博士論文

論文題目　**NMR-based analysis and characterization of minor components in Japanese persimmon (*Diospyros kaki*) cultivars**

（**NMR** 法による日本柿の微量成分分析の検討及び微量成分による品種特徴づけに関する研究）

氏　　名　　劉　晶楽

CONTENTS

ABSTRACT

Diospyros kaki, better known as the Japanese persimmon, is recognized as an outstanding source of biologically active compounds related to many health benefits. About 1000 varieties are estimated to be grown in Japan. However, there are only limited efforts to investigate the metabolites in persimmon fruit compared to other fruits. In addition, the investigations on persimmons have generally been focused on certain selected metabolites (carotenoid, polyphenols, etc.) and the metabolic profiling has not been applied to persimmons for an overview of the chemical compounds. Furthermore, identification of persimmon cultivars has been traditionally performed based on morphological and physiological features and recently genetic methods have been used to distinguish cultivars; however, metabolic profiling for discriminating the cultivars of persimmon has not been reported in the literatures at our knowledge.

In the present study, metabolic profiling of persimmon juice was performed by nuclear magnetic resonance (NMR) spectroscopy. Since the main components in persimmon juice, such as water and sugars, make minor components undetectable or detected with relatively poor signal-to-noise (S/N) ratio due to the limitation of dynamic range. Broad Band WET method was developed to analyze persimmon juice as a new NMR method for detecting minor components in complex mixtures. Detailed signal assignments of Japanese persimmon juice (Taishu cultivar.) were carried out using various 2D NMR techniques incorporated with Broad Band WET or WET sequences and individual compounds in persimmon juice were quantitatively evaluated. Furthermore, NMR spectra combining with multivariate statistical analysis were employed to discriminate 5 different Japanese persimmon cultivars. A better discrimination and a more informative detail of metabolic differences among cultivars were achieved by Broad Band WET spectra than using conventional ^{1}H NMR. On the basis of signal assignments, minor components detected by Broad Band WET method were captured as biomarkers responsible for the cultivar discrimination. Broad Band WET spectra combined with multivariate analysis could be a very useful tool for Japanese persimmon cultivar characterization which will help in selection or modification of better or nutritionally important cultivars combined with genomics researches in the future.

Chapter I

Introduction

1-1 Japanese persimmons

Diospyros kaki, better known as the Japanese persimmon, is the most widely cultivated species of the Diospyros genus. Persimmon is among the oldest plants in cultivation, known for its use in China for more than 1000 years and has been developed in Japan, Korea, Brazil, Spain, and Italy, etc. The Pen Ts'ao Kang Mu, China's most famous materia medica, compiled in 1578 by Li Shi-Zhen (1518–1593) and published in 1597, described how persimmon can be used to improve lung, stomach, spleen, and intestinal conditions. It also describes how persimmon can be used to prevent and treat diseases such as sore throat, aphtha, and insomnia. Additionally, it has the added benefit of relieving alcoholism (Chen *et al*., 2008).

Japanese persimmon is a popular and widely consumed fruit species in Japan and about 1000 varieties are estimated to be grown in Japan (Veberic *et al.*, 2010). Persimmons are classified broadly into two categories: astringent cultivar and non-astringent cultivar. An astringent cultivar is not suitable for eating at harvest time, which is associated with a high amount of soluble tannins in the fruit. It can only be edible after artificial removal of astringency, or in a state of being overripe or dried (Taira *et al*., 1990, 1992, 1996). On the other hand, a non-astringent cultivar is edible because the seeds in the fruit have the ability to insolubilize the soluble tannins during development and remove the astringency at harvest time (Sugiura *et al*., 1983; Yonemori *et al*., 1983; Harada *et al*., 1990).

A lot of studies on Japanese persimmons concerning the antioxidant, radical scavenging activity, antihypertensive activity, anti-atherosclerosis activity and so on have been made in recent years (Uchida *et al*., 1990; Chen *et al*., 2008; Park *et al*., 2008; Jang *et al*., 2010). Lots of efforts have been devoted to investigate the nutritional components contained in this kind of fruits related to these health beneficial properties, most of which generally have focused on certain selected metabolites such as carotenoids, Vitamin C, and polyphenols (Giordani *et al*., 2011). Some polar components associating with the persimmon fruit quality such as sugars and organic acids have also been investigated (Senter *et al*., 1991; Del Bubba *et al*., 2009; Veberic *et al*., 2010).

Japanese persimmon fruits are an important source of sugars. The most abundant sugars in persimmons are glucose, fructose and sucrose with methanol or water

extraction identified by HPLC techniques (Del Bubba *et al*., 2009; Veberic *et al*., 2010). Galactose and arabinose have also been found in some cultivars, and the quantities of which vary significantly by cultivar and maturity by GC analysis (Senter *et al*., 1991). Other sugars, such as rhamnose and mannose, have been identified during a softening-associated modification of Japanese persimmon pectin in chloroform and methanol extraction (Asgar *et al*., 2003).

Some powerful antioxidants, including carotenoids, Vitamin C, and polyphenols, are found to be rich in Japanese persimmon. Carotenoids are higher in skin than those in pulp. β-carotene are the predominant carotenoid in persimmon pulp detected with HPLC techniques, followed by β-cryptoxanthin, α-carotene and zeaxanthin, and the contents varies amongst cultivars (Veberic *et al*., 2010; Takahashi *et al*., 2006). Other carotenoids, such as *cis*-mutatoxanthin, have also been identified by using HPLC technique (Daood *et al*., 1992).

Veberic *et al*. suggested that amongst the phenolic compounds in fully ripen persimmon fruit, catechin and gallic acid are predominant epicatechin, caffeic acid, quercetin-3-rutinoside, quercetin-3-galactoside and quercetin-3-glucoside were also detected in trace amounts in HPLC analysis (Veberic *et al*., 2010). Gorinstein *et al*. reported that protocatechuic, ferulic, gallic, *p*-coumaric, and vanillic acids and epicatechin in whole persimmons are presented as major phenolics using fluorescence emission spectroscopy (Gorinstein *et al*., 2001). The variety of catechin composition identified by HPLC amongst different cultivars has also been discussed (Suzuki *et al*., 2005)

With respect to amino acids, 14 amino acids, such as alanine, aspartic acid, citrulline, glutamic acid, glycine, leucine, serine, threonine, tyrosine, have been identified in Japanese persimmon, which have a variety of functionalities (Mallavadhani *et al*., 1998).

It should be mentioned that a variation of results occurred in the reports mentioned above. All the methods adopted above are all compound-targeted, which means only some specified kind of compounds can be identified in one observation, and require extraction, purification or derivatization of each kind of compounds. The different sample treatments may cause the modification of the original composition of

components, and the intrinsic variance of metabolites among different cultivars also makes the comparison of different studies challenging.

In addition, despite of the above results, however, the knowledge about the metabolites in persimmon fruits is still limited compared to other popular fruits, such as apple, tomato, mango and so on.

Therefore, considering the variety of Japanese persimmon cultivars, a rapid, and un-biased with a minimum destruction of samples, but informative analytical approach is still expected to be developed and applied in analysis of Japanese persimmon.

1-2 Metabolic profiling and Nuclear Magnetic Resonance

Metabolic profiling (Niwa, 1986), also known as metabolomics or metabonomics, provides a detailed and comprehensive overview of food composition, which has been applied in food science for food component analysis, food quality/ authenticity assessment, food consumption monitoring, and physiological monitoring in food intervention, and so on (Wishart, 2008). In other words, with the advent of metabolic profiling, foods are now been analyzed with considerably more chemical details, with many chemical compounds being detected and quantified simultaneously. Comprehensive analysis of metabolic profiles using different analytical techniques, along with suitable statistical analysis, has been established to identify similarities and differences among crop varieties based on their metabolic profiles. Untargeted studies have been carried out in a few number of foods, such as melon (Biais *et al*., 2009), apple (Aprea *et al*., 2011; Vermathen *et al*., 2011), tomato (Gomez *et al*., 2010; Perez *et al*., 2011), potato (Beckmann *et al*., 2007), etc.

There are several techniques performed in metabolic profiling, including mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), etc. (Dunn *et al*., 2005). Among these techniques, NMR spectroscopy represents a powerful technique for food analysis because of many advantages: (1) It provides a faithful overview of many components in a mixture solution; (2) It can be performed without any extraction, purification and chemical derivation of each component; (3) quantitative data can be obtained by integration relative to a standard component without calibration curves; (4) unknown or unexpected chemical components can be identified through

appropriate two-dimensional (2D) NMR measurements (Wishart, 2008). Furthermore, NMR was found to be particularly useful in the identification and quantification of the isomers that could not be distinguished by LC or LC-MS methods (Tiziani *et al*, 2006). In the last decade, NMR has been applied for the comprehensive analysis and characterization of liquid foods, such as wine (Nilsson *et al*., 2004; Hong *et al*., 2011), vinegar (Caligiani *et al*., 2007), milk (Hu *et al*., 2004, 2007), coffee (Wei *et al*., 2012), and non-liquid foods such as soy sauce (Ko *et al*., 2009), tomato (Sobolev *et al*. 2003), apple (Belton *et al*., 1997), and mango (Gil *et al*., 2000**,** Duarte *et al*., 2005, 2006; Koda *et al*., 2012). By these attempts, it has been shown that foods can be chemically characterized with sufficient accuracy using NMR spectra. However, considering the enormous variety of foods, NMR data enough to indicate the characteristics of foods are still very limited. Therefore, it is necessary to enlarge the database of foods including the quantitative information for both major and minor components by the analysis with NMR.

At our knowledge, the metabolic profiling are not applied to Japanese persimmon for an overview of the chemical compounds and a full high field NMR analysis of Japanese persimmon has not been reported in literatures. Therefore, the present study has tried to analyze metabolic profiles of Japanese persimmon by NMR spectroscopy, and characterize the differences amongst cultivars by combining the signal patterns of minor components detected with NMR spectroscopy with statistical analysis.

Chapter 2

Broad Band WET — a new method to quantitatively observe minor components in foods

2-1 Introduction

The high sensitivity of high-field ^{1}H NMR makes it possible to analyze complex mixture directly without any destruction of components, and the spectra provide the qualitative and quantitative information on various components in complex mixtures. When the signals in ^{1}H NMR spectra are fully assigned using 2D experiments, database, and/or spiking experiments (Mannina *et al*., 2012), complex mixtures can be characterized by a rapid and single ^{1}H NMR experiment. .

In the last decade, NMR has been applied to characterize liquid foods such as milk (Hu *et al*., 2004; Hu *et al*., 2007), coffee (Wei *et al*., 2010; Wei *et al*., 2011), wine (Hong *et al*., 2011) and vinegar (Caligiani *et al*., 2007), and non-liquid foods such as mango (Gil *et al*., 2000**,** Duarte *et al*., 2005, 2006; Koda *et al*., 2012), apple (Belton *et al*., 1997) and tomato (Sobolev *et al*., 2003). However, considering the enormous variety of foods, NMR data enough to indicate the characteristics of foods are still very limited. Little information on minor components has also been utilized in the nondestructive NMR analysis so far although the minor components are valuable to characterize the foods (Koda *et al*., 2012; Belton *et al*., 1998). Therefore, it is necessary to enlarge the database of foods including the quantitative information for both major and minor components by the non-destructive analysis with NMR.

The NMR analysis of foods frequently suffers from a problem of dynamic range. In many foods, the main components such as sugars (especially evident in fruits) are observed as strong signals as well as water (Figure 2-1). The signals of water and sugars make the minor components undetectable or detected with very poor signal-to-noise (S/N) ratio due to the limitation of dynamic range. It is indispensable to suppress the strong signals of water and sugars simultaneously to detect the minor components with a better S/N ratio.

Conventional solvent-suppression methods, such as PRESAT, WET (Smallcombe *et al*., 1995; Mo *et al*., 2008), and WATERGATE (Piotto *et al*., 1992), are utilized as effective solvent-suppression methods; however, simultaneous saturation for signals of sugars and water is assumed to be difficult because it is difficult to saturate signals in an extensive range. As one of the efficient methods for saturating wide range of signals, DPFGSE-WATERGATE method has already been reported as a robust method for broad band saturation (Piotto *et al*., 1992; Hwang *et al*., 1995; Dalvit *et al*., 1999; Rutherford

et al., 1999; Kövér *et al*., 2000; Schievano *et al*., 2010); however, distorted and attenuated signals occurs caused by *J*-modulation effect and T_2 relaxation time, which results in a spectrum not suitable for direct integral measurement required in quantitative analysis (Hajduk *et al*., 1993).

In order to solve the above problem, in the present study, we propose Broad Band WET method, which is a modification of WET method, as a new method for saturating a broad bandwidth. The method was in fact applied to simultaneously saturate the resonances of water and sugars in persimmon juice as an example. Moreover, the quantitative characteristics of Broad Band WET method were also systematically studied.

2-2 Materials and Methods

2-2-1 Sample preparation

Fruits of persimmon cultivar “Taishu” were supplied by National Agriculture and Food Research Organization (Hiroshima, Japan) in the middle of November. The fruits are grounded into a pulp and are then centrifuged at 13,000×g for 10 min at 4°C. The supernatant was used as persimmon juice. The juices were stored at –20°C until NMR measurements. 70 μl D_2O (99.9%; Shoko Co., Ltd., Tokyo, Japan) was added to 630 μl of persimmon juice for locking. A final concentration of 0.1 mM 3-(trimethylsilyl) propionic 2,2,3,3-d_4 acid, sodium salt (TSP-d_4) was added to the sample as a reference for chemical shift and concentration. A final concentration of 1.5 mM EDTA (Dojindo, Kumamoto, Japan) was added to the sample to capture the paramagnetic ions that cause a broadening of NMR signals. The mixture then was transferred to a 5 mm NMR tube (Kanto Chemical Co., Inc., Tokyo, Japan).The mixture then was transferred to a 5 mm NMR tube. For plotting working curves, isoleucine, uridine, and trigonelline (special grade; Wako Pure Chemical Industries, Ltd., Osaka, Japan, or Nacalai Tesque Inc., Tokyo, Japan) were used as standard solutions dissolved in D_2O and prepared at 3 concentrations. These standard solutions (700 μL) were transferred to a 5 mm NMR tube for NMR measurements.

2-2-2 NMR Spectroscopy

All NMR spectra were obtained at 20°C on a Varian Unity INOVA-500 spectrometer equipped with a 5 mm triple resonance probe (Agilent Technologies, Inc., CA, USA). The acquisition parameters of the ^{1}H NMR spectra were as follows: spectral width, 8,000 Hz; number of data points, 32K; number of scans, 128; acquisition time, 2.048 s; and delay time, 2.0 s. The water signal was suppressed by NOESYPRESAT method. (Croasmun *et al*., 1994)

The acquisition parameters of DPFGSE-WATERGATE methods were as follows: spectral width, 8,000 Hz; number of data points, 32K; number of scans, 128; acquisition time, 2.048 s; and delay time, 1.0 s; the pulse length of Re-Burp pulse was 4.1 ms.

The acquisition parameters of both conventional WET and Broad Band WET

methods were as follows: spectral width, 8,000 Hz; number of data points, 32K; number of scans, 128; acquisition time, 2.048 s; and delay time, 1.0 s; the pulse length of e-Burp pulse was 3.8 ms.

The acquisition parameters of both conventional WET COSY and Broad Band WET COSY were as follows: spectral width, 6,700 and 6,700 Hz (F1, F2); number of data points, 512 and 2,048 (F1, F2); number of scans, 64; the pulse length of e-Burp, 3.8 ms.

The acquired NMR data were manually phased, carefully baseline corrected, and referenced to TSP at 0.00 ppm. The signal-to-noise (S/N) ratio of the region from 0.7 ppm to 3 ppm was calculated automatically by VNMR 6.1C software of Varian NMR spectrometer systems. The S/N ratio of the strongest signals (citric acid at 2.7 ppm) was compared among the spectra obtained with individual suppression methods.

2-3 Results and Discussion

2-3-1 Pulse sequence of Broad Band WET method

The pulse sequence of Broad Band WET method is a new sequence that incorporates WET sequence on the basis of NOESYPRESAT sequence Figure 2-2a.

PRESAT was firstly incorporated to saturate bulk water signal. Then the following WET scheme was used for further saturation on both water and sugar signals in a broad bandwidth. The tip angles for the four selective pulses in the WET scheme were all adjusted to 90° for the purpose of obtaining a best quality of selectivity; however, this adjustment resulted in an insufficient suppression on water signal which made it necessary to incorporate PRESAT to saturate water signal.

The continued composite 180° pulse after WET scheme was equal to the first and second 90° pulses in NOESYPRESAT sequence, and it was toggled on (180°) and off (0°) by the phase cycling to cancel the off-resonance signals which cannot be excited by this pulse. Moreover, during the sequential mixing time, field gradient pulses (PFGs; g1, g2, g3 and g4) which were the homo spoil pulses were inserted and they had a function of z-filter which were able to dephase the unwanted signals in x,y-plane and store the desired magnetizations in the z-direction.

At this stage, the saturation for the strong resonances of water and sugars was still incomplete; therefore, a sel 90°-180°-sel 90° pulse sequence was newly introduced to further saturate water and sugar resonances, where sel 90° indicates selective 90° pulse. The two selective 90° pulses were toggled on and off sequentially by phase cycling, serving as a selective 180° pulse and a selective 0° pulse, respectively. Taking the difference between these two conditions allowed the signals from minor components to be added up and the signals from water and sugars to be further canceled out. The hard 180° pulse was incorporated to yield a better subtraction for selective pulses. Since the mixing time of the z-filter was extremely short (shorter than several ms), the magnetization in the z-direction is not influenced by the inserted sequence.

In this way, Broad Band WET method combining WET sequence with NOESYPRESAT sequence provided a great ability to saturate the bulk water and sugar signals.

2-3-2 Comparison between the saturation profiles for conventional WET and Broad Band WET method

To confirm the null region and selectivity for saturation, the saturation profiles of the water signal were monitored using the pulse sequences of both conventional WET and Broad Band WET (Figure 2-3). Resonance offsets were recorded at 100 Hz increments. The e-Burp pulse was used for the selective pulse and the saturation range was set as 1,175Hz.

The saturation profile acquired from conventional WET was shown in Figure 2-3 a. The saturation range for conventional WET was set from 3.05 ppm to 5.40 ppm, which were the same as Broad Band WET method. The saturation profile of the conventional WET exhibited a low selectivity for suppression. Many resonances in the vicinity of the suppressed region were attenuated considerably. The reason for the low selectivity is considered to be the influence of variable flip angles of the four selective pulses. The selectivity was lowered when the method applied to a broad bandwidth. To overcome this problem, the four variable-tip-angle selective pulses in the WET scheme were replaced with the selective 90° pulse in the Broad Band WET sequence (Figure 2-2). As shown in Figure 2-3b, the Broad Band WET sequence suppressed the entire resonances spanning 1,175 Hz (3.05–5.40 ppm) quite well. In addition, the resonances outside of this region were not affected by the saturation, and exhibit a flat region with uniform signal intensities in the saturation profile. The results indicate that the Broad Band WET sequence is effective in suppressing with a high selectivity, which could be very helpful in quantitative analysis that requires the accuracy of peak area relative to a standard reference with known concentration.

To confirm the quantification property of Broad Band WET method, the working curves for a standard mixture with three standard chemicals (isoleucine, uridine, and trigonelline) were plotted and the ratio of calculated experimental concentration to theoretical concentration for individual chemicals were compared for Broad Band WET and ^{1}H NMR spectra as shown in Figure 2-4. All chemicals showed good linearity with a high coefficient of determined R^2 value for their working curves; and the slops were calculated as almost 1 for both Broad Band WET and conventional ^{1}H NMR spectra. The resonance of 1,6-CH (5.88 ppm) of uridine is near to the suppression region, however, the intensity of the signals are almost as same as 1, which shows the excellent

selectivity for Broad Band WET method.

2-3-3 Comparison among 1D spectra of DPFGSE-WATERGATE, conventional WET and Broad Band WET sequence

Figure 2-5 showed the ^{1}H NMR spectrum of the persimmon fruit juice without water presaturation. The spectrum was dominated by the strong signals of water and sugars. For this reason, the receiver gain was restricted at 0 and the resonances originated from the low concentration components are inaccurately sampled with poor S/N ratio of 43 in the high-field region (0.0–3.0 ppm), and no signal in the low-field region (6.00–10.00 ppm). On the other hand, the NOESYPRESAT method effectively suppressed the resonance of water (Figure 2-5 b), which elevated the receiver gain to 18 dB. However, the resonances from sugars were still strongly observed in the range from 3.2 to 4.2 ppm and restricted the receiver gain. As a result, signals in high-field region were detected with relatively low S/N ratio of 517, and few signals were observed in the low-field region.

The DPFGSE-WATERGATE method was applied to simultaneously suppress the water and sugar signals of persimmon juice. DPFGSE-WATERGATE method is a DPFGSE sequence (G1-sel 180°-G1-G2-sel 180°-G2) in which the sel 180° is replaced by a sel 180°-hard 180° sequence. The Re-Burp pulse was used for the selective pulse and the length of selective 180° pulse was 4.1 ms. The suppression range was set from 3.05 ppm to 5.40 ppm to saturate both water and sugar signals. The NMR spectrum with DPFGSE-WATERGATE method with presaturation incorporated to saturate the resonance of water was shown in Figure 2-5c. The DPFGSE-WATERGATE method allowed an increase in the receiver gain to 48 dB, and the signals in high-field region were detected with an improved S/N ratio to 1195 and many signals were newly observed in the low-field region (6.00–10.00 ppm). However, the resonances of sugars were remained strongly. These sugar signals were considered to be the strong off-resonance signals of sugars excited by stray magnetic field. (Mo *et al*., 2008)

Figure 2-5d shows the spectrum of persimmon fruit juice by using conventional WET method. The e-Burp pulse was used for the selective pulse and the length of selective 90° pulse was 3.88 ms. The resonances of water and sugars were saturated simultaneously by setting up the saturation range for selective pulse from 3.05 ppm to

5.40 ppm, which allowed an increase in the receiver gain to 40 dB. The S/N ratio was also improved to 1522 in the high-field region, and many peaks were newly observed in the low-field region. However, the suppression level for water signal was still not sufficient. Although they were suppressed to some extent, the signals from sugars were also still strong compared with the signals of minor components. The insufficient saturation was considered to be caused by the off-resonance of the strong signal of water and sugars. The saturation of the strong water signal was still difficult even with incorporating PRESAT.

The typical spectrum of persimmon fruit juice obtained by Broad Band WET sequence was shown in Figure 2−5e. The Broad Band WET method successfully suppressed not only the resonances of water but also those of sugars to very low levels. The suppression effects of Broad Band WET were much better than those of the conventional WET and DPFGSE-WATERGATE methods. Minor components in the high-field region were observed with a good S/N ratio of 1998 and many peaks were observed in the low-field region.

The distorted signals were observed in the region from 0.7 to 3.0 ppm of the spectrum obtained by the DPFGSE-WATERGATE method (Figure 2-6 a), and were assigned to malic acid (at 2.38 ppm). The distortion of signals was due to the effects of J-modulation, especially on the signals with a large J coupling constant, which make it very difficult for quantitative analysis. In addition, the intensities of signals were not proportional to the concentrations directly, which would be caused by the unavoidable signal attenuation of different transverse relaxation time (T_2) during the intervals between selective 180° pulses in DPFGSE-WATERGATE sequence. On the contrary, in the spectrum with Broad Band WET method (Figure 2-6 b), signals of malic acid showed an improved S/N ratio without the distortion of signals influenced by *J* modulation. The undistorted signal makes it possible to measure the intensities of signals from minor components, which allows an identification of minor components with quantification.

2-3-4 Application of Broad Band WET sequence to 2D NMR

The water and sugar signals were strongly observed in persimmon juice, 2D NMR

spectra suppressing such strong signals are necessary to assign the signals of minor components observed only in the ^{1}H NMR spectra with broad band saturation.

In the conventional WET-COSY spectrum (Figure 2-7 a), the saturation for the strong signals of water and sugars was still insufficient although field gradient pulses were applied for coherence selection. Artifact peaks such as t1 noise and P-type cross peaks were observed and are considered to be derived from the strong signals of water and sugars. These peaks made it difficult to observe the cross peaks for minor components.

To improve the suppression of the water and sugar signals, the Broad Band WET sequence was incorporated into COSY sequence (Figure 2-2 b). Coherence selection was used by applying field gradient pulses and phase cycling to obtain N-type cross peaks. In the spectrum with Broad Band WET-COSY shown in Figure 2-7 b, the water and sugar signals were suppressed to a adequate level, which made the cross peaks for the minor components observed clearly. Besides that, in the strong sugar-signal region of the F2 axis, the cross peak of minor components were also observed owing to the suppression of sugars signals. For example, the cross peaks for malic acid at 2.40/2.70/4.32 ppm, and γ-amino butyric acid at 2.30/1.89/3.00 ppm. The suppression made it much easier to assign minor components.

In conclusion, the present results have indicated that the Broad Band WET method is an efficient and highly selective method for the simultaneous suppression of the strong signals derived from water and sugars in foods. This new method provides high-throughput information in a high S/N ratio for minor components in complex mixture both qualitatively and quantitatively, and is universally applied to juices or other foods with high concentrations of sugars or other major components whose resonances spread over a broad bandwidth.

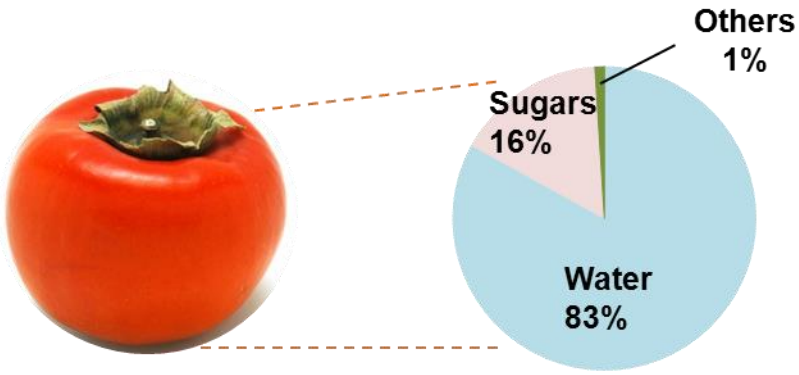


Figure 2-1 Chemical composition of Japanese persimmons (source: Standard Tables of Food Composition in Japan)

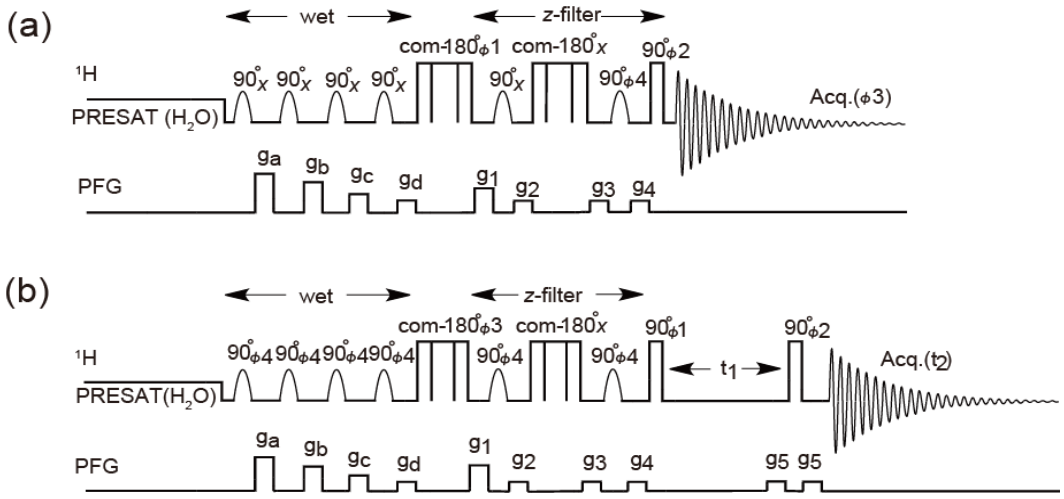


Figure 2-2 Pulse sequence of (a) Broad Band WET 1D NMR, and (b) Broad Band WET-COSY. The sine bell represents a selective pulse. In the pulse sequence (a), the tip angles for four WET e-Burp pulses are 90° along *x*. The duration of the g_a, g_b, g_c, g_d gradients is 1.5 ms and the gradient pulse strengths are set as follows: g_a = –12.0 G/cm; g_b = –6.0 G/cm; g_c = –3.0 G/cm; g_d = –1.5 G/cm. Each pulse is followed by an additional 2 ms delay prior to the next RF pulse. The composition and phase of the first composite pulse (comp-180°φ1) are 90°*x*-180°*y*-90°φ1, and was toggled alternatively on and off. Those of the second composite pulse (comp-180°*x*) are 90°*x*-180°*y*-90°*x*. The e-Burp pulse are 90°, and along *x*-axis. The pulse strengths of PFGs in the *z*-filter are set as follows: g1 = –3.3 G/cm; g2 = –1.0 G/cm; g3 = –11.0 G/cm; g4 = –11.0 G/cm. The duration of the g1, g2, g3, g4 gradient are 1.0 ms. Phase cycling is applied as follows: φ1 = *x*,-*x*, *x*, -*x*; φ2 = *x*, *x* -*x*, -*x*, *y*, *y*,-*y* -*y*; φ3 = *x*,-*x*, *x*,-*x*, *y*, -*y*, *y*, -*y*; φ4 = *x*, *x*, *x*, *x*, -*x*, -*x*, -*x*, -*x*, -*x*, -*x*, -*x*, -*x*, *x*, *x*, *x*, *x*. In the pulse sequence (b), The pulse strengths of g5 is set at –7.0 G/cm. Phase cycling is applied as follows:φ1 = *x*, *x*, -*x*, -*x*, *y*, *y*, -*y*, -*y*; φ2 = *x*, *x*, -*x*, -*x*, *y*, *y*, -*y*, -*y*, -*y*, -*y*, *y*, *y*, *x*, *x*, -*x*, -*x*, -*x*, -*x*, *x*, *x*, -*y*, -*y*, *y*, *y*, *y*, *y*, -*y*, -*y*, -*x*, -*x*, *x*, *x*; φ3 = *x*, -*x*, -*x*, *x*, -*x*, *x*, *x*, -*x*; φ4 = *x*, -*x*; Acq. = -*x*, *x*, -*x*, *x*, *y*, -*y*, *y*, -*y*, *x*, -*x*, *x*, -*x*, -*y*, *y*, -*y*, *y*.

