Identification of volatile compound markers during the ripening and senescence of lulo (*Solanum quitoense* Lam.)

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Abstract Lulo (*Solanum quitoense* Lam.) is an exotic fruit cultivated in Colombia. During ripening and senescence, this climactic fruit undergoes biochemical processes that produce the volatiles responsible for its aroma. This study aimed to evaluate the changes in the volatile content during the ripening and senescence of lulo. Analysis of the volatile composition of lulo harvested in each of its five ripening stages and during its senescence time when stored at 18 ± 2 °C was performed using HS-SPME with GC–MS. Throughout ripening, the most notable change was the transformation of alcohols such as (Z)-3-hexen-1-ol and 1-penten-3-ol to afford esters such as (Z)-3-hexenyl acetate and ketones such as 1-penten-3-one. Some acids reacted with alcohols to produce acetate and hexanoate esters, concentrations which increased more than sixfold between stage one and five. Moreover, all the major compounds were C₆ straight chain compounds related to the lipoxigenase pathway. During senescence, majority of compounds were methyl esters, which increased in

concentration consistently until day eight. Remarkably, the content of methyl butanoate increased from 0.9% of the total amount of volatiles on day two up to 76.4% on day eight. Some of these volatiles are probably contributors to the “off flavor” during senescence.

Keywords Lulo fruit · Markers · Metabolite profiling · Metabolomics · Territorial map · Volatile organic compounds

Introduction

Volatiles are low molecular weight compounds that are components of fruit aroma (Pino 2014). These compounds are produced through different metabolic pathways. Consequently, compounds such as alcohols, esters, aldehydes, and acids can be found among volatiles (Qin et al. 2012). It is necessary to employ an efficient extraction method, such as solid phase microextraction (SPME), which, compared to the traditional methodologies, is faster, easier, more sensitive, and solventless and avoids artifact formation (Cheong et al. 2012).

To identify the volatiles and establish the variation in their concentrations, some researchers focus on the stages preceding the consumption ripeness in different fruits (Vandendriessche et al. 2013). Furthermore, several studies have elucidated the content of volatiles in steps to subsequent the full ripeness of some fruits (Steingass et al. 2014). Nevertheless, there are no studies on lulo (*Solanum quitoense* Lam.), an exotic species that is predominant in Colombia, or on the identification of markers for the ripening and senescence of lulo.

This study aimed to evaluate the behavior of the volatile fraction of lulo pulp by associating the volatile

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concentrations with a specific ripening or senescence stage and identifying the compounds that could be considered markers for these stages.

Materials and methods

Plant material

The experimental units of lulo originated from the “Villa Malicia” farm (coordinates: 75°32'11.85"W and 5°5'8.28"N, altitude of 2160 m), which is located approximately 1 km from Manizales (Caldas, Colombia). The fruit belonged to crops obtained via the reproduction of tissue and in vitro propagation of seedlings from the biotechnological company Agro in vitro S.A.S. which is located in Manizales.

Reagents and materials

Purified distilled water obtained from a Direct-Q3 model ZRQS0P030 of Merck Millipore® (Darmstadt, Germany) was used to prepare the internal standards. Moreover, Carlo Erba Reagents® (Barcelona, Spain) supplied sodium chloride (99%). Likewise, the internal standard 1-octanol was from Sigma–Aldrich® (Saint Louis, MO, USA). Additionally, Supelco® (Bellefonte, PA, USA) manufactured the SPME holder and the fiber used.

Fruit selection and procedure of extraction and analysis

Lulo fruit in all experiments had a diameter between 5 and 6 cm, which corresponds to a previous study (Mejía et al. 2012). These fruits were selected by considering the fruit color according to the Colombian Technical Standards NTC 5093 (ICONTEC 2002) and the degrees on the Brix scale, which were based on Mejía et al. (2012), for stages one to five for fruits analyzed during ripening and for stage five for those analyzed during senescence. After sampling, lulo samples that were harvested in different ripening stages were analyzed immediately, and the senescence analyses were performed every 2 days, from day two to twelve, on fruit stored at 18 ± 2 °C.

