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The kinetics of grape ripening revisited through berry density sorting

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ABSTRACT

Aim: In this study, ripening and heterogeneity in density-sorted berries were investigated, with the aim of more clearly understanding the kinetics of water uptake and sugar, acid and anthocyanin accumulation in the fruit of a new, disease-resistant hybrid.

Methods and results: The 3184-1-9N hybrid, grown in a semi-arid climate in the South of France, was used. Its genotype is the result of several backcrosses of a *Muscadinia* × *Vitis vinifera* F1 hybrid with several *V. vinifera* varieties. From the end of the green plateau to the over-ripening stage, single berries were sampled weekly and sorted by density, to monitor the advancement of ripening and the heterogeneity of grapevine fruit development at population level. Fruit firmness, density, fresh weight, primary metabolite content (sugars and organic acids), secondary metabolite content (anthocyanins), potassium content and pH were measured in berries from each density class. The data showed that softening and hexose accumulation occurred before fruit pigmentation. Based on malic acid and anthocyanin concentration relative to sugar concentration in density-sorted grapes, malic acid breakdown or dilution was promoted and anthocyanin accumulation reduced in late- or slow-ripening berries. In the different population structures in two experimental plots, berries with similar sugar concentration showed considerable heterogeneity in terms of volume and anthocyanin content, whereas pH, potassium content and acid content showed much more homogeneous kinetics.

Conclusions: During ripening, analysis of density-sorted berries provides useful information about sugar concentration heterogeneity and its relation with organic acid and anthocyanin content. The stage at which grapes can be considered ripe may be precisely determined through periodic determination of mean berry volume or weight, to detect the cessation of phloem unloading and the onset of berry shrivelling. However, ripening and shrivelling berries generally coexist in the harvested population. Asynchronous berry development and berry weight heterogeneity greatly complicate determination of the timing of developmental events that are regulated at the single-berry level. Therefore, analysis of individual berries would seem to be an indispensable component of investigations of berry growth and development.

Significance and impact of the study: Plotting water uptake and solute accumulation relative to sugar accumulation (as an internal clock) provides an original way to display grapevine fruit development data. Analysis of density-sorted berries has identified the relatively invariant fruit features, such as the minimum concentration of tartaric acid, the stability of potassium accumulation and pH, and conversely, the huge variations in berry size.

KEYWORDS

fruit ripening, asynchronous development, berry heterogeneity, berry density sorting.

INTRODUCTION

Viticulture plays a considerable socioeconomic role in many countries. Vine-growing areas represent more than 8 million hectares worldwide, making grapes one of the most important fruit crops (Myles *et al.*, 2011; Aleixandre *et al.*, 2014). In France, for example, the wine industry represents a yearly economic activity of about 11.4 billion euros (FranceAgrimer, no date). Wine grape is one of the most sensitive crops to climate changes, due to the influence of meteorological factors in determining wine composition (Webb *et al.*, 2008). Most widely used grapevine cultivars belong to *Vitis vinifera*, which is supposed to have originated from South Caucasus (This *et al.*, 2006; Bacilieri *et al.*, 2013).

Grape is a non-climacteric fleshy fruit. Its growth is characterized by a double-sigmoid growth pattern, with two phases of growth separated by a period of non-growth termed the *lag phase* or *green plateau* (Vicens, 2007; Thomas *et al.*, 2008).

The first phase of growth, termed the *green growth phase*, is the period during which pericarp develops from ovary mesocarp. This occurs by means of a series of cell divisions triggered by fecundation, followed by two cycles of vacuolar expansion (Coombe and Hale, 1973; Coombe, 1976; Ojeda *et al.*, 1999). The green growth phase results from both mitosis, which peaks 5 days after anthesis, and an initial period of cell enlargement (Ojeda *et al.*, 1999). During the green growth phase, the berry is green and hard, and it accumulates tartaric and malic acids, which are the main contributors to its osmotic potential (Terrier and Romieu, 2001; Keller *et al.*, 2015). Tartaric acid is predominantly accumulated at the beginning of the green growth phase, whereas malic acid is accumulated later, until the onset of ripening. The final number of cells in the pericarp is determined definitively by the end of the green growth phase (Ojeda *et al.*, 1999).

In the second phase of growth, termed *ripening*, metabolite concentrations increase or decrease, depending on net biosynthesis or metabolization and growth dilution, both mechanisms being genotype-dependent (Dai *et al.*, 2011; Keller, 2015; Bigard *et al.*, 2018). Ripening starts with berry softening (Coombe, 1984; Robin *et al.*, 1997) mediated by abscisic acid signalling

(Kuhn *et al.*, 2013; Castellarin *et al.*, 2015; Pilati *et al.*, 2017). Considerable changes in gene expression occur simultaneously with berry softening (Terrier *et al.*, 2005; Deluc *et al.*, 2007; Rienth *et al.*, 2016; Balic *et al.*, 2018).

Until recently, most grapevine phenology scaling systems had considered the change in berry skin pigmentation as the starting point of ripening (Baggiolini, 1952; Eichhorn and Lorenz, 1977; Symons *et al.*, 2006; Toffali *et al.*, 2011). The reference most widely used to qualify the onset of grape ripening is termed *mid-véraison* and is that stage at which half the berries are pigmented (Grotte *et al.*, 2001).

