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^{1}H HR-MAS NMR-based metabolomics study of different persimmon cultivars (*Diospyros kaki*) during fruit development

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ABSTRACT

^{1}H HR-MAS NMR spectroscopy was used to track the metabolic changes throughout the whole development of astringent ('Giombo') and non-astringent ('Fuyu') cultivars of persimmon (*Diospyros kaki).* The NMR data revealed the low concentration of amino acids (threonine, alanine, citrulline and GABA) and organic acids (malic acid). In addition, the signals of carbohydrates (sucrose, glucose and fructose) seemed to play the most important role in the fruit development. In both cultivars, the growth was characterized by fluctuating sucrose concentration along with a constant increase in both glucose and fructose. In the initial growth stage, the polyphenol composition was quite different between the cultivars. Gallic acid was detected throughout the growth of 'Giombo', while for 'Fuyu', signals of polyphenols disappeared over time. Additional multivariate analysis suggested that these cultivars share many metabolic similarities during development. These findings might help the comprehension of fruit development, which in turn, impacts the quality of the fruits.

1. Introduction

Persimmon (*Diospyros kaki*) is recognized as the most important species for fruit production in the *Diospyros* genus (Ebenaceae) (Yonemori, Sugiura, & Yamada, 2010). In 2013, world production of persimmons reached 4.6 million tonnes, with China accounting for 43% of this total. Other major producers include the Republic of Korea, Japan, Brazil and Azerbaijan (FAOSTAT, 2013).

In addition to its economic value, some studies have shown that persimmon is one of the most bioactive fruits (Daood, Biacs, Czinkotai, & Hoschke, 1992; Gorinstein et al., 1998; Veberic, Jurhar, Mikulic-Petkovsek, Stampar, & Schmitzer, 2010). In this context, reports have been found regarding antioxidant, cytotoxic and antidiabetic activities, as well as the beneficial effect on coronary diseases (Katsube et al., 2004; Kawase et al., 2003; Lee, Cho, Tanaka, & Yokozawa, 2007; Santos-Buelga & Scalbert, 2000).

The chemical composition of persimmon is the key to understand not only the aforementioned biological activity, but also to ensure the nutritional and organoleptic properties which, in turn, affect consumer satisfaction. The quality and enjoyment of fruits are intimately tied to their chemical composition, in this context, compounds, such as carbohydrates, organic acids, polyphenols and carotenoids, play key roles (Colaric, Veberic, Stampar, & Hudina, 2005; Daood et al., 1992). For instance, the sugar/organic acid ratio is a common quality index for fruits (Bassi & Selli, 1990). Giordani, Doumett, Nin, and Del Bubba (2011) reported a comprehensive review of the primary and secondary metabolites in fresh persimmons, highlighting the analytical methods employed for the determination of sugars, vitamin C, carotenoids and polyphenols. In a critical manner, the authors evaluated the overall significance of literature results concluding that the literature data are affected by a number of sources of variability, such as ripeness stage and analytical methods that should be more controlled and standardized in order to obtain more reliable and comparable results. The situation is further complicated by the vast number of varieties, which adds additional variation in the chemical investigation of persimmons. In Japan alone, over 100 cultivars are grown (Yamagishi, Matsumoto, Nakatsuka, & Itamura, 2005; Zhou, Zhao, Sheng, Tao, & Yang, 2011).

In contrast, a versatile, non-selective, NMR based approach, termed high resolution magic angle spinning (HR-MAS) NMR spectroscopy, has allowed the characterization of foodstuff

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providing information on a wide variety of compounds (Santos, Fonseca, Lião, Alcantara, & Barison, 2015; Valentini et al., 2011). In this technique, with the purpose of eliminating the contribution of dipolar coupling and differences in magnetic susceptibility, samples are submitted to fast spinning about the so-called "magic angle" (54.74°) (Santos et al., 2015). As a result of such a procedure, the line broadening in the spectra is significantly reduced, which results in a boost of signal-to-noise ratio and resolution. In a single NMR experiment, it is possible to identify and quantify non-polar and polar substances; thus, both primary and secondary metabolites can be evaluated. In addition, NMR spectra are acquired directly from samples avoiding the loss of information provoked by the extraction procedures (Santos et al., 2015). These features make such a technique ideal for tracking chemical changes associated with complex processes, such as fruit development.

As such, there is a clear need for a better understanding of the chemical processes (including the metabolites involved) behind fruit growth and ripening. Such processes affect the whole characteristic of fruits, including taste, texture and bioactivity. Thus, knowledge about how the fruits grow can provide important molecular indicators to monitor the crop both during the cultivation or after the harvest (Buesa et al., 2013; Harima et al., 2003; Lee, Kim, Kim, & Park, 2005). The present work aims to evaluate the metabolic changes during the development of persimmon cultivars 'Fuyu' (non-astringent) and 'Giombo' (astringent) through ^{1}H HR-MAS NMR and chemometric analysis. In persimmons, the astringency sensation is produced by the presence of high-molecular-weight tannins in fruit flesh. Such compounds damage the normal lubrication of oral surfaces, at least in part, by the precipitation of salivary protein (Lyman & Green, 1990).

