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Metabolic profiling of a range of peach fruit varieties reveals high metabolic diversity and commonalities and differences during ripening

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ABSTRACT

Peach (*Prunus persica*) fruits from different varieties display differential organoleptic and nutritional properties, characteristics related to their chemical composition. Here, chemical biodiversity of peach fruits from fifteen varieties, at harvest and after post-harvest ripening, was explored by gas chromatography–mass spectrometry. Metabolic profiling revealed that metabolites involved in organoleptic properties (sugars, organic and amino acids), stress tolerance (raffinose, galactinol, maltitol), and with nutritional properties (amino, caffeoylquinic and dehydroascorbic acids) displayed variety-dependent levels. Peach varieties clustered into four groups: two groups of early-harvest varieties with higher amino acid levels; two groups of mid- and late-harvest varieties with higher maltose levels. Further separation was mostly dependent on organic acids/raffinose levels. Variety-dependent and independent metabolic changes associated with ripening were detected; which contribute to chemical diversity or can be used as ripening markers, respectively. The great variety-dependent diversity in the content of metabolites that define fruit quality reinforces metabolomics usage as a tool to assist fruit quality improvement in peach.

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1. Introduction

Peach (*Prunus persica* (L) Batsch), with a production of nearly 20 millions tonnes of fruits per year (FAOSTAT, <http://faostat.fao.org/>), is one of the most economically important fruit crop in the Rosaceae family, mainly because of its broad climate range and relatively high yield. In addition, peach has become one of the reference species for *Prunus* due to its diploid compact and small genome (227.3 Mb), taxonomic proximity to other important species and availability of homozygous doubled haploids (Aranzana, Abbassi, Howad, & Ar3s, 2010; Shulaev et al., 2008). Important genes related to production and fruit quality have been described

and mapped in peach, revealing that many morphological and quality traits have a simple genetic basis (Horn et al., 2005). Furthermore, a peach reference genome sequence, based on a doubled-haploid of the Lovell cultivar, has recently been obtained (The International Peach Genome et al., 2013), which shows high correspondence to the previously obtained physical maps for peach (Dirlwanger et al., 2006; Zhebentyayeva et al., 2008).

There are several hundreds of peach varieties distributed around the world displaying a wide phenotypic variability and producing fruits that offer the consumers a great mixture of flavours, textures, and sweetness/acidity ratios (Okie, 1998; Okie, Bacon, & Bassi, 2008). This high phenotypic diversity is the result of several peach breeding programmes in different countries that focus on the development of new varieties to satisfy diverse demands. Among others, these demands include a higher yield, the expansion of their production zones, disease resistance and the need for a better post-harvest quality of the fruit, this is especially critical given its short post-harvest lifespan (Byrne, 2005; Sansavini, Gamberini, & Bassi, 2006). The wide phenotypic variability of peach contrasts with a restricted genetic diversity, probably as a

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consequence of self-compatibility (Font i Forcada, Oraguzie, Igartua, Moreno, & Gogorcena, 2012; Fresnedo-Ramírez, Martínez-García, Partiff, Crisosto, & Gradziel, 2013; The International Peach Genome, 2013). Although traits affecting yield and disease resistance remain essential, plant breeding efforts should additionally address the needs of consumers, which include fruit flavour, quality, nutritional improvement, and enhanced health-promoting properties. All these characteristics are in close connection with the final chemical composition of the fruit at the time when they reach the consumers. Thus, in order to realign breeding programs to satisfy these demands, it is essential to assess the metabolic diversity of peaches from different varieties. In this sense, all the available high-throughput technologies based on genome, transcriptome, proteome, and metabolome analysis offer new possibilities for *Prunus* breeders in the post-genomic era (Martínez-Gómez, Sánchez-Pérez, & Rubio, 2012).

The fruit is one of the most metabolite-rich plant organs and as such contains a massive range in its metabolic complement, where metabolites involved in taste and flavour, with nutritional or pharmaceutical properties, or even with plant defence properties against biotic and abiotic stress can be found. Moreover, the metabolome represents the ultimate phenotype of the cells, and can also influence gene expression and protein function. Thus, metabolic profiling of fruits is a key tool to identify biomarkers related to quality and could be employed to improve breeding strategies and post-harvest storage of fresh products and optimise processing methods (Hu & Xu, 2013; Oms-Oliu, Odriozola-Serrano, & Martín-Belloso, 2013). Metabolic profiling study of primary metabolites during Dixieland peach development, using gas chromatography–mass spectrometry (GC–MS), revealed the metabolic dynamics during peach development at high resolution, and highlighted the key metabolic processes during the different peach developmental stages (Lombardo et al., 2011). More recently, metabolomics studies of Dixieland peach fruits during post-harvest treatments used to prevent fruit decay, allowed the identification of crucial metabolites, which prime the fruit to cope with different stress situations and, thus, which may be involved in better quality properties for consumers (Lauxmann et al., 2014). In the present work, in order to explore the metabolic biodiversity of peach, we performed a profiling study of primary metabolites, which are essential for survival and modulate secondary metabolism. For this purpose, we selected fifteen different varieties with diverse origins and phenotypic properties. One of the goals of this study was to assess how metabolically different the peach varieties are and to find out if there is any particular metabolic profile which could be associated with given phenotypic properties of the fruit. On the other hand, by evaluating the metabolomic pattern at both harvest and after post-harvest ripening of the fifteen varieties, we explored the common and distinct metabolic processes related to ripening across the different peach varieties. In summary, this study explores a part of the enormous chemical potential available in the biodiversity of peach fruit, which aids in the future construction of a catalogue of metabolites that may correlate to different phenotypic, organoleptic and quality properties of the fruit.

2. Materials and methods

2.1. Plant material

Assays were conducted with peach fruit (*P. persica* L. Batsch) of fifteen different varieties grown in the Estación Experimental Agropecuaria INTA, San Pedro, Argentina (Borsani et al., 2009; Budde, Polenta, Lucangeli, & Murray, 2006). The varieties selected were: Flordaking (FD), Don Agustín (DA), Spring Lady (SL), Goldprince (GP), Don Carlos INTA (DC), Red Globe (RG), Elegant Lady (EL), Fred (FR), 95 ED 1 (9S), María Anna (MA), Texprince

(TX), María Delizia (MD), Limón Marelli (LM), Rojo 2 (R2), and 55 RA 15 (SS) (Okie, 1998; Okie et al., 2008). All the peach varieties used were grafted on Cuaresmillo rootstock (*P. persica*). Trees from the different varieties were trained in a similar way, typically to a vase shape with three to five main branches and three sub-branches each. Three adult trees (of nearly 10 years each) of each variety were used for fruit collection. Relevant agronomic characteristics of each variety are listed in Table 1 and Table S1. Fruits were collected at commercial maturity. Depending on the variety, the flesh firmness of the fruits at harvest was between 55 and 70 N (Fig. 1). Immediately after harvest, fruits were manually selected for uniformity of colour, size and firmness and kept in a chamber at 20 °C and 90% relative humidity from 3 to 8 days depending on the variety and until the fruits reached firmness and organoleptic characteristics suitable for consumption (Fig. 1). Representative mesocarp tissue was collected from at least 20 fruits from the different varieties at harvest (H) or post-harvest ripened stage (R), immediately frozen in liquid nitrogen and stored at –80 °C for further experiments (Lauxmann et al., 2014; Lombardo et al., 2011). Five separate pools, each one composed of three different fruits of each variety at harvest (H) and ripened stage (R), were used for further metabolic analysis. The results shown in the present work correspond to fruits collected during the 2009/2010 season, although similar results were obtained for some peach varieties grown during 2011/2012 (not shown in the present work).