During ripening, the increase in sugar and water content is associated with a decrease in the concentration of organic acids (Davies and Robinson, 1996; Terrier *et al.*, 2005; Vicens, 2007). This period is characterized by a noticeable acceleration of phloem unloading and a parallel decrease in water supply via xylem (Greenspan *et al.*, 1994; Keller *et al.*, 2015). Sucrose is accumulated via the apoplastic phloem-unloading pathway (Zhang *et al.*, 2006), before being cleaved by invertases to produce glucose and fructose (Hawker, 1969; Takayanagi and Yokotsuka, 1997). The considerable osmotic potential resulting from sugar accumulation during berry ripening (Matthews *et al.*, 1987), in association with cell wall modifications, promotes the second wave of cell enlargement due to water influx (Xie *et al.*, 2009). Potassium appears to be the fourth most abundant solute in berry (0.05–0.1 mol/L) (Storey, 1987), and has a very discrete contribution to grape osmotic potential (Rogiers *et al.*, 2017). During the ripening period, berry sugar concentration is proportional to berry growth (Matthews and Nuzzo, 2007). However, the concentration of sugars and other metabolites continues to increase after the cessation of phloem unloading in the fruit, due to water loss (Coombe and McCarthy, 2000; Conde *et al.*, 2007; Bondada *et al.*, 2017). Theoretical models of sugar and water accumulation in the grapevine berry have been proposed (Dupin *et al.*, 2010; Dai *et al.*, 2011). Secondary metabolites, such as anthocyanins (in red grapes) or flavonols, are also accumulated in ripening berries (Toffali *et al.*, 2011).

Phenological shifts must be clearly distinguished from intrinsic physiological changes for the interpretation of genotype × environment effects on fruit traits (Carbonell-Bejerano *et al.*, 2013,

2016). Therefore, it is of major importance that key transition stages, such as the onset of sugar accumulation or the cessation of phloem unloading, are experimentally objectivized (Rienth *et al.*, 2016; Bigard *et al.*, 2018). Unfortunately, few methods are available for monitoring grape development in the field, and most of them are destructive or yield imprecise results. In most studies, determination of the onset of ripening is still performed by monitoring berry colour change (Symons *et al.*, 2006; Parker *et al.*, 2011; Toffali *et al.*, 2011; Arrizabalaga *et al.*, 2018), which has been shown to occur after the start of sugar accumulation (Robin *et al.*, 1997; Castellarin *et al.*, 2015).

Berry heterogeneity complicates the analysis of relations between fruit physiology and wine quality (Nelson *et al.*, 1963; Lund *et al.*, 2008; Böttcher *et al.*, 2011; Rolle *et al.*, 2013; Doumouya *et al.*, 2014; Rienth *et al.*, 2016; Shahood *et al.*, 2017). Berry heterogeneity is determined by a wide range of factors controlling inflorescence and fruit development, particularly fruit radiative and evaporative microenvironments (Kuhn *et al.*, 2013; Doumouya, 2014; Gouthu and Deluc, 2015; Reshef *et al.*, 2017). Grape development is generally described in terms of berry growth, and sugar, acid and anthocyanin concentration, using pooled data from a significant number of randomly sampled berries to obtain an accurate estimation of the composition of the average population (De Montmollin *et al.*, 2004; Geraudie *et al.*, 2009; Parker *et al.*, 2011; Arrizabalaga *et al.*, 2018). This approach enables determination of the optimal technical ripening to predict important oenological features, as influenced by the year. It is widely accepted that berry heterogeneity is so great that hundreds of berries must be sampled to avoid sampling biases. Unfortunately, the genotype × environment plasticity of berry heterogeneity and its impact on wine quality are largely unknown. In some studies, to gain precision and standardize samples, berries have been sorted according to their apparent density or internal sugar concentration (Nelson *et al.*, 1963; Singleton *et al.*, 1966; Lanier and Morris, 1978; Terrier *et al.*, 2001, 2005; Fournand *et al.*, 2006; Kontoudakis *et al.*, 2011; Rio Segade *et al.*, 2013; Carbonell-Bejerano *et al.*, 2016; Friedel *et al.*, 2016).

With the aim of characterizing berry heterogeneity during ripening, density sorting

was used to group berries by homogeneous sugar concentration class. Berries in each class were then analysed to determine mean berry weight, sugar and acid concentrations, pH, and potassium and anthocyanins concentrations.

MATERIALS AND METHODS

1. Plant material

Grapevine berries were sampled outdoors at INRA Pech-Rouge, Gruissan, France (43.14' N latitude and 3°14' W longitude; elevation, 6 m above sea level). The experimental vineyard is subject to a semi-arid Mediterranean climate (Giorgi and Lionello, 2008) and has a sandy-clay soil. Watering was by drip irrigation to keep the predawn leaf water potential (Ψ_{PD}) higher than -0.5 MPa. A new powdery and downy mildew-resistant hybrid, 3184-1-9N (Escudier *et al.*, 2017; Ojeda *et al.*, 2017), named GX, was studied in two experimental plots, GX1 and GX2. GX1 was initially established with Gamay in 1986 and top-grafted with 3184-1-9N in 2007. GX2 was initially established in 2004 with Merlot and top-grafted with 3184-1-9N in 2010. Rows are oriented north–west and east–west for GX1 and GX2, respectively. Both plots are grafted onto SO4 rootstock, cordon pruned and managed by vertical shoot positioning.

2. Sampling

Sampling started when the berries were green, 1–2 weeks before the first signs of berry softening, and stopped when it was no longer possible due to the presence of diseases associated with over-ripening (Table 1). During the sampling period, 600 berries were randomly sampled from each plot once weekly. Each berry was separated from its bunch by cutting the pedicel as near as possible to the berry, to minimize the contribution of the pedicel to the measured volume and to limit leakage of juice.