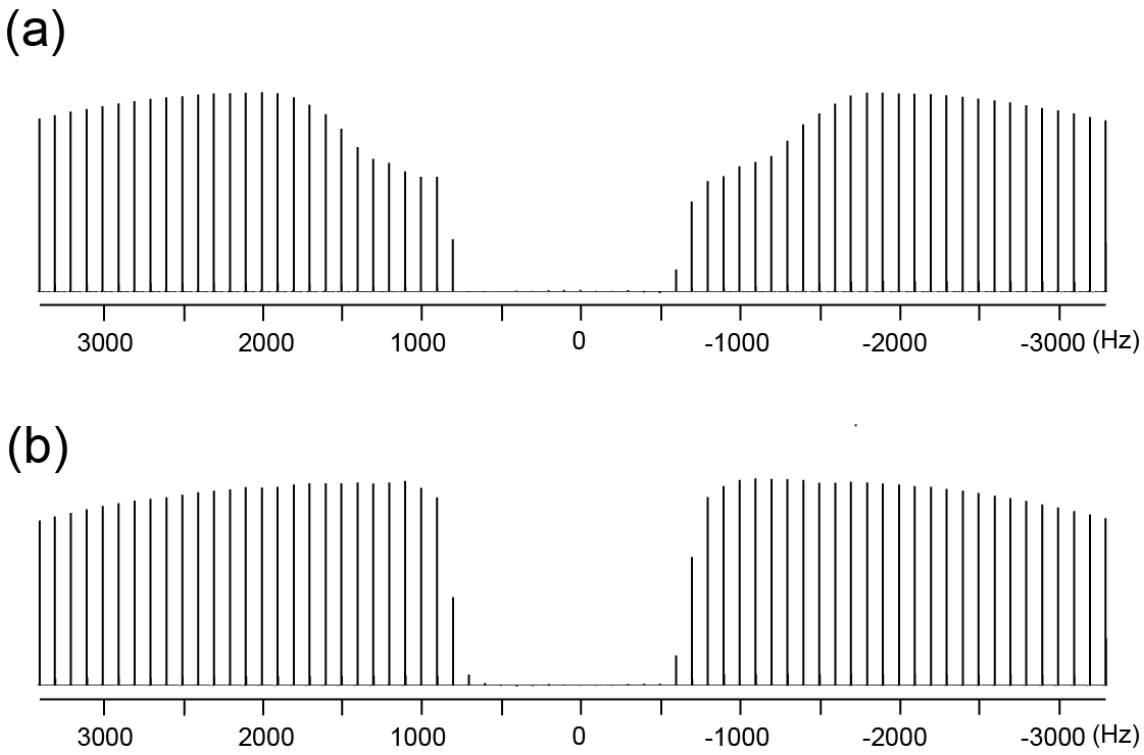


Figure 2-3 The saturation profiles of (a) conventional WET and (b) Broad Band WET pulse sequence. e-Burp = 3.8 ms, Δ = 100 Hz step, H_2O 5%.

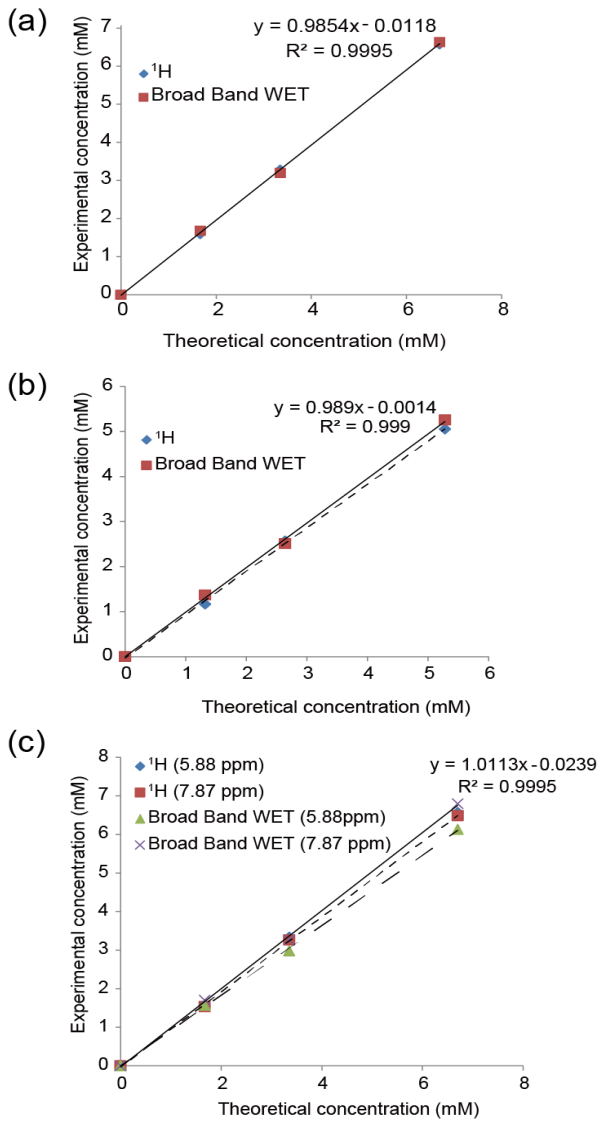


Figure 2-4 Working curves for standard solution containing

(a) isoleucine: δ−CH_3 (0.93 ppm) and γ`−CH_3 (1.00 ppm); Standard solution at three concentration of 1.67mM, 3.35mM, 6.7mM;

(b) trigonelline: 3, 5−CH (8.84 ppm); Standard solution at three concentration of 1.32mM, 2.64mM, 5.28mM;

(c) uridine: 1,6−CH(5.88 ppm) and 7−CH (7.87 ppm); Standard solution at three concentration of 1.67mM, 3.35mM, 6.7mM;

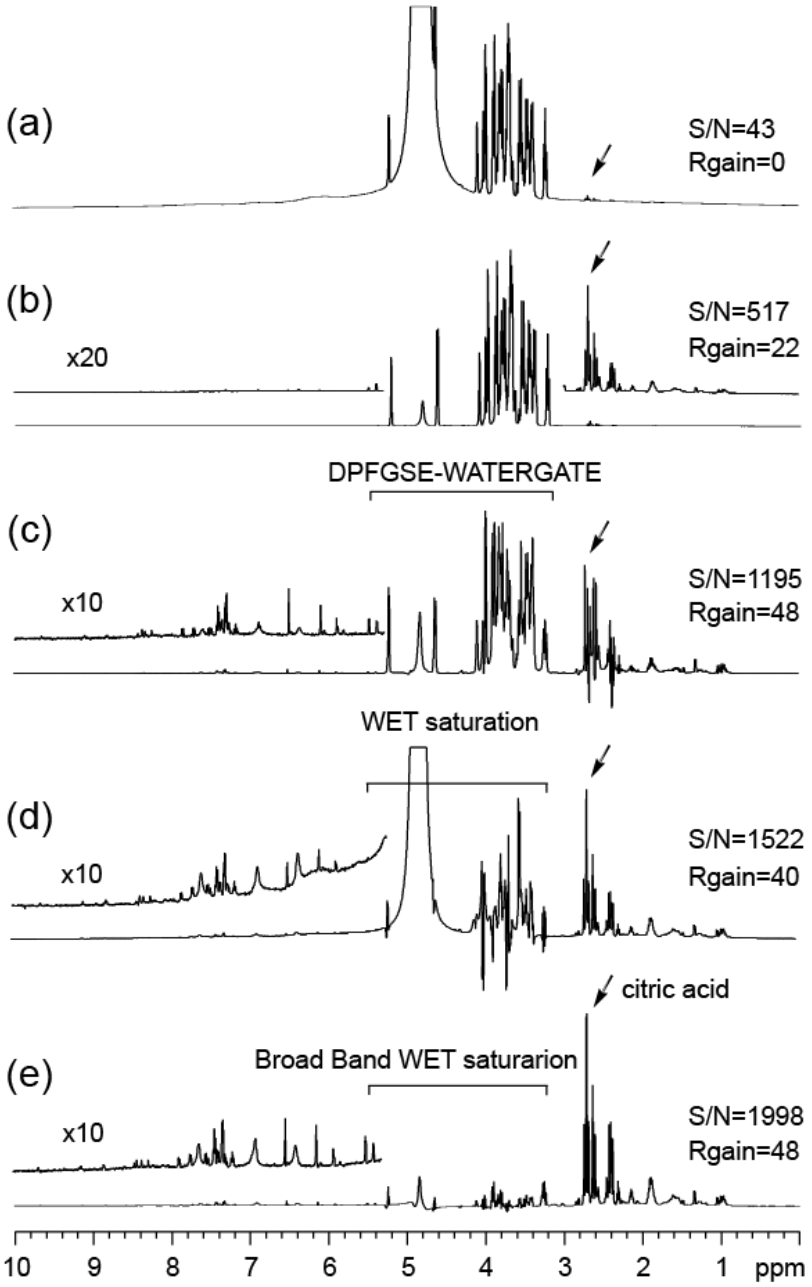


Figure 2-5 ^{1}H NMR spectra of persimmon fruit juice (a) without presaturation (number of scans, 128; receiver gain, 0 dB); (b) with NOESYPRESAT (number of scans, 128; receiver gain, 22 dB); (c) with DPFGSE-WATERGATE (number of scans, 128; receiver gain, 38 dB); (d) with conventional WET (number of scans, 128; receiver gain, 40 dB); and (e) with Broad Band WET method (number of scans, 128; receiver gain, 48 dB).S/N ratio and receiver gain (Rgain) are shown in the individual spectra.

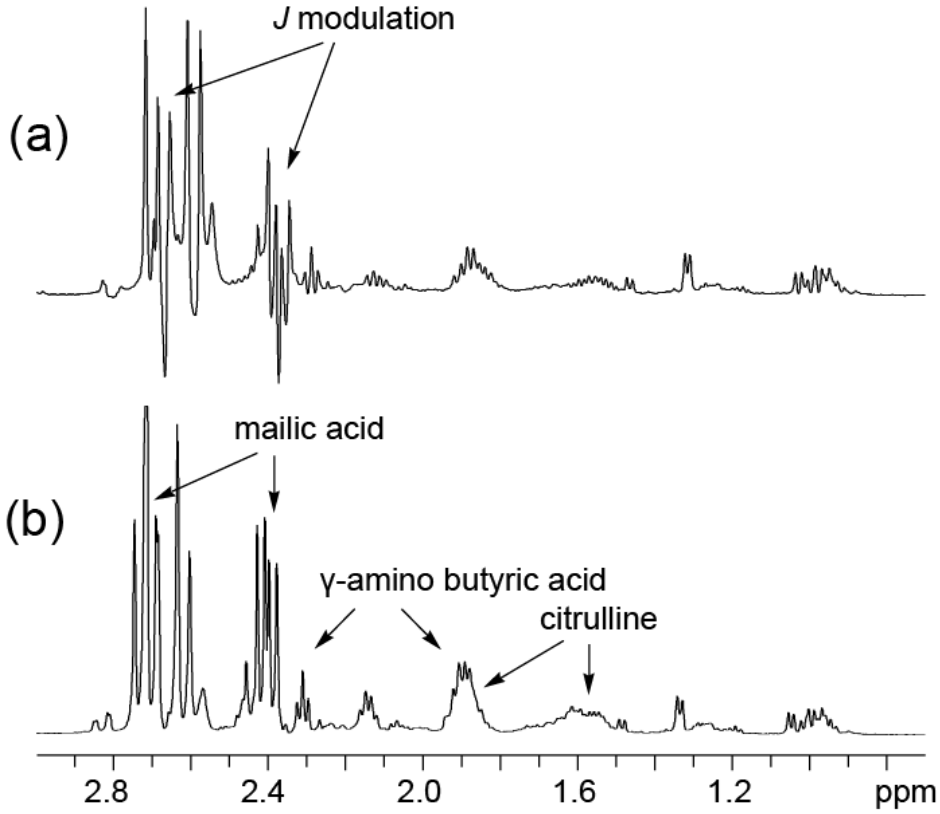


Figure 2-6 Expansion of the region from 0.7 ppm to 3.0 ppm of ^{1}H NMR spectra of persimmon fruit juice (a) with DPFGSE-WATERGATE and (b) with Broad Band WET.

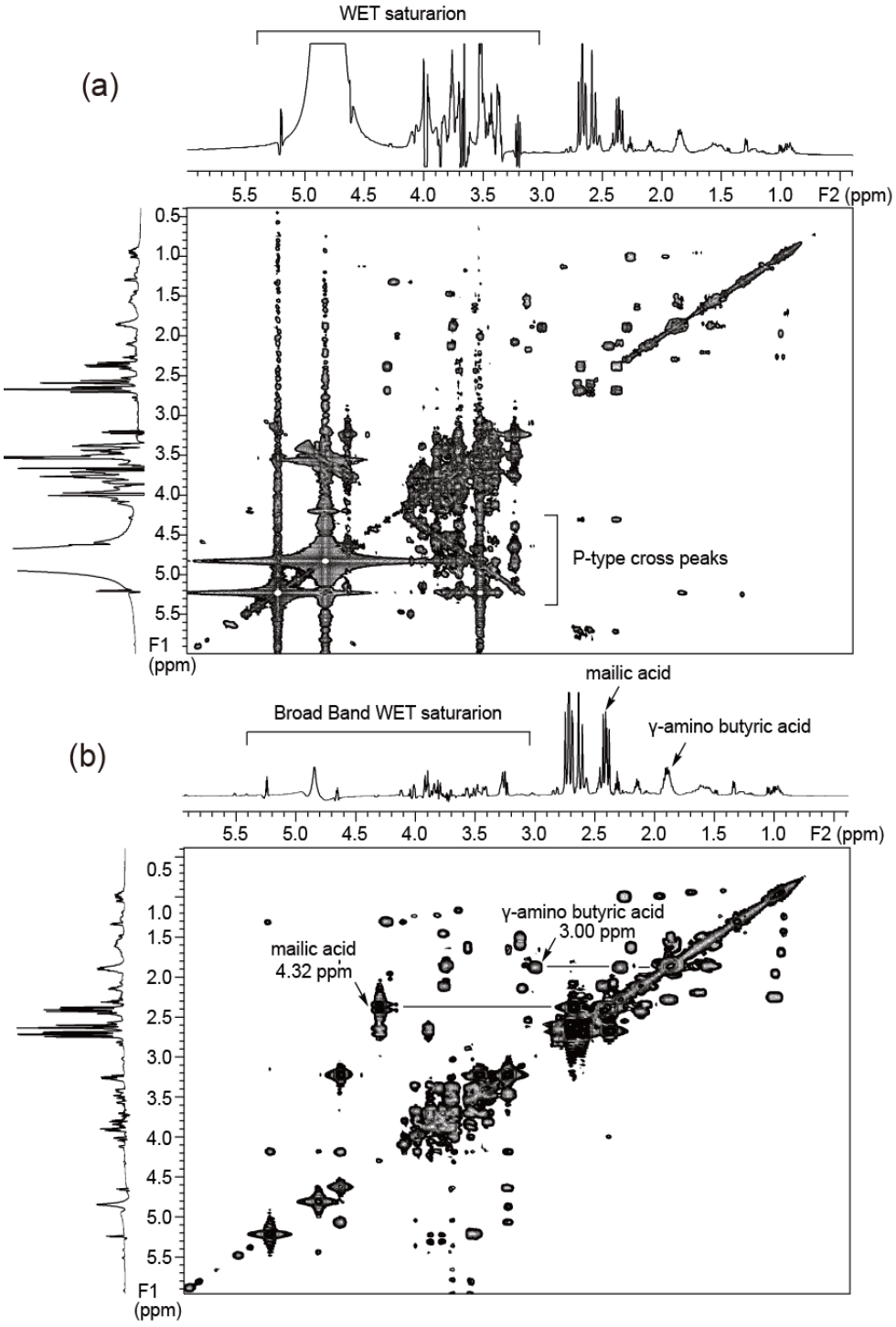


Figure 2-7 2D COSY spectra of persimmon fruit juice (a) with conventional WET-COSY and (b) with Broad Band WET-COSY.

Chapter 3

Spectral analysis of Japanese persimmon juice (Taishu cultivar)

3-1 Introduction

Over recent years, food composition studies have been developed into comprehensive metabolomic analysis, which is applied to many nutraceuticals and pharmaceutical foods and beverages.

Persimmon fruit (*Diospyros kaki* L.) is a popular and widely consumed fruit species in Japan, and about 1000 varieties are estimated to be grown in Japan (Veberic *et al*, 2010). Persimmon is generally recognized as an outstanding source of biologically active compounds related to both nutritional and pharmaceutical values and were traditionally used for medicinal purpose. Japanese persimmons are abundant in sugars (Del Bubba *et al*., 2009; Veberic *et al*., 2010). They are also good sources of vitamin C, phenolic compounds and carotenoids, which are usually considered as powerful antioxidants that protect against free radicals and prevent the risk of cardiovascular disease, diabetes and cancer (Giordani *et al*., 2011). However, there are only limited efforts to investigate the metabolites in persimmon fruit compared to other fruits, such as apple, tomato, mango and so on.

At our knowledge, a full high field NMR study for the metabolic profiling of Japanese persimmon has not been reported in literatures. Since most nutrients can be easily extracted into liquids, and that most chemical detection technologies are optimized for working with liquids, the majority of food-based metabolic profiling published to date have focused on liquid rather than an analysis of food solids (Wishart, 2008). In the present study, persimmon juice was prepared for analysis.

For the successful detection of minor components by suppressing the major components with Broad Band WET method, which was proposed in Chapter 2, in this chapter, we combined Broad Band WET method with ^{1}H NMR and various 2D NMR spectra to identify the components in Japanese persimmon juice. Both major components (sugars) and minor components (such as organic acid, amino acid, etc.) are also quantified simultaneously. This is the first time to accomplish a detailed assignment together with quantitative characterization of chemical components using NMR for Japanese persimmon.

3-2 Materials and Methods

3-2-1 Sample preparation

Fruits of cultivar “Taishu” were supplied by National Agriculture and Food Research Organization (Hiroshima, Japan) in the middle of November. The fruits were grounded into a pulp and then centrifuged at 13,000×g for 10 min at 4°C. The supernatant was used as persimmon juice. The juices were stored at−20°C until NMR measurements.

A final concentration of 1M and pH value of 6.5 phosphate buffer prepared with D_2O (99.9%, 70μl; Shoko Co., Ltd., Tokyo, Japan), was added to persimmon juice (630μl) for locking. TSP-d_4 was added to the sample at a final concentration of 0.1 mM as a reference for both chemical shift and quantitative determination. EDTA (Dojindo, Kumamoto, Japan) was added to the sample at a final concentration of 1.5 mM to capture the paramagnetic ions that cause a broadening of NMR signals. The mixture was transferred to a 5 mm NMR tube. Condensed persimmon juices were prepared with centrifugal thickener (Sakuma, Tokyo, Japan.).

3-2-2 NMR spectroscopy

NMR spectra were obtained at 20°C on a Varian Unity INOVA-500 spectrometer for the ^{1}H NMR, ^{1}H Broad Band WET, ^{13}C NMR, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H Broad Band WET TOCSY, ^{1}H-^{1}H DQF-COSY and ^{1}H-^{1}H Broad Band WET COSY spectra and on a Varian Unity INOVA-600 spectrometer equipped with a cryogenic probe for the ^{1}H-^{13}C CT-HMBC and WET-HMBC spectra.

The acquisition parameters of the ^{1}H NMR spectra were as follows: spectral width, 8,000 Hz; number of data points, 64K; acquisition time, 2.048 s; and delay time, 5.0 s. The water signal was suppressed by a presaturation method. The parameters of the ^{13}C NMR spectrum for the assignments were as follows: number of data points, 16 k; spectral width, 31,422 Hz; acquisition time, 1.043 s; delay time, 2.0 s; and number of scans, 52,896.

The acquisition parameters of Broad Band WET methods were as follows: spectral width, 8,000 Hz; number of data points, 64K; number of scans, 128; acquisition time, 2.048 s; and delay time, 5.0 s. The pulse length of e-Burp pulse in Broad Band WET

method was 3.88 ms.

The acquisition parameters of Broad Band WET COSY and Broad Band WET TOCSY were as follows: spectral width, 6,700 Hz (F1) and 6,700 Hz (F2); number of data points, 512 (F1) and 2,048 (F2); number of scans, 64; and the pulse length of e-Burp, 3.88 ms.

The acquisition parameters of total correlation spectroscopy (TOCSY) were as follows: spectral width, 6,700 Hz (F1) and 6,700 Hz (F2), number of data points, 512 (F1) and 2,048 (F2); number of scans, 32; acquisition time, 0.341s; delay time, 2.0s; field strength of MLEV-17 spin-lock pulse, 7.1 kHz; length of trim pulse, 2 ms; and mixing time, 80 ms.

The acquisition parameters of double-quantum filtered correlation spectroscopy (DQF-COSY) were as follows: spectral width, 6,000 Hz (F1) and 6,000 Hz (F2); number of data points, 512 (F1) and 2,048 (F2); number of scans, 16; acquisition time, 0.341 s; and delay time, 2.0 s.

The acquisition parameters of ^{1}H–^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC) were as follows: spectral width, 6,000 Hz (^{1}H) and 20,742 Hz (^{13}C); number of data points, 512 (F1) and 2,048 (F2); number of scans, 256; acquisition time, 0.341 s; and delay time, 2.0 s.

The acquisition parameters of ^{1}H–^{13}C constant-time heteronuclear multiple-bond correlation spectroscopy (CT-HMBC) (Furihata *et al*., 1998) were as follows: spectral width, 6,000 Hz (^{1}H) and 28,902 Hz (^{13}C); number of data points, 512 (F1) and 2,048 (F2); number of scans,256; acquisition time,0.341s; and delay time, 2.0 s.

3-2-3 NMR data processing and signal assignment

All ^{1}H NMR spectra were manually phased, baseline corrected, and referenced to TSP at 0.00 ppm. Signals were essentially assigned according to the previously described method. The signals in ^{1}H and ^{13}C NMR spectra were tentatively assigned and then verified using different types of 2D NMR spectra as follows: The spin systems were confirmed by the ^{1}H-^{1}H DQF-COSY NMR spectrum; the ^{1}H-^{13}C CT-HMBC NMR spectrum was used to confirm the connections of quaternary carbons to protons through two- or three-bond couplings; and the ^{1}H-^{13}C HSQC NMR spectrum was used to find

the correlations between protons and their neighboring carbons. The ^{13}C signals of minor components were identified by ^{13}C NMR spectra acquired by using 10 mm probe and 2D NMR spectra combined with Broad Band WET or WET sequence (Broad Band WET TOCSY, Broad Band WET COSY and WET HMBC). Finally, the assignments were compared to the data given in several online databases for metabolomics, such as HMDB (Wishart *et al*., 2009), BMRB (Ulrich *et al*., 2008) and MMCD (Cui *et al*., 2008), and in papers on other fruit juice and several candidate components were picked out. Finally, signal assignment was accomplished by spiking authentic standard compounds (special grade; Wako Pure Chemical Industries, Ltd., Osaka, Japan, or Nacalai Tesque Inc., Tokyo, Japan) into the juice and comparing the spectra of the resulting mixtures with those of plain juice.

3-2-4 Quantification of persimmon juice components

In order to obtain sufficient precision in the quantitative analysis, the delay time (d1) should satisfy the following equation.

$$d1 \geq 5 \times T_1 - aq$$

where aq is acquisition time and T_1 is spin-lattice relaxation times. The T_1 values of sugars were measured by the inversion recovery experiments. The d1 of minor components were determined with arrayed d1.

3-3 Results and Discussion

3-3-1 ^{1}H NMR spectra of persimmon juice and signal assignments

The ^{1}H NMR spectrum of persimmon juice (Taishu cultivar) is shown in Figure 3-1(a). The spectrum exhibits a characteristic pattern for Japanese persimmon juice. Because sugars are the most abundant constituents in Japanese persimmon, the spectrum are dominated the strong proton resonances from sugars, which were observed in the region from 3.0 to 5.6 ppm. By means of 2D ^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C CT-HMBC spectra, proton signals in this region were assigned to *α*-glucopyranose, *β*-glucopyranose, *β*-fructopyranose, *α*-fructofuranose and *β*-fructofuranose. Sucrose was also identified by spiking standard compound because of its very low concentration. These are the main sugars in mature fruit of persimmons, which are in agreement with the results reported in some previous papers (Candir *et al*., 2009; Del bubba *et al*., 2009; Veberic *et al*., 2010). However, Ittah *et al*. reported that persimmon contain mainly sucrose and much less glucose and fructose (Ittah *et al*., 1993). These different results on the composition of sucrose are considered to depend on the extraction method adopted. Whether the inhibition procedure for invertase activity has been conducted lead to very different results. (Giordani *et al*., 2011). Therefore, the low content of sucrose in this study may due to the sample preparation without inhibition procedure for invertase activity. Galactose and arabinose have been reported by other groups (Senter *et al*., 1991). However, in the present study, no galactose and arabinose were detected in the persimmon juices.

As mentioned in chapter 2, the resonances from sugars were strongly observed in the ^{1}H NMR spectrum of persimmon juice because of their high concentrations. As a result, signals in high-field region (0.5–3.0 ppm) were detected with relatively poor S/N ratio, and few signals were observed in the low-field region (6.0–11.0 ppm). In order to observe the resonances of minor components in persimmons, Broad Band WET method were incorporated in 1D and several 2D NMR measurement. The typical Broad Band WET spectrum of persimmon fruit juice was shown in Figure 3-1(b). Owing to the successful suppression of both water and sugars, minor components in the high-field region were observed with a good S/N ratio and many peaks were observed in region from 6.0 ppm to 11.0 ppm.

The signals in the region from 0.5 ppm to 3.0 ppm were derived from protons of free amino acids (alanine, aspartic acid, citrulline, 4-aminobutanoic acid (GABA), glutamine, glutamate, isoleucine, leucine, valine and threonine) and organic acids (malic acid and citric acid) as shown in Figure 3-1c. The signals at the region from 6.0 ppm to 11.0 ppm was considered to be derived from the aromatic protons of amino acids, fumaric acid, uridine, adenosine, trigonelline, acetaldehyde and unknown singlet signals assumed to derive from polyphenols (Figure 3-1d). In this region, the broad signals were assigned to the amino protons from glutamine and confirmed by spiking the standard compound. The assignments of the ^{1}H NMR signals are summarized in Table 3-1.

The unknown signals in the region from 7.1 ppm to 7.5 ppm are assumed to be derived from polyphenols in persimmon juice. Total phenols were reported to be higher in whole persimmons than in whole apples (Gorinstein *et al*., 2001). However, since the polyphenols contained in non-astringent persimmons exist in a form of insoluble tannins which highly polymerized in different degree. Therefore, I presume that these signals might derive from terminal units of tannin, and the high molecular weight made the compounds difficult to be identified by NMR spectroscopy.

The addition of EDTA relieved the broadening of NMR signals for citric acid and malic acid, which implied paramagnetic ions bind to these two organic acids. The concentrations of Mg (8.22 mg/100g), Fe (101.4μg/100g), Mn (107.1μg/100g), and other minerals in whole persimmons were reported to be sufficiently high to induce the binding (Gorinstein *et al*., 2001).

3-3-2 ^{13}C NMR spectrum of persimmon juice

The ^{13}C NMR spectrum of persimmon juice is shown in Figure 3-2a. ^{13}C signals of sugars were observed in the region from 60 ppm to 110 ppm. In contrast, the signals from the minor components cannot nearly be detected in both high-field region and low-field region because of the low sensitivity of ^{13}C NMR, which made the assignment of minor components difficult. To resolve this problem, the 10 mm probe was applied to acquire ^{13}C NMR spectrum, combined with concentrated persimmon juice. The acquired spectrum is shown in Figure 3-2b. The signals from minor components were observed clearly by the utilization of 10 mm probe and the persimmon juice

concentrate.

The signals in the ^{13}C NMR spectrum were assigned by utilization of 2D ^{1}H-^{13}C HSQC, CT-HMBC and WET-HMBC spectra. The signals in the region from 10 to 60 ppm were assigned to the primary carbon atoms and the secondary carbon atoms from organic acids, amino acids, and ethanol and so on. In the region from 60 to 110 ppm the signals of tertiary carbon atoms were assigned to be deriveed from aliphatic rings of glucose and fructose. The signals in the low-field region from 110 ppm to 220 ppm were assigned to the aromatic ring of phenylalanine and the carbonyl group (quaternary carbon) atoms of amino acids and organic acids. Assignment of the ^{13}C NMR signals is summarized in Table 3-1 and Figure 3-3.

3-3-3 2D NMR spectra of persimmon juice

Figure 3-4 shows the 2D conventional ^{1}H-^{1}H TOCSY and Broad Band WET-TOCSY spectra of persimmon juice. TOCSY spectra show correlation signals between hydrogen nuclei belong to the same spin system. In the conventional TOCSY spectrum, the correlation peaks of the minor components in the low-field region were not detected, and few peaks were observed in the high-field region. With lowering of threshold, many noises were observed which are considered to be derived from the strong signals of water and sugars. These peaks made it difficult to observe the cross peaks for minor components. As shown in Figure 3-4b, the signal intensities of both water and sugars were suppressed by Broad Band WET sequence and a high-quality TOCSY with a clean background was obtained. Thus the cross peaks for the minor components were clearly observed in the Broad Band WET-TOCSY spectrum. Besides that, in the F2 region observed with strong sugar-signals (3.0- 5.4 ppm), the cross peaks of minor components were also observed owing to the suppression of sugar signals. For example, the cross peaks from α and β-protons of valine and leucine are overlapped by strong signals of sugars in the conventional TOCSY spectrum. This advantage greatly facilitated the assignment of minor components.

Conventional 2D ^{1}H-^{13}C CT-HMBC spectrum and WET-HMBC spectrum were also compared in Figure 3-5. Both of them were measured with a Varian Unity INOVA-600 spectrometer equipped with a cryogenic probe. The HMBC spectrum was used to confirm the connections of quaternary carbons to protons through two- or three-bond

couplings, which is represented as cross-peaks. Conventional ^{1}H-^{13}C CT-HMBC spectrum provided good-quality signals in the high-field region (from 0.5 ppm to 3.0 ppm) in F2. However, the ghost signals, which are considered to be derived from strong signals, were observed in the low-field region (from 5.5 ppm to 7.0 ppm in F2). These signals significantly perturbed the observation of signals from minor components in the low-field region. With the incorporation of WET sequence to HMBC, high-quality spectrum was obtained with clearly observation of signals of minor components. The signals assigned to carboxyl group of some minor components are also shown in Figure 3-6.

In Figure 3-7, the signals in the region between 7.0 ppm to 7.8 ppm at TOCSY, COSY and HMBC spectra were assigned to aromatic rings of tryptophan and phenylalanine. Because of the very low concentration, other minor components, such as adenosine, uridine and trigonelline, were identified by means of matching the chemical shift of cross peaks to those of databases (HMDB and SDBS) or referring to previous papers. The final confirmation was performed by adding the corresponding standard compounds.

3-3-4 Quantitative determination of components in persimmon juice by ^{1}H NMR spectroscopy

Among the observed signals, separated signals were chosen for the integration of peak area. The signal overlap with neighboring resonances was also integrated by using curve deconvolution. TSP was added at a final concentration of 0.1 mM into each NMR sample tube as a concentration standard. The concentration of each component was determined, and the results are summarized in Figure 3-8 and Table 3-2. In this study, the major components (glucose and fructose) were quantified using ^{1}H NMR spectra, and the minor components were quantified using Broad Band WET spectra. The standard deviations of triplicate measurements for each sample were shown in Table 3-2. The P values of the intra-sample shown in Table 3-3 indicate a good reproducibility of measurements.

Sugars accounted for the greatest portion of components in persimmon juice, and β-glucose was the main constituent of sugars. The proportions for β-glucose and α-glucose were 64% and 36%, respectively, which are consistent with the stable ratio

for glucose at equilibrium in solution. Because the anomeric proton signal of sucrose was overlapped with a doublet unassigned signal or almost undetectable in some samples, sucrose was not quantified. Glucose/fructose concentration ratio was about 4:1 in this study, which are different to the results of other reports. The result is considered as a consequence of no inhibition of invertase activity.

Three organic acids were identified and quantified. The predominant organic acids were malic acid followed by citric acid. Lesser amount of fumaric acid was also found. The result is consistent with published data (Senter *et al*., 1991; Veberic *et al*., 2010). Variations in sugars and organic acid content are considered to be important in assessing persimmon fruit quality (Senter *et al*., 1991).