HS-SPME procedure

Every sample of lulo fruit was washed with distilled water. Subsequently, 10 g of lulo pulp and 1 g of sodium chloride were weighed in an extraction vial with a capacity of 20 ml. Then, 5 µl of a solution of 1-octanol, used as an internal standard with a concentration of 0.0018 mol/l, was added to the extraction vial. Later, the container was sealed

with a rubber septum and then placed in a water bath. Subsequently, the CAR/PDMS fiber was manually inserted in the headspace (HS) of the pulp and exposed for 30 min at 60 °C. Next, the fiber was withdrawn, immediately allowed to desorb for 2 min in the injection port of the GC/MS equipment, and removed.

Analysis of volatile compounds

A Shimadzu® GCMS/QP 2010 Plus gas chromatograph system equipped with injector split/splitless was coupled to a mass spectrometer. Volatiles were desorbed at 230 °C in the injector, operated in splitless mode. For focusing volatile metabolites, a 0.75-mm ID SPME liner (Supelco®, Bellefonte, PA) was used. Moreover, helium was used as the carrier gas at a constant flow rate of 4 ml/min. In addition, the analytical column was a semi-polar Shimadzu® of 5% polysiloxane (30 m × 0.25 mm ID × 1.4 µm DF). Furthermore, the heating ramp of the programmed temperature used was as follows: after an initial period of 1 min at 50 °C, the oven temperature was increased at 2.5 °C/min to 150 °C and maintained at that temperature for 7 min; then, the oven temperature was raised at 15 °C/min to 220 °C and held isothermal for 3 min; and finally, it was increased at 15 °C/min to 230 °C, where it was kept for another 2 min.

The mass spectrometer was operated with an ion source temperature of 235 °C; an interface temperature of 240 °C; an equilibration time of 1 min; initial and final masses of 33 and 350, respectively; and an ionization energy of −70 eV. The total time of a single run was 50 min. Moreover, the identification was based on its mass spectra compared to those found at the library of National Institute of Standards and Technology (NIST® library, version 08), accepting a concordance at or above 93%. Additionally, a verification of the Kovats retention index from the analysis of a mixture of alkanes (C7–C24) was performed. In addition, the quantification of volatile compounds of the lulo pulp in both ripening stages and senescence times was performed according to the following equation:

$$C = A_c * FR_c * C_{ie}/A_e \quad (1)$$

where C indicates the concentration of each volatile, A_c is the area of each volatile, FR_c is the response factor of the respective compound (quotient between the area of the compound and the internal standard), C_{ie} is the concentration of the internal standard, and A_e is the area from the internal standard of the respective analysis.

Statistical analysis

The compound data derived from *S. quitoense* in its ripening and senescence stages were generated by

experiments performed in quintuplicate and sextuplicate, respectively. First, the Tukey test was conducted to identify the majoritarian compounds during the ripening stages and senescence times. Second, regression analysis was applied through partial least squares (PLS) to identify markers. Finally, discriminant analysis (DA) allowed the identification of the compounds with high predictive value at the ripening stage and senescence time of lulo. The data were analyzed using statistical programs SPSS® version 22 and MetaboAnalyst® version 3.0. The relative standard deviation (RSD) of the data in each experimental group of both the ripening stages and senescence times was less than or equal to 12%.

Selection of markers for both ripening stages and senescence times

DA allowed to predict both the ripening stage and senescence time from the recorded contents of the volatiles. On the one hand, the Wilks lambda test was applied to determine the compounds with significant differences in their mean contents when moving from one stage to another ($P < 0.05$). On the other hand, the quotient between the variation coefficient of the compound concentration throughout the experiment and the greater variation coefficient of the compound concentration in the interior of the groups (that corresponded to each ripening stage and senescence time) was obtained. Thus, compounds with coefficients lower than 120% were discarded. Additionally, the compounds whose correlation coefficients had high correlations ($P < 0.01$) compared to other compounds were excluded. The selected compounds in the aforementioned full data processing were considered in the subsequent DA.

Results

Volatile compounds obtained during the ripening and senescence of lulo

Regarding the predominance of the different functional groups, the esters were the main chemical class during ripening (between 10 and 12 compounds), followed by alcohols (between 6 and 8 volatiles) and aldehydes (seven compounds). During senescence, there was a predominance of esters (between 16 and 21 metabolites), which were mostly methyl esters and some acetate esters. Furthermore, alcohols (between 2 and 6 compounds) and aldehydes (between 4 and 6 volatiles) preceded the esters in terms of their numbers.

