40 ± 41.13^{gh}	138.32 ± 32.92^{def}	612.25 ± 96.52^{a}	51.05 ± 69.91^{fgh}	94.84 ± 89.92^{efgh}	ND^{h}	ND^{h}	ND^{h}	268.69 ± 37.78^{c}
3'-AMP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	173.12 ± 59.93^{a}	ND^{b}	ND^{b}
AMP	793.83 ± 85.14^{a}	54.54 ± 51.29^{f}	39.87 ± 54.61^{f}	222.41 ± 54.26^{cd}	117.08 ± 177.43^{def}	12.71 ± 28.42^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	263.19 ± 34.15^{c}
IMP	254.71 ± 50.68^{bc}	71.92 ± 26.47^{de}	89.30 ± 37.34^{de}	ND^{e}	941.55 ± 232.62^{a}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	127.87 ± 35.29^{cde}
Prostaglandin E2	ND^{d}	5475.10 ± 3149.50^{b}	3019.30 ± 3040.49^{bc}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	16337.10 ± 4051.33^{a}	ND^{d}	ND^{d}	ND^{d}
Prostaglandin F2alpha	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	84.20 ± 8.52^{a}	ND^{c}	37.96 ± 52.83^{b}	ND^{c}	ND^{c}	ND^{c}
UDP	ND^{c}	ND^{c}	ND^{c}	ND^{c}	435.63 ± 167.84^{a}	ND^{c}	24.43 ± 54.62^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
ADP	ND^{b}	10.58 ± 23.66^{b}	47.48 ± 36.60^{ab}	47.99 ± 31.96^{ab}	86.81 ± 80.62^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	8.34 ± 18.64^{b}
IDP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	621.77 ± 209.00^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
GDP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	120.70 ± 71.88^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}

The values indicate as mean ±SD (nmol g^{-1}) of the metabolite concentration of dry weight (n= 5). ND, not detected. Different superscript letters indicate the significant differences (P<0.05) between each type of species by Tukey HSD analysis. Statistical analysis were compared between all data set (species and extraction methods (Table 3.2, S3.1)).

Table S3.2 Amino acids, sugars and major organic acids in 11 seaweed after methanol-chloroform-water extraction method.

Methanol-Chloroform-Water extraction methods											
	Brown algae				Red algae					Green algae	
Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	Dasya sessilis (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Urea	486.95 ± 33.93^{a}	104.43 ± 16.72^{b}	77.21 ± 5.86^{b}	54.10 ± 36.89^{b}	88.90 ± 22.69^{b}	75.40 ± 7.00^{b}	75.90 ± 7.97^{b}	72.15 ± 12.67^{b}	97.95 ± 20.93^{b}	64.36 ± 30.46^{b}	56.66 ± 19.68^{b}
Isobutylamine	ND^{d}	11.22 ± 2.36^{d}	27.57 ± 3.86^{d}	ND^{d}	ND^{d}	236.50 ± 78.59^{c}	ND^{d}	ND^{d}	620.49 ± 20.75^{b}	ND^{d}	ND^{d}
1,3-Diaminopropane	ND^{b}	ND^{b}	ND^{b}	ND^{b}	4.42 ± 2.74^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Trimethylamine N-oxide	16.49 ± 4.22^{def}	31.36 ± 3.51^{cde}	14.96 ± 12.39^{def}	65.98 ± 5.25^{ab}	4.94 ± 0.66^{f}	37.07 ± 8.79^{cd}	6.09 ± 5.94^{f}	17.91 ± 5.33^{def}	9.04 ± 2.80^{ef}	25.32 ± 19.99^{cdef}	10.15 ± 1.74^{ef}
Isopropanolamine	1.07 ± 1.07^{d}	0.39 ± 0.88^{d}	ND^{d}	3.24 ± 0.62^{d}	0.26 ± 0.57^{d}	12.80 ± 1.34^{bc}	25.69 ± 2.75^{a}	10.69 ± 3.93^{c}	1.59 ± 1.48^{d}	ND^{d}	ND^{d}