2.2. Fruit quality traits determination

Flesh firmness was evaluated with a penetrometer (Effegi 327, Alfonsine, Italy) with a 7.9-mm tip and expressed in newton (N). Measurements were carried out on two opposite sides of each individual fruit after peel removal. Ground colour was measured on the greenest portion of the peel free of red blush and flesh colour was determined on the equatorial zone of a longitudinal section with a chromameter (Minolta CR 300; Budde et al., 2006). Twenty to thirty fruits of each variety at harvest (H) and post-harvest ripened stage (R) were used for each quality trait determination.

2.3. Metabolite measurements

Metabolite analysis by gas chromatography–mass spectrometry (GC–MS) was carried out essentially as described by Roessner-Tunali et al. (2003). Representative mesocarp tissue of peach fruits from the different varieties (250 mg) at harvest (H) or ripened stage (R) were ground using ceramic mortar and pestle pre-cooled with liquid nitrogen and extracted in 3 mL of methanol. Internal standard (180 µL, 0.2 mg ribitol mL⁻¹ water MilliQ) was subsequently added for quantification purposes. The mixture was extracted for 15 min at 70 °C (vortexing every 3 min) and mixed vigorously with pre-cooled water MilliQ (1.5 mL). After centrifugation at 2,200 g, an aliquot of the supernatant (50 µL) was transferred to a reaction tube (1.5 mL) and vacuum dried. Tubes were filled with argon gas and stored at –80 °C. Samples were derivatised using methoxyamine hydrochloride in pyridine followed by *N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide treatment. Derivatisation and GC–MS were performed as described by Roessner-Tunali et al. (2003). Mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005). Five independent determinations composed of three fruits each, and each one repeated three times for methodological replica, were performed for each variety at harvest (H) and ripened stage (R). Metabolite quantification was based on the relative peak response area of each chromatogram and expressed relative to the internal standard (ribitol; Table S2). The relative values were also expressed as log₂ using the MultiExperiment Viewer software with

Table 1

Relevant agronomic characteristics of the fifteen varieties selected for metabolomic studies. Depending on the harvesting date, the varieties were classified as early, mid (light grey) and late season (grey). The colour of the fruits was measured with a chromameter and the % of red colour is indicated. The average weight ($\pm 10\%$) of the fruits at harvest is indicated. ARG: Argentina; MEX: Mexico; IT: Italy.

Variety name	Country origin	Harvest date	Flesh adhesion	Flesh colour	Epidermal ground colour	Epidermal red colour (%)	Average weight (g)
Flordaking (FD)	USA	Early	Semifree-stone	Yellow	Yellow	40–60%	156
Rojo 2 (R2)	USA	Early	Semifree-stone	Yellow	Yellow	80%	130
Don Agustín (DA)	USA	Early	Semifree-stone	Yellow	Yellow	60–70%	150
55 RA 15 (SS)	USA	Early	Cling-stone	Yellow	Yellow	90%	155
Spring Lady (SL)	USA	Mid	Free-stone	Yellow	Yellow	>90%	150
Goldprince (GP)	USA	Early	Free-stone	Yellow	Yellow	40–60%	160
Fred (FR)	MEX	Early	Semifree-stone	Yellow (red strips)	Yellow	80%	150
Texprince (TX)	USA	Mid	Semifree-stone	Yellow (red strips)	Yellow	60–70%	140
Don Carlos INTA (DC)	ARG	Mid	Semifree-stone	White	White	60–80%	150
Red Globe (RG)	USA	Mid	Free-stone	Yellow	Yellow	60–80%	170
María Anna (MA)	IT	Late	Free-stone	Yellow	Yellow	40–70%	155
95 ED 1 (9S)	USA	Late	Free-stone	White (red strips)	Yellow	40%	>200
Elegant Lady (EL)	USA	Late	Free-stone	Yellow	Yellow	70–90%	170
María Delizia (MD)	IT	Late	Free-stone	White (red strips)	White	40%	190
Limón Marelli (LM)	ARG	Late	Cling-stone	Yellow	Yellow	<20%	160