Majoritarian compounds in lulo during the ripening stage and senescence time

The comparative Tukey test designated the following compounds as majoritarian: (Z)-3-hexenyl acetate, hexyl acetate, (Z)-3-hexen-1-ol, 1-hexanol, and (E)-2-hexenal. In addition, the Tukey test in senescence indicated that 8 of the 51 quantified compounds, which were mostly methyl esters, had significantly higher concentrations (methyl butanoate, methyl 2-butenate, methyl hexanoate, methyl acetate, and methyl benzoate).

Markers in lulo during the ripening stages and senescence time

Among the markers of lulo ripening, certain metabolites had significantly different concentrations between each stage. Methyl (E)-2-butenate was one of the compounds with the highest scores. Furthermore, compounds such as 4-heptanone and *o*-xylene, which had higher concentrations only when they reached the final ripening stages, were selected (Fig. 1, left). Additionally, several acetate esters were markers, including (Z)-3-hexenyl acetate and hexyl acetate, because of their differentially increased contents, and 3,7-dimethyl-1,6-octadien-3-ol was the only marker with a significantly decreased concentration. Regarding senescence, most of the compounds that were designated markers were esters, and the majority of these species were methyl esters (Fig. 1, right). Furthermore, the alcohols 1-penten-3-ol and (E)-3-hexen-1-ol, pentanal aldehyde, and 1,7,7-trimethyl-bicyclo[2.1.1]-heptan-2-one ketone were selected.

DA for predictive determination of ripening stages and senescence times in lulo

For the subsequent DA on lulo ripening, the following compounds were selected: methyl propionate, unidentified 2, acetic acid, 1-penten-3-ol, 1-penten-3-one, 3-pentanol, *o*-xylene, (E)-3-hexen-1-ol, 1-hexanol, (E)-2-hexenal, 1-octen-3-one, (Z)-3-hexenyl acetate, (E)-3-hexenoic acid, (E)-2-hexenoic acid, undecane, (Z)-3-hexenyl butanoate, 1,7,7-trimethyl-bicyclo[2.1.1]-heptan-2-one, and decanal. The discriminant functions (DF) produced correlation coefficients with high predictive power: the first two explained 98.3% of the data variability. In addition, these functions were used to obtain the respective territorial map (Fig. 2, left).

In the case of senescence, the following compounds were selected: unidentified 1, methyl propionate, 1-methylethyl acetate, methyl 2-methylpropanoate, methyl 2-methyl-2-propenoate, pentanal, methyl butanoate, methyl 2-butenate, 1-pentanol, ethyl butanoate, (Z)-2-penten-1-

Fig. 1 Identification of markers in lulo pulp during the ripening stages (left) and senescence times (right)

Fig. 2 Territorial map of the volatiles in the ripening stages (left) and senescence times (right) of lulo pulp

ol, butyl acetate, hexanal, methyl-(E)-2-methyl-2-butenate, (E)-2-hexenal, (Z)-2-penten-1-ol acetate, methyl hexanoate, (E,E)-2,4-hexadienal, (E)-3-hexen-1-ol acetate, hexyl acetate, 1-methylethyl hexanoate, hexenoic acid, 5-ethyl-2(5H)-furanone, (E)-3-hexenoic acid, methyl benzoate, (E)-3-hexyl butyrate, and unidentified 5. The two first DF represented 88.9% of the variability obtained and were then used to obtain the territorial map (Fig. 2, right). Finally, to predictively use the territorial map, the first DF must be located on the X axis and the second on the Y axis.

Discussion

Effect of the ripening stage on the volatile concentration in lulo pulp

The major acetate esters, (Z)-3-hexenyl acetate and hexyl acetate, established in the PLS as ripening markers and selected for the DA have also been found in other fruits. In strawberry trees (*Arbutus unedo* L.), these compounds were found to have high concentrations, but the concentrations decreased as the fruit ripened (Oliveira et al. 2011).

Additionally, (Z)-3-hexenyl acetate had relevant levels in raspberry (*Rubus idaeus*), which declined during the ripening process (Robertson et al. 1995), and hexyl acetate predominated in greater quantities in the final stages for the Pink Lady® apple (Villatoro et al. 2008).