There is very few report concerning on the concentration of other minor components, such as amino acids in Japanese persimmon. The reason might be that most researches were focused on some presumed antioxidants such as vitamin C, carotenoids and polyphenols. In the results of this study, the concentrations of amino acids were relatively low comparing with organic acids. The most abundant amino acid was citrulline, which is a precursor to arginine and has been used for cardiovascular disease, reduction of muscle fatigue, etc. (Tarazona-Diaz *et al*., 2013). This result indicates that Japanese persimmon might be a good source of citrulline in addition to watermelon. Various other amino acids were also quantified; GABA and glutamine were found relatively high comparing with other amino acids. Furthermore, low concentrations of trigonelline, uridine and ethanol are also quantified.

In conclusion, the identification and quantification of chemical compounds in persimmon juice (Taishu cultivar.) by one-dimensional (1D) NMR and various 2D NMR spectroscopy was carried out in this Chapter. Because the high concentrations of major components in persimmon juice made the assignment of minor components very difficult, 2D NMR spectroscopy incorporating with Broad Band WET sequence or WET sequence were devised and 10 mm probe was used to acquire ^{13}C NMR spectra. With those attempts, the signals from many minor components were observed clearly, and 26 compounds were identified. In addition, most components were quantified by the integration of signals using conventional NMR and Broad Band WET spectra. The results provided a comprehensive metabolic profile for Japanese persimmon juice with NMR spectroscopy, and presented a useful method in identification of minor

components in fruit juice, with the aid of 1D and 2D NMR cooperating with methods of suppression (Broad Band WET or WET method) selectively on strong signals.

Table 3-1. Summary of the metabolites identified in the Persimmon juices.

Compound	Assignment	Chemical shift (ppm)			Multiplicity: J(Hz)
		^{1}H	^{13}C	^{13}C(HMBC or HSQC)	
Acetaldehyde	2-CH_3	2.23			
	1-CH	9.67			
Adenosine	2-CH	8.31			
	6-CH_3	6.06			
	8-CH	4.43			
	10-CH_2	3.78			
Alanine(Ala)	α-CH	3.78		53.93	
	β-CH_2	1.47		19.33(HSQC)	d:7.0
	COOH			179.39	
Aspartic acid(Asp)	α-CH	3.9	54.89		dd:9.3
	β-CH_2	2.66, 2.81	39.13		dd:2.9, 17.6
	γ-COOH			177.37	
	COOH			180.89	
Citric acid	1-COOH			181.54	
	2-CH_2	2.61,2.72		47.97	d:15.6
	3-C			78.42	
	6-COOH			184.12	
Citrulline	α-CH	3.75		58.22	
	β-CH_2	1.86		30.72	
	γ-CH_2	1.51,1.57		28.57	
	δ-CH_2	3.13		42.07(HSQC)	q:6.2
	ε-NH	6.38			
	COOH			178.1	
	$CONH_2$			164.8	
Ethanol	1-CH_2	3.66		60.8	t:7.0
	2-CH_3	1.17		19.74(HSQC)	
Fumaric acid	2-CH	6.52			
	3-CH_2	6.52			
GABA	α-CH_2	2.29		37.6	t:7.8

(to be continued)

	β-CH_2	1.89	26.86	
	γ-CH_2	3	42.75	t:7.9
	COOH		184.55	
Glutamate(Glu)	α-CH	3.75	58.22	
	β-CH_2	2.05,2.11	29.73	
	γ-CH_2	2.35	36.74	
	δ-COOH		184.55	
	COOH		177.37	
Glutamine (Gln)	α-CH	3.78	57.79	
	β-CH_2	2.12	29.43	
	γ-CH_2	2.43	34.16	
	ε-NH_2	6.92,7.62		
	δ-$CONH_2$		181.11	
	COOH		177.25	
Isoleucine (Ile)	α-CH	3.66	62.95	
	β-CH	1.98		
			39.32	
	γ-CH_2	1.24, 1.47	27.72	
	δ-CH_3	0.93		t:7.8
	γ`-CH_3	1	17.43 (HSQC)	d:7.8
Leucine (Leu)	α-CH	3.7		
	β-CH_2	1.73-1.66	43.18	
	γ-CH	1.71	27.28	
	δ-CH_3	0.95	24.11 (HSQC)	
	δ`-CH_3	0.95	25.35	
Malic acid	1-C		184.12	
	2-CH	4.31	73.69	
	3-CH_2	2.39,2.69	45.47	
	4-C		182.83	
Phenylalanine(Phe)	1-C		138.15	
	2,6-CH	7.32	132.56	d:7.4
	3,5-CH	7.42	132.24	
	4-CH	7.36	130.84	
	7-C		39.75	
Threonine (Thr)	α-CH	3.59	63.8	

(to be continued)

	β-CH	4.24			
				69.39	
	γ-CH_3	1.31		22.77(HSQC)	
	COOH	-	-	-	
Trigonelline	1-CH	9.12			
	3-CH	8.84			
	4-CH	8.09			
	5-CH	8.84			
Tryptophan(Trp)	1-CH	7.31		128.29(HSQC)	
	2-C			111.07	
	3-C			129.98	
	4-C			139.86	
	5-CH	7.52		115.37	
	6-CH	7.73		121.82	
	7-CH	7.19		122.68	
	8-CH	7.26		125.25	
	NH	10.2			
Uridine	1-CH	5.9			
	2-CH	4.34			
	3-CH	4.24			
	6-CH	5.9			
	7-CH	7.87			d:7.8
Valine(Val)	α-CH	3.61		63.81	
	β-CH	2.27		32.44	
	γ-CH_3	1.02		21.27	d:7.4
	γ'-CH_3	0.97		19.98	d:7.3
	COOH			176.82	
α-D-glucopyranose	1-CH	5.22	94.74		
	2-CH	3.52	74.16		
	3-CH	3.71	75.49		
	4-CH	3.38	72.33		
	5-CH	3.82	74		
	6-CH_2	3.76, 3.83	63.28		
β-D-glucopyranose	1-CH	4.64	98.56		
	2-CH	3.23	76.82		

(to be continued)

	3-CH	3.44	78.47
	4-CH	3.38	72.33
	5-CH	3.41	78.47
	6-CH_2	3.72,3.89	63.45
β-D-fructopyranose	1-CH_2	3.54, 3.69	66.7
	2-CH		100.6
	3-CH	3.78	70.36
	4-CH	3.85	72.36
	5-CH		
	6-CH_2	3.99,4.02	66.04
α-D-fructofuranose	1-CH_2	3.64	
	2-CH		107.78
	3-CH	4.09	84.79
	4-CH	3.99	78.74
	5-CH	4.05	83.97
	6-CH_2		
β-D-fructofuranose	1-CH_2	3.53,3.58	65.39
	2-CH		104.19
	3-CH	4.1	77.18
	4-CH	4.1	78.02
	5-CH	3.82	83.29
	6-CH_2		
Sucrose	1-CH	5.4	

Table 3-2 Concentrations of components in persimmon juices quantified by Broad Band WET and ^{1}H NMR spectra.

Components	Concentrations (mM)[a]										
	No[c].1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	Average±SD[b]
Leucine	0.178±0.004	0.391±0.035	0.707±0.059	0.169±0.017	0.277±0.011	0.571±0.016	0.284±0.025	0.048±0.006	0.137±0.023	0.999±0.088	0.376±0.300
Isoleucine	0.117±0.005	0.162±0.030	0.343±0.023	0.126±0.013	0.147±0.021	0.270±0.015	0.165±0.007	0.032±0.005	0.098±0.005	0.316±0.042	0.178±0.104
Valine	0.305±0.011	0.443±0.008	0.783±0.020	0.317±0.000	0.376±0.007	0.700±0.025	0.410±0.002	0.097±0.004	0.262±0.002	0.930±0.012	0.462±0.270
Ethanol	0.065±0.009	0.045±0.004	0.095±0.002	0.148±0.009	0.078±0.007	0.042±0.001	0.063±0.013	0.082±0.006	0.127±0.009	0.280±0.003	0.102±0.067
Threonine	0.281±0.016	0.303±0.004	0.482±0.004	0.257±0.009	0.237±0.003	0.385±0.005	0.340±0.005	0.134±0.000	0.217±0.003	0.586±0.008	0.322±0.138
Alanine	0.108±0.012	0.119±0.013	0.179±0.003	0.129±0.006	0.115±0.006	0.155±0.002	0.116±0.019	0.075±0.006	0.102±0.001	0.220±0.005	0.132±0.043
Citrulline	1.994±0.036	2.155±0.085	2.592±0.109	2.418±0.145	1.547±0.033	2.259±0.178	2.474±0.068	1.269±0.053	1.691±0.099	3.763±0.289	2.216±0.717
Glutamine	0.685±0.048	0.903±0.042	1.311±0.035	0.899±0.032	0.715±0.040	1.017±0.091	0.741±0.019	0.448±0.014	0.633±0.037	1.514±0.110	0.887±0.334
GABA	0.540±0.008	0.371±0.010	0.427±0.033	0.583±0.038	0.299±0.014	0.510±0.034	0.741±0.010	0.551±0.012	0.580±0.023	0.751±0.050	0.535±0.137
Malic acid	3.874±0.076	5.295±0.226	7.563±0.198	5.312±0.292	6.527±0.145	6.187±0.451	4.684±0.024	5.399±0.087	6.783±0.379	7.181±0.506	5.881±1.114
Citric acid	3.925±0.125	4.577±0.046	3.927±0.083	5.339±0.062	4.666±0.085	5.951±0.023	5.049±0.018	4.493±0.060	6.577±0.059	4.498±0.023	4.900±0.816
Aspartic acid	0.321±0.016	0.284±0.019	0.387±0.008	0.305±0.013	0.370±0.012	0.331±0.027	0.282±0.011	0.330±0.008	0.378±0.025	0.378±0.020	0.337±0.038
Fumaric acid	0.082±0.003	0.072±0.002	0.118±0.010	0.073±0.004	0.056±0.003	0.084±0.013	0.113±0.006	0.093±0.005	0.135±0.010	0.139±0.009	0.096±0.027
Phenylalanine	0.135±0.010	0.166±0.007	0.300±0.009	0.122±0.010	0.138±0.001	0.250±0.021	0.100±0.003	0.034±0.004	0.077±0.003	0.272±0.013	0.159±0.091
Tryptophan	0.077±0.007	0.100±0.013	0.164±0.015	0.084±0.006	0.096±0.006	0.144±0.026	0.087±0.018	0.046±0.008	0.081±0.003	0.185±0.002	0.107±0.045
Uridine	0.050±0.013	0.050±0.007	0.079±0.008	0.066±0.004	0.052±0.005	0.076±0.009	0.070±0.003	0.080±0.008	0.070±0.003	0.061±0.012	0.065±0.012
Trigonelline	0.026±0.003	0.000±0.000	0.031±0.002	0.030±0.001	0.033±0.002	0.035±0.003	0.020±0.003	0.042±0.003	0.035±0.000	0.032±0.004	0.012±0.013
α-glucose	202.9±14.5	245.6±20.9	298.2±37.1	216.8±12.3	296.5±5.9	276.9±15.9	244.7±33.0	262.0±25.3	342.2±41.9	240.8±16.6	262.7±39.6
β-glucose	364.5±26.1	434. 8±35.7	528.4±61.1	389.1±22.3	529.2±9.9	491.8±25.9	439.5±60.9	471.8±46.8	611.9±77.9	430.1±27.7	469.1±70.0
Fructose	128.9±9.2	150.1±12.6	191.2±23.0	138.2±7.4	183.0±4.1	179.6±9.2	157.3±21.9	165.7±15.0	218.5±27.9	157.9±10.4	167.1±25.3

a Values expressed by average of the triplet measurements of the same sample ± standard deviation (n = 3)

b Values expressed by average of three separate samples ± standard deviation (n = 10)

c No.1~No.10 means the number of the 10 different samples

Table 3-3 Results of intra-sample sample and inter-sample variability by ANOVA analysis.

	intra-sample		inter-sample	
	P-values	*F*-values	*P*-values	*F*-values
No.1	0.110	3.245	0.016	1.935
No.2	0.109	3.245		
No.3	0.115	3.245		
No.4	0.099	3.245		
No.5	0.149	3.245		
No.6	0.116	3.245		
No.7	0.114	3.245		
No.8	0.104	3.245		
No.9	0.114	3.245		
No.10	0.105	3.245		

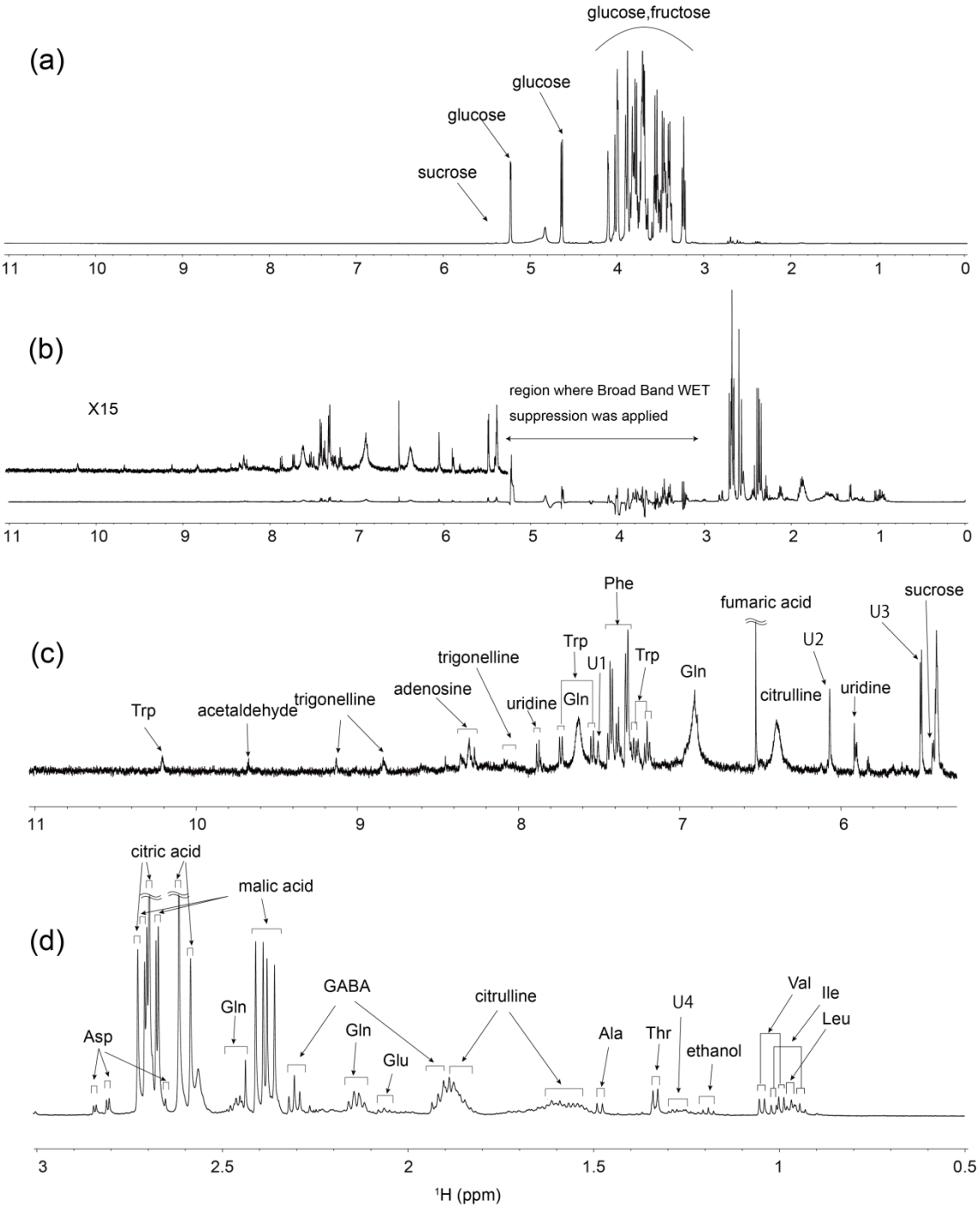


Figure 3-1 Assignment of ^{1}H NMR spectra of persimmon juice (Taishu cultivar). **(a)** ^{1}H NMR spectrum. **(b)** Broad Band WET spectrum. **(c)** Expansion of the Broad Band WET spectrum from 5.4 to 11.0 ppm. **(d)** Expansion of the Broad Band WET NMR spectrum from 0.5 to 3.0 ppm.

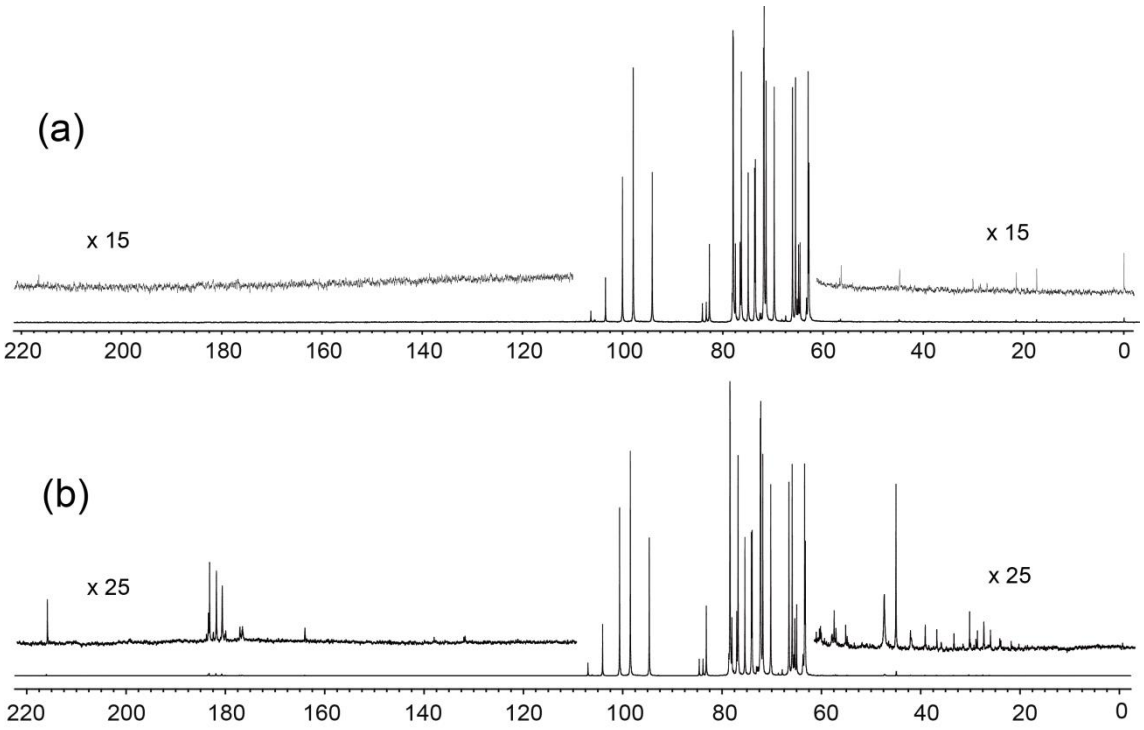


Figure 3- 2 Comparison of the ^{13}C NMR spectra of persimmon juice (Taishu cultivar) measured with **(a)** 5 mm probe and **(b)** 10 mm probe.

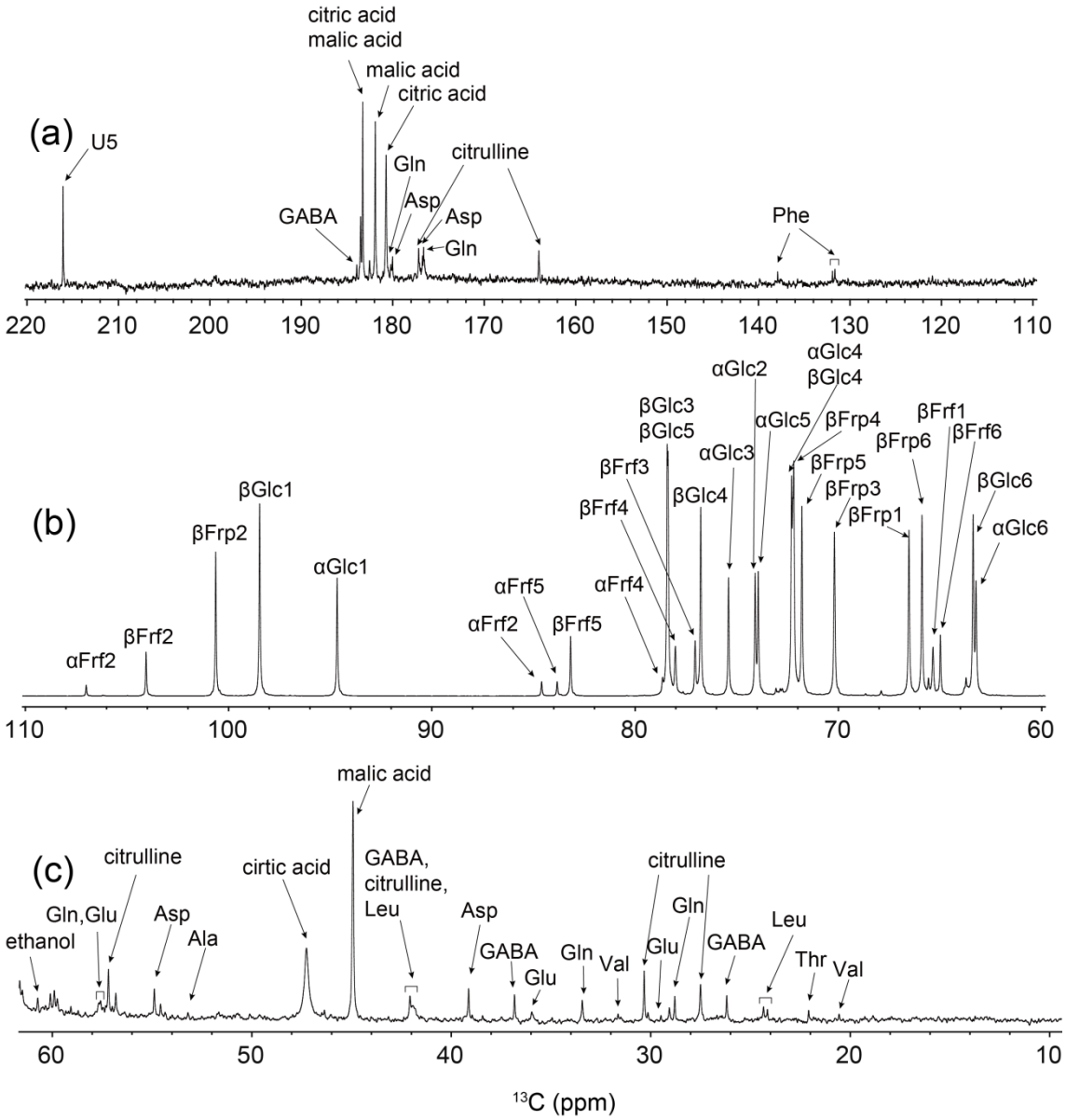


Figure 3-3 Assignment of ^{13}C NMR spectra of persimmon juice (Taishu cultivar). **(a)** Expansion of the ^{13}C NMR spectrum from 110 to 220 ppm. **(B)** Expansion of the ^{13}C NMR spectrum from 60 to 110 ppm. **(C)** Expansion of the ^{13}C NMR spectrum from 10 to 60 ppm.

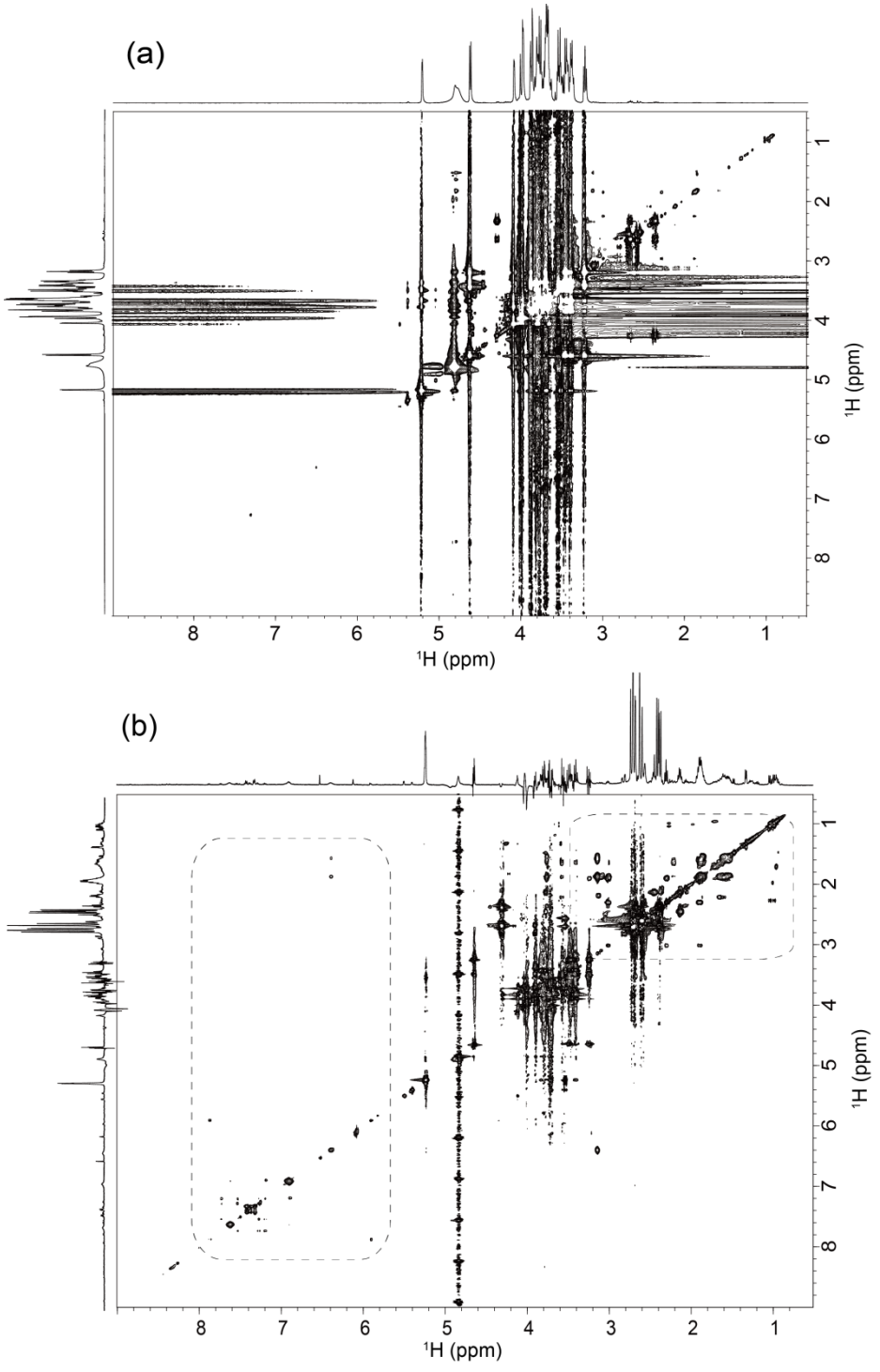


Figure 3- 4 Comparison of the TOCSY spectra of persimmon juice (Taishu cultivar) measured **(a)** without suppression of sugar region and **(b)** with suppression of water and sugar region using Broad Band WET sequence.

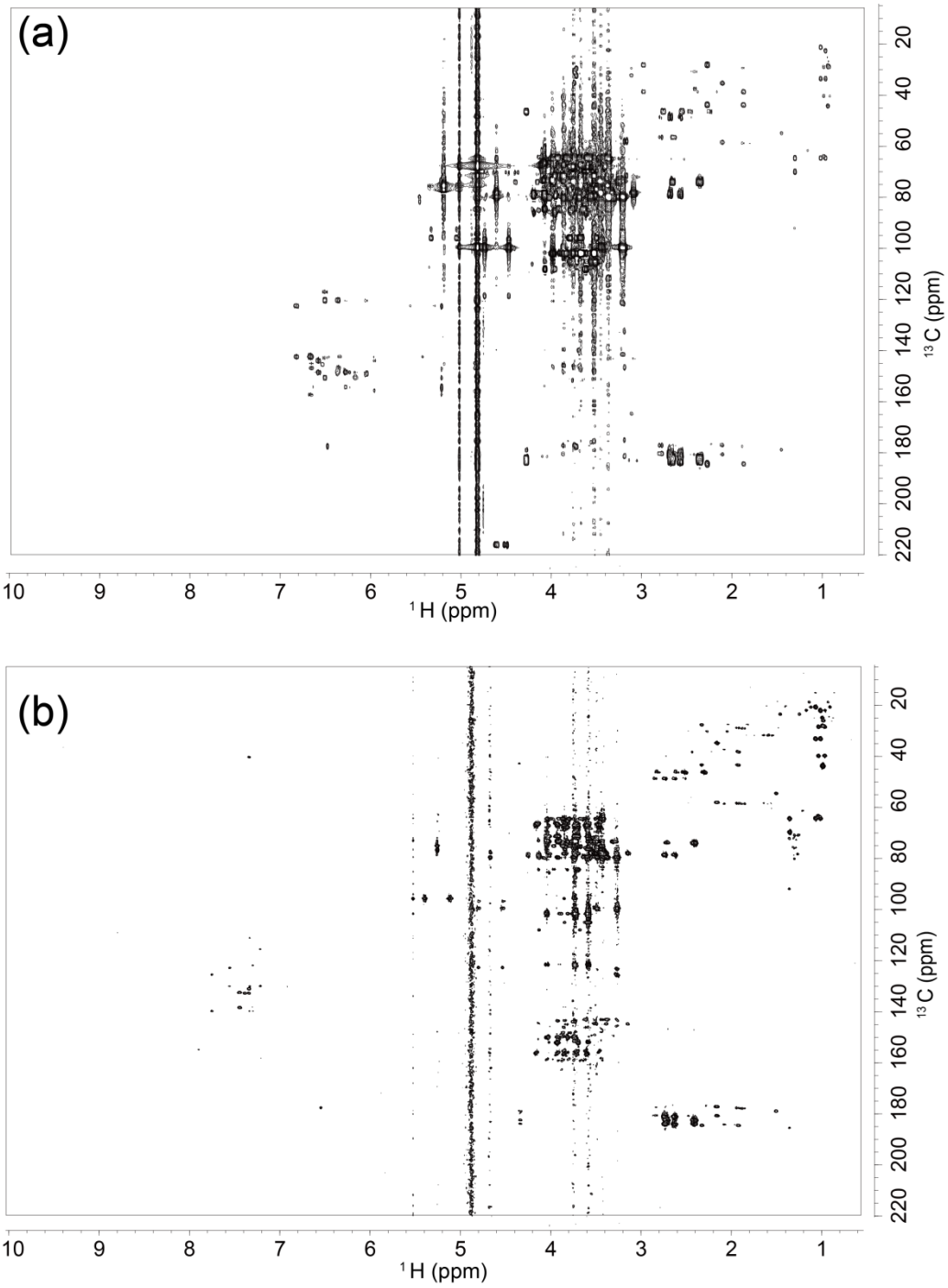


Figure 3-5 Comparison of the HMBC spectra of persimmon juice (Taishu cultivar) measured **(a)** without suppression of sugar region and **(b)** with suppression of water and sugar region using Broad Band WET sequence.