2. Materials and methods

2.1. Samples

Persimmon fruits of cultivars 'Fuyu' and 'Giombo' during the whole period of the development (from September 2012 to March 2013) were provided by Fruticultura Boutin Company (Porto Amazonas, Paraná, Brazil). The fruits were cultivated under the same field conditions. For each cultivar, ten fruits were collected at the medium height of the persimmon tree, covering the entire length of the plantation randomly. The samples were kept in a freezer (-80 °C) until analysis.

2.2. Sample preparation for NMR analysis

For the acquisition of the ^{1}H HR-MAS NMR spectra, the fruits were thawed and split in half, and a slice of one of the parts was pulverized after freezing with liquid nitrogen. Approximately, 12.0 ± 1.0 mg was packed into a 50 μl zirconium rotor followed by the addition of 40 μl of D_2O phosphate buffer (pH 6.4), with 0.5% TMSP-d_4, 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt. The buffer solution was used to prevent the pH-dependent variation of some NMR signals (Shintu, Caldarelli, & Franke, 2007). This is essential as fruit development is characterized by changes in its pH, which could in turn lead to variation in pH sensitive NMR resonances (Gil et al., 2000). In general, molecules with ionization state that change with changing pH also display varying NMR chemical shifts at different pHs (del Campo, Berregi, Caracena, & Santos, 2006).

2.3. NMR measurements

^{1}H HR-MAS NMR spectra were recorded at 293 K on a Bruker AVANCE spectrometer operating at 9.4 T, observing ^{1}H at 400.13 MHz, equipped with a 4 mm four channel (^{1}H, ^{13}C, ^{15}N and ^{2}H) HR-MAS probe. The samples were spun at the magic angle ($\theta = 54.74°$) at 5 kHz. ^{1}H HRMAS-NMR spectra were acquired by using a water suppression pulse sequence, *noesypr1d* (Bruker library), using 64 K data points over a 5999 Hz spectral width averaged over 512 transients. A recycle delay of 1.0 s, power for presaturation of 43 dB and a mixing time of 100 ms were used, the later to help reduce the water signal through T_1 relaxation and chemical exchange. The saturation of the water residual signal was achieved by irradiation during recycle and the NOE mixing time at the H_2O/HOD NMR frequency. The spectra were apodized via an exponential multiplication corresponding to a 0.3 Hz line broadening in the transformed spectrum and zero filled by a factor of 2. The magic angle was adjusted daily using the ^{79}Br signal from a powdered KBr for reference. The samples were locked on the deuterium signal from D_2O, and the magnetic field homogeneity was optimized for each sample. The total experiment time was 60 min for each sample, including the time used for rotor preparation.

In order to support the NMR chemical shift assignments in the ^{1}H HR-MAS NMR spectra, liquid-state NMR experiments were performed on persimmon samples (representing the beginning, middle and end of growth) extracted directly in D_2O. One-bond and long-range ^{1}H$-$^{13}C correlation from HSQC and HMBC NMR experiments were optimized for an average coupling constant $^{1}J_{(C,H)}$ and $^{LR}J_{(C,H)}$ of 140 and 8 Hz, respectively. The experiments were performed on a Bruker AVANCE III 600 NMR spectrometer operating at 14.1 T, observing ^{1}H and ^{13}C at 600.13 and 150.90 MHz, respectively, equipped with a 5 mm inverse detection four channel (^{1}H, ^{13}C, ^{15}N and ^{31}P) probe with an actively shielded field. ^{1}H and ^{13}C NMR chemical shifts are given in ppm referenced to TMSP-d_4 signal at 0.00 ppm.

2.4. Normalized integral areas and statistical analysis

In order to monitor the metabolic changes revealed in the NMR data, the normalized integral areas of identified compounds were calculated using the multi-integration tool-kit of AMIX-viewer (Bruker BioSpin, Rheinstetten, Germany). The results were evaluated using the analysis of variance (ANOVA) single factor analysis with significance level of 0.05. The means were compared using Tukey's test.

2.5. Multivariate data analysis

All ^{1}H HR-MAS NMR spectra were manually phased, baseline corrected and aligned by TopSpin® software. The chemical range between 0.50 and 9.50 ppm represented all ^{1}H NMR resonances in the samples. Such range was segmented in continuous small buckets of 0.02 ppm wide. The areas between 4.5–5.2 and 2.20–2.26 were excluded in the ^{1}H NMR spectra to eliminate, respectively, the residual H_2O/HOD signals, as well as the signal of remaining acetone from the cleaning procedure of the rotor. The area under each bucket was integrated in AMIX® 3.9.12 (Bruker BioSpin, Rheinstetten, Germany) using the special integration mode. The spectra were scaled to total intensity, which in turn divides the measured signal intensity for each bucket by the integral of the complete spectrum. Thus, the spectral differences resulting from the variation of the amount of samples can be corrected. After this procedure, a matrix was created in which each row represented persimmon samples and each column contained the integrated area of the original spectroscopic intensities within each bucket region. The initial matrix was composed of 70 samples (lines) and 446 variables (columns). For the principal components analysis (PCA), the variables were scaled to unit variance (autoscale). This means that all columns have the same weight and all