Concomitant with the increased quantity of esters, several alcohols in lulo pulp showed declining trends during ripening. Similar to lulo, (Z)-3-hexen-1-ol and 1-hexanol had high concentrations in strawberry tree (*Arbutus unedo* L.) (Oliveira et al. 2011) and cherry (*Prunus avium* L.) (Zhang et al. 2007), but their levels declined during ripening. Moreover, (Z)-3-hexen-1-ol integrated the group of majoritarian compounds in raspberry (*R. idaeus*) (Robertson et al. 1995), but in contrast to lulo, its maximum levels were reached at full ripeness. Regarding the minoritarian alcohols, 1-penten-3-ol, which decreased during the ripening of lulo, was an indicator of immaturity in strawberry (*Fragaria × ananassa* Duch.) (Vandendriessche et al. 2013).

Main metabolic pathways related to the volatiles in lulo during ripening

Among the identified metabolites, the ones related to the LOX pathway were hexanal, (E)-2-hexenal, 1-hexanol, (Z)-3-hexen-1-ol, (E)-3-hexen-1-ol, (Z)-3-hexenyl acetate, and hexyl acetate. Furthermore, the quantity of the majoritarian alcohols 1-hexanol and (Z)-3-hexen-1-ol decreased as the concentrations of (Z)-3-hexenyl acetate and hexyl acetate increased, which occurs via the LOX pathway and suggests that LOX is a distinctive pathway during the ripening of lulo.

The analysis of the behavior of ketones, acids, and hydrocarbons during the ripening process inspired some interpretations. Most ketones had statistically significant increases at the end of the ripening of *S. quitoense*. Considering the decrease in the alcohols and increase in the ketones at the end of ripening, some alcohols, such as 1-penten-3-ol, were likely transformed into ketones, such as 1-penten-3-one. Similar to the oxidation of primary alcohols such as hexanol and aldehydes such as hexanal, these reactions would originate from the LOX pathway. However, some acids, such as acetic acid and hexanoic acid, had similar concentrations in the early stages and increased at the end of ripening. These metabolic products may have been used together with alcohols to produce acetate and hexanoate esters, which are predominant in the last ripening stage.

Effect of the senescence time on the volatile concentration in lulo pulp

The increase in both hexanoates and benzoates during senescence was also described in melon (*Cucumis melo* L.) (Obando-Ulloa et al. 2008). Additionally, in ripe strawberries (*F. × ananassa* Duch.) after three days of storage, metabolites such as ethyl butanoate, methyl butanoate, and ethyl hexanoate were among the most prevalent compounds (Larsen and Watkins 1995). Moreover, in apple pulp (*Malus domestica*), the butanoates with lower levels at the optimal quality time increased their concentrations later, although these variations did not affect its acceptability (López et al. 2007).

Regarding the alcohols, both (Z)-2-penten-1-ol and pentan-1-ol, which had a decreasing trend in their levels during lulo senescence, had the same trend in watermelon senescence [*Citrullus lanatus* (Thunb.) Matsum. and Nakai, 'Sugar Heart') (Saftner et al. 2007]. On the other hand, for 1-penten-3-ol and (E)-3-hexen-1-ol, no prior references of changes in their concentrations during the lifespans of fruits were found; nonetheless, they could be considered markers of loss of volatile integrity in lulo due to their limited or absent concentration starting in the intermediate stage of senescence.

The environmental conditions under which the ripening and senescence of *S. quitoense* occur affect the volatile profile in this fruit. Therefore, this study constitutes a baseline for understanding the changes in the concentrations of these metabolites in this fruit, starting from the normal conditions of ripening on the tree to senescence at room temperature. Additionally, these compounds could be analyzed under different environmental conditions to establish the effect of these variations on their concentrations and to contrast their potential as markers.

Conclusion

Lulo ripening was characterized by the reduction in the concentration of the alcohols concomitant with the increase in the ester content, mainly C₆ straight chain compounds, which probably originated from the LOX pathway. In senescence, the total content of volatiles mainly comprised methyl esters, whose maximum increase occurred on day eight. Similarly, the majoritarian compounds during ripening were hexyl acetate, 1-hexanol, (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, and (E)-2-hexenal. These last three compounds were also majoritarian, along with various methyl esters, during senescence. Furthermore, the ripening markers established through the PLS were mainly acetate esters and acids, whereas the markers of senescence were mainly methyl esters. The DA from the contents of

volatiles allowed us to obtain territorial maps with high predictive power models for both ripening stages and senescence times in lulo.

The ripening and senescence markers could be analyzed during the times after flowering in a later study to achieve a more accurate understanding of the behaviors of these metabolites in this fruit and to compare the results with the changes obtained in their concentrations during ripening and senescence.

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