3. Density sorting

Directly after sampling, the berries were sorted by their apparent density, as described by Nelson *et al.* (1963) and Singleton *et al.* (1966), with slight modifications, for example using NaCl instead of sucrose (Rolle *et al.*, 2011; Carbonell-Bejerano *et al.*, 2013). Twelve NaCl solutions were prepared, at concentrations ranging from 80 to 190 g/L, with the same increments as used by Carbonell-Bejerano *et al.* (2013) (Table 2).

4. Weight measurement

Berries were weighed using the Ohaus® scale, to a precision of ± 0.01 g (OHAUS, Parsippany, NJ, USA). Mean berry weight was then calculated for each density class.

5. Colour measurement

The colour of each berry was measured using the Dyostem® tool (Vivelys, Villeneuve-lès-Maguelone, France).

TABLE 1. Sampling dates in 2014 for each experimental plot, GX1 and GX2

Week	Sampling date	
	GX1	GX2
1	11 July	11 July
2	18 July	18 July
3	24 July	24 July
4	30 July	30 July
5	6 August	8 August
6	13 August	14 August
7	20 August	22 August
8	27 August	29 August
9	3 September	5 September
10	10 September	12 September
11	17 September	19 September

6. Measurement of pH and primary and secondary metabolites

Each density class was analysed as a separate sample, except for density classes with (batches with less than 20 berries were pooled with the class with the most similar density). To avoid variations in skin and flesh extractability due to marked changes in texture during berry development, and thereby achieve a more quantitatively accurate monitoring of the accumulation of organic and inorganic solutes in the fruit, samples were prepared by crushing rather than pressing. Samples were crushed in a domestic fruit grinder for 15 s at room temperature, immediately after berry sorting. The pH of the crushed material was then measured.

Anthocyanin content was measured as follows. One aliquot of fresh sample (10–20 mL) was weighed and then diluted in a 1:3 ratio with hydro-alcoholic solution (2.63 mol/L ethanol plus 0.01 mol/L HCl). After 1 h of orbital stirring, 10 mL of the solution was centrifuged for 5 min at 12000 g (at 20°C). The supernatant was then diluted with hydro-alcoholic solution in a ratio of 1:20–50 (depending on colour), before its absorbance at 520 nm was measured using a 1-cm optical path Evolution® 300 UV-VIS spectrometer (Thermo Scientific, Waltham, MA, USA). Anthocyanin concentration (mg/L) was calculated as total dilution \times OD 520 nm \times 22.76 (Ribéreau-Gayon *et al.*, 1998).

TABLE 2. Bath NaCl concentration and corresponding apparent berry density and theoretical sugar concentrations.

Bath no.	NaCl (g/L)	Density	Sugar (g/L)	Sugar (mmol/L)
1	190	1.12	279	1549
2	180	1.11	264	1465
3	170	1.11	248	1377
4	160	1.10	233	1293
5	150	1.09	218	1210
6	140	1.09	202	1121
7	130	1.08	187	1038
8	120	1.08	172	955
9	110	1.07	156	866
10	100	1.06	141	783
11	90	1.06	126	699
12	70	1.05	110	611
13	< 70	< 1.05	< 110	< 610

Primary metabolites (glucose, fructose, malic acid and tartaric acid) were measured in a separate aliquot of fresh juice (2 mL), which had been centrifuged as described above. The supernatant was diluted in a 1:10 ratio with 0.2 N HCl and filtered through cellulose acetate 0.2- μ m membranes, before being injected into a high-performance liquid chromatography column (Bio-Rad[®] Aminex HPX-87H; Bio-Rad, Hercules, CA, USA), according to the method described by Bories *et al.* (2011) and under the same conditions described by Bigard *et al.* (2018). For measurement of potassium content, the supernatant was diluted in a 1:1000 ratio with water and the resulting solution injected into a Solaar S Series AA[®] spectrometer (Thermo Scientific).

7. Data analysis

R-software version 3.4.4 was used to visualize the data (package: ggplot2, version 3.0.0) (R Core Team, 2017). Excel[®] was also used to record the data.

RESULTS AND DISCUSSION

1. Determination of onset of ripening

Ripening begins with the accumulation of sugars, when the berry has reached its maximum content and concentration of organic acids. At this stage, the cell divisions of the green growth phase are complete (Ojeda *et al.*, 1999; Fernandez *et al.*, 2006) and final berry size can already be estimated (Coombe, 1984; Houel *et al.*, 2013; Bigard *et al.*, 2018).

Onset of ripening did not occur simultaneously throughout the berry population but instead occurred over several weeks, as shown in Figure 1. Skin coloration was delayed from the start of sugar accumulation, which is indicated by berry softening (Robin *et al.*, 1997; Castellarin *et al.*, 2015). It took 3 weeks for all the berries of the population to soften, but the change in colour took another week. Consequently, a bunch considered to be at mid-véraison (the stage at which half the berries are pigmented) actually contained more ripening berries than unripe berries. Mixing of ripening berries (in which organic acids are being metabolized and diluted) with hard green berries (in which organic acids are being accumulated) inevitably leads to underestimation of peak acidity at onset of ripening. Therefore, and because the difference in firmness between hard and soft berries is great enough to be detected even by hand (Terrier *et al.*, 2005; Bigard *et al.*, 2018), we separated the hard and soft berries in our samples. Determination of berry firmness by hand is as quick as monitoring changes in berry colour and provides much more accurate information.