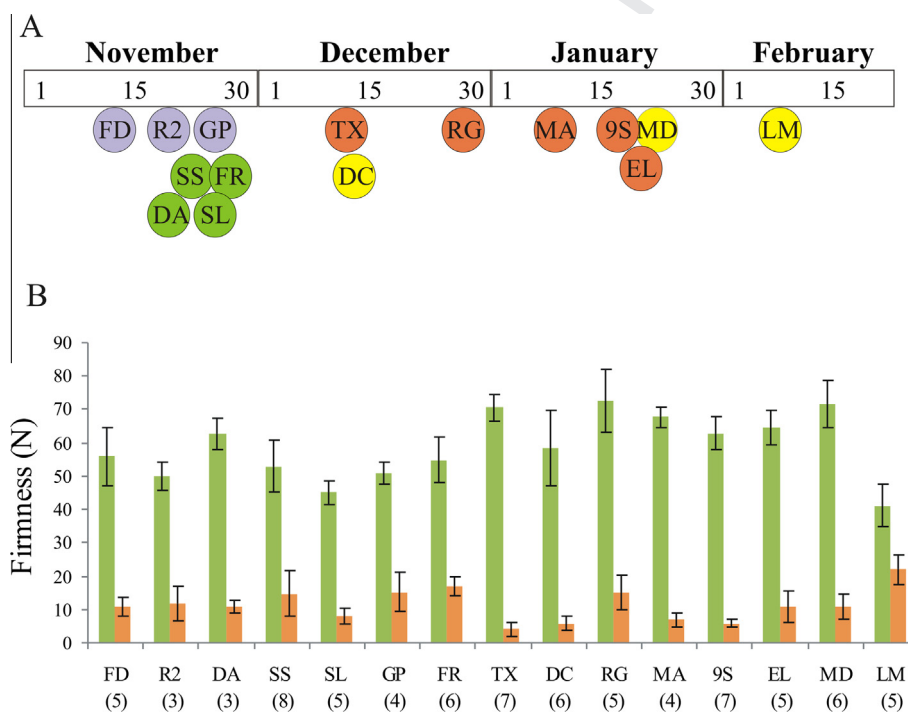


Fig. 1. Harvest time and post harvest softening of peach fruit varieties. (A) Harvest time of peach varieties. The 15-peach varieties (Flordaking, FD; Rojo 2, R2; Don Agustín, DA; 55 RA 15, SS; Goldprince, GP; Spring Lady, SL; Fred, FR; Texprince, TX; Don Carlos INTA, DC; Red Globe, RG; María Anna, MA; 95 ED, 9S; Elegant Lady, EL; María Delizia, MD; and Limón Marelli, LM) are placed chronologically according to typical date of harvest, from November to February. The different colours used for each variety indicate the four different metabolic groups (groups 1–4) identified in the present work according to the relative levels of metabolites. (B) Post harvest softening of peach fruit varieties. Fruits from the indicated 15-varieties were harvested and kept at 20 °C for softening until reaching an edible state. The days taken for the softening process depend on the variety and are indicated in parenthesis in the x-axes, below the name of each variety. Values represent the mean \pm SD, $n = 15$ for firmness determinations. Standard deviations are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

204 a colour scale (MeV v4.4.1, <http://www.tm4.org/>, Saeed et al., 2003,
205 Fig. 2). Table S3 provides technical data from these experiments in
206 accordance to recommendations for reporting metabolomic data
207 (Fernie et al., 2011).

Determination of the absolute concentrations of 19 identified
208 metabolites using three peach fruit varieties (GP, DC and MD)
209 was performed by comparison with calibration standard curve
210 response ratios of various concentrations of standard solutions,
211

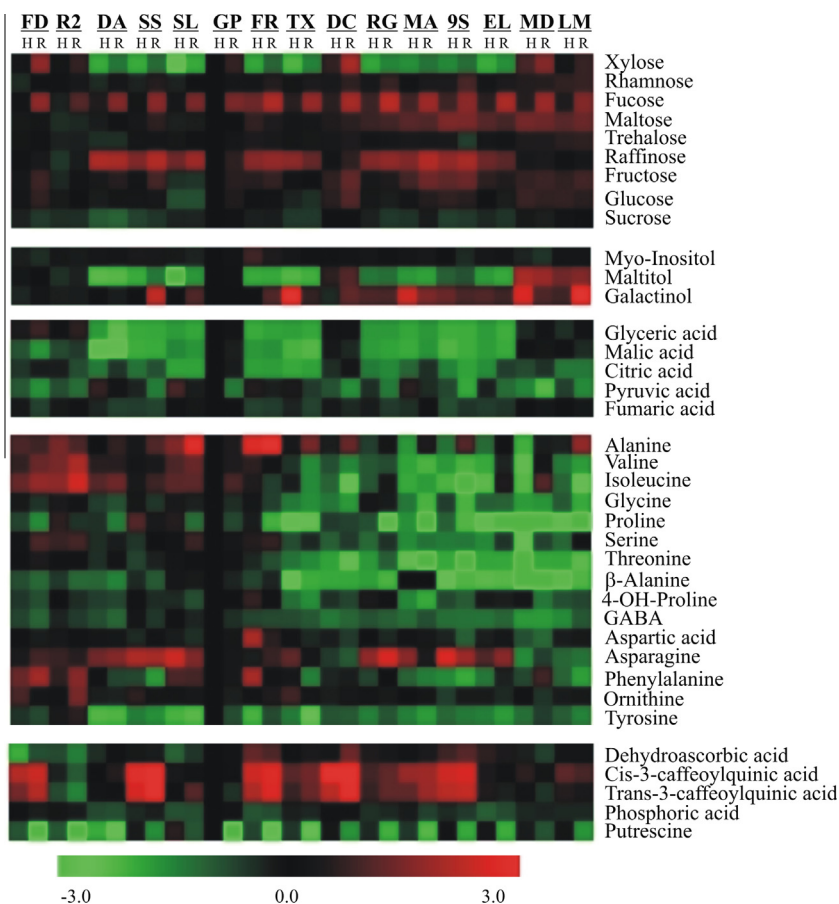


Fig. 2. Distribution of polar metabolite levels analysed by GC–MS in fifteen peach fruit varieties. The mesocarp of fifteen peach varieties were analysed at harvest (H) and after post-harvest ripening (R): Flordaking (FD-H and FD-R); Rojo 2 (R2-H and R2-R); Don Agustín (DA-H and DA-R); 55 RA 15 (SS-H and SS-R); Spring Lady (SL-H and SL-R); Goldprince (GP-H and GP-R); Fred (FR-H and FR-R); Texprince (TX-H and TX-R); Don Carlos INTA (DC-H and DC-R); Red Globe (RG-H and RG-R); María Anna (MA-H and MA-R); 95 ED 1 (9S-H and 9S-R); Elegant Lady (EL-H and EL-R); María Delizia (MD-H and MD-R); and Limón Marelli (LM-H and LM-R). The graph shows the relative level of each metabolite to its amount found in the sample GP-H, which was randomly selected. Normalised values are shown on a colour scale (shown at the bottom of the figure), which is proportional to the content of each metabolite. Mean values of 5 independent determinations for each stage were normalised to sample GP-H and expressed as log₂ using the MultiExperiment Viewer software (MeV v4.4.1, Saeed et al., 2003). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including the internal standard ribitol, which were derivatised concomitantly to tissue samples (Table S4).

2.4. Statistical analysis

Principal component analysis was performed on data sets obtained from metabolite profiling using the software package XLSTAT (Microsoft Excel). The data were log₂ transformed and normalised to the median of the entire sample set for each metabolite before analysis. Correlation analysis between metabolites based on Pearson correlation was performed using R software (Ihaka & Gentleman, 1996). For the correlation analysis, all metabolic data were used.

Data presented were analysed using one-way analysis of variance (ANOVA). Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunnett and Duncan tests ($\alpha = 0.05$) using the SigmaStat package.

3. Results

3.1. Peach varieties characteristics and post-harvest ripening

Fifteen peach (*P. persica* L. Batsch) varieties were selected for this work: Flordaking (FD), Rojo 2 (R2), Don Agustín (DA), 55 RA

15 (SS), Spring Lady (SL), Goldprince (GP), Fred (FR), Texprince (TX), Don Carlos INTA (DC), Red Globe (RG), María Anna (MA), 95 ED 1 (9S), Elegant Lady (EL), María Delizia (MD), and Limón Marelli (LM). The principal agronomic characteristics of each variety are listed in Table 1, and additional information about the varieties is indicated in Table S1. Depending on the harvest date (Fig. 1A), the varieties were divided into categories of early, mid and late harvesting date (Table 1). Phenotypic characteristics also varied among the selected peach varieties, including flesh adhesion (freestone, semi-freestone or clingstone), flesh and epidermal colours and average weight (Table 1).