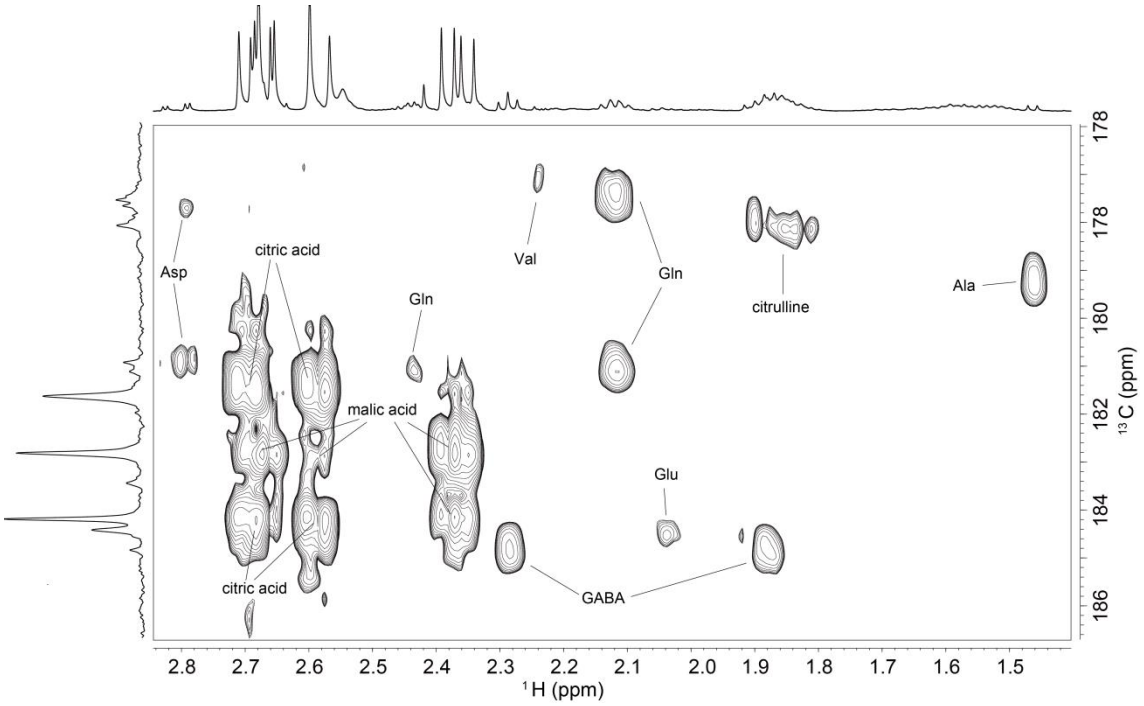
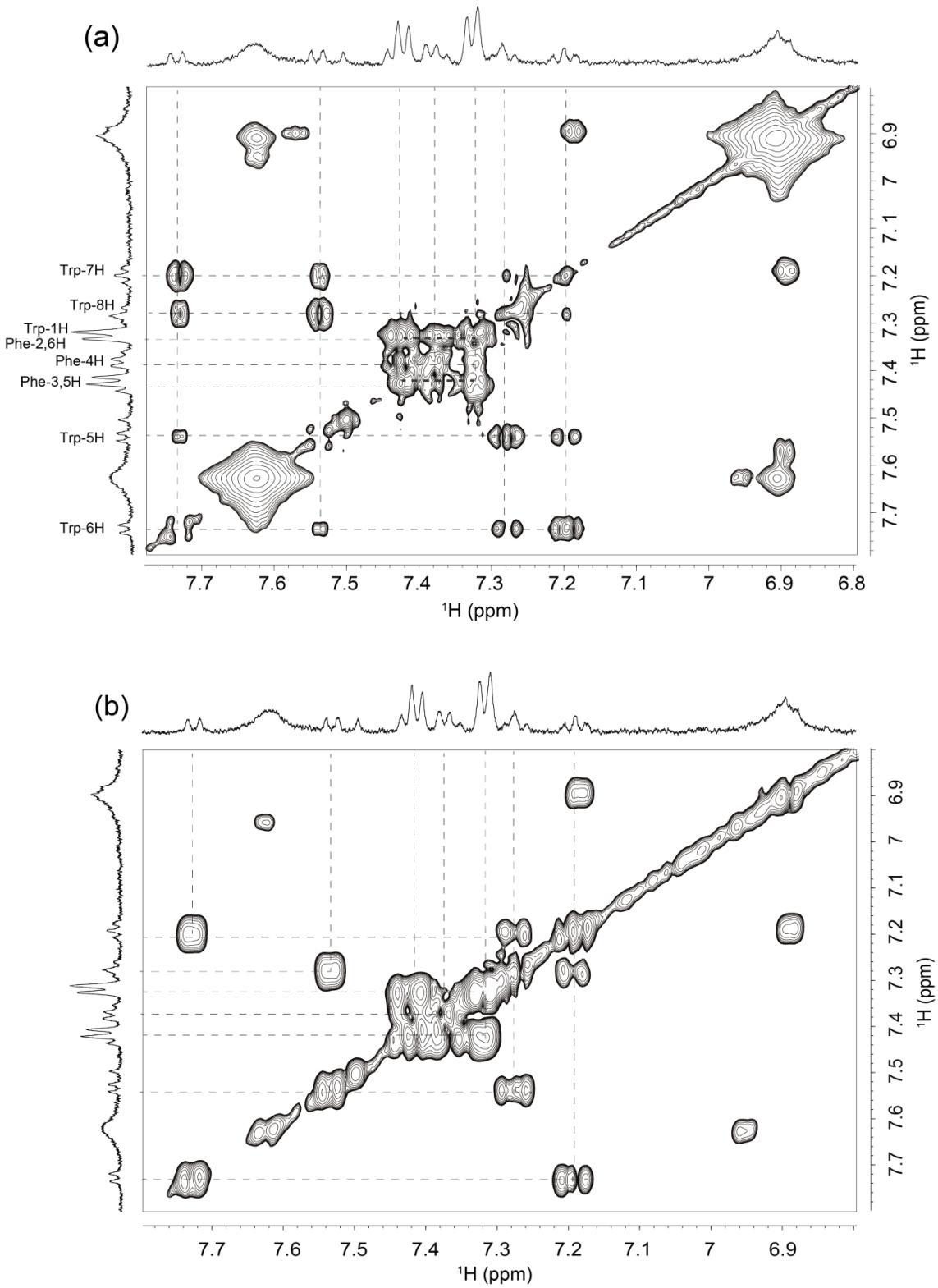


Figure 3-6 Expansion of WET-HMBC spectrum for signals assigned to carboxyl group of minor components.


(a)
Trp-7H
Trp-8H
Trp-1H
Phe-2,6H
Phe-4H
Phe-3,5H
Trp-5H
Trp-6H
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7
6.9
6.8
¹H (ppm)
(b)
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7
6.9
¹H (ppm)

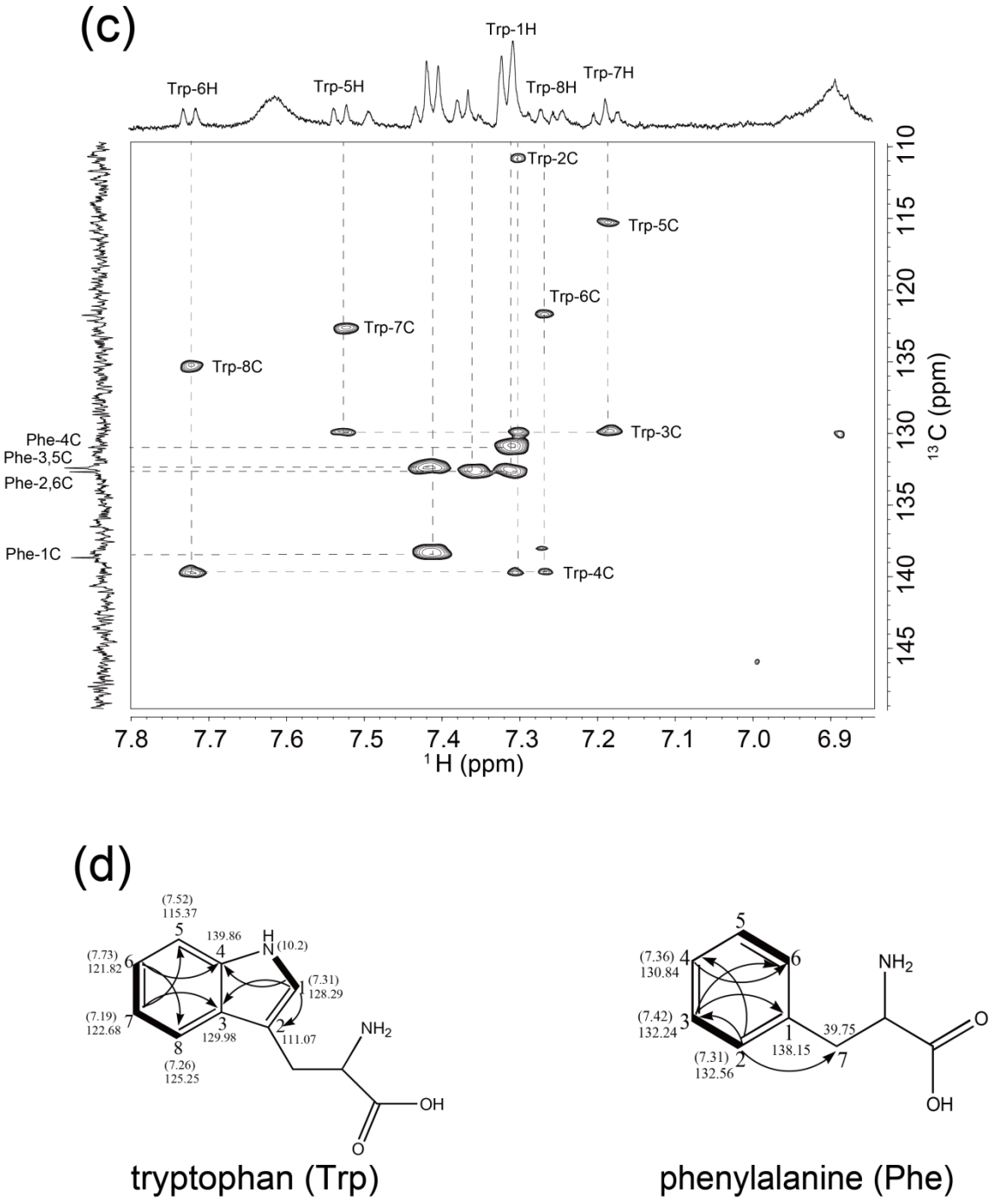


Figure 3-7 Expansion of Broad Band WET-TOCSY **(a),** Broad Band WET-COSY **(b)** and WET-HMBC spectra **(c)** of persimmon juice (Taishu cultivar) showing the assignment of the aromatic ring of tryptophan and phenylalanine. The bold-bonds indicate the possible correlations in the ^{1}H-^{1}H Broad Band WET-COSY spectrum. The arrows indicate the possible correlations in the ^{1}H-^{13}C WET-HMBC spectrum.

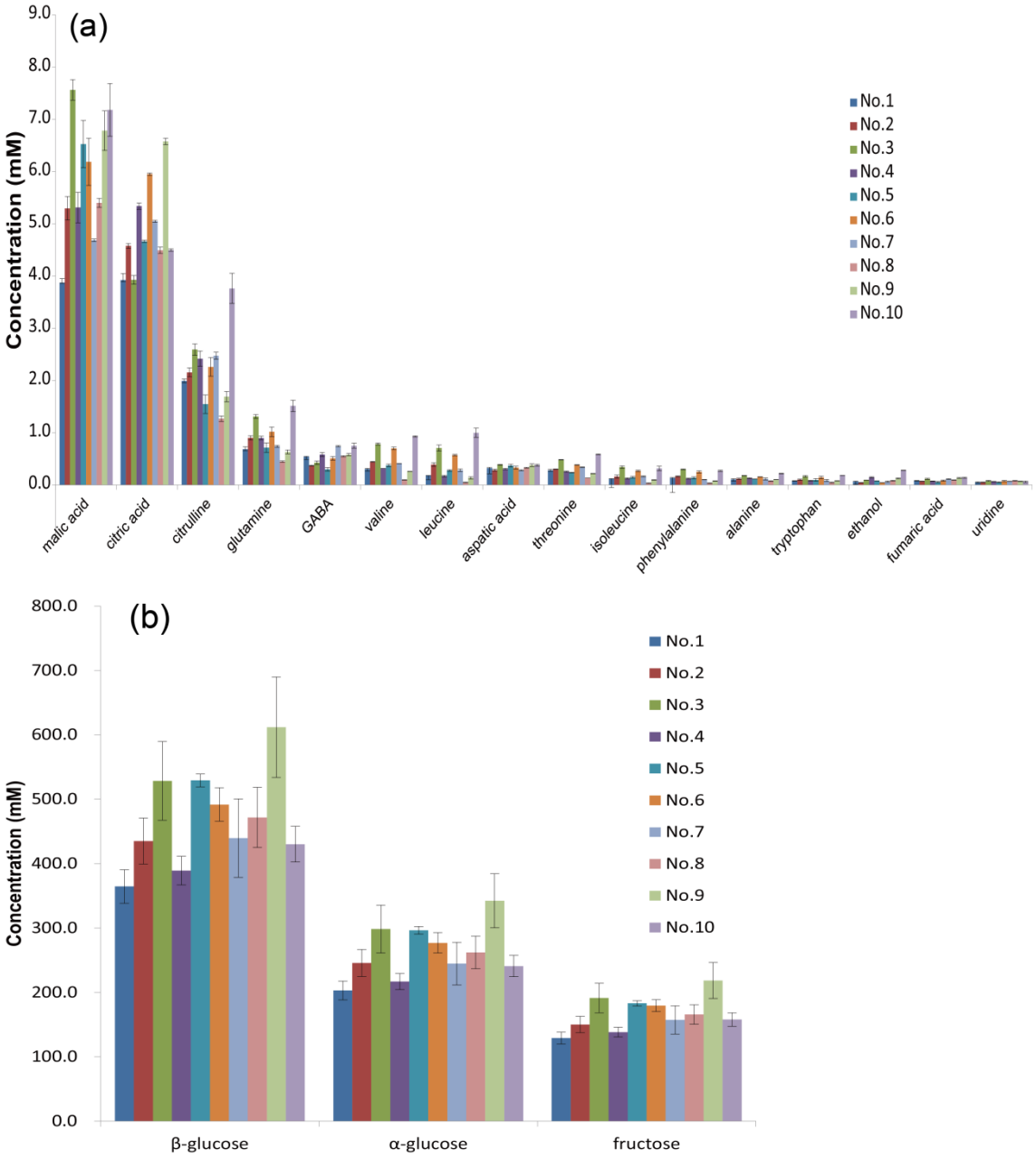


Figure 3-8 Concentrations of components quantified using the Broad Band WET NMR spectra **(a)** and ^{1}H NMR spectra **(b)**. No.1~No.10 means the number of the 10 different samples.

Chapter 4.

Investigation of different Japanese persimmon cultivars by NMR spectroscopy combined with multivariate analysis

4-1 Introduction

The differences in the concentrations of primary and secondary components determine the nutritional importance of the selected cultivars of crops. Improved crop varieties can play an important role in the commercial success. Metabolic profiling provides a robust method to support the selection and breeding of high quality crops. Furthermore, the knowledge of nutritional profile of crops is very important for the food industries which extract specific components from some crops to obtain additives for other foodstuffs.

Comprehensive analysis of metabolic profiles using different analytical techniques, along with suitable statistical analysis, has been established to identify similarities and differences among crop varieties. Untargeted studies have been carried out in a few number of crops, such as melon (Biais *et al*., 2009), apple (Aprea *et al*., 2011; Vermathen *et al*., 2011), tomato (Gomez *et al*., 2010; Perez *et al*., 2011), potato (Beckmann *et al*., 2007), etc. The varietal characteristics of different cultivars have relations to their metabolic composition. It was highlighted that many metabolites significant for compositional differences among cultivars were closely linked to quality traits in potato tubers (Beckmann *et al*., 2007; Dobson *et al*., 2008). The content in taste-relevant metabolites such as fructose and organic acid was proved to be variety-dependent in study on tomato. Variations in the abundant, polar metabolites (sugars, acids, etc.) are responsible for the obvious tomato taste variations (Perez *et al*., 2011).

NMR is a valuable tool for metabolic profiling of food, because it has the advantages of the simultaneous analysis of metabolites in complex solution in a single experiment. Combined with multivariate statistical analysis such as the Principal Component Analysis (PCA) and the partial least-squares discriminate analysis (PLS-DA), NMR has been developed into a more attractive tool for varieties classification, origin determination, etc. (Ryu *et al*., 2010; Mikros *et al*., 2009; Ohno *et al*., 2011). PCA is a dimensional reduction technique that allows one to easily plot, visualize and cluster multiple metabolomics data sets based on linear combinations of their shared features. As a clustering technique, PCA is most commonly used to identify how one sample is different from another, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated)

or independently (i.e. uncorrelated) from each other. In contrast to PCA, PLS-DA is a supervised classification technique that can be used to enhance the separation between groups of observations by rotating PCA components such that a maximum separation among classes is obtained. The basic principle behind PLS-DA is similar to that of PCA, but in PLS-DA a second piece of information is used, namely, the labeled set of classes identities. While PCA and PLS-DA, on their own, do not permit the direct identification or quantification of components they still allow an unbiased (or untargeted) chemically comprehensive comparison to be made among different samples (Wishart, 2008).

About 1000 varieties are estimated to be grown in Japan (Veberic *et al*., 2010). Traditionally, identification of persimmon cultivars has been performed based on morphological and physiological features including fruit size, fruit shape, leaf shape, specific spots on calys, fruit ripening characteristics, and taste (Maki *et al*., 2001). Recently, genetic methods have been used to distinguish cultivars by evaluating the genetic diversity, such as RAPD (Random amplified polymorphic DNA) analysis (Luo *et al*., 1995), AFLP (amplified fragment length polymorphism) (Kanzaki *et al*., 2000), RFLP (restriction fragment length polymorphis manalysis) (Maki *et al*., 2001), microsatellite analysis (Naval *et al*., 2010), etc. With respect to those methods, it is important to choose appropriate probes, and the repeatability is affected by many elements. At our knowledge, metabolic profiling for discriminating the cultivars of Japanese persimmon has not been reported in the literatures.

Therefore, in this chapter, we tried to provide a detailed and comprehensive overview of the differences among five leading commercial Japanese persimmon cultivars in Japan using NMR spectroscopy. Three non-astringent persimmons, `Taishu (TS)`, `Matsumotowase-Fuyu (MF)` and `Maekawa Jiro (MJ)` and two astringent cultivars `Hiratanenashi (HN)` `Yotsumizo (YM)` were collected from the same organization in the same year (Figure 4-1). All of five cultivars have a strong sweetness but each has a different texture. TS is a new cultivar released in 1995, is a mid-ripening type with very large fruit (about 400g), with large fruits of excellent quality (very soft, juicy flesh). Fruit can develop shallow, concentric cracking, which lead to high sugar content in the flesh just under the cracks (Yamane *et al*., 2001; Iwanami *et al*., 2002). MF is an early-maturing strain selected from `Fuyu` persimmon (Niikawa *et al*., 2005), which is about 250 g in weight and has a comparatively soft texture. MJ is a bud

mutation of `Jiro` persimmon. It has a weight of about 200g and a moderate firmness. HN and YM are two well-known astringent persimmons. HN is a high quality cultivar with soft and juicy flesh after treatment to remove astringency with a weight of about 250g. YM is relatively small in size with about 150g in weight and has a hard flesh texture. It is often used to prepare for died persimmon (Matsui *et al*., 1957).

The metabolic profiles for the five cultivars were established and compared, using NMR spectroscopy combined with statistical analysis. Whether the minor components identified by Broad Band WET NMR spectroscopy are important in differentiation of cultivars are also evaluated in details. .

4-2 Material and Methods

4-2-1 Sample preparation

Three non-astringent persimmons (TS, MF, MJ), and 2 astringent persimmons (HN, YM) were supplied by National Agriculture and Food Research Organization (Hiroshima, Japan). 10 fruits from each of the 5 cultivars were collected in the middle of November in the same year. The 2 astringent persimmons were prepared after constant temperature short duration (CTSD) treatment to remove astringency.

The fruits were grounded into a pulp and then centrifuged at 13,000×g for 10 min at 4°C. The supernatant was used as persimmon juice. The juices were stored at −20°C until NMR analysis.

A final concentration of 1M and pH value of 6.5 phosphate buffer prepared with D_2O (99.9%, 70μl; Shoko Co., Ltd., Tokyo, Japan), was added to persimmon juice (630μl) for locking. TSP-d_4 was added to the sample as both chemical shift and quantitative reference with a final concentration of 0.1mM. A final concentration of 1.5mM EDTA (Dojindo, Kumamoto, Japan) was added to the sample to capture the paramagnetic ions that cause a broadening of NMR signals. The mixture then was transferred to a 5mm NMR tube.

4-2-2 NMR spectroscopy

NMR spectra were obtained at 20°C on a Varian Unity INOVA-500 spectrometer for

the ^{1}H, ^{1}H Broad Band WET, ^{13}C, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H TOCSY spectra, ^{1}H-^{1}H Broad Band WET TOCSY spectra, ^{1}H-^{1}H DQF-COSY spectra, and ^{1}H-^{1}H Broad Band WET COSY spectra , and a Varian Unity INOVA-600 spectrometer equipped with a cryogenic probe for the ^{1}H-^{13}C CT-HMBC spectrum and WET-HMBC spectrum.

The acquisition parameters of the ^{1}H NMR spectra were as follows: spectral width, 8,000 Hz; number of data points, 64K; acquisition time, 2.048 s; and delay time, 5.0 s. The water signal was suppressed by a presaturation method. The parameters of the ^{13}C NMR spectrum for the assignments were as follows: number of data points, 16 k; spectral width, 31,422 Hz; acquisition time, 1.043 s; delay time, 2.0 s; and number of scans, 52,896.

The acquisition parameters of Broad Band WET methods were as follows: spectral width, 8,000 Hz; number of data points, 64K; number of scans, 128; acquisition time, 2.048 s; and delay time, 5.0 s. The pulse length of e-Burp pulse in Broad Band WET method was 3.88 ms.

The acquisition parameters of Broad Band WET COSY and Broad Band WET TOCSY were as follows: spectral width, 6,700 Hz (F1) and 6,700 Hz (F2); number of data points, 512 (F1) and 2,048 (F2); number of scans, 64; and the pulse length of e-Burp, 3.88 ms.

The acquisition parameters of total correlation spectroscopy (TOCSY) were as follows: spectral width, 6,700 Hz (F1) and 6,700 Hz (F2), number of data points, 512 (F1) and 2,048 (F2); number of scans, 32; acquisition time, 0.341s; delay time, 2.0s; field strength of MLEV-17 spin-lock pulse, 7.1 kHz; length of trim pulse, 2 ms; and mixing time, 80 ms.

The acquisition parameters of double-quantum filtered correlation spectroscopy (DQF-COSY) were as follows: spectral width, 6,000 Hz (F1) and 6,000 Hz (F2); number of data points, 512 (F1) and 2,048 (F2); number of scans, 16; acquisition time, 0.341 s; and delay time, 2.0 s.

The acquisition parameters of ^{1}H–^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC) were as follows: spectral width, 6,000 Hz (^{1}H) and 20,742 Hz (^{13}C); number of data points, 512 (F1) and 2,048 (F2); number of scans, 256; acquisition time, 0.341 s; and delay time, 2.0 s.

The acquisition parameters of ^{1}H–^{13}C constant-time heteronuclear multiple-bond

correlation spectroscopy (CT-HMBC) (Furihata *et al*., 1998) were as follows: spectral width, 6,000 Hz (^{1}H) and 28,902 Hz (^{13}C); number of data points, 512 (F1) and 2,048 (F2); number of scans,256; acquisition time,0.341s; and delay time, 2.0 s.

4-2-3 NMR data processing and signal assignments

All ^{1}H NMR spectra were manually phased, baseline corrected, and referenced to TSP at 0.00 ppm.

Signal assignments were made essentially according to the previously described method. The signals observed in 1D and 2D NMR spectra were compared with the data given in several online databases for metabolomics, such as HMDB,(Wishart *et al*., 2009), BMRB(Ulrich *et al*., 2008), MMCD (Cui *et al*., 2008) and in papers on other fruit juice and several candidate components were picked out. Finally, signal assignment was accomplished by spiking authentic standard compounds (special grade; Wako Pure Chemical Industries, Ltd., Osaka, Japan, or Nacalai Tesque Inc., Tokyo, Japan) into the juice and comparing the spectra of the resulting mixtures with those of plain juice.

4-2-4 Multivariate statistical analysis

The ^{1}H NMR spectral data were reduced into 0.04 ppm spectral buckets, and all spectra were normalized by MestRe Nova. The resulting data sets were then imported into SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) for further multivariate statistical analysis

Prior to PCA, data were mean-centered and then scaled using Pareto scaling. Hotelling's *T2* region, shown as an ellipse in the score plots, defined the 99% confidence interval of the modeled variation. The quality of the model was described by Rx^2 *and* Q^2 values. Rx^2 was defined as the proportion of variance in the data explained by the model and indicates goodness of fit. Q^2 was defined as the proportion of variance in the data predictable by the model and indicates predictability.

OPLS-DA gives the maximum covariance between the measured data (X) and the response variable (Y). For OPLS-DA model, the confidence level for membership probability was considered to be 95%; observations at < 5% are considered to be outliers. The overall predictive ability of the model is assessed by cumulative Q^2 representing the fraction of the variation of Y that can be predicted by the model, which

was extracted according to the internal cross-validation default method of SIMCA-P software.

4-3 Results and Discussion

4-3-1 Comparison of ^{1}H NMR spectra and Broad Band WET spectra of Japanese persimmons of 5 cultivars

Figure 4-2 shows a comparison of ^{1}H spectra of persimmon juice of 5 different cultivars. As shown in Figure 4-2a, the signal patterns for ^{1}H NMR spectra of 5 cultivars are similar, and the spectra region between 3.10 and 5.40 ppm contains signals mainly from sugars (glucose, fructose), which are the dominant components in all cultivars. Components were assigned through analyses of 2D NMR and spiking experiments mentioned in Chapter 3. The expanded spectra of high-field region (0.5~3.0ppm) and low-field region (6~10ppm) are shown in Figures 4-1b and 4-1c. In the high-field region, the peaks mainly from organic acids and amino acids were observed. In contrast, few peaks were observed in the low-field region for all cultivars. Here we used Broad Band WET method to measure the spectra as described in Chapter 2. The full and expansion of Broad Band WET spectra are shown in Figure 4-3. As expected, new signals were detected in the low-field region with the same scans (128 scans).

4-3-2 Comparison of the suitability of Broad Band WET spectra with ^{1}H NMR spectra for multivariate statistical analysis on cultivar discrimination

To investigate the overall composition variation, PCA was performed on the dataset for persimmon juice of 5 cultivars (each 10 samples), and the results of multivariate statistical analysis are compared between ^{1}H NMR and Broad Band WET spectra. The score plots are shown in Figure 4-4. Firstly, the ^{1}H NMR spectra except for water suppression region between 4.5 ppm and 5.2 ppm do not show any statistic differences among cultivars in the score plot shown in Figure 4-4a. Secondly, PCA was applied to the ^{1}H NMR spectra except for the region of water and sugars between 3.0 ppm and 5.6 ppm, and the score plot showed that HN-CTSD was divided from other 4 cultivars by PC2 (Figure 4-4b), which indicates that this cultivar is the most significantly different from the other cultivars. The exclusion of sugars regions of ^{1}H NMR spectra before

applying to PCA made an improvement in the discrimination among cultivars. The reason is considered to be that the concentrations of sugars are affected by ripeness and de-astringency treatments rather than cultivars. The differences amongst cultivars might be more contributed by other metabolites than sugars. For this reason, PC2 explained only a low percentage of 17.2% of the total variance for five cultivars because only limited components were detected in ^{1}H NMR spectra, which indicates that the discrimination result is not informative enough for cultivars. PC1 explained 56.0% of the total variance, but no information on cultivars was provided. PCA was then applied to the Broad Band WET spectra, except for water and sugar saturation region from 3.0 ppm to 5.6 ppm. The score plot (Figure 4-4c) showed the first two PCs (PC1 and PC2) explain 33.9% and 26.2% of the total variance obtained for fruit cultivars. HN-CTSD is clearly separated from other 4 cultivars explained by PC1, and two cultivars of YM-CTSD and TS are divided from cultivars of MF and MJ, which is explained by PC2. The results are improved by using Broad Band WET spectra, because more minor components are detected in Broad Band WET spectra than in ^{1}H NMR spectra. Combining with the exception of the sugar region, more minor components that might be responsible for cultivar discrimination are revealed, which provides a better discrimination result.

The PCA analysis of 5 cultivars indicated that the 4 cultivars except for HN-CTSD are similar to each other. To further explore if the multivariate statistical analysis combined with Broad Band WET spectra can extract the features of similar cultivars more effectively than that with ^{1}H spectra, another PCA analysis was performed using the 4 cultivars (YM-CTSD, TS, MF and MJ) except for HN-CTSD. The PCA score plots for PCA model are shown in Figures 4-4a and 4-4b for ^{1}H NMR and Broad Band WET spectra except for the region of water and sugars between 3.0 ppm and 5.6 ppm, respectively. The PC1 and PC2 with ^{1}H NMR spectra are 59.6% and 14.5% respectively, and the score plot showed nearly no separation among cultivars. In contrast, 4 cultivars were separated on the first two principle components, PC1 (37.2%) and PC2 (29.6%), in the score plot with Broad Band WET spectra. PCA analysis shows the discrimination depends on differences intrinsic to the persimmon cultivars when using Broad Band WET spectra. The results indicate that Broad Band WET spectra are very useful for persimmon cultivars characterization.

In order to find out the details of the different components among cultivars, the supervised method, OPLS-DA, was used to isolate the variables (the components assigned to the corresponding buckets and integration intensity of the corresponding buckets, in this study) responsible for differences between astringent and non-astringent cultivars. The score plots are shown in Figure 4-6. The goodness-of-fit parameter R^2 and the predictive ability parameter Q^2 were also compared. R^2 describes how well the data of the training set is mathematically reproduced, and varies between 0 and 1, where 1 indicates a perfect fit between the model and the data. A prediction model is considered to be good when Q^2 >0.5, and excellent if Q^2 >0.9. Figure 4-6a is the OPLS-DA score plot performed with ^{1}H spectra with exclusion of water suppression region between 4.5 ppm and 5.2 ppm, and shows roughly separation between the astringent (HN-CTSD,YM-CTSD) and non-astringent (TS, MF, MJ) cultivars with total variance of 48.7% (20% for OPLS1 and 28.7% for OPLS2). The OPLS-DA modeling revealed Rx^2, Ry^2, and Q^2 values of 0.488, 0.557, and 0.505 for OPLS1. The S-plot (Figure 4-6b) for OPLS1 shows the most relevant variable for differentiation of astringent and non-astringent cultivars with ^{1}H NMR spectra with exclusion of water suppression region. S-plot reveals the contributions of particular variables toward either an increase or a decrease in integration intensities. Each variable is represented as a peak with a particular chemical shift in the ^{1}H NMR spectrum. The increased or decreased components could be identified, and the variables of the chemical shifts associated with the largest changes in integration intensity are indicated farther away from the center of the PLS1 coordinate axis. Thus, as a result of the S-plots analysis, the above components contribute the most to the difference between astringent and non-astringent cultivars in OPLS1. The results in Figure 4-6b show that the separation is mainly attributable to the sugars (α-glucose, β-glucose, and fructose) between astringent and non-astringent cultivars by OPLS1 in the OPLS-DA score plot.