Isoamylamine	3.27 ± 1.46^{e}	4.43 ± 1.65^{e}	7.94 ± 2.39^{e}	4.82 ± 0.90^{e}	1.85 ± 0.07^{e}	352.58 ± 250.77^{cd}	112.39 ± 39.07^{de}	855.79 ± 114.51^{b}	964.28 ± 54.21^{ab}	5.95 ± 2.02^{e}	8.53 ± 4.44^{e}
Putrescine	3.99 ± 0.22^{bcde}	ND^{e}	0.12 ± 0.28^{e}	0.28 ± 0.62^{de}	0.12 ± 0.27^{e}	2.19 ± 0.79^{cde}	5.29 ± 0.66^{bc}	13.82 ± 3.98^{a}	ND^{e}	6.49 ± 1.28^{b}	3.96 ± 0.49^{bcde}
beta-Ala	102.94 ± 12.57^{bcd}	20.17 ± 2.01^{d}	20.23 ± 7.74^{d}	9.61 ± 2.02^{d}	2254.20 ± 211.11^{a}	27.01 ± 4.86^{d}	26.97 ± 8.40^{d}	237.20 ± 18.76^{b}	12.08 ± 0.94^{d}	42.76 ± 7.32^{cd}	32.87 ± 2.95^{d}
1-Aminocyclopropane-1-carboxylate	2.38 ± 0.43^{a}	ND^{c}	ND^{c}	ND^{c}	0.23 ± 0.51^{c}	1.80 ± 0.33^{a}	0.17 ± 0.39^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Hexylamine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.35 ± 0.33^{bc}	ND^{c}	1.77 ± 0.44^{a}	ND^{c}	ND^{c}	ND^{c}
Cadaverine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	179.22 ± 31.27^{a}	ND^{c}	0.35 ± 0.54^{c}	8.41 ± 1.42^{c}
GABA	1036.92 ± 72.82^{b}	24.50 ± 13.15^{cd}	10.36 ± 1.49^{d}	4.83 ± 0.84^{d}	486.57 ± 56.15^{cd}	13.09 ± 1.20^{d}	17.25 ± 18.08^{d}	3152.45 ± 722.45^{a}	11.88 ± 3.83^{d}	7.57 ± 3.17^{d}	17.24 ± 2.25^{d}
3-Aminoisobutyrate	0.87 ± 1.24^{ef}	6.74 ± 1.16^{b}	3.17 ± 0.68^{cd}	1.39 ± 0.46^{ef}	ND^{f}	1.59 ± 0.71^{def}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	8.01 ± 0.62^{ab}
2AB	187.70 ± 12.68^{b}	60.21 ± 7.43^{cd}	9.78 ± 0.90^{e}	9.09 ± 1.97^{e}	419.53 ± 46.50^{a}	7.30 ± 3.05^{e}	6.07 ± 1.25^{e}	15.91 ± 4.29^{de}	3.24 ± 0.64^{e}	3.00 ± 0.57^{e}	3.74 ± 0.84^{e}
N-Methylalanine	1.18 ± 1.08^{de}	15.39 ± 1.34^{a}	7.32 ± 0.92^{bc}	4.27 ± 3.25^{cd}	1.84 ± 0.38^{de}	3.38 ± 0.26^{d}	3.53 ± 0.99^{d}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
N,N-Dimethylglycine	ND^{c}	6.30 ± 1.24^{bc}	4.76 ± 0.59^{c}	ND^{c}	ND^{c}	0.56 ± 1.26^{c}	0.40 ± 0.90^{c}	1.01 ± 2.26^{c}	ND^{c}	13.50 ± 8.51^{ab}	0.45 ± 1.00^{c}
Choline	197.88 ± 19.02^{f}	546.76 ± 141.98^{cd}	717.76 ± 208.89^{bc}	170.68 ± 67.24^{f}	1554.97 ± 235.78^{a}	882.61 ± 106.15^{b}	632.58 ± 179.68^{bc}	677.73 ± 122.27^{bc}	641.91 ± 26.54^{bc}	23.07 ± 6.69^{f}	291.72 ± 45.15^{def}
Diethanolamine	136.10 ± 92.12^{ab}	54.84 ± 12.83^{ab}	36.26 ± 17.68^{b}	105.18 ± 34.69^{ab}	36.98 ± 6.76^{b}	46.49 ± 10.44^{ab}	64.82 ± 16.90^{ab}	61.76 ± 16.41^{ab}	65.51 ± 39.35^{ab}	65.99 ± 16.20^{ab}	89.53 ± 37.37^{ab}
Hypotaurine	12.80 ± 7.38^{bc}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	14.41 ± 18.33^{bc}	ND^{c}	ND^{c}	ND^{c}	69.35 ± 11.93^{a}	21.48 ± 4.32^{b}
Cytosine	5.49 ± 1.14^{ab}	ND^{e}	0.15 ± 0.34^{e}	3.19 ± 0.49^{bcd}	7.02 ± 1.40^{a}	ND^{e}	ND^{e}	1.15 ± 1.07^{cde}	0.92 ± 0.93^{de}	2.51 ± 1.48^{cde}	3.19 ± 0.83^{bcd}
Histamine	0.39 ± 0.68^{ab}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	8.64 ± 16.77^{a}	ND^{b}	0.61 ± 0.57^{ab}	ND^{b}	ND^{b}	ND^{b}
Uracil	67.24 ± 9.04^{ab}	ND^{c}	ND^{c}	ND^{c}	97.43 ± 12.43^{a}	ND^{c}	ND^{c}	43.09 ± 40.30^{abc}	ND^{c}	45.69 ± 42.47^{abc}	ND^{c}