Fruits from the different peach varieties were harvested at physiological maturity and kept in a chamber at 20 °C and 90% relative humidity for post-harvest ripening. Depending on the variety, the ripe stage was reached after 3–8 days in the chamber (Fig. 1B). Considering that one of the best indicators of the ripening stage is flesh firmness, this parameter was measured for each variety at harvest (H) and after post-harvest ripening (R) (Fig. 1B). At the ripe stage, fruits from all the varieties exhibited all the characteristics of an edible fruit, such as an increase in the amount of juice and enhanced aroma. At the ripe stage, the firmness of the fruits from the different varieties was typically below 15 N (Fig. 1B). The only exception was LM, which is a non-melting flesh variety and, because of this, this variety must be harvested at firmness lower

than 50 N and reaches a value of approximately 20 N at the ripe stage (Fig. 1B).

3.2. Metabolic profiling of peach varieties at harvest and ripe stages

Representative mesocarp of the fifteen selected peach varieties was collected at harvest (H) and after post-harvest ripening (R), and used for metabolite extraction followed by GC–MS analysis, in order to detect primary metabolite levels. By this technique, 37 polar primary metabolites were unambiguously identified and divided into sugars (9), sugar alcohols (3), organic acids (5), amino acids (15) and miscellaneous compounds (5) (Table S2). In order to easily visualise the metabolic differences among the varieties, the peak response areas of each metabolite, relative to internal standard, were normalised to sample GP-H, which was arbitrarily selected for this purpose. Fig. 2 shows the normalised values (expressed as log₂) for each metabolite and sample using a colour scale which is proportional to the content of each identified metabolite. A high metabolic diversity is clearly observed among all the samples analysed; this diversity depends on both the variety considered and the maturity stage (H or R; Fig. 2).

The comparison of the relative levels of the different soluble sugars analysed indicates that the content of sucrose, fructose and glucose among samples is quite similar, especially when considering the higher variability of other sugars such as xylose, fucose and raffinose (Fig. 2 and Table S2). In the case of sucrose, a 2.2-fold difference between the sample with highest relative level (DC-H) and the sample with the lowest (DA-R) was observed. In the case of glucose, a 3.8-fold difference between the sample with highest relative level (DC-R) and the sample with the lowest (SL-H) was detected, whilst in the case of fructose a 3.7-fold difference between the sample with highest relative level (MA-R) and the sample with the lowest (SL-R) was found. Comparing all the varieties, DC is the variety with the highest relative levels of both sucrose and glucose (Table S2). On the other hand, xylose, raffinose and fucose vary dramatically among the varieties (Fig. 2). In the case of xylose, a 27-fold difference was detected between the sample with highest (DC-R) and lowest relative levels (SL-H) (Table S2). It is noticeable that the relative level of xylose depends most on the variety considered and not on the maturity state (H or R) (Fig. 2). However, this is not the case for fucose, which dramatically increases after post-harvest ripening in all fifteen varieties studied (Fig. 2). In the case of fucose, a sixfold difference is observed between the varieties with the highest (RG-R) and lowest (R2-H) relative levels (Table S2). Regarding raffinose, a 6.5-fold difference between the sample with highest (MA-R) and lowest (R2-H) relative levels was observed (Table S2). The variation of maltose among the samples analysed is similar to that of glucose (Fig. 2), with a 3.9-fold difference between the sample with highest (9S-H) and the sample with the lowest (R2-H) relative levels (Table S2).

With regard to sugar alcohols, maltitol and galactinol relative levels were considerably different among the samples (Fig. 2). In the case of maltitol, a 21.4-fold difference is observed between the sample with the highest (MD-H) and lowest (DA-H) levels (Table S2). An even greater variance was observed in the case of galactinol (Fig. 2 and Table S2). No detectable levels of galactinol were observed in four samples: DA-H, SS-H, SL-H and FR-H (Table S2). Galactinol variation among the samples with detectable levels is up to 28.6-fold, with the highest relative level measured in TX-H and the lowest in DC-H (Table S2). Sorbitol peak could not be identified in our system analysis.

Considering the organic acids detected, pyruvic and glyceric acids greatly vary among the samples (Fig. 2). In both cases, a nearly 9.7-fold difference is observed between the samples with the highest (FD-R for glyceric and SL-H for pyruvic acids) and the

lowest relative levels (DA-R for glyceric and MD-R for pyruvic acids) (Table S2). Regarding malic and citric acids, which are considered the major acids related to flavour in peach fruit, a higher variability is observed for malic than for citric acid (Fig. 2). For malic acid, a 7.1-fold difference is observed between the sample with the highest (GP-H) and the lowest (DA-H) relative levels, whilst in the case of citric acid, a 4.0-fold difference is observed (GP-H is the sample with highest and TX-H is the one with lowest relative levels) (Table S2).

A dramatic variation was also clearly observed in the relative amino acid levels among the samples analysed (Fig. 2). Large differences were observed in the case of Ile, Ala, Pro, Val, Asn and Phe, for which relative levels vary 52.0-, 46.8-, 24.3-, 23.2-, 18.9- and 16.1-fold, respectively, between the samples with the highest (R2-R for Ile and Val, FR-R for Ala, SS-H for Pro, 9S-H for Asn and FR-H for Phe) and the lowest (9S-R for Ile and Phe, and MD-H for Ala, Pro, Val and Asn) relative levels (Table S2). In the case of β -Ala and Orn, there are samples with undetectable levels (MA-H and MA-R in the case of β -Ala; and MA-H in the case of Orn) (Table S2). If these samples are not considered, the variation in β -Ala and Orn, between the samples with the highest and the lowest relative levels were 16.7- and 4.3-fold, respectively (Table S2). In general, the relative levels of all the amino acids are highly variable among the samples; the lowest fold of difference between the sample with lowest and highest levels is 3.7-fold for GABA, followed by 6.0-fold for Gly (Table S2).