2. Berry growth, accumulation of acids and anthocyanins relative to sugar accumulation: heterogeneity and timing of berry development

In most genetic and physiological studies, berries at different individual developmental stages are mixed, which prevents determination of the timing of flesh expansion (in response to

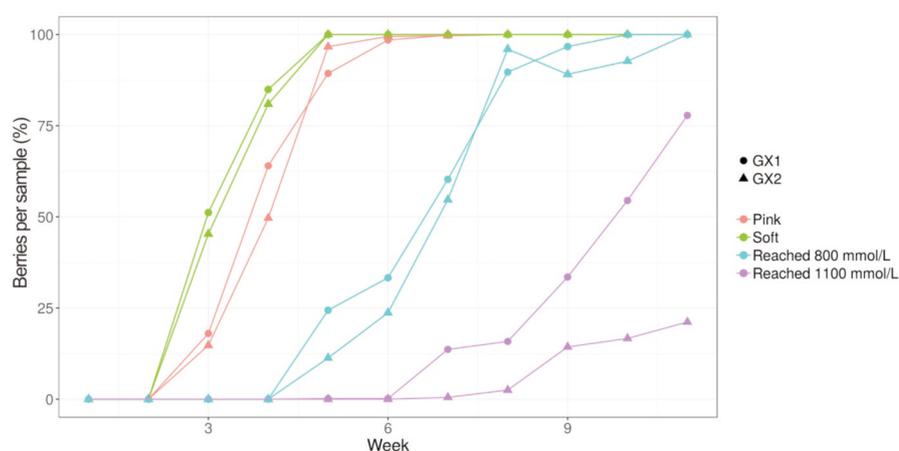


FIGURE 1. Changes in berry characters during ripening, observed at population level, in fruits from two experimental plots, GX1 and GX2.

Green curve, percentage of softening berries; orange curve, percentage of pigmented berries; blue curve, percentage of berries with sugar concentration > 800 mmol/L; violet curve, percentage of berries with sugar concentration > 1100 mmol/L

import of water), sugar accumulation and malic acid breakdown, and their relations with anthocyanin synthesis. In the present study, randomized samples of 600 berries were assumed to be representative of the berry diversity of an experimental plot (De Montmollin *et al.*, 2004; Geraudie *et al.*, 2009; Parker *et al.*, 2011; Arrizabalaga *et al.*, 2018).

At each sampling date, berries were sorted according to their apparent density, which depends mostly on sugar concentration during ripening, as reported by Nelson *et al.* (1963), Singleton *et al.* (1966), Böttcher *et al.* (2011), Friedel *et al.* (2016) and others. Berry weight (Figure 2), tartaric and malic acid concentration (Figures 3 and 4, respectively), potassium concentration (Figure 5), pH (Figure 6) and anthocyanin concentration (Figure 7) were plotted against the concentration of sugars (glucose plus fructose) as a proxy for the

advancement of ripening, as described by Rienth *et al.* (2016). The results shown in Figures 2–7 confirm that berries do not ripen simultaneously, which is consistent with the findings of Coombe (1984) and the results of several transcriptomic analyses (Terrier *et al.*, 2005; Lund *et al.*, 2008; Rienth *et al.*, 2016).

Berry growth is a critical factor determining yield. It depends largely on early events affecting fecundation, seed number and cell division, and possibly also cell expansion in the green growth phase. Figure 2 shows that resumption of growth and sugar accumulation occurred almost simultaneously at the onset of ripening, with a rapid initial period of water accumulation until the concentration of sugars (glucose plus fructose) reached 800 and 625 mmol/L in GX1 and GX2, respectively. Above these concentrations, growth clearly decreased. However, these general trends mask noticeable

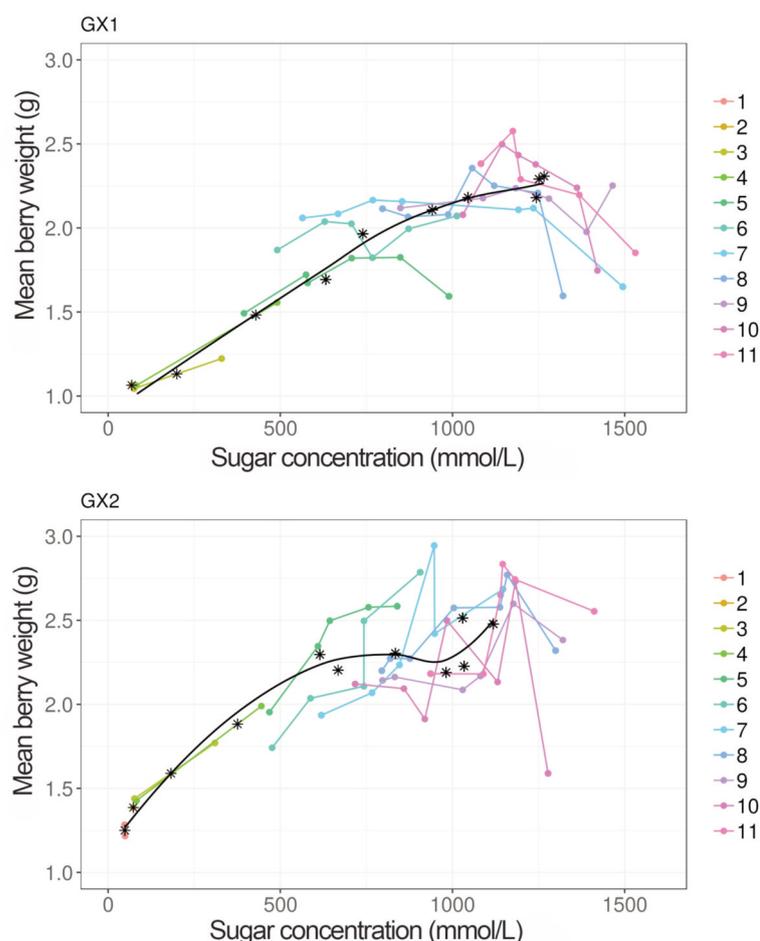


FIGURE 2. Relation between berry weight and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 and GX2. Each dot corresponds with a density class at a sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

discrepancies in population structures between the two experimental plots: in GX1, sugar concentration remained low in large berries compared with average-sized berries (indicating greater dilution in the former), whereas sugar concentration was higher in large berries from the GX2 plot compared to average-sized berries. Berries from the GX1 plot continued to expand at sugar concentration > 800 mmol/L, in contrast with those from the GX2 plot.