Betaine	129.90 ± 61.46^{b}	1678.60 ± 330.71^{b}	758.10 ± 52.56^{b}	43.30 ± 14.74^{b}	10.30 ± 1.23^{b}	276.50 ± 143.16^{b}	71.70 ± 21.47^{b}	258.20 ± 17.96^{b}	45.60 ± 3.79^{b}	516.00 ± 107.95^{b}	38664.10 ± 3975.78^{a}
2,4-Diaminobutyrate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	6.65 ± 1.13^{a}	ND^{c}	2.09 ± 0.38^{b}	ND^{c}	ND^{c}	ND^{c}

Methanol-Chloroform-Water extraction methods											
	Brown algae				Red algae					Green algae	
Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	Dasya sessilis (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Alpha-Methylserine	0.69 ± 1.55^{c}	ND^{c}	ND^{c}	1.45 ± 1.20^{c}	10.83 ± 0.75^{b}	15.19 ± 7.04^{ab}	0.29 ± 0.65^{c}	0.26 ± 0.59^{c}	ND^{c}	0.28 ± 0.62^{c}	ND^{c}
Phenethylamine	99.27 ± 9.34^{b}	5.73 ± 1.39^{b}	3.32 ± 0.84^{b}	ND^{b}	ND^{b}	16.57 ± 6.51^{b}	469.03 ± 297.07^{a}	402.48 ± 86.22^{a}	11.15 ± 1.98^{b}	ND^{b}	ND^{b}
Nicotinamide	0.92 ± 0.84^{e}	0.20 ± 0.46^{e}	ND^{e}	ND^{e}	6.34 ± 0.54^{de}	2.79 ± 0.38^{e}	ND^{e}	11.64 ± 3.75^{cd}	0.18 ± 0.41^{e}	21.51 ± 6.78^{b}	ND^{e}
Taurine	138.70 ± 13.69^{f}	164.80 ± 40.96^{f}	105.00 ± 32.39^{f}	ND^{f}	10563.20 ± 654.85^{b}	235.10 ± 80.41^{f}	4282.50 ± 463.28^{d}	39.40 ± 3.49^{f}	1812.10 ± 172.05^{e}	98.60 ± 43.79^{f}	163.60 ± 21.06^{f}
5-Methylcytosine	ND^{c}	ND^{c}	ND^{c}	2.96 ± 0.36^{b}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Thymine	52.41 ± 15.73^{e}	78.57 ± 8.33^{e}	72.90 ± 4.29^{e}	ND^{e}	429.16 ± 42.43^{cde}	292.77 ± 37.48^{cde}	664.68 ± 93.95^{cd}	309.87 ± 11.18^{cde}	192.79 ± 20.60^{de}	1920.39 ± 244.82^{b}	7352.73 ± 646.51^{a}
Pipecolate	2.18 ± 0.40^{c}	12.49 ± 1.34^{c}	10.37 ± 1.15^{c}	22.58 ± 12.12^{c}	90.09 ± 8.49^{c}	44.79 ± 2.89^{c}	102.87 ± 10.57^{c}	18.33 ± 1.55^{c}	5.06 ± 0.38^{c}	1445.22 ± 252.84^{b}	51.41 ± 2.99^{c}
N-Acetylputrescine	13.92 ± 0.79^{a}	0.17 ± 0.23^{e}	0.08 ± 0.17^{e}	0.18 ± 0.41^{e}	3.27 ± 0.61^{c}	0.92 ± 0.19^{de}	ND^{e}	ND^{e}	1.47 ± 0.36^{d}	ND^{e}	ND^{e}
Agmatine	4.25 ± 0.40^{cd}	ND^{d}	2.21 ± 1.61^{cd}	ND^{d}	ND^{d}	0.63 ± 0.65^{d}	1.89 ± 2.61^{cd}	119.39 ± 17.54^{a}	3.45 ± 3.25^{cd}	1.97 ± 1.68^{cd}	11.71 ± 1.68^{c}
Hydroxyproline	2.58 ± 1.63^{de}	5.69 ± 3.40^{cd}	1.39 ± 1.27^{de}	ND^{e}	ND^{e}	3.76 ± 1.12^{de}	2.77 ± 0.65^{de}	10.21 ± 0.86^{bc}	0.92 ± 1.28^{de}	11.76 ± 4.05^{b}	56.22 ± 5.70^{a}
5-Aminolevulinate	0.23 ± 0.52^{cd}	ND^{d}	ND^{d}	2.36 ± 1.12^{ab}	ND^{d}	ND^{d}	1.92 ± 0.33^{abc}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Creatine	0.17 ± 0.39^{d}	1.84 ± 1.45^{cd}	1.84 ± 2.18^{cd}	ND^{d}	ND^{d}	5.14 ± 5.60^{abcd}	2.01 ± 2.91^{cd}	5.74 ± 1.01^{abc}	1.12 ± 0.13^{cd}	7.47 ± 1.07^{a}	1.21 ± 0.69^{cd}
Gly-Gly	ND^{c}	ND^{c}	ND^{c}	ND^{c}	19.64 ± 1.21^{a}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Ornithine	100.99 ± 13.71^{cde}	122.58 ± 9.16^{bcd}	ND^{g}	13.40 ± 2.87^{g}	133.49 ± 16.43^{bc}	60.64 ± 11.84^{defg}	27.80 ± 3.85^{fg}	373.69 ± 77.57^{a}	23.23 ± 1.31^{fg}	4.69 ± 7.74^{g}	20.72 ± 2.55^{fg}