The relative content of the two quinic acid derivatives identified (free quinic acid could not be identified) is highly variable among the samples considering the varieties but not the maturity state (Fig. 2). *Trans*- and *cis*-3-caffeoylquinic acids vary 34.4- and 25.2-fold between the samples with the highest (SS-R in both cases) and the lowest (R2-R in both cases) relative levels (Table S2). Dehydroascorbic acid is also highly variable among the samples analysed, although to a lesser extent than the quinic acid derivatives (Fig. 2), with a 10.6-fold difference between the sample with highest (DC-R) and the lowest (FD-H) relative levels (Table S2). Putrescine is also highly variable but the major differences are observed when comparing H versus R-samples and not when comparing the varieties (Fig. 2). A 13.3-fold difference in the relative content of putrescine is observed between SL-H and R2-R, which display the highest level and lowest level, respectively, among the samples analysed (Table S2).

3.3. Clustering peach fruit varieties on the basis of their metabolite profiles at harvest and fully ripe stages

In order to cluster the fifteen selected varieties by their intrinsic metabolic properties, but not by their maturity state, the data set of the relative content level of the 37 primary metabolites, at either harvest or fully ripe states (Table S2), were independently examined by principal component analysis (PCA).

At harvest, two principal components explaining 62.27% of the overall variance of the metabolite levels (36.37% and 25.9% for PC1 and PC2, respectively) separated the varieties in four distinct metabolic groups, which were named groups 1–4 (Fig. 3A). The contribution of metabolites to each principal component is shown in Table S5. The metabolites that most contributed to PC1 (positive side) were principally amino acids: Val (6.85%); Thr (6.82%); Ile (6.26%) and β -Ala (6.02%); and the disaccharide maltose (6.11%) to the negative side. On the other hand, the main contributors to PC2 (positive side) were organic acids: glyceric (9.88%), malic (8.20%), and citric (7.24%) acids; as well as the soluble sugars xylose (7.99%); whilst raffinose (9.73%) contributed to the negative side (Table S5).

Interestingly, at the fully ripe stage and considering two principal components explaining 53.24% of the overall variance of the

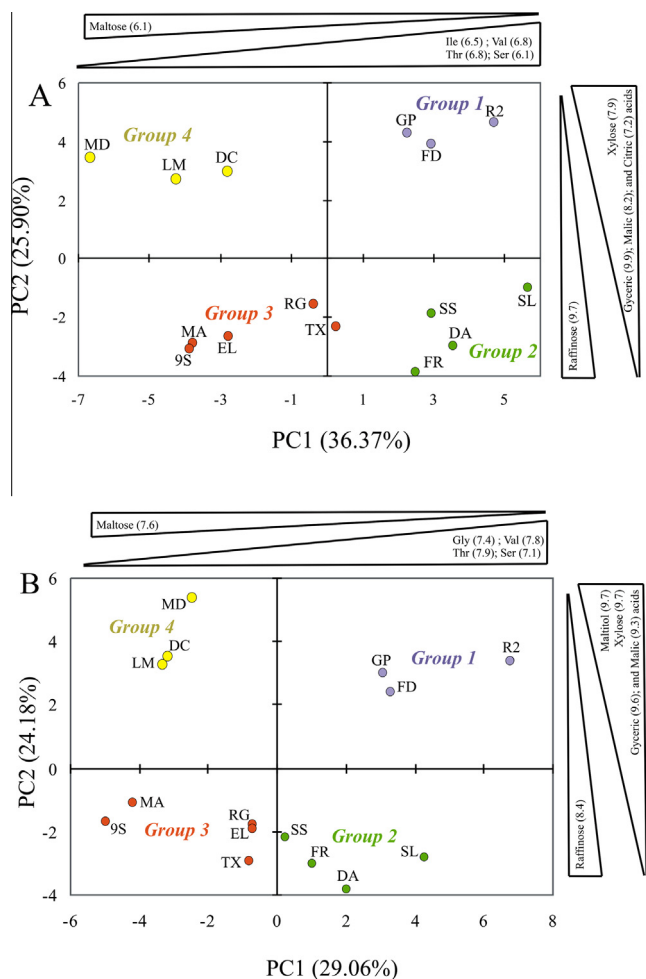


Fig. 3. Principal component analysis of GC-MS data at harvest (A) and after post-harvest ripening (B) of fruits from fifteen peach varieties. Four separated groups, named group 1–4, can be easily distinguished considering the metabolic content at both harvest (A) and after ripening (B). Group 1 clusters the varieties FD, GP and R2; group 2 includes the varieties DA, FR, SL and SS; group 3 clades the varieties EL, MA, RG, TX and 9S; and group 4 clusters the varieties DC, LM and MD. The contribution of each metabolite to the principal components in Fig. 3A and B are shown in Tables S5 and S6, respectively. The variance explained by each component (%) is given within parentheses. The five metabolites that contribute the most to the separation of the varieties in PC1 and PC2 are indicated. The variable contribution to each principal component are indicated in brackets (Tables S5 and S6).

metabolite levels – 29.06% and 24.18% for PC1 and PC2, respectively –, the PCA analysis separated the varieties in the same four metabolic groups (groups 1–4) found at harvest stage (Fig. 3B). The contribution of metabolites to each principal component is shown in Table S6. Again, the metabolites that most contributed to PC1 were principally amino acids (positive side): Val (7.84%); Thr (7.88%); Gly (7.46%), and Ser (7.11%); and the disaccharide maltose (7.64%) to the negative side. On the other hand, some of the main contributors to PC2 (positive side) were, again, organic acids: glyceric (9.66%) and malic (9.28%) acids and the soluble sugar xylose (9.68%); whilst raffinose (8.34%) contributed to the negative side. In this case, maltitol (9.72%) appears as one of the most important contributors to the positive side of PC2 (Table S6).

3.4. Correlation analysis of metabolite content among the varieties

Correlation analysis was also performed on the entire data set of metabolites of the fifteen different varieties at both harvest and after post-harvest ripening. This analysis allows the

identification of associations of metabolites, with a more detailed evaluation of the behaviour of the metabolite network. Correlation analysis was carried out by the calculation of the Pearson correlation coefficients for each metabolite pair. Out of 1,369 pairs of metabolites analysed, 452 resulted in significant correlations ($p < 0.05$). From these, 288 were positive and 164 negative (Fig. S1). The amino acids, especially Val, Ile, Leu, Pro, Ser, Thr, GABA and Phe, showed the highest number of positive correlations among them (Fig. S1). Negative correlations were detected between the group of amino acids and soluble sugars, especially maltose, rhamnose, trehalose, fructose and glucose (Fig. S1). Xylose displays positive correlation with the organic acids, glyceric, malic and citric acids (Fig. S1). Dehydroascorbic acid displays positive correlation with soluble sugars, but negative correlation with some amino acids (Fig. S1).