Because water availability was similar in both plots, these discrepancies could be due to differences in the distribution of berry sizes in the population (e.g. early development of large berries in the GX2 plot due to flowering or fecundation events) or variation in evapotranspiration. The considerable heterogeneity of sugar concentration in the early August samples (see Table 1), which ranged from 500 to 1200 mmol/L, may indicate marked

asynchrony at this stage. Thereafter, heterogeneity in sugar concentration tended to decrease, mostly in berries from the GX1 plots (from 1000 to 1400 mmol/L), consistent with the results of Kontoudakis *et al.* (2011), Gouthu *et al.* (2014) and Belviso *et al.* (2017). However, heterogeneity in sugar concentration remained very large, indicating that the development of late berries does not catch up with that of early ones. Clearly, such differences in the development of subpopulations of berries are not apparent when, as is usual, unsorted samples are analysed, although it is not possible to know whether the development of these subpopulations depends on their interaction. Non-destructive monitoring of the relation between sugar concentration and the volume of single fruits seems essential to achieve a better understanding of the origin of differences in

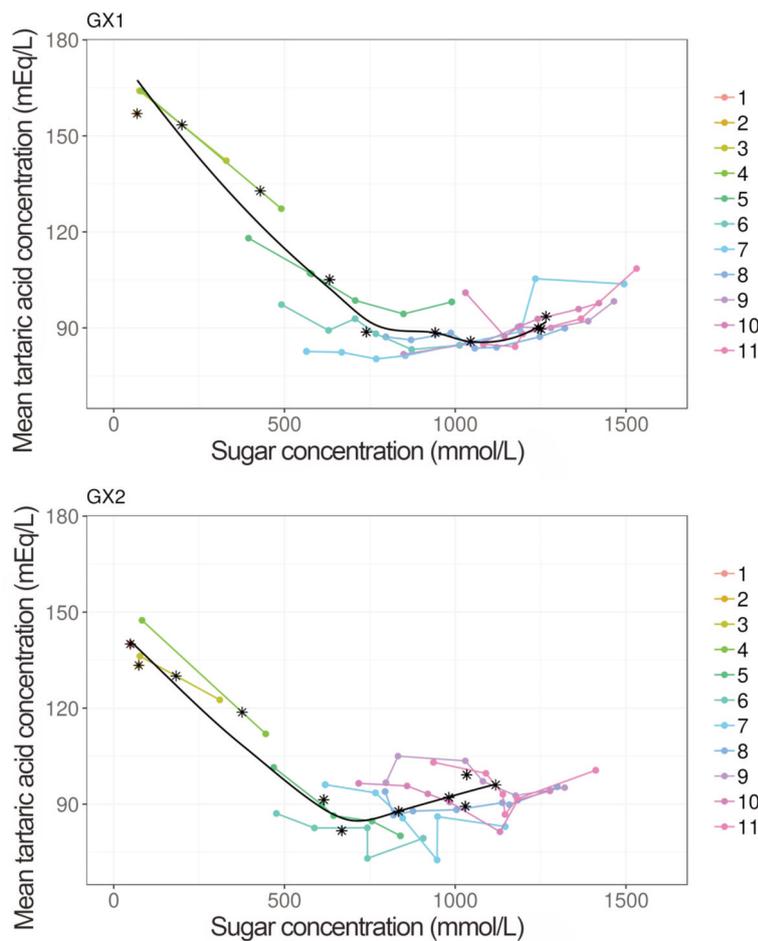


FIGURE 3. Relation between tartaric acid and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 and GX2.

Each dot corresponds with a density class of a sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