Adenine	14.82 ± 2.63^{c}	3.27 ± 0.95^{c}	3.49 ± 0.44^{c}	3.95 ± 2.18^{c}	10.51 ± 1.68^{c}	3.66 ± 1.06^{c}	ND^{c}	ND^{c}	19.08 ± 4.16^{c}	561.51 ± 49.40^{b}	26.20 ± 2.80^{c}
Hypoxanthine	14.65 ± 1.67^{bcdef}	24.30 ± 9.79^{abc}	2.41 ± 5.39^{def}	1.32 ± 2.94^{ef}	13.21 ± 2.57^{bcdef}	11.49 ± 4.07^{cdef}	ND^{f}	9.69 ± 6.49^{cdef}	10.65 ± 6.18^{cdef}	11.40 ± 11.50^{cdef}	18.81 ± 10.76^{abcde}
Tyramine	1279.37 ± 133.35^{a}	1.46 ± 0.15^{c}	1.82 ± 0.50^{c}	1.57 ± 3.03^{c}	3.39 ± 0.20^{c}	77.51 ± 21.64^{bc}	64.79 ± 13.37^{bc}	6.33 ± 1.99^{c}	73.80 ± 11.13^{bc}	0.57 ± 0.79^{c}	1.30 ± 2.91^{c}
Urocanate	ND^{b}	2.79 ± 4.38^{b}	ND^{b}	7.06 ± 4.23^{b}	6.06 ± 2.13^{b}	ND^{b}	ND^{b}	7.24 ± 8.86^{b}	16.15 ± 2.74^{a}	3.97 ± 6.86^{b}	22.76 ± 3.07^{a}
Ectoine	ND^{b}	22.37 ± 11.21^{b}	78.12 ± 38.20^{a}	ND^{b}	ND^{b}	1.15 ± 0.63^{b}	ND^{b}	0.43 ± 0.96^{b}	0.17 ± 0.38^{b}	7.29 ± 1.07^{b}	ND^{b}
Proline betaine	50.31 ± 12.92^{c}	15.30 ± 2.38^{c}	18.78 ± 3.35^{c}	1.15 ± 0.45^{c}	0.43 ± 0.12^{c}	27.31 ± 3.75^{c}	2.97 ± 2.18^{c}	5009.97 ± 787.55^{a}	29.15 ± 13.03^{c}	94.14 ± 12.94^{c}	821.23 ± 63.87^{b}
gamma-Guanidinobutyrate	3.40 ± 0.10^{cdef}	ND^{f}	9.71 ± 2.61^{b}	0.18 ± 0.41^{ef}	ND^{f}	1.18 ± 1.64^{def}	4.79 ± 1.20^{c}	ND^{f}	0.69 ± 0.63^{ef}	ND^{f}	0.69 ± 0.64^{ef}
gamma-Butyrobetaine	42.00 ± 6.96^{c}	617.40 ± 16.35^{c}	23.50 ± 8.85^{c}	0.98 ± 0.30^{c}	16866.70 ± 1178.12^{b}	9.10 ± 1.70^{c}	6.40 ± 2.39^{c}	56.40 ± 10.69^{c}	8.50 ± 2.02^{c}	61.10 ± 6.53^{c}	15.90 ± 17.71^{c}
O-Acetylserine	7.88 ± 17.63^{de}	2.61 ± 2.84^{e}	2.81 ± 2.59^{e}	ND^{e}	3.47 ± 4.76^{e}	5.83 ± 4.04^{e}	0.67 ± 1.50^{e}	4.80 ± 6.08^{e}	ND^{e}	ND^{e}	ND^{e}
Guanine	10.23 ± 1.13^{c}	7.13 ± 7.27^{c}	3.72 ± 3.64^{c}	36.33 ± 4.50^{c}	2.58 ± 0.35^{c}	3.46 ± 2.00^{c}	ND^{c}	9.73 ± 4.93^{c}	9.97 ± 7.02^{c}	269.15 ± 60.22^{b}	4.16 ± 2.62^{c}
Dopamine	8.18 ± 1.21^{ab}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	6.52 ± 3.32^{ab}	ND^{c}	ND^{c}	ND^{c}

Methanol-Chloroform-Water extraction methods											
	Brown algae				Red algae					Green algae	
Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	Dasya sessilis (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Ala-Ala	7.70 ± 0.81^{c}	7.74 ± 1.17^{c}	3.20 ± 0.50^{c}	ND^{c}	52.50 ± 17.19^{ab}	1.63 ± 0.97^{c}	58.31 ± 54.88^{a}	ND^{c}	ND^{c}	70.91 ± 3.85^{a}	8.27 ± 4.77^{c}
Tryptamine	ND^{d}	0.13 ± 0.30^{d}	0.39 ± 0.53^{d}	ND^{d}	2.08 ± 0.41^{cd}	13.86 ± 11.56^{bc}	2.71 ± 1.76^{bcd}	ND^{d}	40.82 ± 13.35^{a}	ND^{d}	ND^{d}
alpha-Aminoadipate	ND^{c}	3.67 ± 8.21^{c}	1.47 ± 3.29^{c}	ND^{c}	3.07 ± 0.55^{c}	12.97 ± 2.61^{ab}	1.15 ± 1.07^{c}	17.02 ± 2.08^{a}	2.60 ± 1.57^{c}	0.91 ± 1.29^{c}	2.61 ± 1.59^{c}
N-Methylglutamate	0.37 ± 0.84^{b}	0.92 ± 1.28^{b}	0.60 ± 0.88^{b}	22.50 ± 17.08^{a}	ND^{b}	3.59 ± 0.68^{b}	ND^{b}	6.64 ± 0.67^{b}	4.72 ± 0.78^{b}	ND^{b}	ND^{b}
Carnitine	56.42 ± 4.20^{efg}	887.83 ± 76.32^{b}	136.49 ± 11.38^{cd}	1.04 ± 0.95^{h}	1.89 ± 0.19^{h}	37.08 ± 5.58^{fgh}	7.67 ± 3.60^{gh}	24.22 ± 1.81^{fgh}	4.16 ± 0.61^{gh}	77.96 ± 5.11^{ef}	5.71 ± 0.80^{gh}