3.5. Absolute metabolite concentration of selected compounds in three peach varieties

For three peach fruit varieties distributed from early to late harvest season, which according to PCA analysis clustered in two distinct groups (GP in group 1, and DC and MD both in group 4), quantitative determination of the concentration of 19 metabolites was performed at both harvest and ripe stage, using compound specific calibration curves for each metabolite by GC-MS (Table S4). This evaluation allows the comparison of the relative levels among metabolites in the varieties. The comparison of the average absolute content (in $\mu\text{g/g}$ FW) of the soluble sugars indicates that the relative content ratio of glucose to fructose varies from 9.1 to 15.5 among the three varieties (Table S4); which indicates that glucose concentration is nearly, and consistently, one order of magnitude higher than that of fructose. With regards to organic acids, the average content ratio of malic to citric acid varies from 7.0 to 10.4, indicating that malic acid is the major organic acid in peach fruit mesocarp (Table S4). Regarding the amino acid content, Asn is, by far, the one with the highest concentration in the three varieties, followed by Asp and Ser which vary quite considerably among the samples (Table S4).

3.6. Metabolite changes associated with post-harvest ripening in peach varieties

In order to identify the metabolic processes in which behaviour is similarly affected during the post-harvest ripening of the fifteen peach varieties, the content of the 37 primary metabolites at the mature state (R) were normalised to the content measured at harvest (H) in each variety (Table S7). Among the metabolites that show statistically significant changes ($p < 0.05$) when comparing the R and H samples of each variety, we can find metabolites that increase during ripening (ratios higher than 1); metabolites that decrease during post-harvest ripening (ratios lower than 1); or metabolites with no significant changes when comparing H and R samples and thus, they are not modified during ripening (Table S7). Overall, the results reveal both conserved levels of metabolites and distinct changes in the dynamics of metabolic processes during post-harvest ripening across the different peach varieties (Table S7).

Regarding the changes in sugar content during post-harvest ripening, the only sugars that consistently increase in all the fifteen peach varieties are xylose and fucose (Fig. 4A), with rises of up to 3.6-fold (in FD) and 3.4-fold (in SS), respectively (Table S7). Rhamnose, fructose and raffinose significantly increase in 10, 6 and 5 varieties, respectively, with no significant changes in the rest of the varieties (Fig. 4A). Five varieties show significant increases in the level of glucose during ripening, whilst only one shows decrease of this monosaccharide (EL) (Table S7 and Fig. 4A). It is

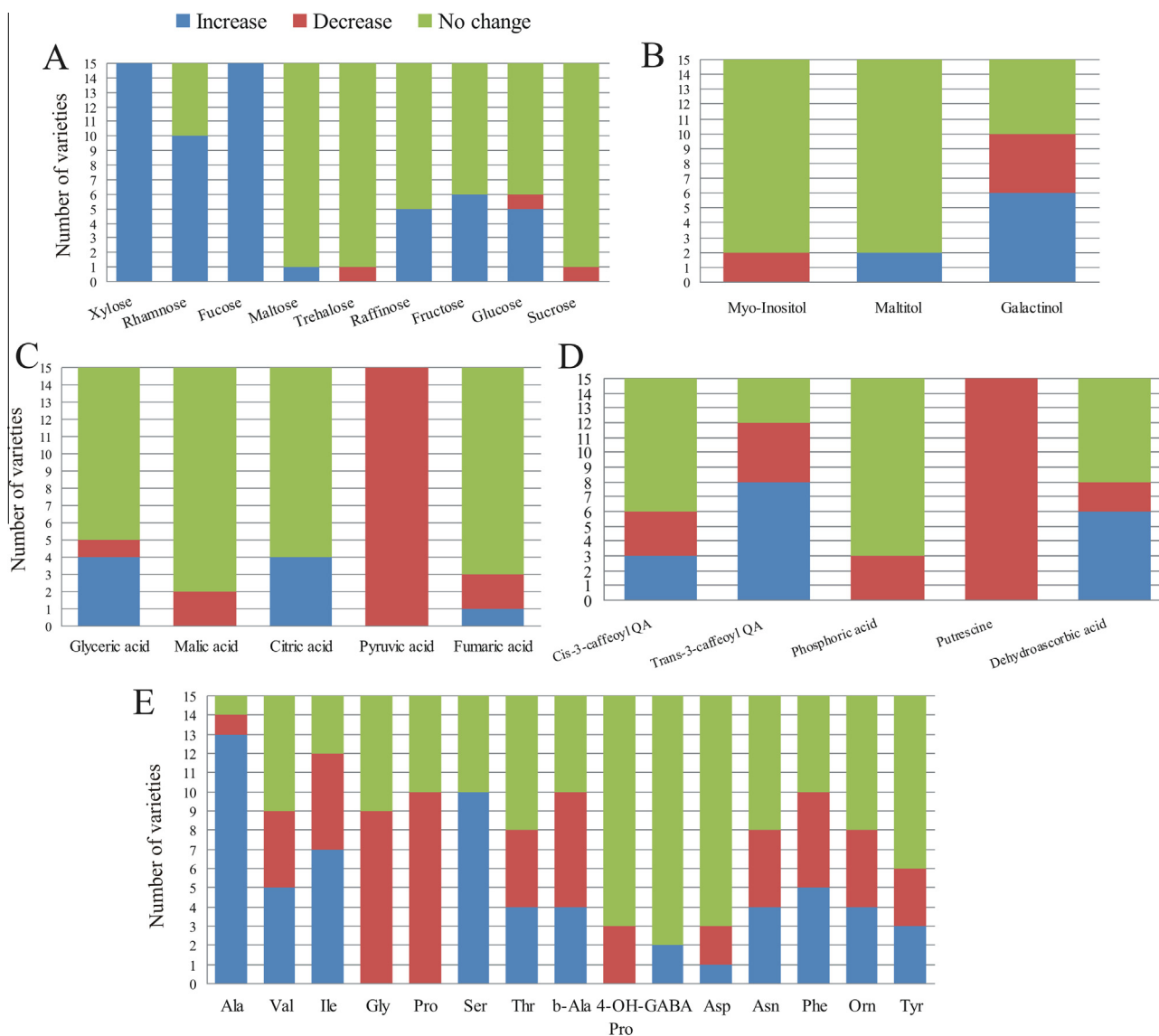


Fig. 4. Modification of metabolite levels after post-harvest ripening across peach varieties. The number of varieties which show significant increases (in blue), decreases (in red) or no significant modifications (in green) in the level of sugars (A), sugar alcohols (B), organic acids (C), miscellaneous compounds (D) and amino acids (E) during ripening are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interesting to note that the varieties that exhibit increases in the level of glucose during post-harvest ripening (FD, SS, GP, DC and MA) also show rises in the level of fructose (Table S7). In the case of sucrose, only one variety displays significant decrease during ripening (FD), whilst the other 14 do not show significant changes (Table S7 and Fig. 4A). The sugar alcohols *myo*-inositol and maltitol do not show significant changes in 13 varieties during post-harvest ripening (Fig. 4B). The behaviour of galactinol during ripening was very variable among the varieties, with 6 varieties showing increases, 4 decreases and 5 showing no changes (Fig. 4B).