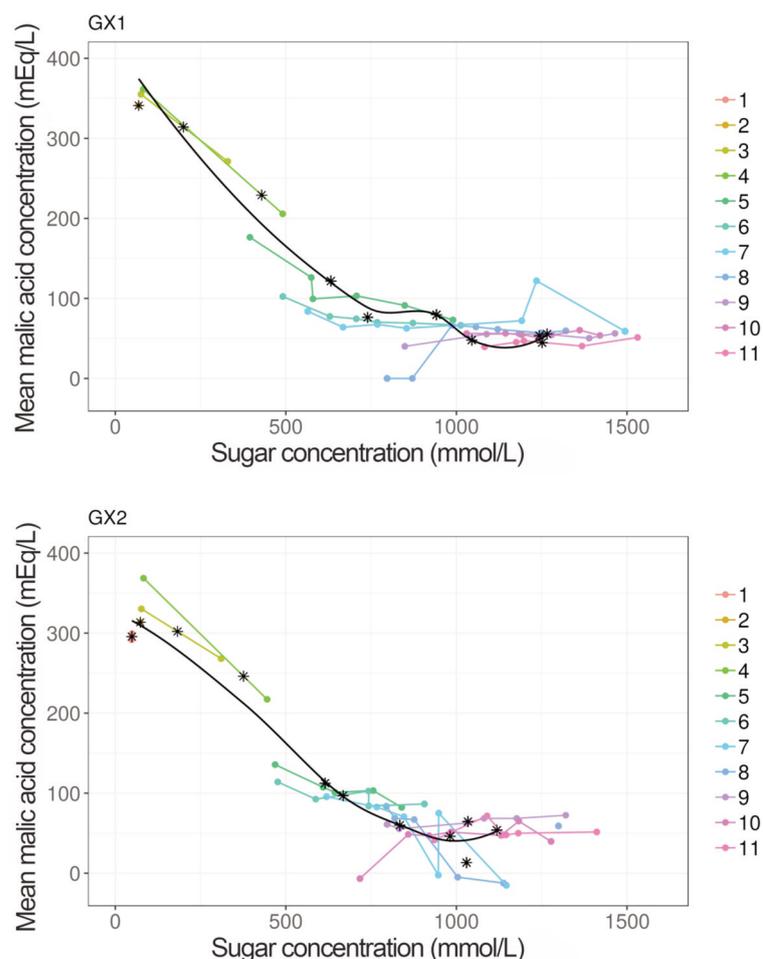


FIGURE 4. Relation between malic acid and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 et GX3.

Each dot corresponds with a density class ρ a sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

berry development, which are currently difficult to interpret.

Tartaric and malic acids are determinants of berry acidity. It is largely accepted that tartaric acid content remains stable in terms of quantity per fruit during ripening (Lang and Thorpe, 1989; Terrier and Romieu, 2001; Rösti *et al.*, 2018). Tartaric acid concentration ranged between 150 and 160 mEq/L at the beginning of ripening before decreasing to 80 mEq/L at a sugar concentration of 800 mmol/L in berries from both plots (see Figure 3). As expected, tartaric acid concentration then increased, either immediately (in berries from GX2) or at sugar concentration > 1200 mmol/L (in berries from GX1), a result caused by shrivelling of the berry due to transpiration. One should note that all berries from GX1, but not those from GX2, apparently followed a unique pattern of changes

in tartaric acid concentration, with small berries starting to shrivel at a higher concentration of tartaric acid (due to lower expansion) and also at a lower sugar concentration (500–600 mmol/L), when compared with larger berries (compare Figures 2 and 3).

Malic acid concentration, initially in the range 300–380 mEq/L, decreased to < 50 mEq/L at a sugar concentration of 800 mmol/L, after which it remained stable (see Figure 4). In grapes from both plots, both tartaric and malic acid concentration showed lower heterogeneity than berry weight, which is consistent with the results of previous studies (Rienth *et al.*, 2016; Shahood, 2017). Acidity tends to decrease during the season in all density classes, as originally suggested by Singleton *et al.* (1966) and recently confirmed by Friedel *et al.* (2016). This suggests that during sugar accumulation, early-ripening

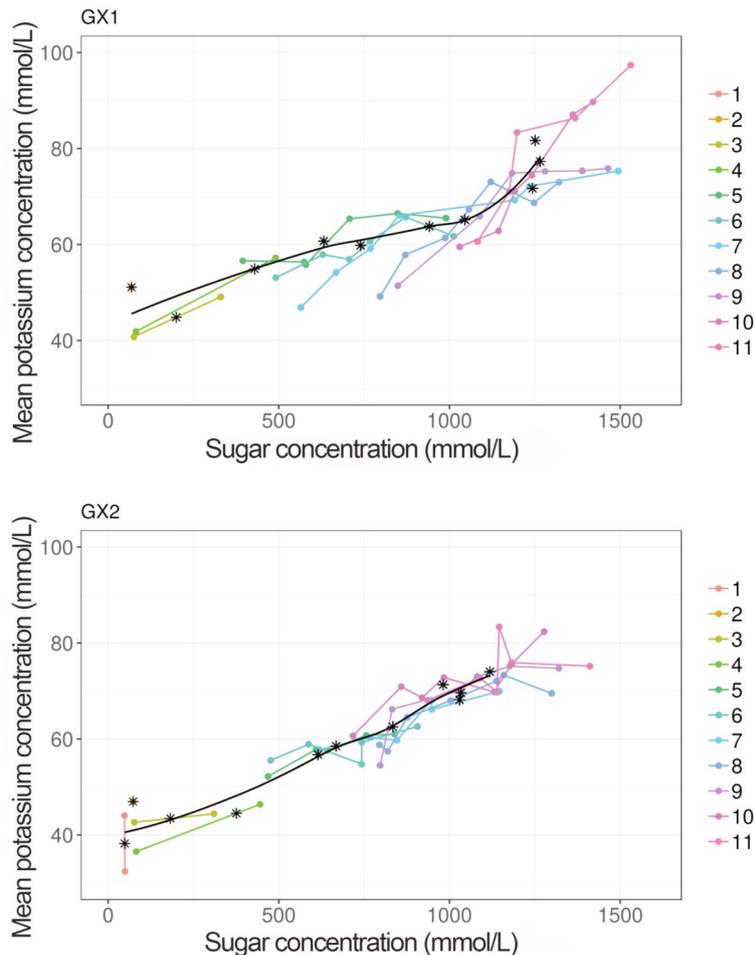


FIGURE 5. Relation between potassium and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 and GX2.

Each dot corresponds with a density class 'a' sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

berries consume less malic acid than later-ripening berries, or that the latter accumulate more water. Various mechanisms could explain this shift in primary metabolites: for example, sugar availability may be higher and water availability may be lower for early-ripening berries, fruits with more comfortable glucidic status may have delayed or reduced malic acid breakdown, and smaller berries may start ripening first (Rienth *et al.*, 2016; Shahood, 2017). Alternatively, changes in malic acid content may be modulated by progressive changes in environmental factors through the season (Davies and Robinson, 1996; Vicens, 2007; Rienth *et al.*, 2016).