Nicotine	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Methionine sulfoxide	97.74 ± 6.06^{ab}	102.22 ± 17.94^{a}	39.52 ± 10.22^{efg}	4.14 ± 2.46^{i}	21.25 ± 1.72^{fghi}	ND^{i}	9.99 ± 1.59^{hi}	17.47 ± 16.10^{ghi}	ND^{i}	68.06 ± 7.05^{cd}	16.42 ± 15.00^{ghi}
Taurocyamine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Pyridoxal	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.37 ± 0.35^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	4.14 ± 0.65^{b}	ND^{c}
Pyridoxamine	ND^{e}	0.44 ± 0.42^{de}	ND^{e}	ND^{e}	0.66 ± 0.37^{cd}	0.13 ± 0.29^{de}	ND^{e}	ND^{e}	ND^{e}	1.90 ± 0.36^{a}	1.42 ± 0.15^{ab}
3-Methylhistidine	3.38 ± 1.08^{cde}	4.59 ± 0.71^{cd}	3.01 ± 0.57^{de}	ND^{i}	2.83 ± 0.34^{def}	9.95 ± 0.92^{b}	2.02 ± 0.41^{efgh}	ND^{i}	0.49 ± 0.69^{ghi}	0.71 ± 1.01^{ghi}	ND^{i}
N-Acetylornithine	ND^{c}	1.94 ± 2.68^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	0.71 ± 1.58^{c}	ND^{c}	ND^{c}	3.42 ± 0.85^{c}
Citrulline	68.90 ± 6.72^{e}	61.10 ± 6.95^{e}	26.40 ± 7.95^{e}	3.80 ± 0.97^{e}	11763.90 ± 729.98^{b}	50.90 ± 17.94^{e}	1852.70 ± 650.59^{cd}	11363.50 ± 1227.90^{b}	840.90 ± 116.19^{de}	12.50 ± 2.72^{e}	17.70 ± 4.22^{e}
Phosphorylcholine	0.92 ± 2.05^{c}	242.66 ± 39.03^{ab}	44.50 ± 14.56^{c}	293.94 ± 116.71^{ab}	6.81 ± 1.00^{c}	ND^{c}	352.68 ± 131.13^{a}	24.38 ± 2.18^{c}	6.13 ± 3.54^{c}	ND^{c}	1.36 ± 3.04^{c}
N-alpha,N-alpha-Dimethylhistidine	ND^{c}	ND^{c}	ND^{c}	ND^{c}	14.30 ± 1.52^{a}	0.80 ± 0.76^{c}	0.70 ± 1.05^{c}	3.34 ± 0.62^{b}	0.41 ± 0.56^{c}	ND^{c}	ND^{c}
Gly-Leu	65.68 ± 7.30^{c}	9.67 ± 0.62^{d}	3.57 ± 0.41^{d}	1.26 ± 0.52^{d}	172.00 ± 23.16^{ab}	4.39 ± 1.41^{d}	1.04 ± 0.39^{d}	4.89 ± 0.33^{d}	1.37 ± 0.23^{d}	169.40 ± 10.31^{b}	15.94 ± 5.61^{d}
N-epsilon-Acetyllysine	2.54 ± 0.21^{fg}	5.35 ± 0.47^{cde}	3.76 ± 0.84^{efg}	ND^{h}	10.50 ± 1.12^{b}	2.25 ± 0.41^{g}	0.21 ± 0.47^{h}	ND^{h}	ND^{h}	5.60 ± 1.17^{cde}	ND^{h}
N6,N6,N6-Trimethyllysine	6.05 ± 0.80^{b}	151.56 ± 22.55^{b}	2.60 ± 0.70^{b}	892.16 ± 288.56^{a}	32.81 ± 2.51^{b}	3.46 ± 1.46^{b}	31.63 ± 2.99^{b}	10.22 ± 1.64^{b}	123.05 ± 23.60^{b}	20.41 ± 2.22^{b}	81.45 ± 12.02^{b}
SAH	1.24 ± 0.75^{b}	0.19 ± 0.42^{b}	ND^{b}	1.15 ± 1.10^{b}	12.72 ± 1.70^{a}	0.63 ± 0.87^{b}	ND^{b}	0.65 ± 0.90^{b}	0.67 ± 0.93^{b}	1.04 ± 1.42^{b}	2.36 ± 1.44^{b}
DOPA	2.35 ± 0.64^{b}	1.74 ± 1.25^{b}	1.35 ± 0.78^{b}	ND^{b}	4.74 ± 0.51^{b}	ND^{b}	ND^{b}	541.99 ± 158.53^{a}	ND^{b}	ND^{b}	ND^{b}
SAM+	11.70 ± 3.13^{cdef}	8.13 ± 0.74^{cdefgh}	3.33 ± 0.58^{fgh}	5.92 ± 0.62^{defgh}	47.75 ± 7.76^{b}	2.11 ± 0.16^{fgh}	ND^{h}	1.35 ± 0.28^{gh}	9.70 ± 1.71^{cdefgh}	5.45 ± 0.39^{defgh}	14.07 ± 2.05^{cde}
ADMA	ND^{e}	ND^{e}	ND^{e}	4.49 ± 1.43^{cde}	11.58 ± 1.05^{c}	ND^{e}	ND^{e}	3.95 ± 0.48^{de}	ND^{e}	ND^{e}	86.44 ± 12.78^{a}
SDMA	4.16 ± 1.39^{bc}	0.72 ± 1.61^{de}	ND^{e}	8.75 ± 1.51^{a}	ND^{e}	ND^{e}	ND^{e}	6.54 ± 0.66^{ab}	3.56 ± 0.86^{bcd}	ND^{e}	ND^{e}
o-Acetylcarnitine	28.91 ± 0.76^{cde}	112.83 ± 65.58^{b}	54.52 ± 12.28^{cd}	ND^{e}	ND^{e}	24.94 ± 5.02^{de}	1.58 ± 0.23^{e}	0.26 ± 0.58^{e}	ND^{e}	ND^{e}	ND^{e}