In relation to the modification of organic acids during post-harvest ripening, a consistent reduction in the content of pyruvic acid was observed in all the varieties, with decreases up to 0.38-fold in GP (Table S7 and Fig. 4C). The other organic acids measured did not significantly change in the majority of the varieties: glyceric acid was unaltered in 10 varieties, malic acid in 13, citric acid in 11, and fumaric acid in 12 varieties (Fig. 4C). By contrast, the five organic acids analysed in this work were all modified during the post-harvest ripening process of the FD variety, in which an increase of nearly 1.3-fold of glyceric and citric acid is

observed, along with decreases of nearly 0.6-fold of malic, pyruvic and fumaric acids (Table S7).

The polyamine putrescine displayed a consistent decrease during post-harvest ripening in all varieties analysed, with dramatic decreases (up to 0.2-fold) in some cases (Table S7 and Fig. 4D). Dehydroascorbic acid increased in 6 varieties during ripening but decreased in 2; whilst the quinic acid derivatives display variable patterns of modification during post-harvest ripening across the 15-peach varieties analysed (Fig. 4D).

The behaviour of the amino acids during post-harvest ripening is diverse, and none of the 15 amino acids display the same trend in all the 15 varieties analysed (Fig. 4E). Among the amino acids, Ala displayed increases in 13 varieties (up to 5.2-fold increase in 9S) and Ser in 10 (up to 3.7-fold increase in MD) (Table S7 and Fig. 4E). Pro and Gly showed decreases, of up to 0.3-fold in some cases, in 10 and 9 varieties, respectively (Table S7 and Fig. 4E). GABA and 4-OH-Pro did not significantly change during post-harvest ripening in 13 and 12 varieties, respectively (Fig. 4E). In all the other cases, the pattern of amino acid modifications was more diverse along the varieties (Fig. 4E).

4. Discussion

4.1. The content of key metabolites underlying peach fruit quality properties is variety-dependent

Higher plants have the remarkable ability to synthesise a huge diversity of compounds that differ in chemical structure and biological activity. In particular, fruits contain a vast array of metabolites, which are responsible for their organoleptic properties, nutritional value and pharmaceutical activities. Peach represents a particular model fruit in terms of physiology, anatomy and metabolism, different from other model fruits, such as tomato, strawberry or grape; and with particular metabolic networks during development, which are related to its morphology (a drupe with seeds covered by a lignified endocarp and a juicy mesocarp; Lombardo et al., 2011). Within this general morphology, there is an extensive variability in flavours, textures, and sweetness/acidity ratios among the several hundreds of varieties that exist around the world (Byrne, 2005; Sansavini et al., 2006). Taking these facts into consideration, in the present work, we evaluated the variance in metabolite content of fifteen peach varieties grafted on the same rootstock, and with trees trained in a similar way, as it was shown that rootstock or training systems may influence phytochemical composition of the fruits (Orazem, Mikulic-Petkovsek, Stampar, & Hudina, 2013; Tavarini et al., 2011). This is an approach with great potential for the improvement of the compositional quality of the fruits; especially in light of the high diversity of the content of metabolites detected across the varieties analysed here (Fig. 2 and Table S2).

Regarding the relative content of sugars along the peach varieties, a very high diversity in the content of the trisaccharide raffinose was detected (Fig. 2 and Table S2). High levels of raffinose were detected during pit hardening of Dixieland peach fruit (Lombardo et al., 2011) and marked increases of this trisaccharide were found following post-harvest treatments applied to prevent chilling injury in Dixieland peach fruit (Lauermann et al., 2014). In other plant species, raffinose, along with galactinol, were identified as key antioxidants or molecule signals mediating stress responses, and they are often increased during cold acclimatisation (Korn et al., 2010; Nishizawa, Yabuta, & Shigeoka, 2008; Valluru & van den Ende, 2011). Other compounds related to stress protection, such as maltitol and galactinol, were also highly diverse in content among the varieties (Fig. 2 and Table S2), with maltitol playing roles as osmoprotector in stress situations (Erxleben, Gessler, Vervliet-Scheebaum, & Reski, 2012). Considering the high diversity in the content of raffinose, galactinol and maltitol across peach varieties and the role of these compounds in plant stress tolerance, we are currently analysing if there is a correlation between their content and the degree of susceptibility to stress in each variety, as e.g. chilling injury induced by cold storage after harvest of peach fruits.

The content of taste-relevant metabolites, such as organic acids and sugars, was also variety-dependent in peach fruit. In the case of organic acids, the highest levels of both malic and citric acids were detected in GP variety; whilst in the case of sucrose and glucose, the highest levels were detected in DC variety (Fig. 2 and Table S2). The content of fructose, which greatly contributes to fruit sweetness, was also variable across peach varieties (Fig. 2). The diversity in the content of sugars and organic acids detected in the present work is in agreement with the extensive variability in sweetness/acidity ratios found in the different peach varieties.

A great diversity in the level of amino acids was detected across peach varieties (Fig. 2 and Table S2). Amino acid content in peach fruit may be related to several different and important fruit quality traits. For example, Ile, Leu, Tyr and Phe are precursors of volatile

compounds that may contribute to final aroma and flavour (Tieman et al., 2006); Pro is involved in the response to different stress situations in plants (Kavi Kishor & Sreenivasulu, 2014); Tyr is substrate for polyphenol oxidases, which are involved in the undesirable browning of the fruits (Tran, Taylor, & Constabel, 2012); moreover, several different amino acids have key nutritional importance (Wu, 2013).

Finally, a high diversity in the content of caffeoylquinic acids and dehydroascorbic acid was detected across the peach varieties (Fig. 2 and Table S2). Caffeoylquinic acids represent an important group of plant-based bioactive polyphenols, with significant anti-oxidant activity, and have been reported as having an important beneficial effect in human health (Luo et al., 2008).

To evaluate the interactions among metabolites and their coordinated variations across peach varieties, a correlation analysis was performed on the entire data set of metabolites of the fifteen different varieties (Fig. S1). This analysis indicates that the amino acids show the highest number of positive correlations among them; and display negative correlations with some soluble sugars, especially maltose, rhamnose, trehalose, fructose and glucose (Fig. S1). Some of these correlations were also found during Dixieland peach development (Lombardo et al., 2011), indicating their relevance in peach metabolism and, thus, could be useful to identify co-regulated pathways and biochemical regulatory mechanisms in peach fruit.

In summary, the present work clearly shows that the fruits from different peach varieties display a great variation in the content of key metabolites involved in organoleptic properties, protection against stress situation, nutritional value and pharmaceutical activities, which reinforces the use of metabolomics as a tool for functional genomics and fruit quality improvement in peach fruit.