OPLS-DA was also applied to the ^{1}H NMR spectra with region for water and sugars (between 3.0 ppm and 5.6ppm) excluded. The OPLS-DA score plot in Figure 4-7a shows the separation between the astringent and non-astringent cultivars with total variance of 63.2% (8.8% for OPLS1 and 54.4% for OPLS2). By excluding the sugar region of the ^{1}H NMR spectra, the goodness-of-fit parameter Rx^2 and Ry^2 were improved to 0.940, 0.966, respectively, and predictive ability parameter Q^2 values was

also increased from 0.505 to 0.761 for OPLS1, which indicate that the quality of the OPLS regression can be improved by removing the major bulk signals. The results of S-plot for OPLS1 (Figure 4-7b) show that the most relevant variables for the differentiation of astringent and non-astringent cultivars are the components in astringent cultivars, such as ethanol, threonine, fumaric acid, and some characteristic signals in astringent cultivars.

OPLS-DA was applied to the Broad Band WET spectra, with water and sugar saturation region from 3.0ppm to 5.6 ppm excluded. The OPLS-DA score plot (Figure 4-8a) shows total variance of 50.6% (23.1% for OPLS1 and 27.5% for OPLS2). The OPLS-DA modeling revealed Rx^2, Ry^2, and Q^2 values of 0.870, 0.965, and 0.936 for OPLS1, which indicate an excellent fit and prediction ability for this OPLS regression model.

The S-plot for OPLS1 (Figure 4-8b and 4-8c) shows that, the two astringent cultivars after constant temperature short duration treatment (HN-CTSD and YM-CTSD) are similar with non-astringent cultivars in amino acids and organic acids components. The separation between astringent and non-astringent cultivars is contributed by ethanol, fumaric acid, threonine, GABA, alanine and some characteristic signals in astringent cultivars, such as the broad signals at 1.1−1.14 ppm, 1.26−1.30 ppm, and 2.02−2.30 ppm, and singlet signals at 7.05−7.09 ppm and 7.21−7.29 ppm which are assumed to be signals for polyphenols. More biomarker components were identified using Broad Band WET spectra than that using ^{1}H NMR spectra, such as threonine, GABA, alanine, and signals assumed to be derived from polyphenols. The result of cultivar discrimination becomes more informative and detailed because threonine, GABA, alanine can be detected more clearly (with a good S/N ratio) in Broad Band WET spectra than ^{1}H NMR spectra and singlet signals at 7.05−7.09 ppm and 7.21−7.29 ppm can only be observed in Broad Band WET spectra,

In summary, the results clearly demonstrate the cultivar discrimination of Japanese persimmons by ^{1}H NMR spectra can be improved by removing the major bulk signals (water and sugars). A more informative detail of metabolic differences among cultivars can be achieved by using Broad Band WET spectra.

4-3-3 Comparison of samples among 3 non-astringent cultivars

Since Broad Band WET spectra represent a suitable methodology in the study of cultivar differentiation, OPLS-DA was applied to spectra of 3 non-astringent cultivars, and the variables that are mainly responsible for the separation were also determined.

Firstly, for the two cultivars of TS and MJ, S-plots for OPLS1 (Figure 4-9) show the relevant variables for the differentiation of the two groups. Some amino acids and organic acids, were analyzed as variables of the S-plot, and are considered to contribute to the difference between TS and MJ cultivars in OPLS1. As shown in Figure 4-9b, valine, leucine, isoleucine, citrulline, malic acid, aspartic acid, tryptophan, phenyalanine and several unknown signals (1.24 ~1.29 ppm, and 2.18 ~2.25 ppm) were observed as typical components in TS. Meanwhile, ethanol, citric acid and fumaric acid were detected as biomarkers in MJ. Therefore, these results show that the separation between TS and MJ cultivars by OPLS1 in the OPLS-DA score plot is mainly attributable to the above components, and the biomarkers of TS cultivar can be considered as amino acids, and malic acid, whereas the biomarkers of MJ cultivar are ethanol, citric acid and fumaric acid. The Xvar values (integration intensities) associated with component variables are shown in Figure 4-9c, and reveal either an increase or a decrease in integration intensities for those particular variables.

In the same way, the comparisons were performed between TS and MF. The result is shown in Figure 4-10. The total variance of the separation among the two cultivars is 0.763 (0.564 for OPLS1 and 0.199 for OPLS2) (Figure 4-10a). S-plots (Figure 4-10b) for OPLS1 show the most relevant variables for the differentiation of the two groups. In TS, amino acids (such as valine/leucine/isoleucine, threonine, citrulline, GABA, glutamine, phenylalanine, tryptophan, etc.) and malic acid are obviously higher than MF. In addition, the signals at 1.148−1.22 ppm are found to be the biomarkers of MF cultivar. These peaks were partially assigned to a series of ethyl glycosides by the aid of 2D NMR spectra (^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C CT-HMBC, and ^{1}H-^{1}C HSQC), and a part of the 2D NMR spectra are shown in Figure 4-11. Ethyl moieties were confirmed by cross peaks in ^{1}H-^{1}H DQF-COSY spectrum, which are 1.22 (t)/3.71 ppm, 1.22(t)/3.95 ppm, 1.148(t)/3.75 ppm, 1.154(t)/3.75 ppm, and 1.17 (t)/3.65 ppm. The ^{13}C chemical shifts of -CH_3 are obtained by ^{1}H-^{1}C HSQC spectrum, which are

1.22/17.05 ppm, 1.154/17.71 ppm, and there are overlap for the cross peaks at 1.148 /20.36 ppm and 1.17/19.70 ppm. From ^{1}H-^{13}C CT-HMBC spectrum, the cross peaks at 1.22/69.16 ppm, 1.148/82.63 ppm, 1.154/73.65 ppm, 1.17/60.18 ppm are identified and interpreted to be the coupling between the protons of $-CH_3$ and the carbons of $-CH_2$ in ethyl moieties. Furthermore, $-CH_2$ in ethyl moieties are indicated to be linked to a glycoside via a glycosidic bond in ^{1}H-^{13}C CT-HMBC spectrum from the cross peaks such as 4.46/69.16 ppm, 4.52/82.63 ppm, and 4.49/73.65 ppm, of which the ^{1}H are considered to be the anomeric protons. Meanwhile, in the ^{1}H-^{1}H TOCSY spectrum, the *J*-coupled spin systems were identified as 4.46/3.25/3.46/3.38 ppm, 4.52/3.26/3.48/3.39 ppm, 4.49/3.31/3.48/3.38 ppm, and the cross peak at 4.46/3.25 ppm was detected in ^{1}H-^{1}H DQF-COSY spectrum which means the two protons coupled with each other. The spin systems are very similar to glucose and are considered to be glycosides derived from saccharides. It was reported that MF cultivar loses the fruit firmness quickly after harvest, which relates to degradation of cell wall polysaccharides by polygalacturonase (PG) (Niikawa *et al*., 2005). The glycoside may originate from the degradation of cell wall polysaccharides such as polyuronide, xyloglucans during softening (Wakabayashi, 2000). Yoshioka *et al*. suggested that de-esterification of polyuronides with a high degree of methoxylation during softening of apples and pears (Yoshioka *et al*., 1992). In addition, a sugar compound named ethyl-β-glucopyranoside was found in sea buckthorn fruit in a previous paper (Tiitinen *et al*. 2006), which has a very similar chemical shifts with the results found in this study, Therefore, we assigned those signals as ethyl glycosides, maybe in the form of monosaccaride or polysaccharide, which need further study (Figure 4-11). The comparison of integration intensities is shown in Figure 4-10c.

The comparison between MF and MJ cultivars was also performed, and the result is shown in Figure 4-12. The S-plot for OPLS1 showed that the variables contribute to the separation are amino acids and organic acids (malic acid and citric acid), and ethyl glycosides. The comparison of the integration intensities showed that the largest differences in integration intensities are ethyl glycosides and malic acid, which are obviously abundant in MF cultivar, and citric acid, which is higher in MJ cultivar.

From the results depicted above, amino acids are higher in TS than the other two cultivars (MF and MJ), and ethyl glycosides are most abundant in MF cultivar as a

characteristic component. In addition, the fumaric acid and citric acid are relatively higher in MJ cultivar than the other two cultivars.

Variations in these metabolites may be responsible for the variations amongst cultivars in flavor, texture, ripening, softening and so on. The reports on the metabolic detail concerning cultivar differentiation are not found at our knowledge; however, the results of metabolite-based analysis will provide a meaningful data on Japanese persimmon studies and help in selection or modification of better or nutritionally important cultivars combined with genomics researches.

4-3-4 Comparison of samples between 2 astringent cultivars

HN and YM are well-known as astringent persimmons. The results in Figure 4-6 indicated that these two astringent cultivars become similar to non-astringent in amino acids, and organic acids after CTSD treatment to remove astringency. The separation between astringent and non-astringent cultivars is contributed by some characteristic components in astringent cultivars.

In purpose of comparing the two astringent cultivars, OPLS-DA was performed on the two astringent cultivars. The results are shown in Figure 4-13. In HN cultivar, ethanol is significantly higher than in YM cultivar, and the signals which are unassigned at 0.86-0.98 ppm and 1.38-1.42 ppm are the marker components in YM cultivar. In contrast, citric acid in HN cultivar is relatively higher than in YM cultivar; however, there are more malic acid and fumaric acid in YM cultivar than in HN cultivar.

It is interesting to find that HN cultivar contains a high concentration of ethanol but very low concentration in YM cultivar. Figure 4-14 shows the metabolic pathway for acetaldehyde and ethanol production following CO_2 and ethanol de-astringency treatment reported by Yamada *et al*., and significant variety of treatment reaction was found among cultivars (Yamada *et al*., 2002). This result indicates that the two cultivars might have different activities of enzymes or metabolic pathway during CTSD treatment, which needs further studies.

4-3-5 Comparison of samples between 2 astringent cultivars before and after CTSD treatment

In order to investigate the differences in metabolic profiles before and after the

CTSD treatment between the two astringent cultivars, the comparison using OPLS-DA was also performed. However, although the pH values were prepared to the same value for all samples and the measurements were performed at the same temperature, remarkable shifts of many signals across the spectra of HN and YM (without CTSD treatment) occurred. The signals include glucose, fructose and signals derived from organic acid (malic acid, citric acid, etc), amino acids (aspartic acid, GABA, etc.), and unassigned signals in both ^{1}H NMR and Broad Band WET spectra, as shown in Figure 4-15. Therefore, alignment of peaks was conducted, which is a preprocessing step to correct for variations in the position of peaks across NMR (Vu and Laukens, 2013). However, the shifts were strong for several peaks that overlapped with other peaks across NMR spectra, and led to an incorrect result of alignment.

For the above reason, the spectra were compared directly without multivariate statistical analysis. The spectra are shown in Figure 4-16. The signal patterns of the spectra are quite different for HN and YM cultivars before and after CTSD treatment. In the low-field region (6.0~10.0 ppm), there are some strong peaks that are not observed in spectra after CTSD treatment for both HN and YM cultivars. The singlet signals at 7.15 ppm in spectra of HN and 7.12 ppm in spectra of YM are confirmed to be gallic acid with the aid of 2D NMR spectra and spiking experiment. The signals at 7.26~7.40 ppm in spectra of HN and 7.24~7.34 ppm in spectra of YM have a similar signals pattern with gallic acid in ^{1}H-^{13}C CT-HMBC spectrum. Those signals are presumed to be derived from the terminal unit of tannins, and the differences in the signals pattern may due to the differences in the condensed pattern of tannins in these two cultivars. However, because of the high molecular weight and various condensed patterns of tannins, it is very difficult to confirm the component structures for these signals even with spiking experiment. In addition, signal from acetaldehyde are found only in the spectra after CTSD treatment in both cultivars, which indicate the production of this components during de-astringency treatment.

In the high-field region (0.5~3.0 ppm), most of the signals are considered to be derived from the amino acids, organic acids, etc., according to the results of assignments on TS cultivar in Chapter 3. There are also some unassigned signals that different between spectra of before and after CTSD treatment, such as the signals between the 1.3 ppm to 1.5 ppm, and so on. We are conducting further studies on those

unassigned signals, and trying to find out the difference between the two cultivars and the metabolic pathway during de-astringency treatment.

With regard to the shifts of peaks, it may be caused by some elements, such as fluctuations in pH, temperature, instrument factors, ion contents (Vu *et al*., 2013) or the formation of non-covalent complex between components (Wei *et al*., 2012), etc. The reason for the shifts of signals in NMR spectra of astringent Japanese persimmons without CTSD treatment was uncertain yet. Considering soluble tannins have the ability to bind proteins as precipitating agents (Henson *et al*., 2004), and are also able to bind bile acids (Matsumoto *et al*., 2011), we presumed that the shifts of those peaks might concern with the high concentrations of soluble tannins in astringent persimmon juice without CTSD treatment, and the degree of shifts might be associated with the different concentrations of tannins in samples. This presumption needs further investigation in the future.

In conclusion, the Broad Band WET spectra, combined with multivariate analysis were employed to discriminate cultivars and identify the significantly different metabolites among 5 commercial Japanese persimmon cultivars. The PCA models clearly demonstrated that the cultivar discrimination of Japanese persimmons by ^{1}H NMR spectra can be improved by removing the major bulk signals (water and sugars). A better discrimination and a more informative detail of metabolic differences among cultivars can be achieved when using Broad Band WET spectra than using conventional ^{1}H NMR. Biomarkers contributing to the cultivar discrimination were also proposed by OPLS-DA models. Some amino acids (citrulline, GABA, tryptophan, phenylalanine, etc.), organic acids (malic acid, citric acid, etc.), ethanol, etc. were captured as biomarkers responsible for the cultivar discrimination. The results depicted in this Chapter indicate that the minor components are important and valuable to characterize the cultivars, and Broad Band WET spectra combined with multivariate analysis could be a very useful tool for Japanese persimmon cultivar characterization. The metabolite-based analysis of cultivar discrimination with NMR spectroscopy will provide a meaningful data on Japanese persimmon studies, and help in selection or modification of better or nutritionally important cultivars combined with genomics researches in the future.

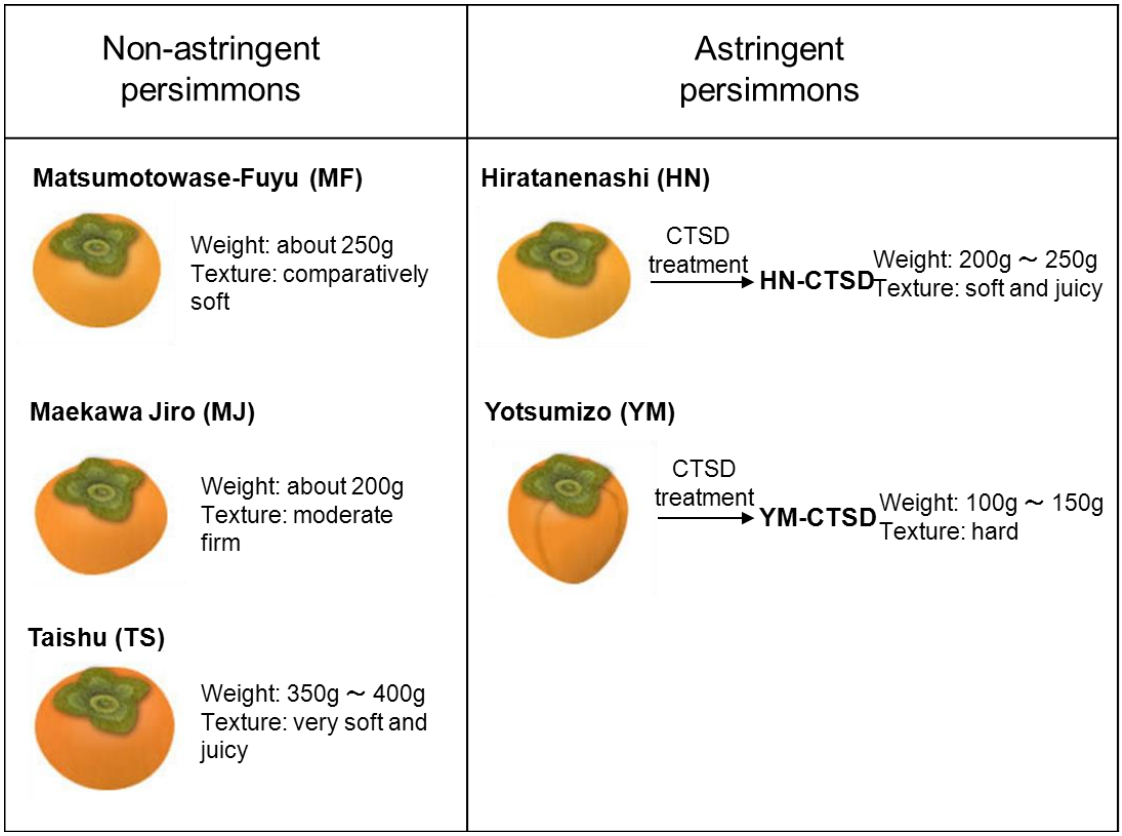


Figure 4-1 Japanese persimmons used in the present study.

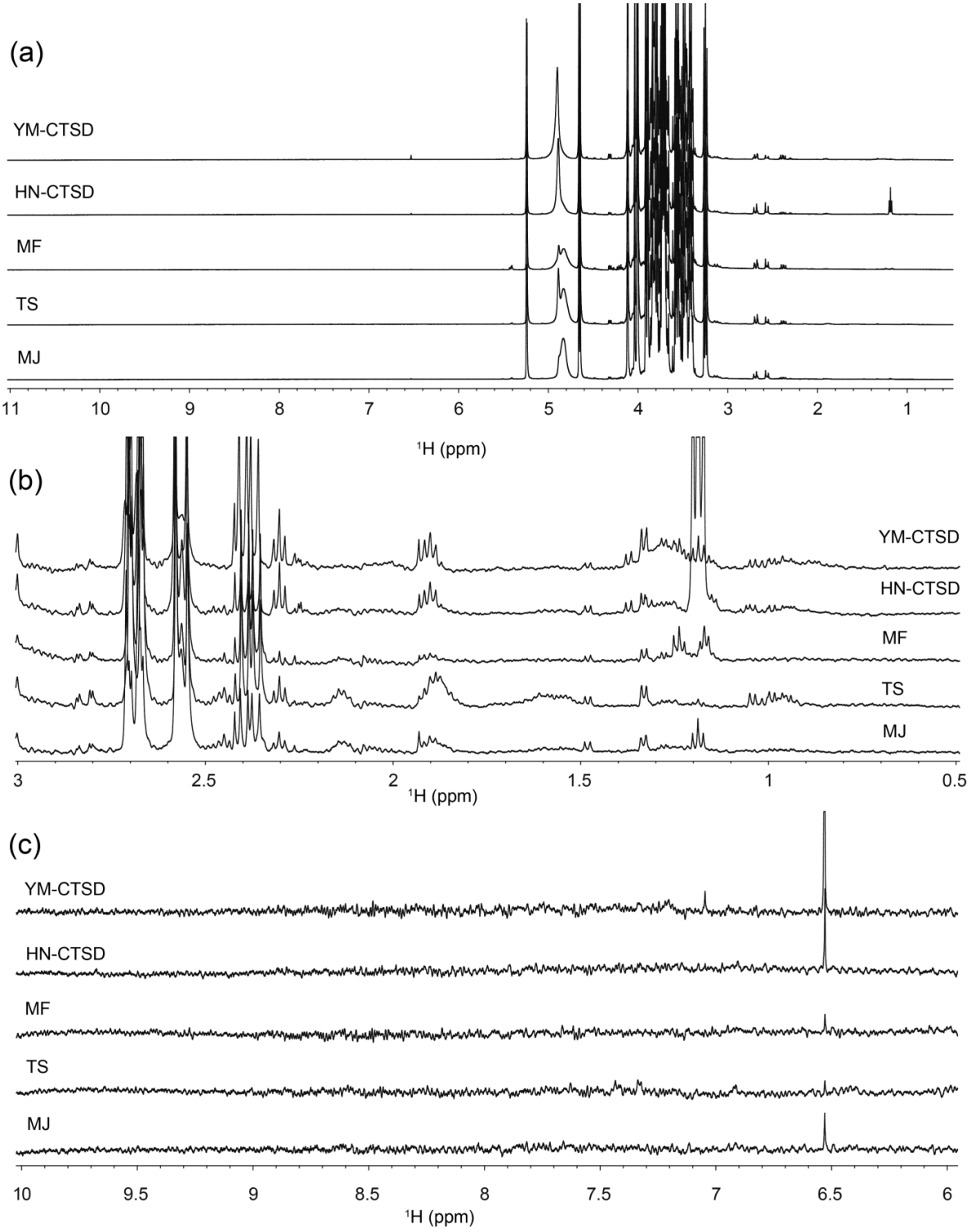


Figure 4-2 Full ^{1}H NMR spectra **(a)** and expansion of high-field (0.5~3.0 ppm) **(b)** and low-field (6.0~10.0 ppm) **(c)** regions for five cultivars.

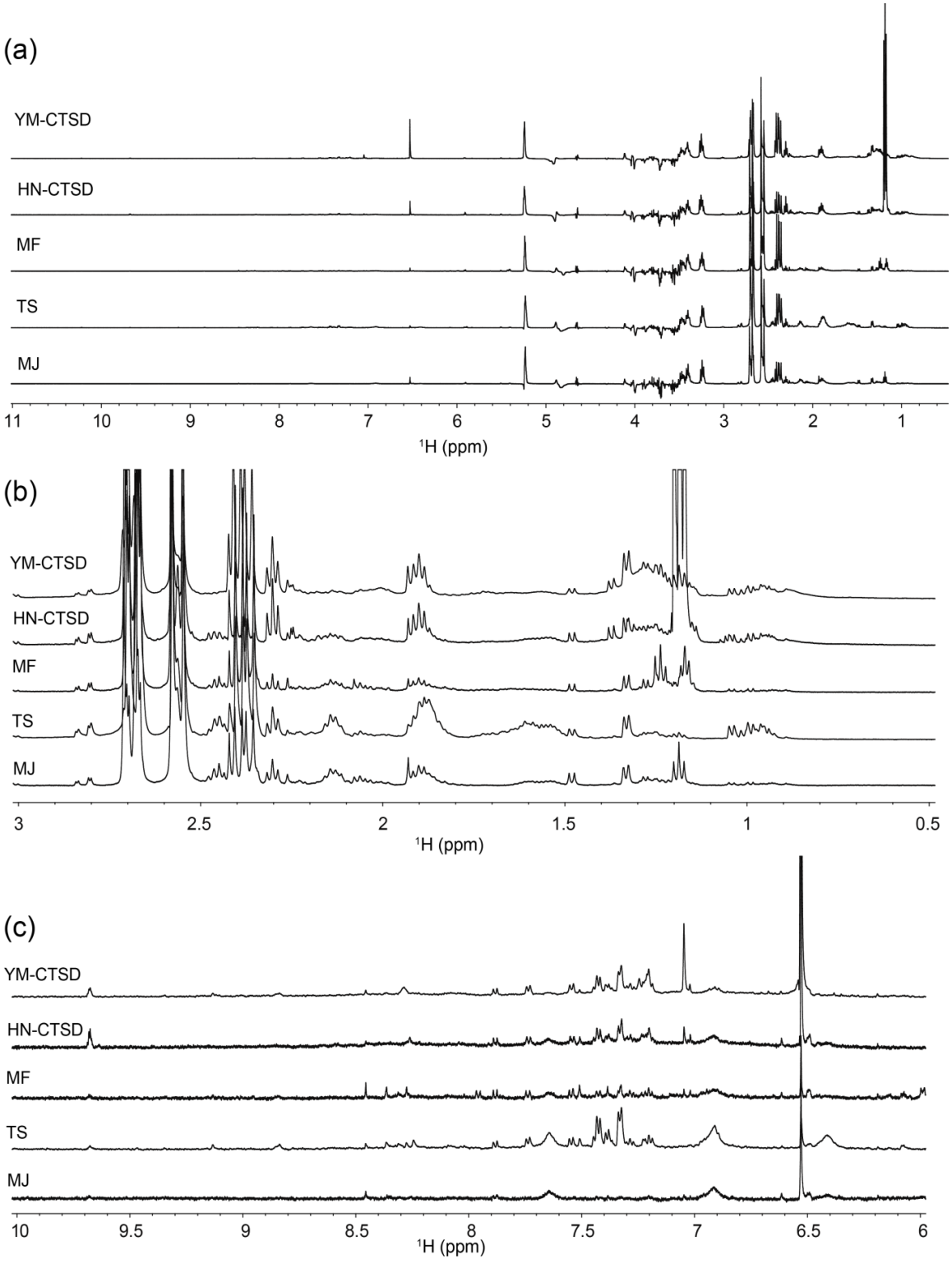
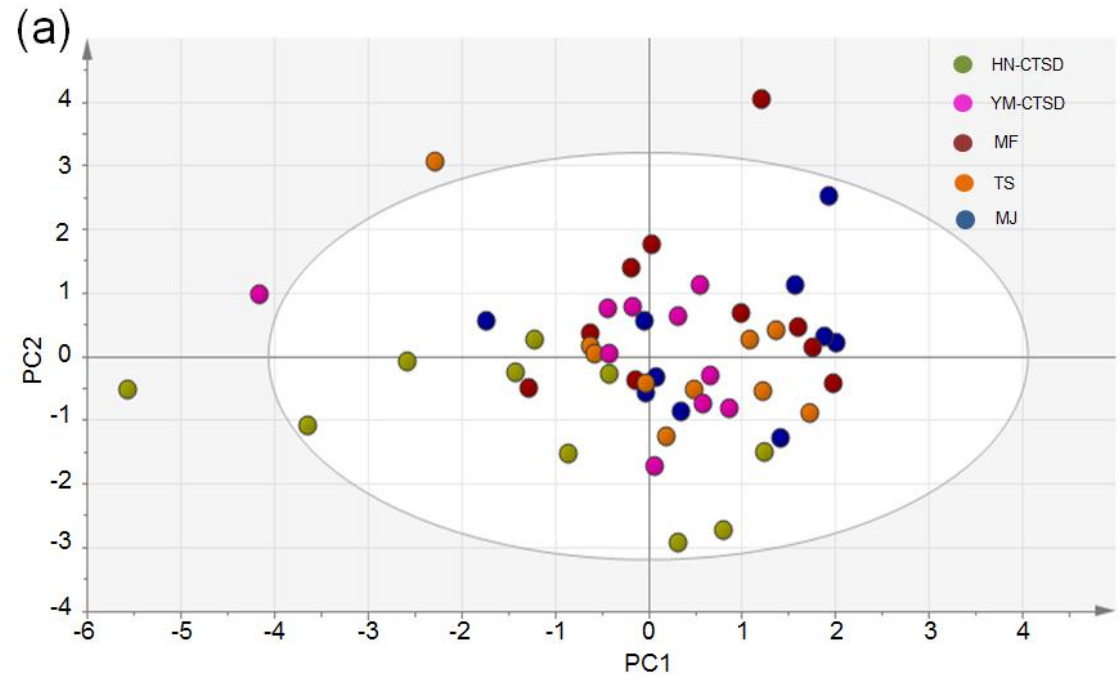
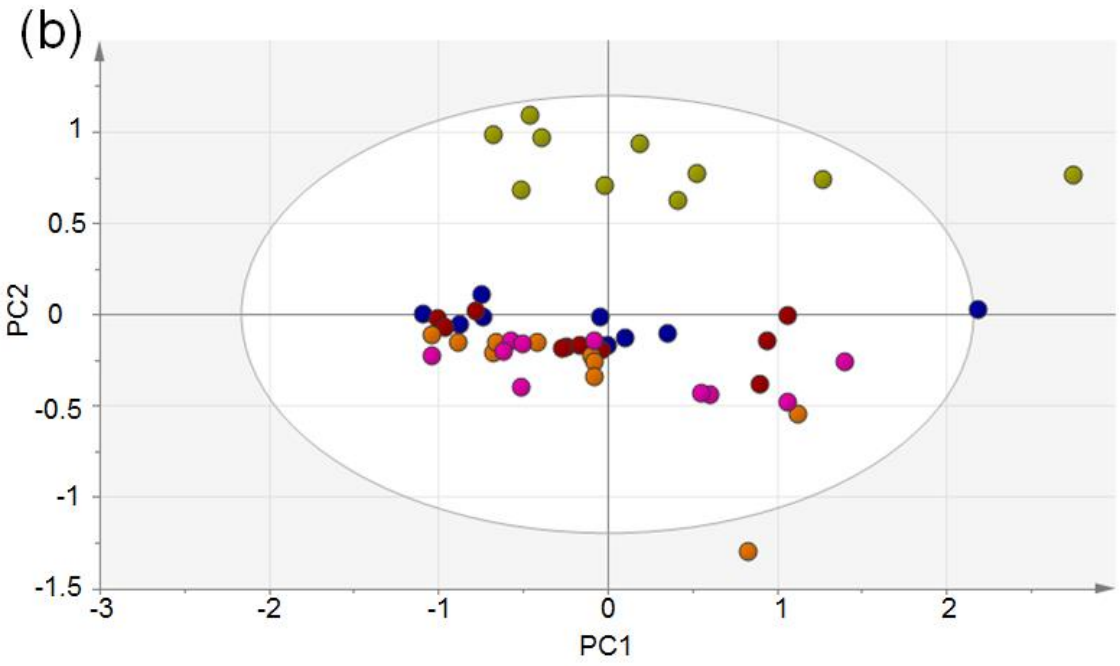


Figure 4-3 Full Broad Band WET NMR spectra **(a)** and expansion of high-field **(b)** and low-field **(c)** regions for five cultivars.


(a)
HN-CTSD
YM-CTSD
MF
TS
MJ
PC2
PC1
4
3
2
1
0
-1
-2
-3
-4
-6
-5
-4
-3
-2
-1
0
1
2
3
4


(b)
PC2
PC1
1
0.5
0
-0.5
-1
-1.5
-3
-2
-1
0
1
2

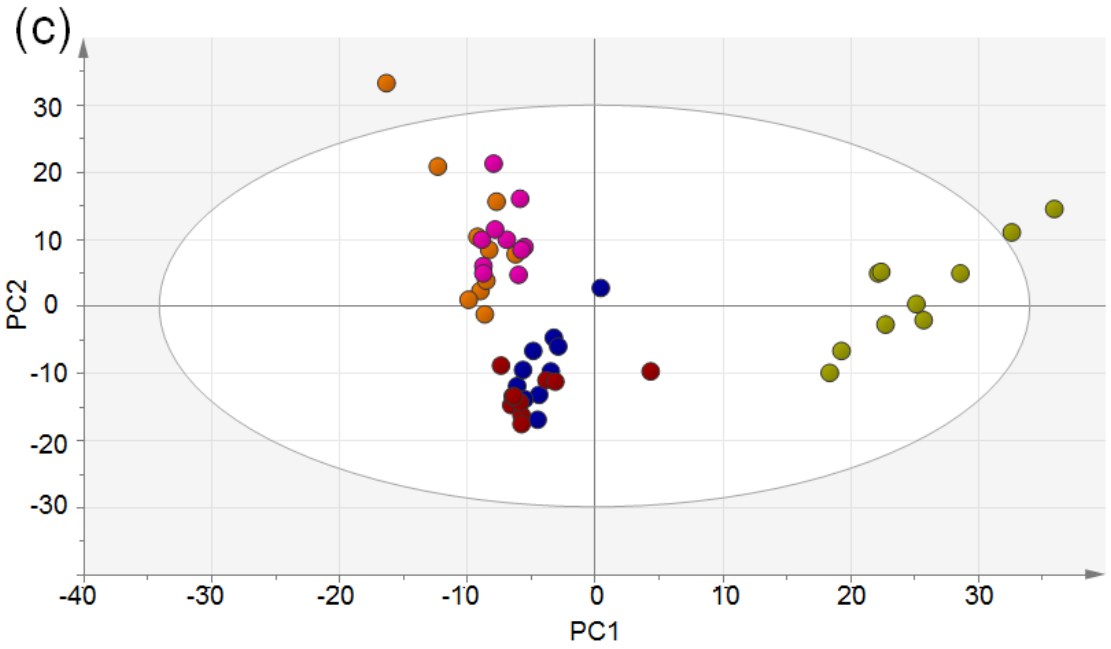


Figure 4-4 The PCA score plots of 5 cultivars. **(a)** ^{1}H spectra with exclusion of water suppression region between 4.5 ppm and 5.2ppm. **(b)** ^{1}H spectra with region for water and sugars (between 3.0 ppm and 5.6ppm) excluded. **(c)** Broad Band WET spectra, with water and sugar saturation region from 3.0ppm to 5.6 ppm excluded.