Cysteine-glutathione disulphide	20.78 ± 1.97^{defg}	12.24 ± 1.50^{fghijk}	4.30 ± 0.69^{ijk}	14.79 ± 2.28^{efghij}	33.49 ± 13.98^{abcd}	6.75 ± 3.49^{ghijk}	37.92 ± 10.49^{abc}	5.40 ± 0.77^{ijk}	0.22 ± 0.49^{k}	20.14 ± 3.18^{defgh}	6.41 ± 1.06^{hijk}
N-Acetylglucosamine	40.70 ± 2.42^{abcd}	31.45 ± 3.70^{bcde}	19.00 ± 1.63^{efghijk}	3.83 ± 8.56^{jk}	7.80 ± 4.42^{hijk}	ND^{k}	11.82 ± 6.74^{fghijk}	24.72 ± 8.54^{cdefgh}	9.34 ± 8.58^{hijk}	42.04 ± 6.26^{abc}	15.83 ± 9.02^{efghijk}

Methanol-Chloroform-Water extraction methods											
	Brown algae				Red algae					Green algae	
Metabolites	*Sargassum micracanthum* (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	Dasya sessilis (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Cystathionine	171.88 ± 23.15^{bc}	947.60 ± 88.58^{a}	83.15 ± 24.93^{cd}	68.92 ± 24.79^{cd}	8.95 ± 1.32^{d}	5.77 ± 0.34^{d}	2.69 ± 2.52^{d}	13.84 ± 1.35^{cd}	0.60 ± 1.33^{d}	ND^{d}	ND^{d}
gamma-Glu-2AB	ND^{b}	ND^{b}	ND^{b}	ND^{b}	1.43 ± 2.40^{ab}	2.04 ± 1.33^{ab}	ND^{b}	ND^{b}	0.48 ± 0.69^{b}	ND^{b}	ND^{b}
7,8-Dihydrobiopterin	10.21 ± 3.27^{d}	ND^{e}	ND^{e}	ND^{e}	15.04 ± 4.05^{c}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
Thymidine	3.74 ± 8.36^{c}	6.60 ± 7.60^{bc}	24.66 ± 16.44^{bc}	ND^{c}	22.53 ± 25.68^{bc}	ND^{c}	6.72 ± 15.03^{bc}	16.38 ± 22.68^{bc}	81.67 ± 71.13^{b}	11.42 ± 13.63^{bc}	25.52 ± 29.31^{bc}
Cytidine	4.94 ± 0.72^{e}	9.62 ± 2.35^{e}	9.01 ± 0.68^{e}	1.47 ± 0.96^{e}	45.89 ± 7.71^{cd}	9.59 ± 2.57^{e}	ND^{e}	8.71 ± 0.70^{e}	7.43 ± 0.93^{e}	150.54 ± 37.59^{b}	3.99 ± 0.52^{e}
Uridine	36.17 ± 5.49^{klm}	85.17 ± 10.99^{hijk}	58.24 ± 5.33^{ijklm}	ND^{m}	120.33 ± 7.81^{ghi}	18.49 ± 11.64^{lm}	64.70 ± 10.38^{ijkl}	267.84 ± 18.56^{cd}	148.88 ± 13.55^{fgh}	462.54 ± 56.79^{b}	198.24 ± 17.50^{ef}
Pyridoxamine 5'-phosphate	ND^{d}	ND^{d}	ND^{d}	ND^{d}	27.98 ± 2.26^{b}	4.79 ± 3.86^{c}	ND^{d}	1.75 ± 2.52^{d}	ND^{d}	ND^{d}	ND^{d}
Glycerophosphorylcholine	29.95 ± 4.17^{i}	3108.49 ± 277.33^{c}	1013.49 ± 477.79^{efg}	639.49 ± 115.35^{ghi}	4677.46 ± 400.29^{a}	1262.99 ± 213.69^{e}	411.31 ± 146.05^{hij}	884.76 ± 198.04^{efgh}	131.11 ± 6.51^{i}	ND^{i}	33.99 ± 6.05^{i}
Thiamine	3.50 ± 0.57^{d}	2.30 ± 0.38^{def}	1.63 ± 0.27^{defg}	2.42 ± 1.03^{de}	0.10 ± 0.23^{g}	ND^{g}	ND^{g}	ND^{g}	0.24 ± 0.54^{fg}	9.18 ± 1.19^{a}	6.99 ± 1.09^{bc}
Adenosine	13.29 ± 1.35^{def}	13.64 ± 3.81^{def}	14.53 ± 2.10^{def}	6.10 ± 4.02^{efgh}	2.83 ± 0.52^{fgh}	3.51 ± 1.50^{fgh}	ND^{h}	5.94 ± 1.27^{efgh}	63.10 ± 5.89^{c}	0.82 ± 0.85^{gh}	84.98 ± 17.38^{b}
Inosine	9.19 ± 2.32^{cd}	34.52 ± 5.31^{cd}	37.39 ± 6.47^{cd}	ND^{d}	29.62 ± 5.55^{cd}	38.63 ± 16.20^{cd}	ND^{d}	314.71 ± 61.87^{a}	35.70 ± 3.48^{cd}	ND^{d}	127.60 ± 20.02^{b}
Glu-Glu	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
1-Methyladenosine	0.41 ± 0.57^{b}	ND^{b}	ND^{b}	ND^{b}	0.24 ± 0.35^{b}	3.85 ± 1.55^{a}	0.19 ± 0.43^{b}	0.10 ± 0.23^{b}	ND^{b}	0.40 ± 0.56^{b}	ND^{b}
Guanosine	5.92 ± 0.92^{fg}	11.44 ± 1.57^{ef}	13.18 ± 1.87^{ef}	ND^{g}	7.06 ± 1.15^{fg}	7.16 ± 1.43^{fg}	ND^{g}	66.67 ± 5.35^{a}	37.26 ± 4.85^{d}	2.25 ± 2.18^{g}	48.04 ± 10.42^{b}