4.2. Metabolic clustering of the fifteen peach varieties fruits reveals common chemical patterns associated with particular properties

According to their metabolic content, the fifteen peach varieties cluster into four major groups at both harvest and ripe stage (Fig. 3). Hence, this division is independent on the metabolic changes that do take place during post-harvest ripening (Figs. 4 and 5 and Table S7). The first PC groups of all the varieties consistently with their harvesting date; early varieties (groups 1 and 2) were separated from mid- and late season varieties (groups 3 and 4; Figs. 1 and 3). Among the metabolites contributing the most to the separation between early and mid- and late season varieties, several amino acids and the disaccharide maltose can be found (Fig. 3, Tables S4 and S5). Thus, early varieties display higher relative levels of several amino acids (Ile, Val, Thr, β -Ala, Gly, Ser), whilst mid- and late varieties display higher levels of maltose (Fig. 5A). The separation of the varieties according to their harvesting date may be linked to genetic difference in metabolic pathways, or may also be related to the fact that these contrasting varieties have different growing periods in different climatic conditions.

The early, mid- and late season varieties are further separated by PC2 into two different groups (Fig. 3). The major contributors to this second separation are organic acids and the trisaccharide raffinose (Fig. 3, Tables S4 and S5). Groups 1 and 4 display higher levels of organic acids such as glyceric, malic and citric acid, whilst groups 2 and 3 display higher relative levels of raffinose (Fig. 5A). No particular phenotypic characteristic could be associated with this second separation of the early and mid-late varieties (Table 1); and further genomic characterisation of the varieties analysed in the present work could help to identify the basis of this second clustering (Fig. 5A).

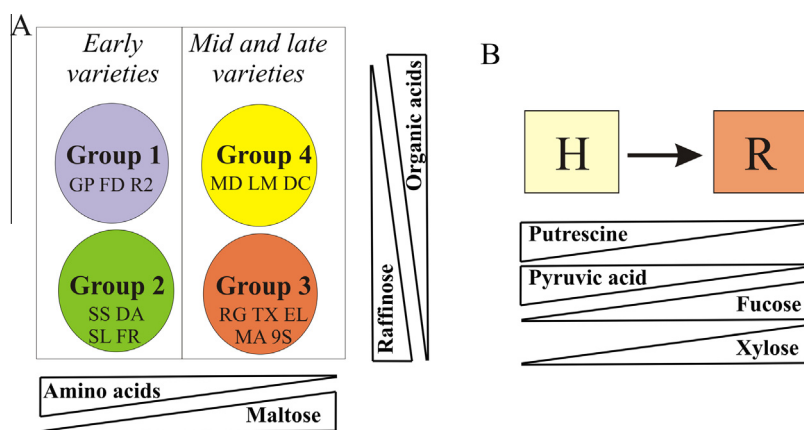


Fig. 5. Simplified scheme showing the most relevant metabolic variation among the fifteen peach varieties and during post-harvest ripening. (A) Principal metabolites contributing to the clustering of the fifteen peach varieties in groups 1–4. The varieties are first divided into either early or mid- and late varieties depending on the content of amino acids and maltose and further divided by the content of organic acids and raffinose. (B) Metabolic changes during post-harvest ripening which are common to the fifteen peach varieties analysed in the present work.

4.3. Metabolite changes associated with peach post-harvest ripening reveal variety common and variety distinct processes

During the post-harvest ripening process, the peach fruit is transformed into a palatable product for consumers. All the modifications that take place during peach ripening affect the nutritional quality, flavour and aroma, as well as the ratio between sugars and organic acids, which contributes significantly to the overall quality of the fruit (Borsani et al., 2009; Lombardo et al., 2011). In the present work, in order to provide insights into the pathways that are essential during the post-harvest ripening of peach fruit and the ones that are specific to each peach variety, the content of primary metabolites at harvest was compared to the content of metabolites at the ripe stage in the fifteen peach varieties (Fig. 1 and Table S7).

The fifteen peach varieties displayed increases of fucose and xylose during post-harvest ripening (Fig. 4A and Table S7), indicating that the increase in these sugars is a common process that take place independently of the genetic background or phenotypic characteristics of the fruits. The increase in these two sugars may be associated with the softening process, which is linked to dramatic cell wall changes, due to the action of several hydrolases and transglycosylases (Brummell & Harpster, 2001; Bustamante et al., 2012). The content of fructose and glucose, metabolites that greatly contribute to fruit sweetness, is increased only in six and five varieties, respectively (Fig. 4A).

The peach varieties also displayed a decrease in the content of pyruvic acid during post-harvest ripening (Fig. 4C and Table S7). The content of malic and citric acids, related to the final flavour of the fruits, varied in only two and four varieties, respectively (Fig. 4C). Thus, the changes in the degree of fruit acidity during ripening are more dependent on the variety than on the ripening process.

The modification of the content of amino acids during post-harvest ripening is very diverse (Fig. 4E). The same amino acid modification did not take place in all fifteen varieties; however, an increase in Ala content was observed in 13 varieties (Fig. 4E). In all other cases, changes in amino acids content during post-harvest ripening seem to be essentially variety-dependent (Fig. 4E). Considering that many amino acids are precursors of bioactive compounds, this great variability in amino acid content may be related to an important variation in the levels of compounds of importance for fruit quality.

Putrescine levels are reduced in all the fifteen varieties analysed (Fig. 4D), as previously found for Dixieland peach variety (Lombardo et al., 2011). Putrescine is a polyamine that has been implicated in a wide range of developmental processes. The decrease found during post-harvest ripening, is in accordance with previous results (Ziosi, Bregoli, Fregola, Costa, & Torrigiani, 2009), and with the potential role of polyamine as a senescence inhibitor. Therefore, low levels of putrescine and a high release of ethylene would favour the post-harvest onset of the senescence process in all the varieties analysed.

In previous studies, the proteomic changes associated with ripening were assessed in Dixieland peach fruit (Lara et al., 2009), and later, in five different peach varieties, in order to find out common proteomic changes in different peach varieties (Nilo, Campos-Vargas, & Orellana, 2012). The results indicated that the proteome is conserved among varieties and, also, during the transition from a firm to a soft fruit, which was associated with the narrow genetic background of the commercial peach varieties (Nilo et al., 2012). In the present work, by using a metabolomic approach, both common changes associated with all peach varieties (Fig. 5B), as well as variety-dependent particular metabolic modifications were detected, which contribute to the high diversity of organoleptic properties of peach fruits. Moreover, it indicates that metabolomics analysis is a more powerful tool than proteomics to assess differences among varieties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.06.043>.

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