Potassium, the fourth and third most important contributor to berry osmotic potential and acidity, respectively, should accumulate just after the onset of ripening (Storey, 1987; Rogiers *et*

al., 2017). During ripening, potassium concentration increased from 40 mmol/L to > 75 mmol/L in berries from GX1 and GX2, respectively, with a slight acceleration after sugar concentration reached 1000 and 800 mmol/L in berries from GX1 and GX2, respectively (see Figure 5). Compared with berries from GX2, potassium concentration was more hetero-geneous in berries from GX1 at the start of ripening, being lower in larger berries, in which it tended to decrease at the start of sugar accumulation.

This finding could be explained by dilution if the small berries started ripening first, and assuming that all berries accumulated sugars at the same rate. When berry growth is accounted for, it is estimated that roughly 20 hexose molecules would accumulate in the ripening berry for each potassium ion (K^+). Phloem would therefore

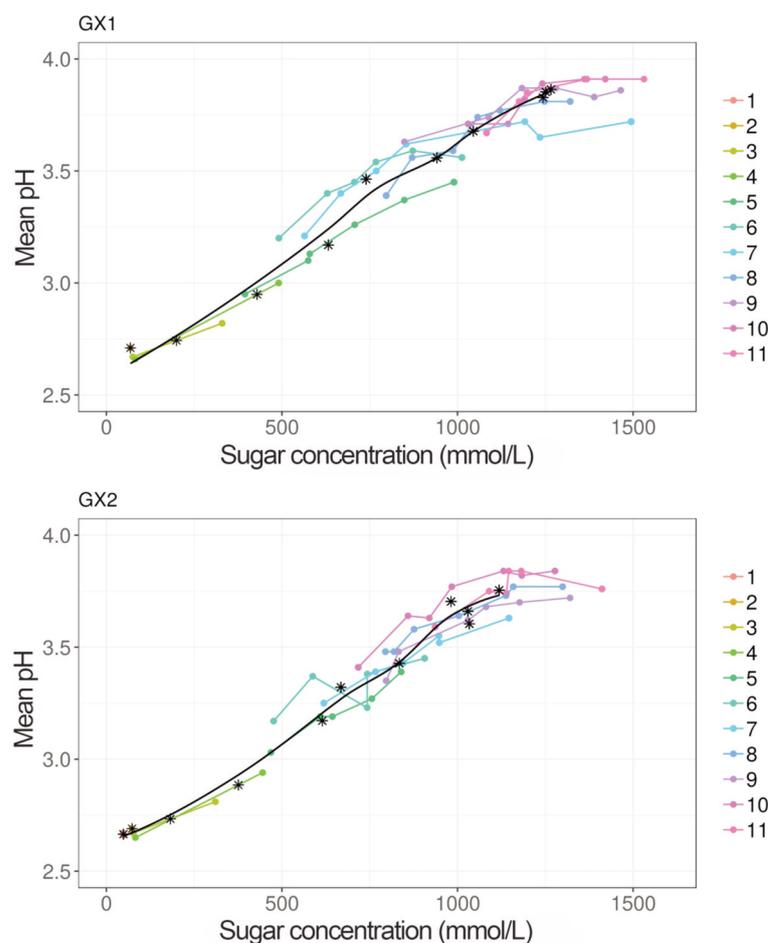


FIGURE 6. Relation between pH and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 and GX2.

Each dot corresponds with a density class 'a' sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

release 10 sucrose molecules into berry apoplast for each K^+ , which casts doubt on the recent claim that the K^+ battery could energize sugar retrieval by 'respiration-restricted' grape mesocarp cells (Nieves-Cordonés *et al.*, 2019).

During berry ripening, changes in pH followed a similar pattern in berries from both plots, increasing from 2.7 to 3.7 at a sugar concentration of 1400 mmol/L (see Figure 6). During the season, pH tends to increase in berries from the same density class, due to changes in organic acid and potassium content. Additionally, because the concentration of acids remained relatively stable at sugar concentration > 800 mmol/L, most changes in pH would have been due mainly to potassium accumulation during late ripening.

Accumulation of secondary metabolites such as anthocyanins is critical for optimizing wine quality. Anthocyanin synthesis started once sugar concentration had increased to 400 mmol/L, reaching > 4 mmol/L at a sugar concentration of 1200 mmol/L (see Figure 7). Late-ripening berries tended to accumulate less anthocyanin than early ones with similar sugar concentration. This may be due to exposure to higher temperatures, which can impair anthocyanin accumulation (Mori *et al.*, 2007). In grapes from both plots, anthocyanin concentration was maximal at the end of the sugar-accumulation phase (sugar concentration, > 1200 mmol/L). However, it did not increase further during berry shrivelling (see Figures 2 and 3), as had been expected, indicating that by that stage these secondary metabolites had degraded or been metabolized.