Ophthalmate	4.67 ± 0.64^{ab}	0.83 ± 1.13^{bc}	ND^{c}	ND^{c}	0.53 ± 1.17^{c}	ND^{c}	6.91 ± 4.55^{a}	0.64 ± 1.42^{bc}	ND^{c}	ND^{c}	ND^{c}
5-Methylthioadenosine	3.45 ± 0.74^{bc}	0.89 ± 1.35^{bc}	ND^{c}	0.64 ± 0.60^{bc}	7.55 ± 9.28^{bc}	0.81 ± 0.53^{bc}	ND^{c}	0.74 ± 1.02^{bc}	1.97 ± 0.42^{bc}	1.51 ± 0.99^{bc}	ND^{c}
Glutathione(ox)	146.96 ± 14.88^{cd}	24.90 ± 3.15^{fg}	12.15 ± 2.64^{fg}	66.70 ± 17.08^{ef}	9.90 ± 9.58^{fg}	19.83 ± 10.16^{fg}	168.91 ± 68.07^{cd}	113.56 ± 19.83^{de}	1.07 ± 0.38^{g}	28.62 ± 4.06^{fg}	157.72 ± 15.85^{cd}
Glutathione(red)	2.49 ± 1.44^{c}	ND^{c}	ND^{c}	17.17 ± 10.56^{a}	2.81 ± 2.57^{c}	6.76 ± 4.19^{bc}	2.78 ± 2.73^{c}	2.63 ± 3.61^{c}	ND^{c}	ND^{c}	ND^{c}
Phe-Phe	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	5.70 ± 0.42^{b}	ND^{c}
AICAR	ND^{c}	ND^{c}	ND^{c}	ND^{c}	10.72 ± 0.56^{c}	2017.88 ± 508.39^{a}	21.70 ± 2.21^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
S-Lactoylglutathione	0.27 ± 0.60^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	22.23 ± 3.67^{c}	46.62 ± 6.60^{a}	ND^{d}	ND^{d}	ND^{d}
5-Methyltetrahydrofolate	51.74 ± 9.24^{de}	342.02 ± 111.96^{bc}	18.51 ± 5.38^{de}	4.51 ± 6.24^{e}	321.12 ± 54.69^{bc}	522.83 ± 263.67^{a}	2.83 ± 6.34^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}
Glycolate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	2424.17 ± 489.13^{b}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Isethionate	79.40 ± 54.30^{d}	1636.90 ± 217.20^{d}	231.60 ± 57.60^{d}	276.50 ± 35.50^{d}	22525.70 ± 890.50^{c}	29523.70 ± 1869.50^{bc}	72403.50 ± 9191.10^{a}	ND^{d}	36678.10 ± 3241.10^{b}	355.00 ± 100.10^{d}	ND^{d}
5-Oxoproline	876.74 ± 144.08^{ef}	271.81 ± 74.77^{fg}	46.64 ± 18.12^{g}	139.97 ± 41.95^{fg}	1943.57 ± 156.40^{d}	4714.39 ± 848.95^{b}	120.13 ± 43.69^{g}	373.52 ± 23.39^{efg}	291.92 ± 61.75^{fg}	109.93 ± 37.76^{g}	4516.23 ± 580.89^{b}
Threonate	1656.00 ± 221.60^{b}	1234.00 ± 452.70^{b}	2740.00 ± 177.30^{b}	170.00 ± 92.70^{b}	458.00 ± 81.00^{b}	281.00 ± 124.20^{b}	125.00 ± 68.50^{b}	606.00 ± 54.80^{b}	1208.00 ± 305.60^{b}	234677.00 ± 39766.60^{a}	9391.00 ± 383.20^{b}

Metabolites	Methanol-Chloroform-Water extraction methods										
	Brown algae				Red algae					Green algae	
	Sargassum micracanthum (Togemoku)	*Sphaerotrichia firma* (Ishimozuku)	*Papenfussiella kuromo* (Kuromo)	*Saccharina japonica* (Kombu)	*Pyropia pseudolinearis* (Uppurui Nori)	*Gelidium elegans* (Makusa)	*Neodilsea yendoana* (Akaba)	Dasya sessilis (Enashi Dajia)	*Botryocladia wrightii* (Taoyagisou)	*Ulva australis* (Ana Aosa)	*Chaetomorpha moniligera* (Tamajuzumo)
Citramalate	ND^{e}	30.37 ± 29.67^{cde}	46.24 ± 7.37^{bc}	ND^{e}	ND^{e}	76.99 ± 20.96^{ab}	ND^{e}	ND^{e}	107.35 ± 18.31^{a}	ND^{e}	ND^{e}
2-Hydroxyglutarate	30.85 ± 32.54^{bc}	ND^{c}	ND^{c}	ND^{c}	61.10 ± 38.48^{abc}	ND^{c}	29.29 ± 65.49^{bc}	ND^{c}	ND^{c}	88.09 ± 60.65^{ab}	ND^{c}
p-Hydroxyphenylacetate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	1491.51 ± 190.98^{b}	ND^{c}	ND^{c}	ND^{c}