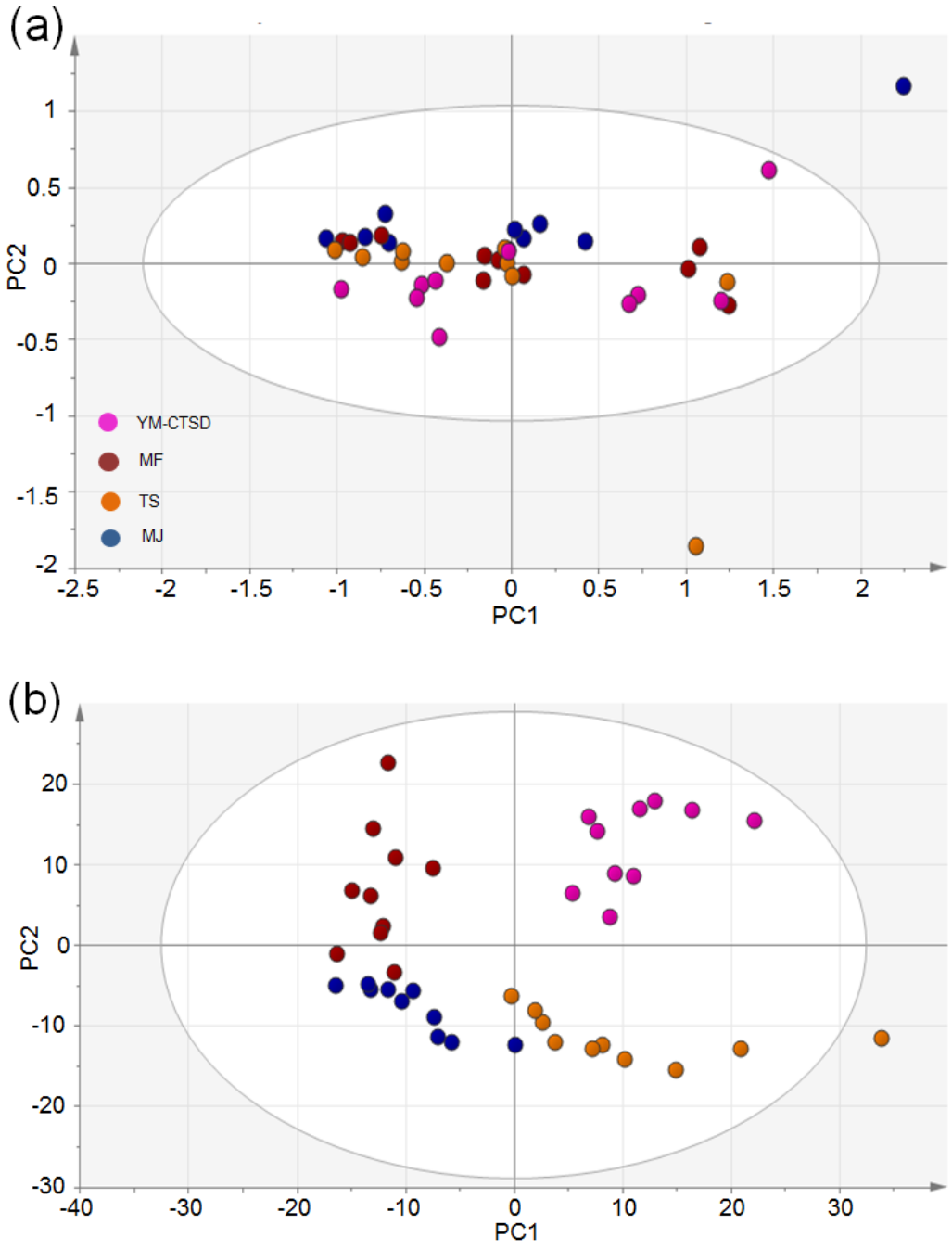


Figure 4-5 The PCA score plot of 4 cultivars samples (YM-CTSD, TS, MF and MJ) **(a)** ^{1}H spectra with region for water and sugars (between 3.0 ppm and 5.6ppm) excluded and (b) Broad Band WET ^{1}H spectra, with water and sugar saturation region from 3.0ppm to 5.6 ppm excluded.

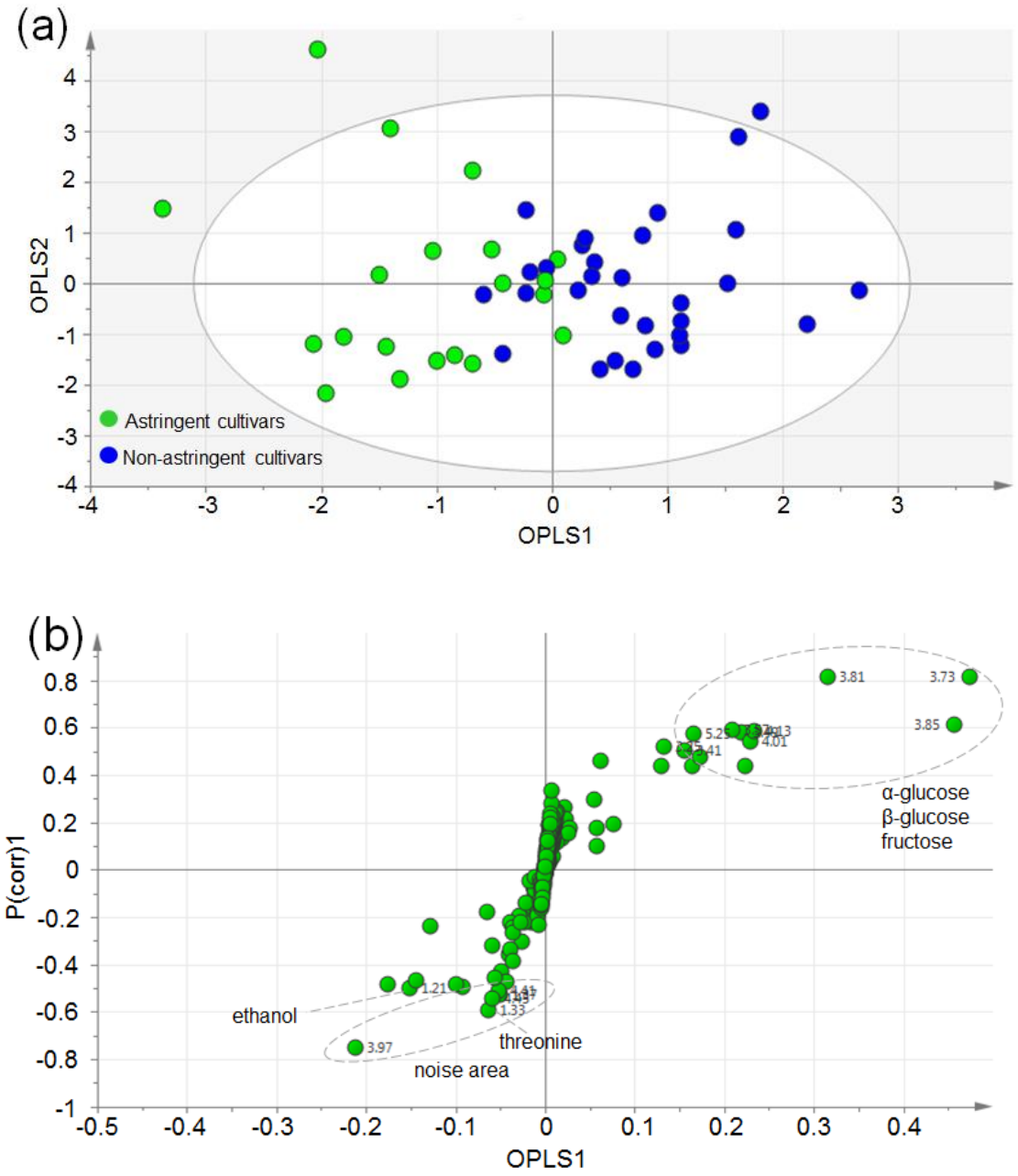


Figure 4-6 The score plot **(a)** and S-plot **(b)** of OPLS1 for5 cultivars with ^{1}H NMR spectra with exclusion of water suppression region between 4.5 ppm and 5.2ppm.

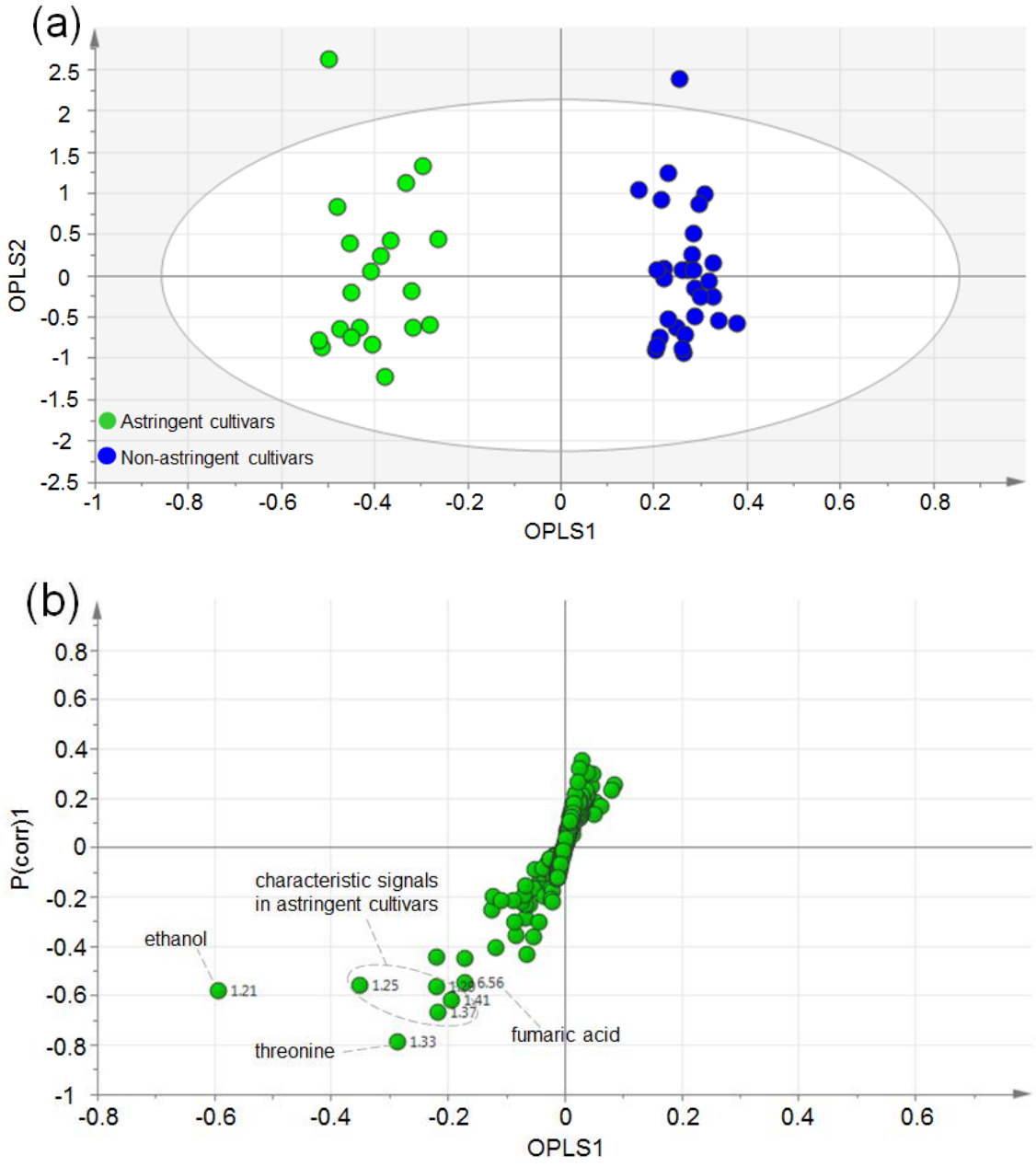
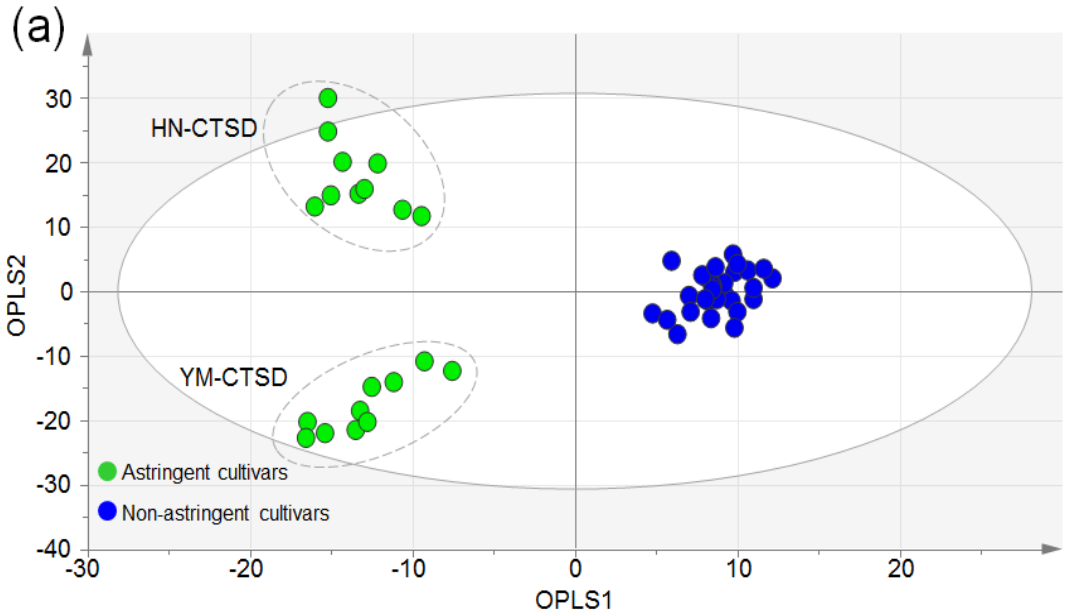
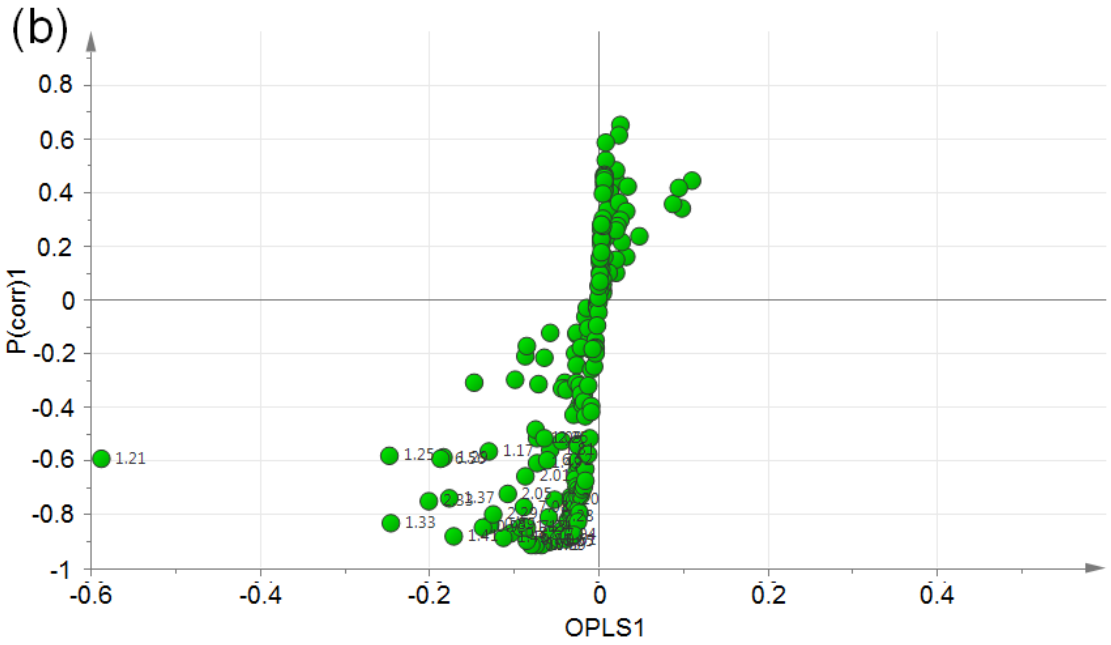


Figure 4-7 The score plot **(a)** and S-plot **(b)** of OPLS1 for 5 cultivars with ^{1}H NMR spectra of region for water and sugars (between 3.0 ppm and 5.6ppm) excluded.


(a)
HN-CTSD
YM-CTSD
Astringent cultivars
Non-astringent cultivars
OPLS2
OPLS1
30
20
10
0
-10
-20
-30
-40
-30
-20
-10
0
10
20


(b)
P(corr)1
OPLS1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-0.6
-0.4
-0.2
0
0.2
0.4
1.21
1.25
1.17
1.33
1.37

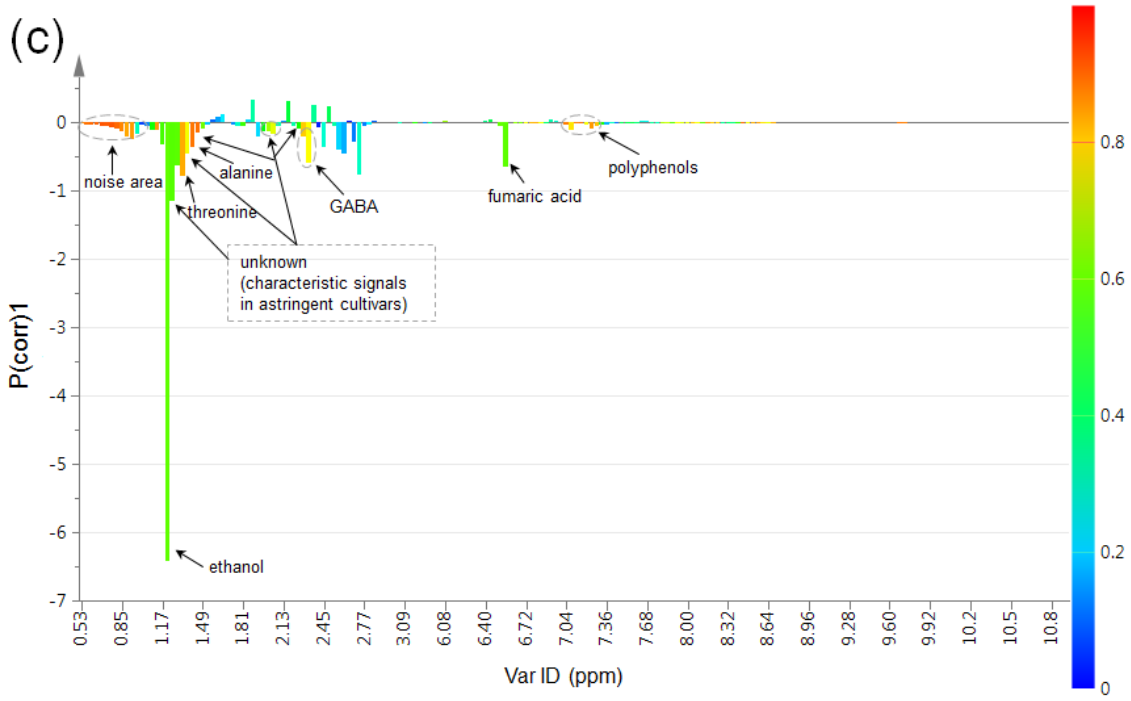
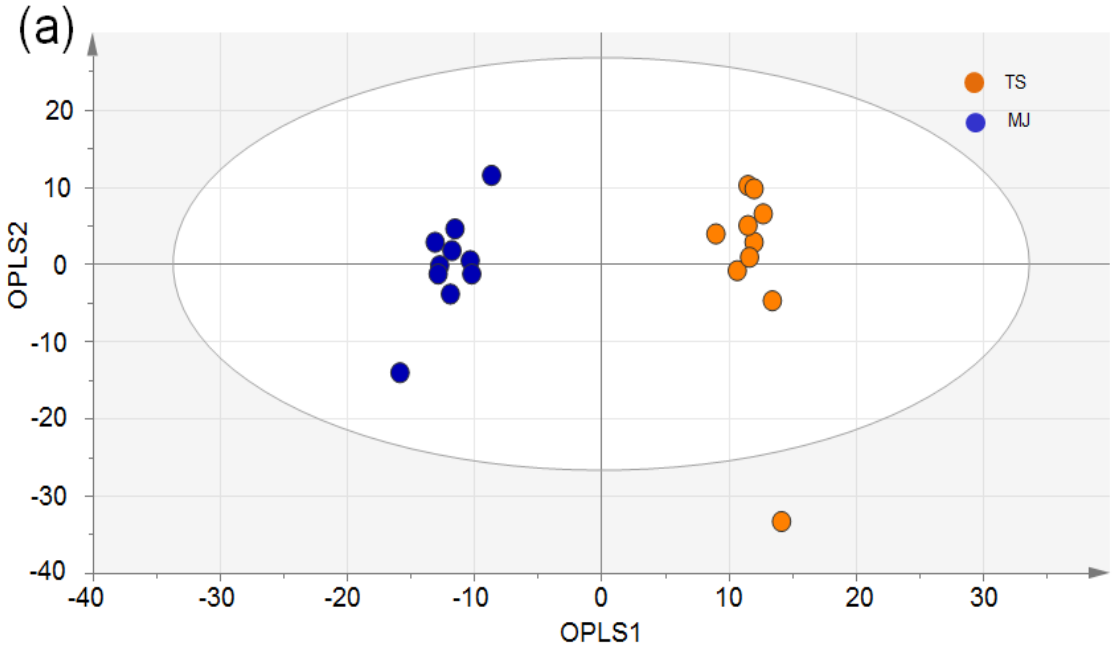
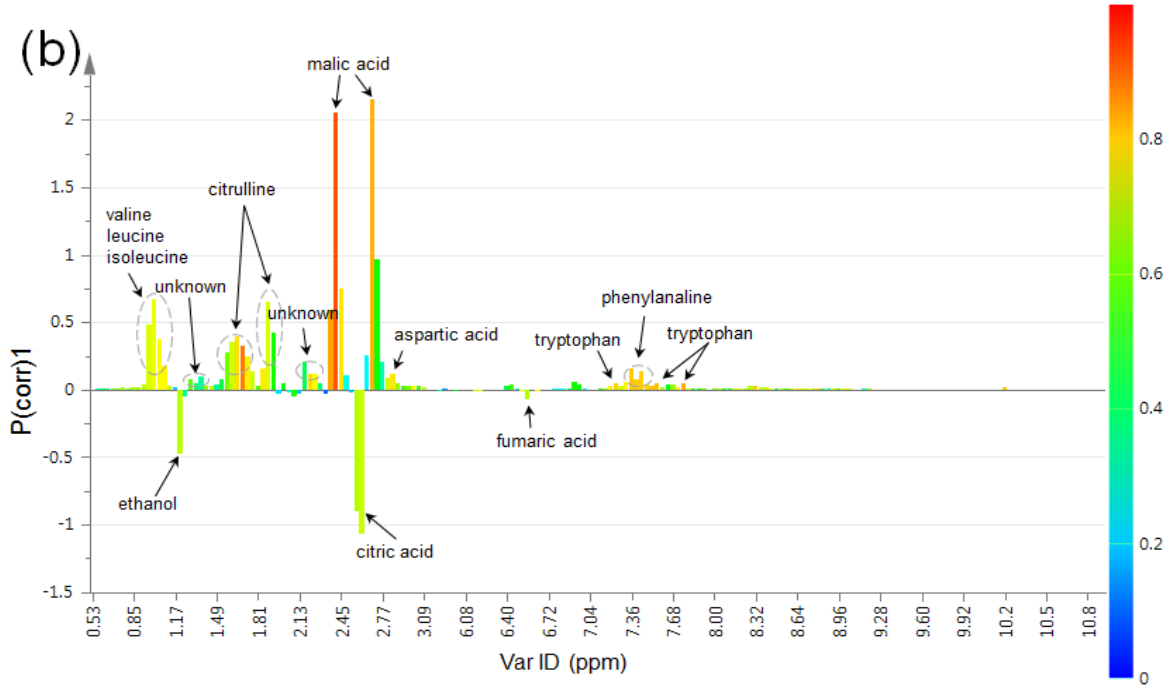


Figure 4-8 The score plot **(a)** and S-plot **(b)** S-column plot **(c)** of OPLS1 for 5 cultivars with Broad Band WET spectra, with water and sugar saturation region from 3.0ppm to 5.6 ppm excluded.


(a)
TS
MJ
OPLS2
OPLS1
20
10
0
-10
-20
-30
-40
-40
-30
-20
-10
0
10
20
30


(b)
malic acid
citrulline
valine
leucine
isoleucine
unknown
unknown
aspartic acid
phenylanaline
tryptophan
tryptophan
fumaric acid
ethanol
citric acid
P(corr)1
Var ID (ppm)
0.8
0.6
0.4
0.2
0

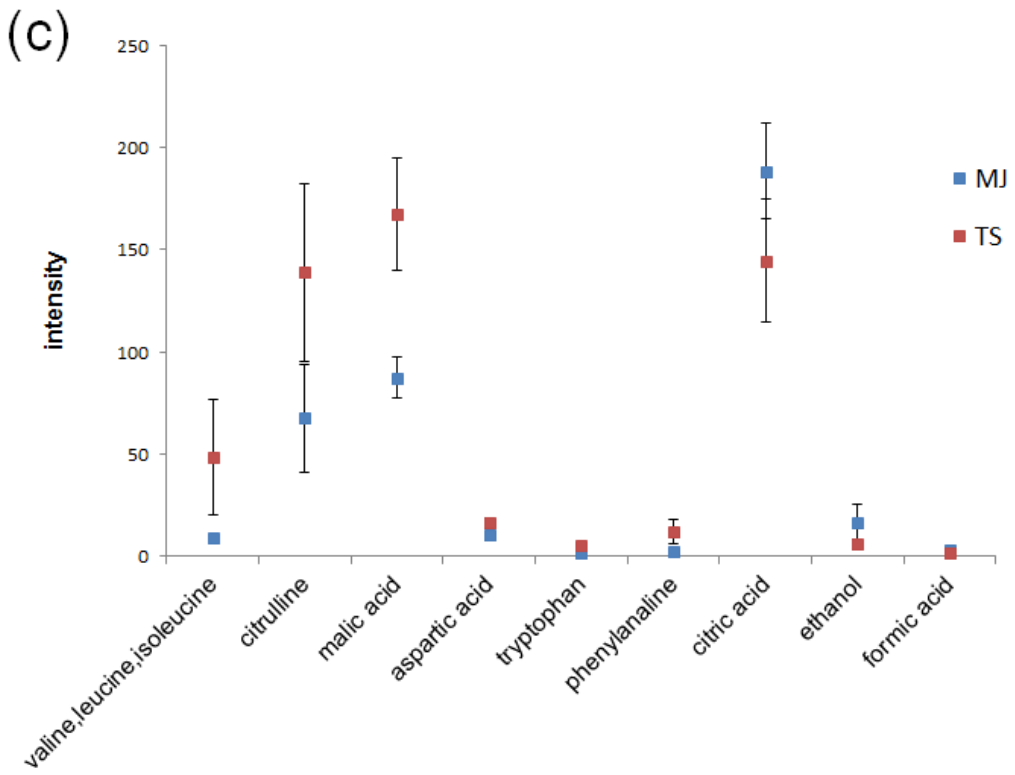
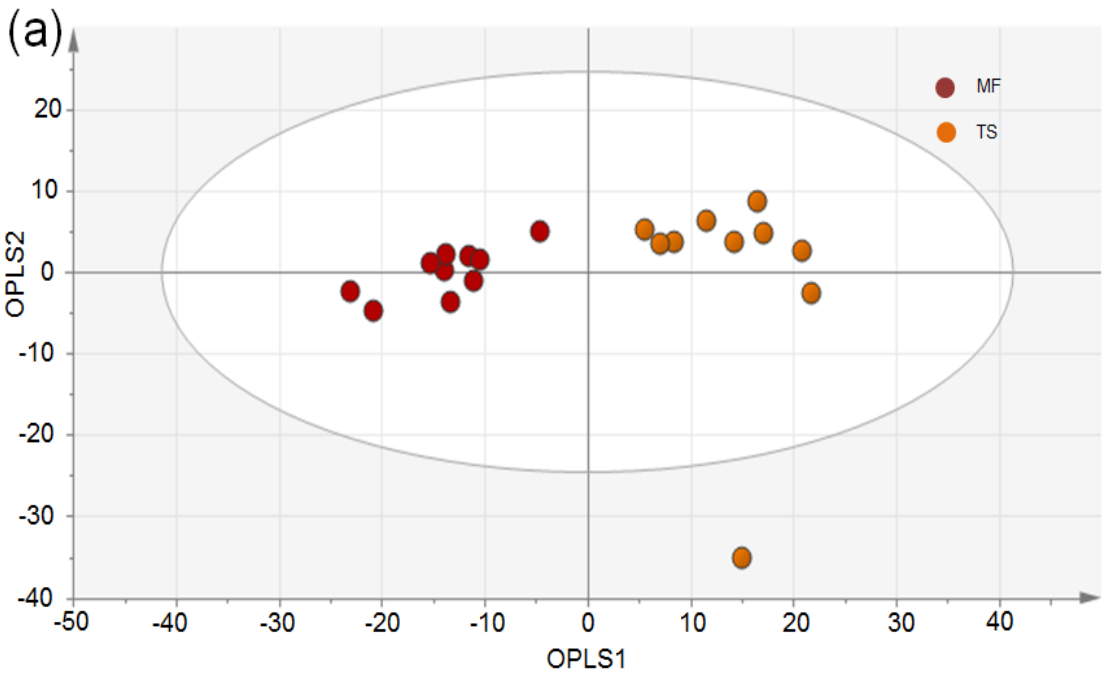
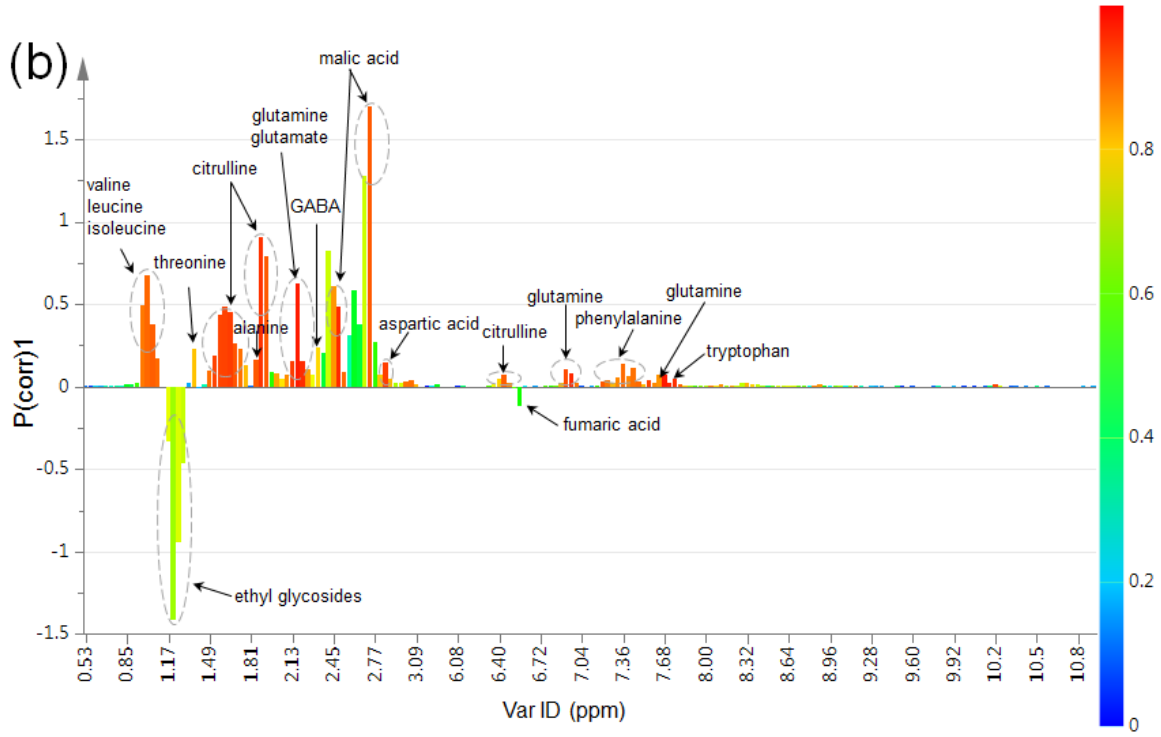


Figure 4-9 The score plot **(a)** and S-plot **(b)** S-column plot **(c)** of OPLS1 for 2 non-astringent cultivars (MJ and TS). **(c)** The integral intensities of biomarkers in MJ and TS cultivars.