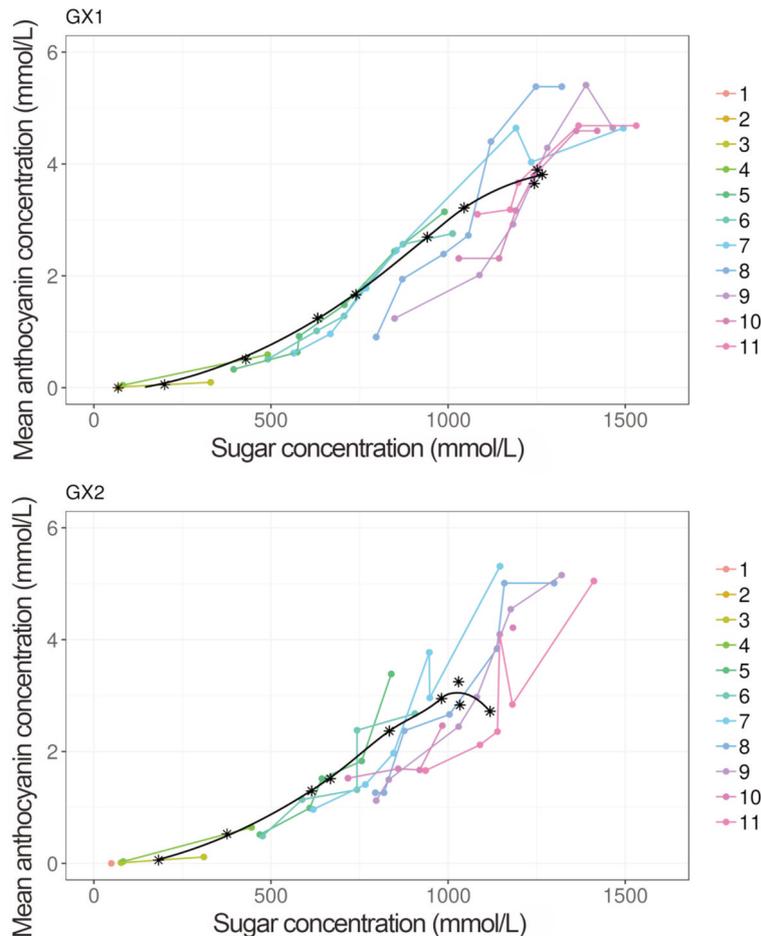


FIGURE 7. Relation between anthocyanin and sugar concentration (glucose plus fructose) in fruits from two experimental plots, GX1 and GX2.

Each dot corresponds with a density class 'a' sampling date. Each colour corresponds with a sampling date (1–11; see corresponding dates in Table 1). Black stars, the values per date that would result from unsorted samples.

Density sorting is a widely accepted method for preparing batches of berries with homogeneous sugar concentration (Nelson *et al.*, 1963; Singleton *et al.*, 1966; Lanier and Morris, 1978; Terrier *et al.*, 2001, 2005; Fournand *et al.*, 2006; Kontoudakis *et al.*, 2011; Rio Segade *et al.*, 2013; Carbonell-Bejerano *et al.*, 2016; Friedel *et al.*, 2016). However, the impact of berry heterogeneity is not considered. In addition to differences in water content and growth, the content and concentration of all ripening-related metabolites relative to sugar concentration may be subject to plasticity, as discussed by Nelson *et al.* (1963), Singleton *et al.* (1996) and Friedel *et al.* (2016). During ripening, especially at the beginning, small berries appeared to ripen first (as evidenced by their high acid, potassium and anthocyanin content relative to sugar concentration), and this phenomenon can complicate interpretation of the results of

analyses of unselected berries. Potential changes in dilution and water balance mean that density and sugar concentration cannot be assumed to correspond with developmental stage.

3. Determination of the end of the growing phase

Maximum berry volume may be considered a marker for the cessation of phloem unloading, and therefore an objective standard with which to define physiological maturity. Thereafter, reduction in berry volume as a result of shrivelling is responsible for continuation of the increase in sugar concentration previously due to mass flow through phloem.

Berry growth, inferred from increased berry weight or increased tartaric acid concentration, reached a plateau at a sugar concentration of 750

and 800 mmol/L in berries from GX1 and GX2, respectively (see Figures 2 and 3). Tartaric acid content per fruit remains stable during ripening, therefore any decrease in its concentration can be regarded as corresponding to growth (Lang and Thorpe, 1989; Terrier and Romieu, 2001; Rösti *et al.*, 2018). Figure 1 shows that some berries had already reached physiological maturity by the beginning of August (see Table 1), suggesting that individual berries can ripen faster than is generally accepted (Coombe, 1992; Shahood, 2017; Shahood *et al.*, 2019). Moreover, monitoring of softening indicated a 20-day delay before the onset of ripening between earliest and latest berries, a phenomenon previously reported for texture and coloration (Coombe and McCarthy, 2000; Vondras *et al.*, 2016). The results of the present study show that 31 days is enough for any berry of the GX genotype (a low sugar accumulator, Ojeda *et al.*, 2017) to reach physiological maturity (i.e. maximum berry volume and a sugar concentration of 800 mmol/L), although this genotype is generally harvested 45–55 days after veraison. We have also observed features similar to those of other varieties (i.e. Grenache, Morrastel, Merlot and the 3197-81B hybrid) that have been studied using the same protocol (Bigard, 2018, data not shown). The observed delay in onset of ripening between early and late berries suggests that technological ripening corresponds with the end of sugar accumulation in the latest berries of a population.

CONCLUSION

To better understand the effects of environmental factors on grape quality, and to properly phenotype genetic resources, critical transitions in berry development require objective standards other than mean sugar concentration calculated from data pooled from a berry population. However, there is an inherent paradox in looking for precise stages of development in a non-synchronous population, because it is necessary to account for the dynamic structure of the population (Shahood *et al.*, 2019). Berry softening proved a much more pertinent indicator of the onset of ripening than colour change. Changes in berry firmness can be determined either manually or by using electronic devices, in a destructive or non-destructive way. During ripening, heterogeneity of berry development can be characterized by a combination of density sorting and quantification of metabolites. It appears that

organic acid and anthocyanin concentration relative to sugar concentration are subject to significant plasticity. Furthermore, temporal berry population structures differed in the two investigated plots, possibly due to early fecundation events. Therefore, accurate determination of berry population structure requires statistical evaluation of these metabolites in hundreds of berries, as described by Rienth *et al.* (2016) and Shahood *et al.* (2019). These aspects will be examined in a forthcoming paper.

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