Orotate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Urate	2761.35 ± 301.55^{b}	102.20 ± 97.34^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
Cysteate	ND^{d}	ND^{d}	ND^{d}	ND^{d}	287.55 ± 37.61^{c}	414.28 ± 26.53^{b}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Glycerophosphate	89.36 ± 20.56^{d}	2122.85 ± 365.92^{c}	2984.67 ± 633.31^{ab}	316.37 ± 105.16^{d}	2300.29 ± 121.77^{bc}	128.21 ± 29.35^{d}	280.05 ± 55.80^{d}	149.49 ± 24.68^{d}	309.32 ± 65.32^{d}	ND^{d}	730.82 ± 63.02^{d}
Allantoate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	942.07 ± 538.09^{a}	ND^{b}	ND^{b}	ND^{b}	173.26 ± 100.80^{b}	90.41 ± 124.05^{b}
N-Acetylglutamate	57.42 ± 7.21^{ab}	29.64 ± 7.49^{bcde}	16.92 ± 3.95^{cde}	ND^{e}	27.52 ± 6.38^{bcde}	22.63 ± 4.78^{cde}	11.88 ± 4.55^{de}	33.87 ± 11.99^{bcd}	ND^{e}	42.81 ± 2.93^{abc}	30.62 ± 3.51^{bcd}
Mucate	193.36 ± 26.68^{ef}	104.93 ± 21.44^{fgh}	236.03 ± 27.29^{de}	66.35 ± 15.86^{gh}	68.27 ± 9.89^{gh}	64.11 ± 12.77^{gh}	ND^{h}	32.10 ± 7.52^{gh}	310.40 ± 62.03^{cd}	93.42 ± 25.19^{fgh}	927.85 ± 102.43^{b}
Galacturonate 1-phosphate	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
Argininosuccinate	ND^{c}	ND^{c}	ND^{c}	ND^{c}	144.76 ± 23.35^{b}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	15.76 ± 35.24^{c}	ND^{c}
CMP	39.71 ± 5.39^{d}	ND^{e}	ND^{e}	3.39 ± 7.58^{e}	89.86 ± 10.19^{b}	3.80 ± 8.50^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	79.45 ± 9.00^{bc}
UMP	210.17 ± 27.59^{cde}	100.94 ± 15.41^{efgh}	59.39 ± 24.82^{fgh}	127.46 ± 28.22^{defg}	478.81 ± 52.83^{b}	65.22 ± 3.27^{fgh}	132.39 ± 26.97^{defg}	81.33 ± 18.68^{fgh}	ND^{h}	ND^{h}	267.38 ± 30.95^{c}
3'-AMP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	168.29 ± 70.69^{a}	ND^{b}	ND^{b}
AMP	561.65 ± 51.71^{b}	102.23 ± 13.53^{ef}	62.08 ± 9.30^{f}	200.64 ± 17.26^{cde}	282.38 ± 48.20^{c}	52.58 ± 27.32^{f}	ND^{f}	ND^{f}	ND^{f}	ND^{f}	241.20 ± 29.35^{c}
IMP	147.15 ± 34.96^{cd}	74.05 ± 29.89^{de}	65.98 ± 27.14^{de}	ND^{e}	358.55 ± 90.40^{b}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	ND^{e}	133.41 ± 18.44^{cde}
Prostaglandin E2	ND^{d}	ND^{d}	ND^{d}	ND^{d}	226.10 ± 505.62^{cd}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
Prostaglandin F2alpha	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
UDP	ND^{c}	ND^{c}	ND^{c}	ND^{c}	226.02 ± 38.17^{b}	ND^{c}	31.75 ± 70.99^{c}	ND^{c}	ND^{c}	ND^{c}	ND^{c}
ADP	ND^{b}	17.49 ± 25.08^{b}	13.72 ± 30.69^{b}	11.25 ± 16.07^{b}	86.24 ± 26.05^{a}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
IDP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}
GDP	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}

The values indicate as mean ±SD (nmol g^{-1}) of the metabolite concentration of dry weight (n= 5). ND, not detected. Different superscript letters indicate the significant differences (P<0.05) between each type of species by Tukey HSD analysis. Statistical analysis were compared between all data set (species and extraction methods (Table 3.2, S3.1)).