(a)
MF
TS
OPLS2
OPLS1
20
10
0
-10
-20
-30
-40
-50
-40
-30
-20
-10
0
10
20
30
40


(b)
malic acid
glutamine
glutamate
citrulline
valine
leucine
isoleucine
GABA
threonine
alanine
aspartic acid
citrulline
glutamine
phenylalanine
glutamine
tryptophan
fumaric acid
ethyl glycosides
P(corr)1
Var ID (ppm)
1.5
1
0.5
0
-0.5
-1
-1.5
0.53
0.85
1.17
1.49
1.81
2.13
2.45
2.77
3.09
6.08
6.40
6.72
7.04
7.36
7.68
8.00
8.32
8.64
8.96
9.28
9.60
9.92
10.2
10.5
10.8
0.8
0.6
0.4
0.2
0

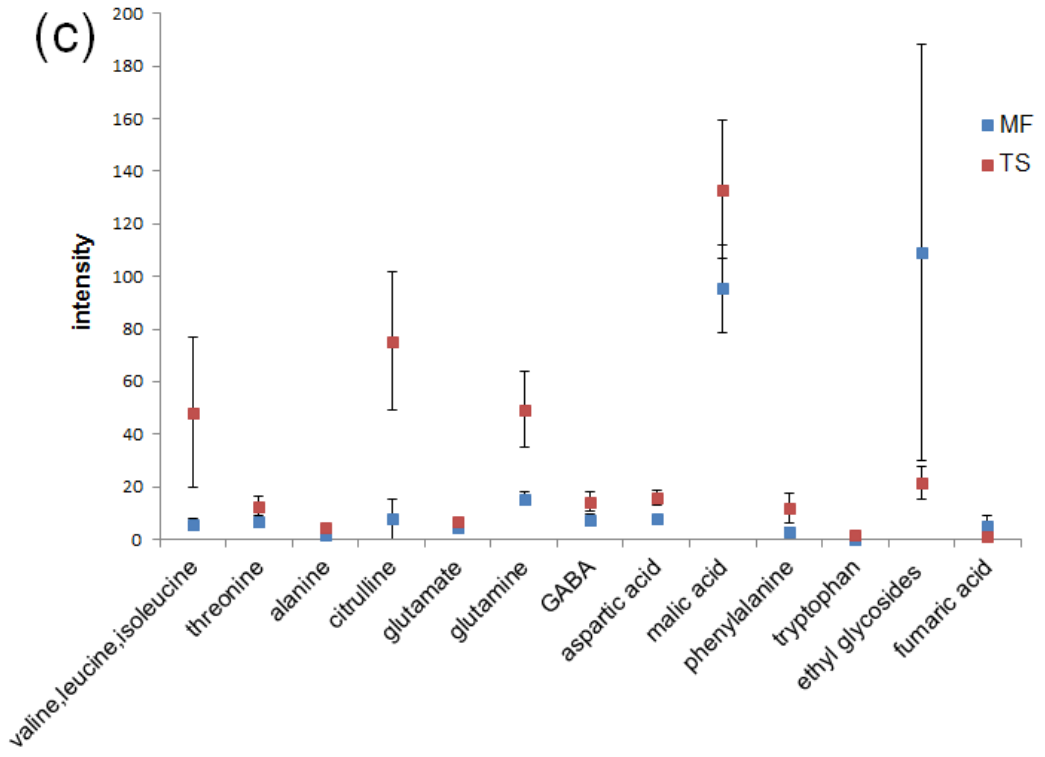
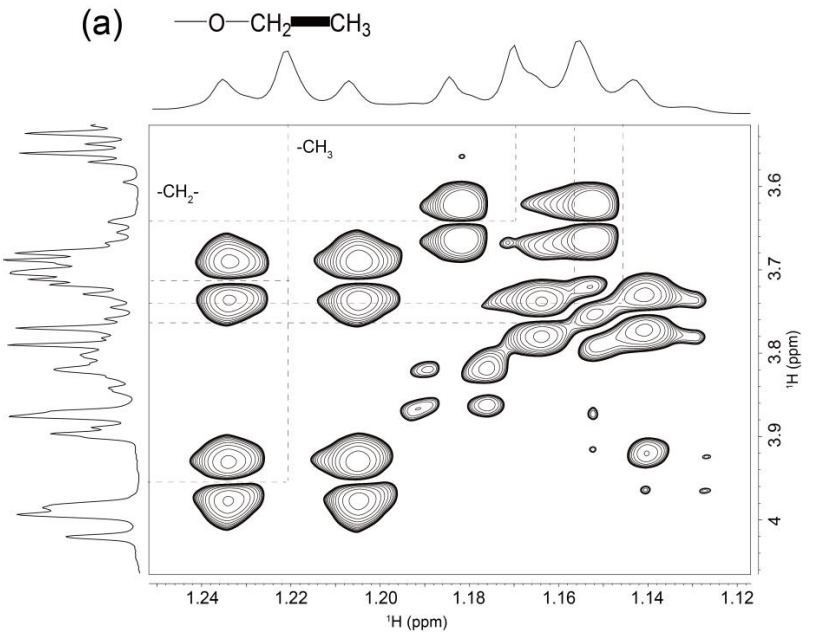
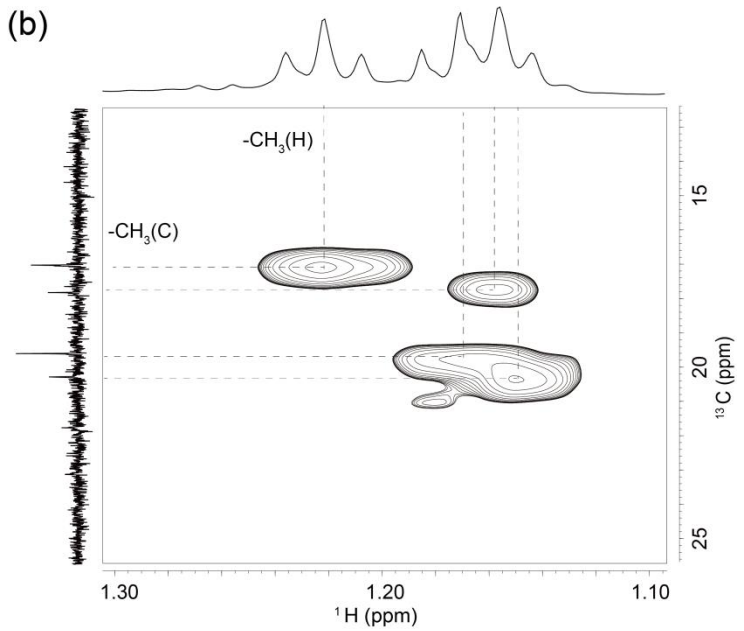


Figure 4-10 The score plot **(a)** and S-plot **(b)** S-column plot **(c)** of OPLS1 for 2 non-astringent cultivars (MF and TS). **(c)** The integral intensities of biomarkers in MF and TS cultivars.


(a)
—O—CH2—CH3
-CH3
-CH2-
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1H (ppm)
3.6
3.7
3.8
3.9
4
1H (ppm)


(b)
-CH3(H)
-CH3(C)
1.30
1.20
1.10
1H (ppm)
15
20
25
13C (ppm)

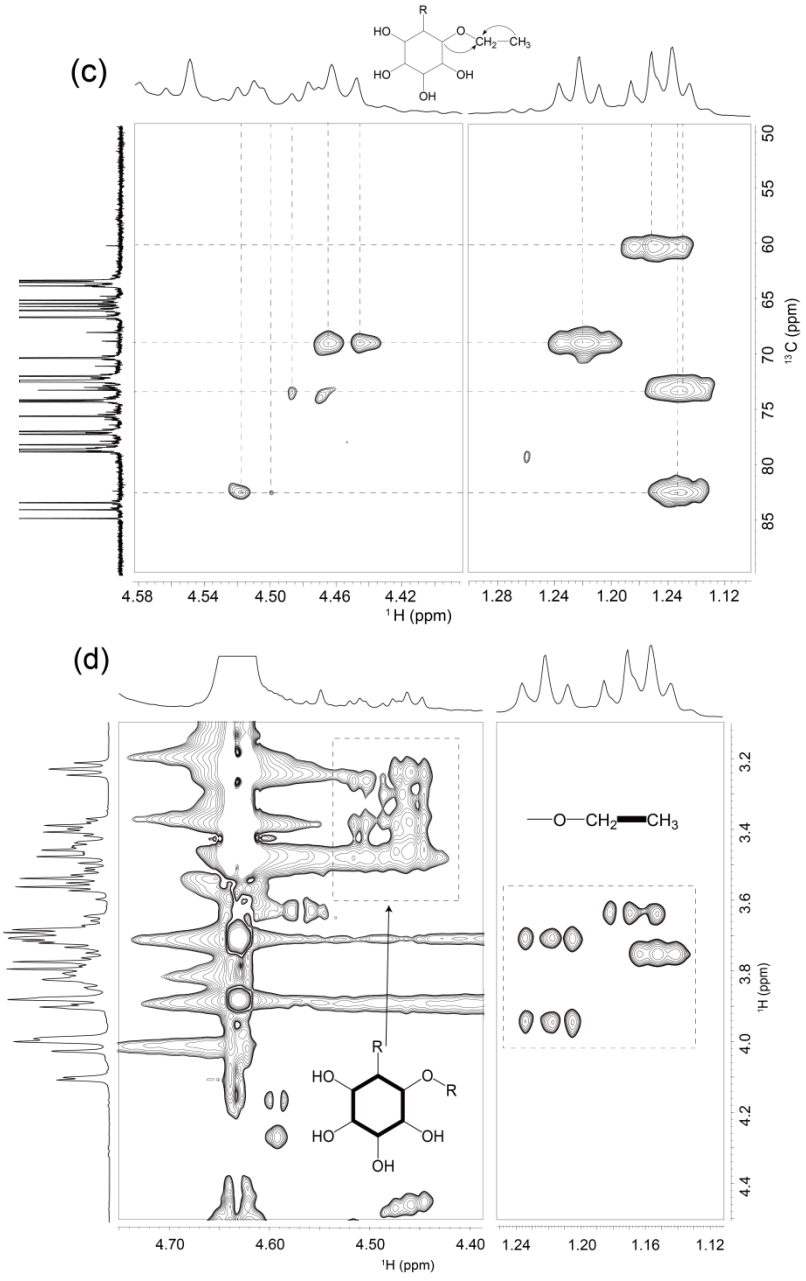
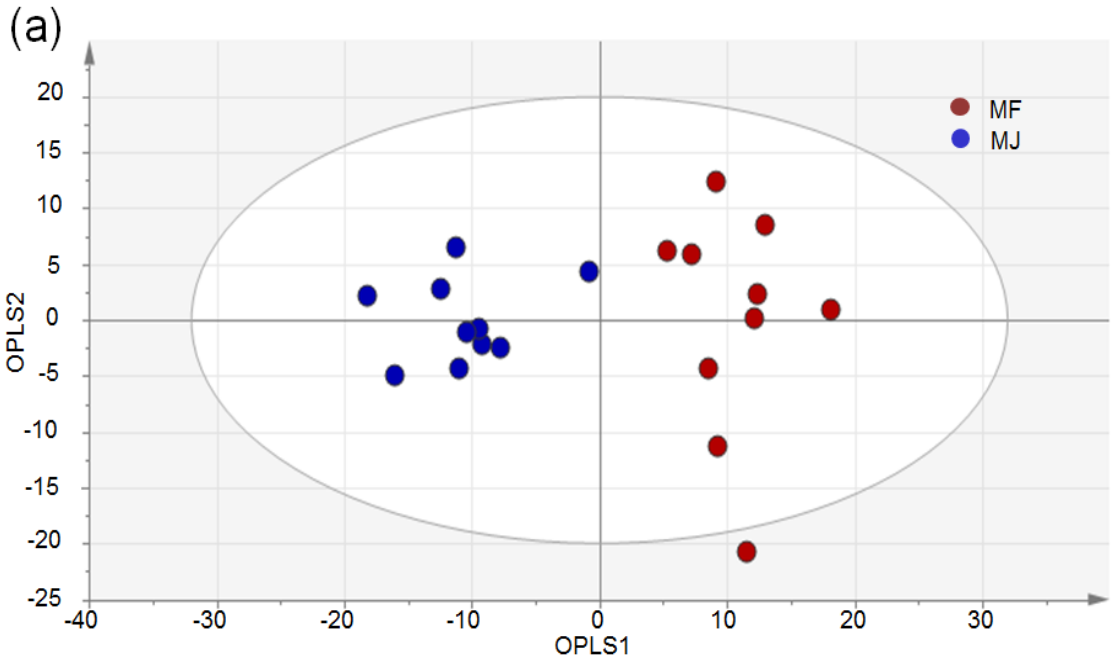
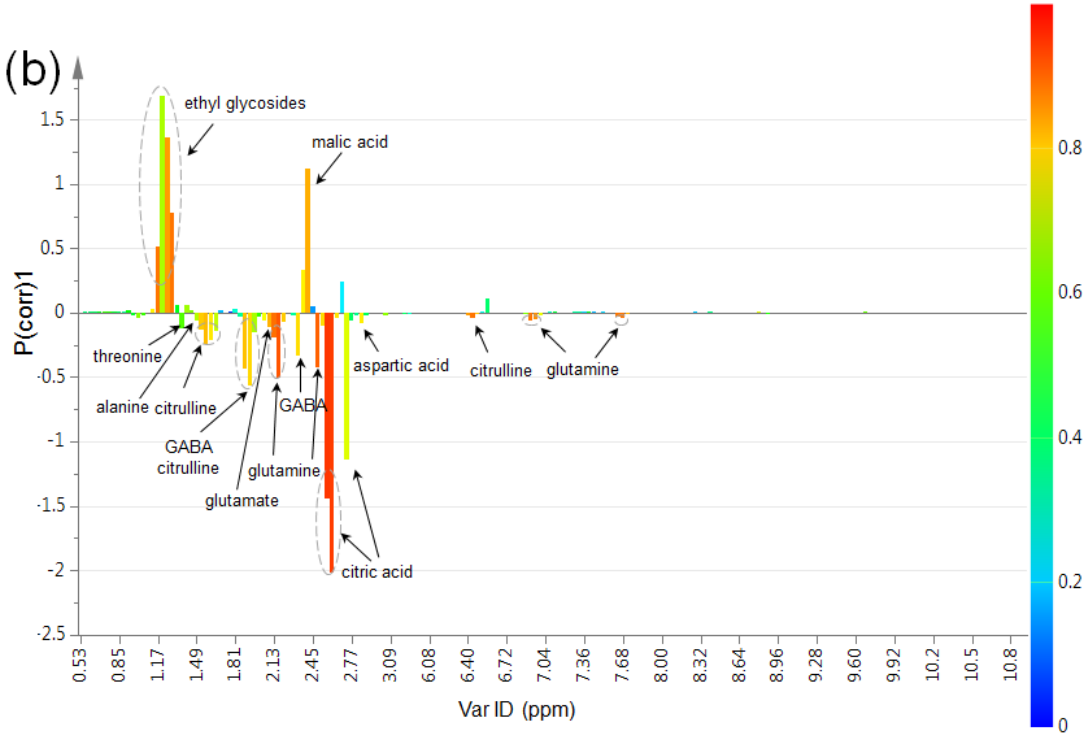


Figure 4-11 Expansion of the 2D NMR spectra of MF cultivar with assignment of ethyl glycosides. **(a)** ^{1}H-^{1}H DQF-COSY spectrum, the bold-bonds in chemical structure indicate the possible correlations in the ^{1}H-^{1}H DQF-COSY spectrum; **(b)** ^{1}H-^{13}C HSQC spectrum; **(c)** ^{1}H-^{13}C CT-HMBC spectrum, the arrows in chemical structure indicate the possible correlations in the ^{1}H-^{13}C CT-HMBC spectrum. **(b)** ^{1}H-^{1}H TOCSY spectrum, the bold-bonds in chemical structure indicate the possible correlations in the possible correlations in the ^{1}H-^{1}H TOCSY spectrum.


(a)
MF
MJ
OPLS2
OPLS1
20
15
10
5
0
-5
-10
-15
-20
-25
-40
-30
-20
-10
0
10
20
30


(b)
P(corr)1
Var ID (ppm)
ethyl glycosides
malic acid
threonine
alanine
citrulline
GABA
citrulline
GABA
glutamate
glutamine
aspartic acid
citrulline
glutamine
citric acid
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
0.53
0.85
1.17
1.49
1.81
2.13
2.45
2.77
3.09
6.08
6.40
6.72
7.04
7.36
7.68
8.00
8.32
8.64
8.96
9.28
9.60
9.92
10.2
10.5
10.8
0.8
0.6
0.4
0.2
0

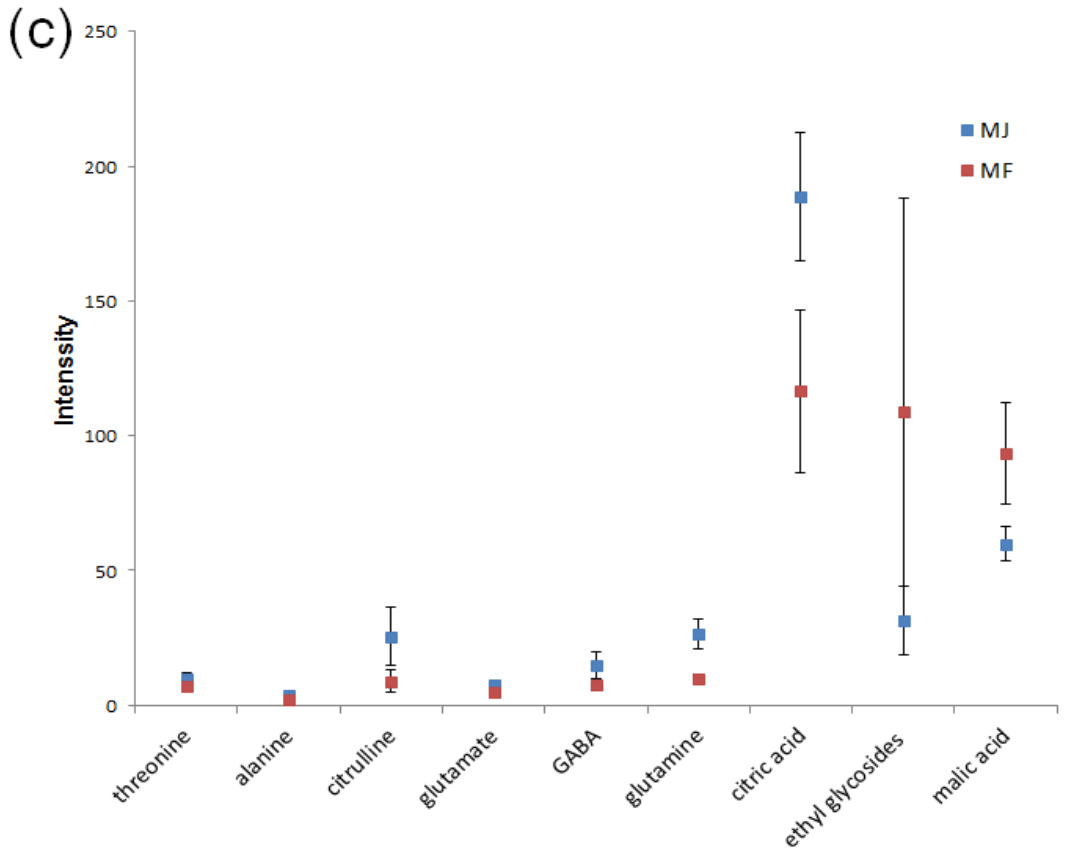
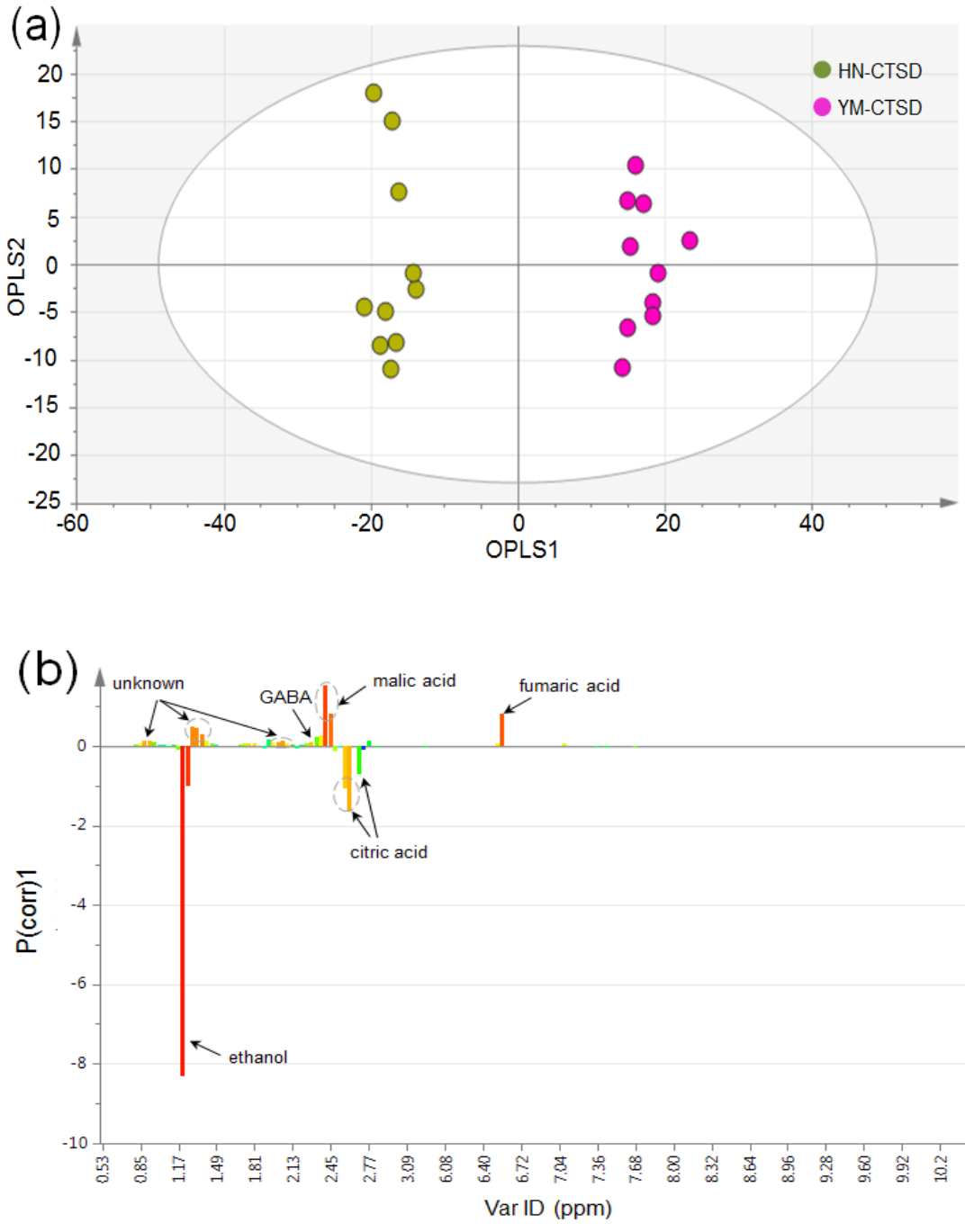


Figure 4-12 The score plot **(a)** and S-plot **(b)** S-column plot **(c)** of OPLS1 for 2 non-astringent cultivars (MF and MJ). **(c)** The integral intensities of biomarkers in MF and MJ cultivars.


(a)
HN-CTSD
YM-CTSD
OPLS2
OPLS1
20
15
10
5
0
-5
-10
-15
-20
-25
-60
-40
-20
0
20
40
(b)
unknown
GABA
malic acid
fumaric acid
citric acid
ethanol
P(corr)1
Var ID (ppm)
0
-2
-4
-6
-8
-10
0.53
0.85
1.17
1.49
1.81
2.13
2.45
2.77
3.09
6.08
6.40
6.72
7.04
7.36
7.68
8.00
8.32
8.64
8.96
9.28
9.60
9.92
10.2
10.5
10.8
0.8
0.6
0.4
0.2
0

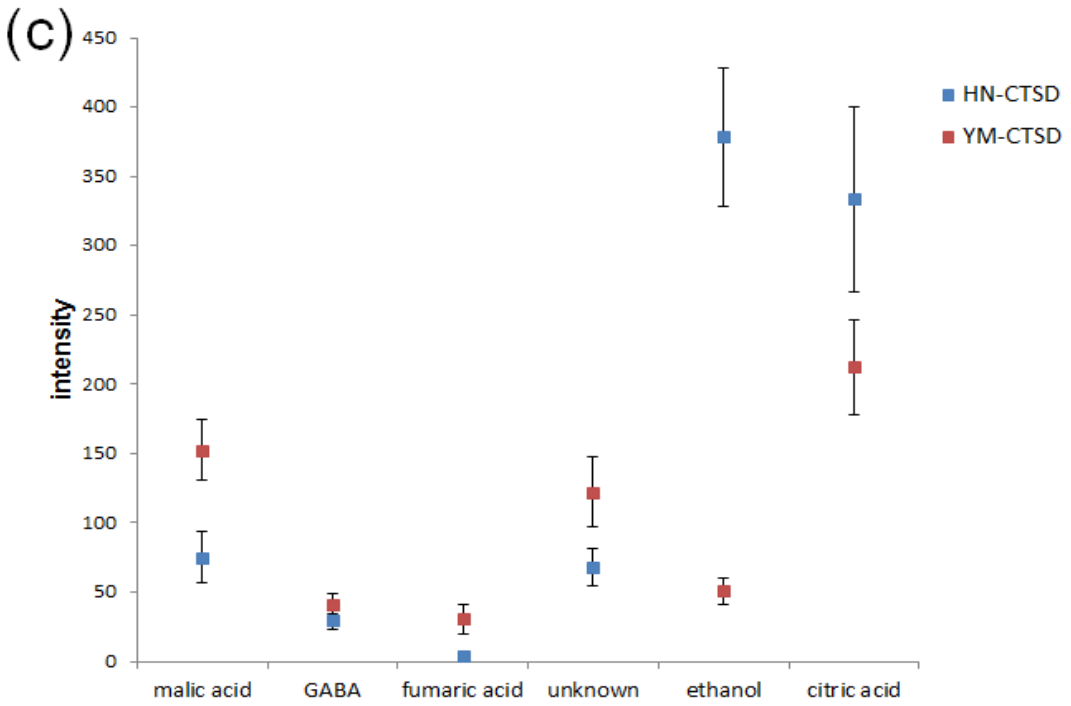


Figure 4-13 The score plot **(a)** and S-plot **(b)** S-column plot **(c)** of OPLS1 for 2 astringent cultivars (HN-CTSD and YM-CTSD). **(c)** The integral intensities of biomarkers in HN-CTSD and YM-CTSD cultivars.

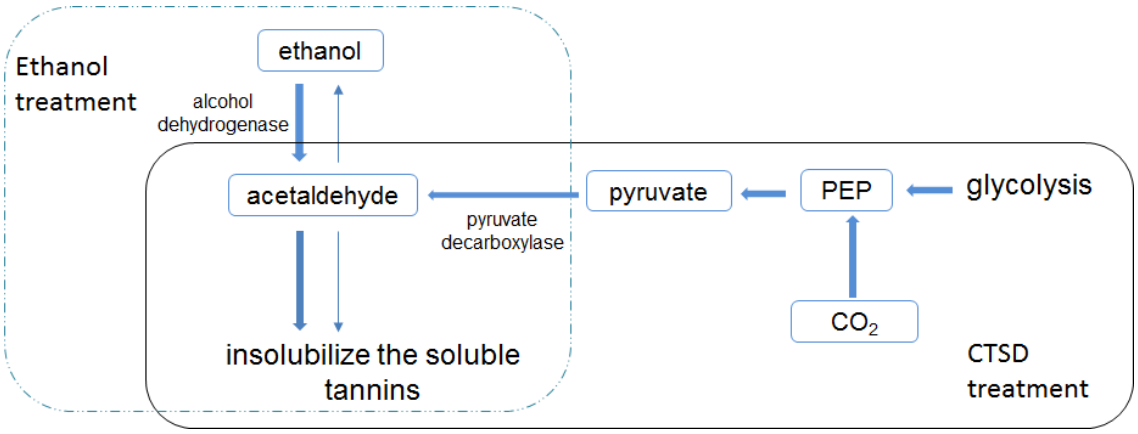


Figure 4-14 Metabolic pathway for CTSD and Ethanol treatment.

