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Changes in the composition of the polar fraction of Persian lime (*Citrus latifolia*) during fruit growth by LC–QTOF MS/MS analysis

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ABSTRACT

Citrus possess a large number of bioactive compounds mainly studied in ripe fruits. Few studies have focused on evolution of metabolites during fruit growth. In this study, fruits were sampled from weeks 1–14 of the ripening process. Polar extracts were obtained from all collected samples and analysed by liquid chromatography–tandem mass spectrometry. Analysis of variance applied to the dataset indicated that the relative concentration of 394 out of 423 molecular entities changed significantly during maturation. Principal component analysis showed a clear separation among samples from different weeks and revealed the main compounds responsible for differentiation. Additionally, 72 metabolites were tentatively identified and changes in their relative concentration during growth were individually analysed. The observed trends in relative concentrations of representative metabolites during the growth process are discussed.

1. Introduction

A large number of studies has demonstrated that citrus fruits possess a high content of bioactive compounds, such as vitamins C and B, phenolic compounds and carotenoids. Few of these studies have evaluated changes in chemical or physical properties during fruit ripening (Lin et al., 2015; Moulehi, Bourgou, Ourghemmi, & Tounsi, 2012; Olmo, Nadas, & García, 2000; Yoo & Moon, 2016) and, when chemical changes were considered, few compounds were monitored. The ripening indicators were based on indices such as the ratio between titratable acidity (% of citric acid) and soluble solids (°Brix), and the colour, although this constitutes an ambiguous ripening estimator highly dependent on temperatures at night (Olmo et al., 2000). Additionally, these studies were focused on advanced maturation stages and the first growing weeks were ignored. Moulehi et al. (2012), and Yoo and Moon (2016) evaluated changes in the content of phenolic compounds, vitamin C and carotenoids in three varieties of citrus fruits, where only three stages of maturation – and based on fruit colour – were sampled. No significant changes in the total content of flavonoids or phenolic acids during the maturity stages under study were observed, and only the contents of vitamin C and carotenoids increased during this maturation period (Moulehi et al., 2012; Yoo & Moon, 2016).

The analysis of sugars and carboxylic acids in mandarins (*Citrus reticulata*) at three ripening stages (120, 195 and 205 days) pointed out an increase in sugars and decrease in organic acids during fruit ripening (Lin et al., 2015). Metabolomics analysis based on volatile compounds from tangerine was used for differentiation and characterisation of samples from different ripening stages. The results of this study revealed that profiles of volatile compounds are characteristic of different ripening times and provide information on specific volatile metabolites strongly associated with the aroma/flavour (Yi, Dong, Liu, Yi, & Zhang, 2015).

In an attempt to expand the knowledge on the changes in polar compounds during citrus fruit maturation, the present research was aimed at establishing similarities/dissimilarities in Persian lime (*Citrus latifolia*) sampled during eight ripening stages (from weeks 1 to 14 of maturation), based on the use of liquid chromatography–tandem mass spectrometry (LC–QTOF MS/MS) without consideration of traditional ripening indices.

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2. Materials and methods

2.1. Sampling methodology

Persian lime (*Citrus latifolia*) samples at different growth stages (1, 3, 5, 7, 9, 12, and 14 weeks – named as samples W01 to W14) were collected in Martínez de la Torre, Veracruz, México (geographical coordinates: 20°04′00″N, 97°03′00″W) from September 2012 to January 2013. The experimental field had 500 mature trees (at least 4 years old) uniformly distributed over an area of 1250 m^2 (40 m length and 31 m wide). The field was monitored weekly from the beginning of flowering and the branches that showed new-born fruits were labelled to identify their birthday. Twelve samples were collected from each growth stage, and those corresponding to week 14 (fully mature fruits) were harvested in duplicate; half of them were stored at 25 °C for 2 weeks to analyse the short-time storage behaviour of mature fruits. The sampling method was based on random rectangular coordinates within the limits of the experimental field. A total of 96 samples, selected by proximity to the corresponding random coordinate, were generated by the following MATLAB algorithm: $n = 96$; for $I = 1:96$; × (i,1) = random('unif',0,1) * 40; x(i,2) = random('unif',0,1) * 31; end. All samples were manually harvested and immediately stored under liquid nitrogen to preserve their constant weight until lyophilisation. Dehydrated samples were ground (particle diameter ≤0.5 mm), then stored in the dark at −20 °C until use.

2.2. Reagents

All solvents were LC grade or higher if required. Ethanol and formic acid were from Scharlab (Barcelona, Spain); acetonitrile and methanol from Fluka (Buchs, Switzerland). Deionised water (18 MΩ.cm) from a Millipore Milli-Q water purification system (Bedford, MA) was used to prepare the mobile chromatographic phases and extractant mixtures.