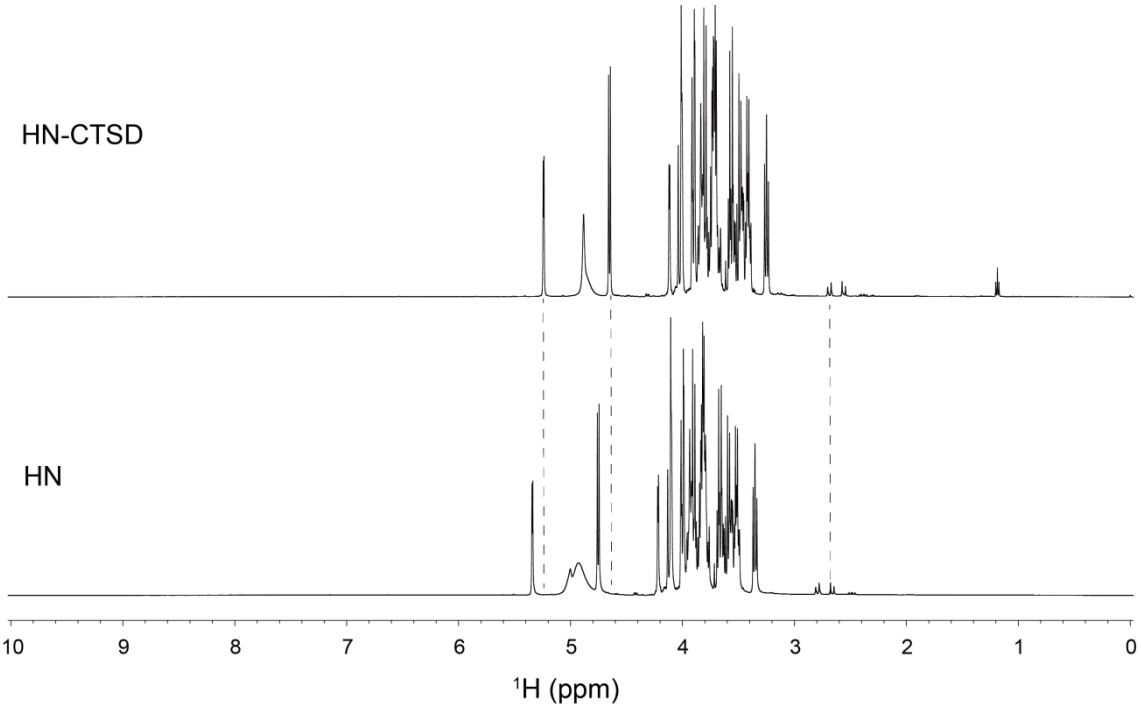


Figure 4-15 The shifts of peaks in spectrum of HN without CTSD treatment.

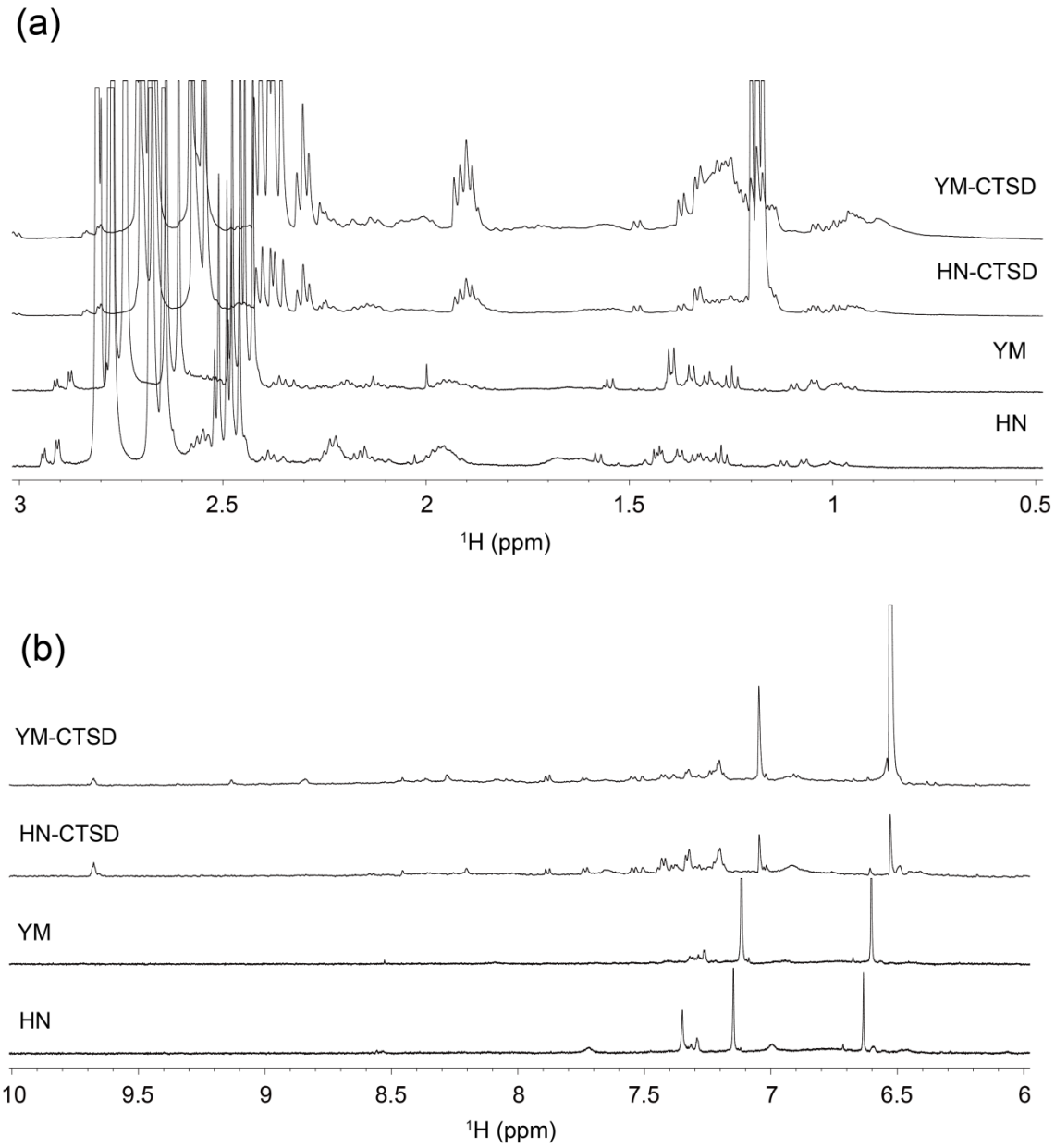


Figure 4-16 Expansion of high-field **(a)** and low-field **(b)** regions for HN and YM cultivars before (HN and YM) and after CTSD treatment (HN-CTSD and YM-CTSD).

Overall Discussion

The present study proposed Broad Band WET method, which is a modification of WET method, as a new method for saturating signals with a broad bandwidth. This new method was proved to be an efficient and highly selective method for the simultaneous suppression of the strong signals derived from water and sugars in persimmon juice, and provided high-throughput information in a high S/N ratio for minor components in complex mixture both qualitatively and quantitatively. It is a promising method to be applied to analysis of juices or other foods universally, which contain high concentrations of sugars or other major components.

Furthermore, the identification and quantification of chemical compounds in persimmon juice (Taishu cultivar.) by one-dimensional (1D) NMR and various 2D NMR spectroscopy was carried out. In order to make an unambiguous identification of minor components, 2D NMR spectroscopy incorporating with Broad Band WET sequence or WET sequence were devised and 10 mm probe was used to acquire ^{13}C NMR spectra. With those attempts, the signals from many minor components were observed clearly. A comprehensive metabolic profile for Japanese persimmon juice with NMR spectroscopy was provided and 26 compounds were identified. In addition, most components were quantified by the integration of signals using conventional ^{1}H NMR and Broad Band WET spectra.

Moreover, cultivar discriminations among 5 commercial Japanese persimmons were applied, using Broad Band WET NMR spectra combined with multivariate analysis. The PCA models clearly demonstrated that the cultivar discrimination of Japanese persimmons by ^{1}H NMR spectra can be improved by removing the major bulk signals (water and sugars). A better discrimination and a more informative detail of metabolic differences among cultivars can be achieved when using Broad Band WET spectra than using conventional ^{1}H NMR. Biomarkers contributing to the cultivar discrimination were proposed by OPLS-DA models. Some amino acids (citrulline, GABA, tryptophan, phenylalanine, etc.), organic acids (malic acid, citric acid, etc.), ethanol, etc. were captured as biomarkers responsible for the cultivar discrimination. The results indicated that the minor components are valuable to characterize the persimmon cultivars, and Broad Band WET spectra combined with multivariate analysis could be a very useful

tool for Japanese persimmon cultivar characterization. The metabolite-based analysis of cultivar discrimination with NMR spectroscopy will provide a meaningful data on Japanese persimmon studies, and help in selection or modification of better or nutritionally important cultivars combined with genomics researches in the future.

REFERENCES

Aprea, E.; Gika, H.; Carlin, S.; Theodoridis, G.; Vrhovsek, U.; Mattivi, F., Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry. Journal of Chromatography, A, 2011, 1218, 4517-4524

Asgar, M.A.; Yamauchi, R.; Kato, K., Modification of pectin in Japanese persimmon fruit during the sun-drying process, Food Chemistry, 2003, 81, 555-560

Beckmann, M.; Enot, D. P.; Overy, D. P.; Draper, J., Representation, comparison, and interpretation of metabolome fingerprint data for total composition analysis and quality trait investigation in potato cultivars, Journal of Agricultural and Food Chemistry, 2007, 55, 3444-3451.

Belton, P. S.; Delgadillo, I.; Gil, A. M.; Roma, P.; Casuscelli, F.; Colquhoun, I. J.; Dennis, M. J.; Spraul, M., High-field proton NMR studies of apple slices. Magnetic Resonance in Chemistry, 1997, 35, S52-S60

Belton, P. S.; Colquhoun, I. J.; Kemsley, E. K.; Delgadillo, I.; Roma, P.; Dennis, M. J.; Sharman, M.; Holmes, E.; Nicholson, J. K.; Spraul, M., Application of chemometrics to the ^{1}H NMR spectra of apple juices: discrimination between apple varieties. Food Chemistry, 1998, 61, 207-213

Biais, B.; Allwood, J. W.; Deborde, C.; Deborde, C.; Xu, Y.; Maucourt, M.; Beauvoit, B.; Dunn, W. B.; Jacob, D.; Goodacre, R.; Rolin, D.; Moing, A., H-1 NMR, GC-EI-TOFMS, and data set correlation for fruit metabolomics: application to spatial metabolite analysis in melon. Analytical Chemistry, 2009, 81, 2884-2894.

Caligiani, A.; Acquotti, D.; Palla, G.; Bocchi, V., Identification and quantification of the main organic components of vinegars by high resolution ^{1}H NMR spectroscopy. Analytica Chimica Acta, 2007, 585, 110-119.

Candir, E. E.; Ozdemir, A. E.; Kaplankiran, M.; Toplu, C., Physico-chemical changes during growth of persimmon fruits in the East Mediterranean climate region. Scientia Horticulturae, 2009, 121, 42-48

Chen, X. N.; Fan, J. F.; Yue, X.; Wu, X. R.; Li, L. T., Radical scavenging activity and phenolic compounds in persimmon (Diospyros kaki L. cv. Mopan). Journal of Food Science, 2008, 73, C24-C28

Croasmun W. R.; Carlson R. M. K., Two-dimensional NMR spectroscopy: applications for chemists and biochemists, second edition, Wiley-VCH, New York, 1994

Cui, Q.; Lewis, I. A.; Hegeman, A. D.; Anderson, M. E.; Li, J.;Schulte, C. F.; Westler, W. M.; Eghbalnia, H. R.; Sussman, M. R.;Markley, J. L. Metabolite identification via the Madison Metabolomics Consortium Database. Nature Biotechnology, 2008, 26, 162−164.

Dalvit. C.; Shapiro, G.; Böhlen, J. M.; Parella, T., Technical aspects of an efficient multiple solvent suppression pulse sequence, Magnetic Resonance in Chemistry, 1999, 37, 7−14.

Del Bubba, M.; Giordani, E.; Pippucci, L.; Cincinelli, A.; Checchini, L.; Galvan, P., Changes in tannins, ascorbic acid and sugar content in astringent persimmons during on-tree growth and ripening and in response to different postharvest treatments. Journal of Food Composition and Analysis, 2009, 22, 668-677

Dobson, G.; Shepherd, T.; Verrall, S. R.; Conner, S.; McNicol, J.W.; Ramsay, G.; Shepherd, L. V. T.; Davies, H. V.; Stewart, D., Phytochemical diversity in tubers of potato cultivars and landraces using a GC-MS metabolomics approach, Journal of Agricultural and Food Chemistry, 2008, 56, 10280-10291

Duarte, I. F.; Goodfellow, B. J.; Gil, A. M.; Delgadillo, I., Characterization of mango juice by high-resolution NMR, hyphenated NMR, and diffusion-ordered spectroscopy. Spectroscopy Letters, 2005, 38, 319-342

Duarte, I. F.; Delgadillo, I; Gil, A. M., Study of natural mango juice spoilage and microbial contamination with Penicillium expansum by high resolution ^{1}H NMR spectroscopy, Food Chemistry, 2006, 96, 313-324

Dunn, W. B.; Bailey, N. J. C.; Johnson, H. E., Measuring the metabolome: current analytical technologies, Analyst, 2005, 130, 606-625

Furihata, K.; Seto, H. Constant time HMBC (CT-HMBC), a new HMBC technique useful for improving separation of cross peaks. Tetrahedron Letters, 1998, 39, 7337−7340.

Gil, A. M ; Duarte, I. F.; Delgadillo, I.; Colquhoun, I. J.; Casuscelli, F.; Humpfer, E.; Spraul, M .,Study of the compositional changes of mango during ripening by use of nuclear magnetic resonance spectroscopy. Journal of Agricultural and Food Chemistry, 2000, 48, 1524-1536

Giordani, E.; Doumett, S.; Nin, S.; Del Bubba, M., Selected primary and secondary metabolites in fresh persimmon (Diospyros kaki Thunb.): A review of analytical methods and current knowledge of fruit composition and health benefits. Food Research International, 2011, 44, SI 1752-1767

Gomez-Romero, M.; Segura-Carretero, A.; Fernandez-Gutierrez, A., Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. Phytochemistry, 2010, 71, 1848-1864

Gorinstein, S.; Kulasek, G. W.; Bartnikowska, E.; Leontowicz, M.; Zemser, M.; Morawiec, M.; Trakhtenberg, S., The effects of diets, supplemented with either whole persimmon or phenol-free persimmon, on rats fed cholesterol. Food Chemistry, 2000, 70, 303-308

Gorinstein, S.; Zachwieja, Z.; Folta, M.; Barton, H.; Piotrowicz, J.; Zemser, M.; Weisz, M.; Trakhtenberg, S.; Martin-Belloso, O., Comparative contents of dietary fiber, total phenolics, and minerals in persimmons and apples. Journal of Agricultural and Food Chemistry, 2001, 49, 952-957

Gorinstein, S; Zachwieja, Z; Folta, M; Barton, H.; Piotrowicz, J.; Zemser, M.; Weisz, M.; Trakhtenberg, S.; Martin-Belloso, O., Comparative contents of dietary fiber, total phenolics, and minerals in persimmons and apples. Journal of Agricultural and Food Chemistry, 2001, 49, 952-957

Hajduk, P. J.; Horita, D. A.; Lerner, L. E., Theoretical-analysis of relaxation during shaped pulses. 1. The effects of short T_1 and T_2. Journal of magnetic resonance, series A, 1993, 103,40-52

Harada, H.; Itamura, H.; Taira, S; Zheng, G. H., Natural removal of astringency in PCNA persimmon fruit CV Jiro grown in some different districts of Japan. Journal of the Japanese Society for Horticultural Science, 1990, 58, 807-811

Henson, G. L.; Niemeyer, L.; Ansong, G.; Forkner, R.; Makkar, H. P. S.; Hagerman, A. E., A modified method for determining protein binding capacity of plant polyphenolics using radiolabelled protein. Phytochemical Analysis, 2004, 15, 159-163

Hong, Y. S., NMR-based metabolomics in wine science. Magnetic Resonance in Chemistry, 2011, 49, SI, S13-S21

Hu, F.; Furihata, K.; Ito-Ishida, M.; Kaminogawa, S.; Tanokura, M., Nondestructive observation of bovine milk by NMR spectroscopy: analysis of existing States of

compounds and detection of new compounds. Journal of Agricultural and Food Chemistry, 2004, 52, (16), 4969-74.

Hu, F.; Furihata, K.; Kato, Y.; Tanokura, M., Nondestructive quantification of organic compounds in whole milk without pretreatment by two-dimensional NMR spectroscopy. Journal of Agricultural and Food Chemistry, 2007, 55, (11), 4307-11.

Hwang, T. L.; Shaka, A. J., Water suppression that works-Excitation sculpting using arbitrary wave-forms and pulsed-field gradients. Journal of magnetic resonance, series A, 1995, 112, 275−279.

Ittah, Y., Sugar content changes in persimmon fruits (*Diospyrose Kaki L.*) during artificial ripening with CO_2- A possible connection to deasringency mechanisms. Food Chemistry, 1993, 48, 25-29

Iwanami, H.; Yamada, M.; Sato, A., A great increase of soluble solids concentration by shallow concentric skin cracks in Japanese persimmon. Scientia Horticulturae, 2002, 94, 251-256.

Jang, I. C.; Jo, E. K.; Bae, M. S.; Lee, H. J.; Jeon, G. I.; Park, E.; Yuk, H. G.; Ahn, G. H.; Lee, S. C., Antioxidant and antigenotoxic activities of different parts of persimmon (Diospyros kaki cv. Fuyu) fruit. Journal of Medicinal Plants Research, 2010, 4, 155-160

Kanzaki, S.; Yonemori, K.; Sato, A.; Yamada, M.; Sugiura, A ., Analysis of the genetic relationships among pollination-constant and non-astringent (PCNA) cultivars of persimmon (Diospyros kaki Thunb.) from Japan and China using amplified fragment length polymorphism (AFLP). Journal of the Japanese Society for Horticultural Science, 2000, 69, 665-670

Ko, B. K.; Ahn, H. J.; van den Berg, F.; Lee, C. H.; Hong, Y. S., Metabolomic Insight into Soy Sauce through H-1 NMR Spectroscopy. Journal of Agricultural and Food Chemistry, 2009, 57, 6862-6870.

Koda, M.; Furihata, K.; Wei, F.; Miyakawa, T.; Tanokura, M., Metabolic discrimination of mango juice from various cultivars by band-selective NMR spectroscopy. Journal of Agricultural and Food Chemistry, 2012, 60, 1158-1166

Kövér K. E., Fehér, K.; Szilágyi, L.; Borbás, A.; Herczegh, P.; Lipták, A., 2D NMR spectra of oligosaccharides enhanced by band-selective suppression of unwanted signals. Tetrahedron Letters, 2000, 41, 393−396.

Luo, Z. R.; Yonemori, K.; Sugiura, A., Evaluation of RAPD analysis for cultivar

identification of persimmons. Journal of the Japanese Society for Horticultural Science, 1995, 64, 535-541

Maki, S.; Oyama, K.; Kurahashi, T.; Nakahira, T.; Kawabata, T.; Yamada, T., RFLP analysis for cultivar identification of persimmons. Scientia Horticulturae, 2001, 91, 407-412.

Mallavadhani, U. V.; Panda, A. K.; Rao, Y. R., Pharmacology and chemotaxonomy of Diospyros. Phytochemistry, 1998, 49, 901-951

Mannina, L.; Sobolev, A. P.; Viel, S., Liquid state H-1 high field NMR in food analysis. Progress in nuclear magnetic resonance spectroscopy, 2012, 66, 1-39

Matsui, S.; Ito, S.; Murata, N.; Banba, Y., The relations of the chemical constituents to the qualitied in died persimmons. 園芸学会雑誌、1957, 26, 105-110

Matsumoto, K.; Kadowaki, A.; Ozaki, N.; Takenaka, M.; Ono, H.; Yokoyama, S .; Gato, N., Bile acid-binding ability of kaki-tannin from young fruits of persimmon (*Diospyros kaki*) in vitro and in vivo. Phytotherapy Research, 2011, 25, 624-628.

Mikros, E.; Anastasiadi, M.; Zira, A.; Magiatis, P.; Haroutounian, S. A.; Skaltsounis, A. L., ^{1}H NMR-based metabonomics for the classification of greek wines according to variety, region, and vintage. Comparison with HPLC data. Journal of Agricultural and Food Chemistry, 2009, 57, 11067-11074.

Mo, H.; Raftery, D., Improved residual water suppression: WET180. Journal of Biomolecular NMR, 2008, 41, 105-111.

Mo H.; Raftery D., Pre-SAT180, a simple and effective method for residual water suppression, Journal of magnetic resonance, 2008, 190, 1−6.

Naval, M. D.; Zuriaga, E.; Pecchioli, S.; Llacer, G.; Giordani, E.; Badenes, M. L., Analysis of genetic diversity among persimmon cultivars using microsatellite markers. Tree Genetics & Genomes, 2010, 6, 677-687

Niikawa, T.; Inari, T.; Ozeki, T.; Mitsui, B., Effects of 1-methylcyclopropene on flesh firmness during storage of pollination-constant and non-astringent cultivars of Japanese persimmon (Diospyros kaki). Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi, 2005, 52, 68-73.

Nilsson, M.; Duarte, I. F.; Almeida, C.; Delgadillo, I.; Goodfellow, B. J.; Gil, A. M.; Morris, G. A., High-resolution NMR and diffusion-ordered spectroscopy of port wine.

Journal of Agricultural and Food Chemistry, 2004, 52, 3736-43.

Niwa, T., Metabolic profiling with gas chromatography-mass spectrometry and its application to clinical medicine. Journal of Chromatography, 1986, 379, 313-345

Ohno, A.; Oka, K.; Sakuma, C.; Okuda, H.; Fukuhara, K., Characterization of tea cultivated at four different altitudes using ^{1}H NMR analysis coupled with multivariate statistics. Journal of Agricultural and Food Chemistry, 2011, 59, 5181-5187.

Park, Y. S.; Leontowicz, H.; Leontowicz, M.; Namiesnik, J.; Jesion, I.; Gorinstein, S., Nutraceutical value of persimmon (Diospyros kaki Thunb.) and its influence on some indices of atherosclerosis in an experiment on rats fed cholesterol-containing diet. Advances in Horticultural Science, 2008, 22, 250-254

Perez, E. M. S.; Lopez, J. G.; Iglesias, M. J.; Ortiz, F. L.; Toresano, F.; Camacho, F., HRMAS-nuclear magnetic resonance spectroscopy characterization of tomato "flavor varieties" from Almeria (Spain), Food Research International, 2011, 44, 3212-3221

Piotto, M.; Saudek, V.; Sklenar, V., Gradient-tailored excitation for single-quantum NMR-spectroscopy of aqueous-solutions. Journal of Biomolecular NMR, 1992, 2, 661-665.

Rutherford, T.J.; Kartha, K.P.R.; Readman, S.K.; Cura, P.; Field, R. A., Iodine and its' interhalogen compounds: Useful reagents in carbohydrate chemistry - X. Adaptation of an NMR signal suppression pulse sequence for the selective removal of benzylic methylene signals of benzyl ether protected carbohydrates. Tetrahedron Letters, 1999, 40, 2025-2028

Ryu, D. H.; Jung, Y.; Lee, J.; Kwon, J.; Lee, K. S.; Hwang, G. S., Discrimination of the geographical origin of beef by ^{1}H NMR-based metabolomics. Journal of Agricultural and Food Chemistry, 2010, 58, 10458-10466.

Schievano, E.; Peggion, E.; Mammi, S., H-1 nuclear magnetic resonance spectra of chloroform extracts of honey for chemometric determination of its botanical origin. Journal of Agricultural and Food Chemistry, 2010, 58, 57−65.

Senter, S. D.; Chapman, G. W.; Forbus, W. R.; Payne, J. A., Sugar and nonvolatile acid composition of persimmons during maturation. Journal of Food Science, 1991, 56, 989-991

Smallcombe, S. H.; Patt, S. L.; Keifer, P. A., WET solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy. Journal of magnetic

resonance, series A, 1995, 117, 295-303

Sobolev, A. P.; Segre, A.; Lamanna, R., Proton high-field NMR study of tomato juice. Magnetic Resonance in Chemistry 2003, 41, 237-245.

Sugiura, A.; Tomana, T., Relationships of ethanol-production by seeds of different types of Japanese persimmons and their tannin content. Hortscience, 1983, 18,319-321

Taira, S.; Abe, K.; Ooi, K.; Watanabe, S., Influence of fruit size, defoliation, gibberellin, and growing regions on the ease of removal of astringency in Japanese persimmon. Journal of the Japanese Society for Horticultural Science, 1990, 59, 299-305

Taira, S.; Oba, S.; Watanabe, S., Removal of astringency from 'Hiratanenashi' persimmon fruit with a mixture of ethanol and carbon dioxide. Journal of the Japanese Society for Horticultural Science, 1992, 61, 437-443

Taira, S; Ooi, M; Watanabe, S, Volatile compounds of astringent persimmon fruits. Journal of the Japanese Society for Horticultural Science, 1996, 65, 177-183

Takahashi, M.; Watanabe, H.; Kikkawa, J.; Ota, M.; Watanabe, M.; Sato, Y.; Inomata, H.; Sato, N., Carotenoids extraction from Japanese persimmon (Hachiyakaki) peels by supercritical CO2 with ethanol. Analytical Sciences, 2006, 22, 1441-1447

Tarazona-Diaz, M. P.; Alacid, F.; Carrasco, M.; Martinez, I.; Aguayo, E., Watermelon juice: potential functional drink for sore muscle relief in athletes. Journal of Agricultural and Food Chemistry, 2013, 61, 7522-7528.

Tiitinen, K. M.; Yang, B. R.; Haraldsson, G. G.; Jonsdottir, S.; Kallio, H. P., Fast analysis of sugars, fruit acids, and vitamin C in sea buckthorn (Hippophae rhamnoides L.) varieties. Journal of Agricultural and Food Chemistry, 2006, 54, 2508-2513

Tiziani, S; Schwartz, S. J.; Vodovotz, Y., Profiling of carotenoids in tomato juice by one- and two-dimensional NMR. Journal of Agricultural and Food Chemistry, 2006, 54, 6094-6100

Uchida, S.; Ohta, H.; Niwa, M.; Mori, A.; Nonaka, G.; Nishioka, I.; Ozaki, M., Prolongation of life span of stroke-prone spontaneously hypertensive rats (SHRSP) ingesting persimmon tannin. Chemical and Pharmaceutical Bulletin (Tokyo), 1990,38,1049-1052.

Ulrich, E. L.; Akutsu, H.; Doreleijers, J. F.; Harano, Y.;Ioannidis, Y. E.; Lin, J.; Livny, M.; Mading, S.; Maziuk, D.; Miller, Z.;Nakatani, E.; Schulte, C. F.; Tolmie, D. E.; Kent Wenger, R.; Yao, H.;Markley, J. L. BioMagResBank. Nucleic Acids Research, 2008, 36,

D402−408

Veberic, R.; Jurhar, J.; Mikulic-Petkovsek, M.; Stampar, F.; Schmitzer, V., Comparative study of primary and secondary metabolites in 11 cultivars of persimmon fruit (Diospyros kaki L.). Food Chemistry, 2010, 119, 477-483

Vermathen, M.; Marzorati, M.; Baumgartner, D.; Good, C.; Vermathen, P., Investigation of different apple cultivars by high resolution magic angle spinning NMR. A feasibility study, Journal of Agricultural and Food Chemistry, 2011, 59, 12784-12793

Wakabayashi, K., Changes in cell wall polysaccharides during fruit ripening. Journal of Plant Research, 2000, 113, 231-237

Wei, F.; Furihata, K.; Hu, F.; Miyakawa, T.; Tanokura, M., Complex mixture analysis of organic compounds in green coffee bean extract by two-dimensional NMR spectroscopy. Magnetic Resonance in Chemistry, 2010, 48, 857-865.

Wei, F.; Furihata, K.; Hu, F.; Miyakawa, T.; Tanokura, M., Two-dimensional ^{1}H-^{13}C nuclear magnetic resonance (NMR)-based comprehensive analysis of roasted coffee bean extract. Journal of Agricultural and Food Chemistry, 2011, 59, 9065-9073

Wei, F.; Furihata, K.; Koda, M.; Hu, F.; Kato, R.; Miyakawa, T.; Tanokura, M., ^{13}C NMR-based metabolomics for the classification of green coffee beans according to variety and origin. Journal of Agricultural and Food Chemistry, 2012, 60,10118-10125

Wei, F.; Furihata, K.; Koda, M.; Hu, F.; Miyakawa, T.; Tanokura, M., Roasting process of coffee beans as studied by nuclear magnetic resonance: time course of changes in composition, Journal of Agricultural and Food Chemistry, 2012, 60, 1005-1012

Wishart, D.S., Metabolomics: applications to food science and nutrition research. Trends in Food Science & Technology, 2008, 19, 482-493

Wishart, D. S. Quantitative metabolomics using NMR, TrAC Trends in Analytical Chemistry, 2008, 27, 228–237

Wishart, D. S.; Knox, C.; Guo, A. C.; Eisner, R.; Young, N.;Gautam, B.; Hau, D. D.; Psychogios, N.; Dong, E.; Bouatra, S.;Mandal, R.; Sinelnikov, I.; Xia, J.; Jia, L.; Cruz, J. A.; Lim, E.; Sobsey,C. A.; Shrivastava, S.; Huang, P.; Liu, P.; Fang, L.; Peng, J.; Fradette,R.; Cheng, D.; Tzur, D.; Clements, M.; Lewis, A.; Souza, A. D.;Zuniga, A.; Dawe, M.; Xiong, Y.; Clive, D.; Greiner, R.; Nazyrova, A.;Shaykhutdinov, R.; Li, L.; Vogel, H. J.; Forsythe, I. HMDB: Aknowledgebase for the human metabolome. Nucleic Acids Research, 2009, 37, D603−D610.

Vu, T. N. and Laukens, K., Getting your peaks in line: A review of alignment methods for NMR spectral data. Metabolites, 2013, 3, 259-276

Yamada, M.; Taira, S.; Ohtsuki, M.; Sato, A.; Iwanami, H.; Yakushiji, H.; Wang, R. Z.; Yang, Y.; Li, G. C., Varietal differences in the ease of astringency removal by carbon dioxide gas and ethanol vapor treatments among Oriental astringent persimmons of Japanese and Chinese origin. Scientia Horticulturae, 2002, 94, 63-72. Yamane, H.; Yamada, M.; Kurihara, A.; Sato, A.; Yoshinaga, K.; Nagata, K.; Matsumoto, R.; Hirakawa, N.; Kakutani, M.; Ozawa, T.; Sumi, T.; Hirabayashi, T.; Iwanami, H., New Japanese persimmon cultivar 'Taishuu'. Bulletin of the National Institute of Fruit Tree Science, 2001, 35, 57-73.

Yonemori, K.; Matsushima, J.; Sugiura, A., Differences in tannins of non-astringent and astringent type fruits of Japanese persimmon (Diospyros kaki Thunb.). Journal of the Japanese Society for Horticultural Science, 1983, 52,135-144

Yoshioka, H.; Aoba, K.; Kashimura, Y., Molecular-weight and degree of methoxylation in cell-wall polyuronide during softening in pear and apple fruit. Journal of the American Society for Horticultural Science, 1992, 117, 600-606

Zheng, G. H.; Sugiura, A., Changes in sugar composition in relation to invertase activity in the growth and ripening of persimmon (*Diospyros kaki*) fruits. Journal of the Japanese Society for Horticultural Science, 1990, 59, 281-287

ABBREVIATIONS

CT-HMBC	constant time hetero-nuclear multiple-bond connectivity
d_1	delay time
DQF-COSY	double quantum filtered correlated spectroscopy
FID	free-induction decay
FT NMR	fourier transform NMR
GABA	γ-aminobutyrate
HSQC	hetero-nuclear single quantum coherence
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
OPLS-DA	orthogonal PLS-DA
PC1	first principal component
PC2	second principal component
PCA	*principal component analysis*
PLS-DA	partial least-squares discriminate analysis
T_1	spin-lattice relaxation time
TSP-d_4	[2,2,3,3-d4] sodium 3-(trimethylsilyl) propanoic acid
WET	water suppression enhanced through T_1 effects
CTSD	constant temperature short duration treatment to remove astringency
TS	Taishu
MF	Matsumotowase-Fuyu
MJ	Maekawa Jiro
HN	Hiratanenashi
YM	Yotsumizo

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