2.3. Metabolites extraction

Persian lime samples (1 g dry weight each) were extracted by a Branson (Fisher Scientific) 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium-alloy probe (12.70 mm diameter). Twenty mL of 53% of ethanol in water were used as extractant, and 5 min were required for extraction with ultrasound assistance (70% amplitude and 0.9 s s^{-1} duty cycle). The extraction method was previously developed by the authors, using a desirability model to maximise the concentration of ten major compounds in the extracts from lemon (Ledesma-Escobar, Priego-Capote, & Luque de Castro, 2015a).

2.4. LC–QTOF MS/MS analysis

Chromatographic separation of the extract components was performed by using an Agilent 1200 series LC provided with an Inertsil ODS-2 C18 analytical column (250 × 4.6 mm i.d., 5 μm particle) from GL Science (Tokyo, Japan). The chromatograph was coupled to an electrospray ionisation source and a 6540 quadrupole–time-of-flight detector (LC–QTOF MS/MS; Agilent Technologies, Santa Clara, CA) for detection. The injection volume was 10 μL, and the mobile phases were 0.1% formic acid in deionised water (phase **A**) and acetonitrile (phase **B**) at a constant flow rate of 1 mL min^{-1}. The gradient was as follows: 4% to 10% **B** in 5 min, change from 10% to 25% **B** in 30 min, from 25% to 100% **B** in 15 min and constant at 100% **B** for 5 min. After analysis, the column was equilibrated to the initial conditions for 5 min. The dual ESI source operated in both positive and negative ionisation modes under the following conditions: nebuliser gas at 40 psi, drying gas flow rate and temperature at 12 L min^{-1} and 325 °C, respectively. The capillary voltage was set at 3500 V, while the Q1, skimmer, and octapole voltages were fixed at 130, 65, and 750 V, respectively. The data were acquired in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at 6 spectra s^{-1} within the m/z range of 40–1700, with subsequent activation of the three most intense precursor ions (allowed charge: single or double) by MS/MS using a collision energy of 20 eV and 40 eV at 3 spectra s^{-1} within the m/z range 30–1700. An active exclusion window was programmed after the first spectrum and released after 0.75 min, to avoid repetitive fragmentation of the most intense precursor ions, thus increasing the detection coverage. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses with the use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine or HP-921) in positive ion mode; and m/z 112.9856 (trifluoroacetic acid anion) and m/z 1033.9881 (HP-921) in negative ion mode.

2.5. Data processing

MassHunter Workstation software (version B6.00 Profinder; Agilent Technologies, Santa Clara, CA) was used to process the data obtained by LC–QTOF in auto-MS/MS mode. Treatment of the raw data file started by extraction of potential molecular features (MFs) by the applicable algorithm included in the software. The recursive extraction algorithm considered all ions exceeding 5000 and 10,000 counts as cut-off in both positive and negative modes, respectively. Additionally, the isotopic distribution to consider a molecular feature as valid should be defined by two or more ions (with a peak spacing tolerance of m/z 0.0025, plus 10.0 ppm in mass accuracy). Apart from $[M+H]^+$ and $[M-H]^-$ ions, adducts formation in the positive (Na^+) and negative ionisation ($HCOO^-$, Cl^-) modes, as well as neutral loss by dehydration, were included, to identify features corresponding to the same potential metabolite. Thus, ions with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as entities characterised by their retention time (RT), intensity at the apex of the chromatographic peak and accurate mass. Background contribution was removed by subtraction of MFs linked to the blank. Then, the recursion step assured correct integration of the entities in all analyses. Raw data files containing the peak area for each entity characterized by m/z and RT were created in compound exchange format (.cef files) for each analysis and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies) for further processing. Normalisation by logarithmic transformation (log2) was used as a preprocessing step. Statistical analysis included the ANOVA test applied to find the number of significant molecular entities ($p \leq 0.01$), and pairwise combinations (Tukey HSD), to identify significant differences in relative concentration of identified compounds, between samples belonging to different growth times. Also, unsupervised analysis by principal component analysis (PCA) was used to find out the main sources of variability in the data set and to detect clusters.

Once all MFs were extracted and aligned, the software MassHunter Qualitative was used for the targeted extraction of MS/MS information associated with the monitored MFs in the whole set of analyses. This information was used for tentative identification of metabolites by searching in the METLIN MS and MS/MS (http://metlin.scripps.edu), MassBank MS/MS (http://www.massbank.jp) and ReSpect MS/MS (http://spectra.psc.riken.jp) databases.