Research Article

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Effect of cultivar, maturation stage, and year on sugar and phenolic composition of elderberries

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Abstract

BACKGROUND: The chemical composition, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) activity of the three main Portuguese elderberry cultivars were determined for the first time through five stages of maturation, in different harvesting years, to gain a deeper understanding of the effect of climatic conditions and enzymatic activity involved in the synthesis and degradation of phenolic compounds on the final quality of elderberries.

RESULTS: Simple sugar and anthocyanin content increased with maturation but total acidity and flavonoids content decreased, and cinnamic acids did not show a clear trend. Climatic conditions seem to have a decisive influence on the elderberry maturation, namely the total number of hot (>30 °C) days. The PAL, PPO, and POD activity can explain the differences observed in elderberry phenolic content.

CONCLUSION: These results highlighted the influence of climatic conditions in each harvesting season on elderberry development and quality.

Supporting information may be found in the online version of this article.

Keywords: *Sambucus nigra* L.; maturation stages; sugar content; polyphenol content; phenylalanine ammonia-lyase; polyphenol oxidase

INTRODUCTION

Sambucus nigra L., also known as European elderberry, black elderberry, or elderberry, grows in sunlight-exposed locations and tolerates the poor soil conditions widespread throughout the temperate and subtropical regions in the northern hemisphere, mainly in Europe but also in Asia and North Africa.[1,2]

The development of elderberries begins in July, reaching optimum maturity at the end of August,[3-5] or at the beginning of September,[6] when 75% of the berries show a dark-violet color and when berries present a total soluble solids (TSS) content of at least 12 °Brix.[4] In Portugal the elderberry plantations are mainly located in the Varosa Valley (in the north of Portugal) due to the excellent edaphoclimatic conditions of this region. They have been increasing in recent decades,[3,5] yielding elderberries with quality recognized by the food industry due to their anthocyanins and sugar content. Some dietary supplements are also available on the market.[2] Due to the importance of *S. nigra* in these industries, it is essential to increase knowledge about the development of elderberries from an immature stage to full/commercial optimum maturity. Salvador *et al.*[3] reported the influence of ripening stage, cultivar, and season in the lipophilic profile of elderberries.

Nevertheless, as far as we know, no studies are available concerning the polyphenols and sugar profile in elderberries during ripening. Knowledge about the evolution of these compounds is valuable because the two most important quality parameters in elderberries are their sugar content and color.

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The accumulation of phenolic compounds in fruits is strongly dependent on the ripening stage, environmental factors (e.g. irradiation, soil condition, viral infection, fungal attack and infestation by insects), and is also dependent on the biosynthesis and catabolism balance.[7] Phenylpropanoid compounds are a large class of secondary metabolites synthesized by different enzymatic reactions from the primary metabolites phenylalanine or tyrosine synthesized from the shikimate pathway.[8] In the first step of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL) catalyzes the non-oxidative deamination of phenylalanine to cinnamic acid. Then, other intermediates are formed, and the carbon flow can follow specific pathways to produce phenolic acids, flavonoids, coumarins, stilbenes, and monolignols.

On the other hand, fruits also contain enzymes that can transform the phenolic compounds into degradation products – for example, polyphenol oxidase (PPO) and peroxidase (POD). Polyphenol oxidase plays a central role in the degradation of phenolic compounds in the presence of molecular oxygen. At the same time, POD promotes the oxidation of phenolic compounds in the presence of hydrogen peroxide.[9]

Thus, the primary purpose of this study was to evaluate the chemical profile of elderberries during maturation of the three most important Portuguese *S. nigra* L. cultivars, namely 'Sabugueiro', 'Sabugueira', and 'Bastardeira', in two consecutive harvesting years. To quantify chemical parameters related to the quality of the elderberries during the development of the fruit, elderberries were harvested in Varosa Valley, grown under the same edaphoclimatic conditions, and five maturation stages were analyzed: green-immature, two immature stages, the mature stage, and the last stage of optimum commercial maturity. The PAL, PPO, and POD activity in elderberries during ripening was also evaluated.

As far as we know, this is the first work that studies the evolution of the most important chemical components of elderberries, namely simple sugars and phenolic compounds, during maturation, and the possible relation between the phenolic composition of elderberries, PAL, PPO and POD activity, and the climatic conditions of the harvesting years.

MATERIALS AND METHODS

Experimental field and elderberry samples

Elderberry plants used in this study were cultivated in the same field, located in Varosa Valley, Moimenta da Beira, Viseu, Portugal (40° 59′ 6″ N, 7° 37′ 4″ W). The solar exposure of all the plants in this field was the same. During elderberry production, no irrigation, soil fertility management, or pesticide applications were used. Depending on the harvesting years, elderberries were collected during maturation in July, August, or September in five different maturation stages. Starting the sampling (first maturation stage) 1 week after the end of the flowering stage (fall of the flowers), and then for the following maturation stages, elderberry harvests were made with 15 day intervals, as shown in Fig. S1 in the supporting information. For each maturation stage and for each biological replicate of the three cultivars ('Sabugueiro': 5, 'Sabugueira': 5 and 'Bastardeira': 8), elderberries were collected from four branches of the same shrub in 2013 and 2014. The samples were freeze dried, milled, and stored until further analysis.

Elderberry berries fresh weight, dry matter, total soluble solid content and titratable acidity

The weight of elderberries was determined by measuring the fresh weight of 20 randomly selected berries. The dry matter (DM) of elderberries was determined after water removal by freeze drying. Total soluble solids content was measured using a refractometer (pocket refractometer, ATAGO, Tokyo, Japan) in fresh material. The TSS was expressed in Brix degree (°Brix). With some modification, titratable acidity (TA) was performed as described by Benjakul and Chuenarrom.[10] Briefly, 5 g of fresh elderberries were blended with 50 mL of water in a blender. Then, after filtration, 25 mL of this extract was titrated with 1.0 N NaOH until pH = 8.1, and titratable acidity was expressed as citric acid mg/g. Salts and reagents were acquired from Sigma-Aldrich/Merk (Algés, Portugal).

Free sugar profile by high-performance anion-exchange chromatography with pulsed amperometric detection

The free sugar content in freeze-dried elderberries (0.1 g) was extracted in ethanol (50% v/v, 9 mL) in an orbital shaker for 30 min. Then it was incubated in a water bath for 20 min at 60 °C, centrifuged, and 1 mL of internal standard (2-deoxy-D-glucose) was added. Samples were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; ICS-3000 system; Dionex, Sunnyvale, CA, USA) as described in Silva *et al.*[11] Salts and reagents were acquired from Sigma-Aldrich/Merk (Algés, Portugal).

Polyphenols profile of elderberries by Reversed-Phase High Performance Liquid Chromatography with Photodiode Array Detector (RP-HPLC-DAD)

Elderberries were extracted exhaustively (eight times) from 250 mg of freeze-dried elderberries using 5 mL acidified methanol (1% HCl) in an orbital shaker (Orbital Shaker GFL 3005 series, Hanover, Germany), for 20 min at 200 rpm, and then centrifuged for 5 min at 8 000 *g* (Sigma Centrifuges 3–30 K, St. Louis, MO, USA), collecting the supernatant and adjusting for a final volume of 50 mL. The extracts were concentrated by rotary evaporation (five times) (Stuart RE300, Staffordshire, UK) and resuspended in aqueous formic acid (5%). The polyphenol profile of the elderberries along the maturation stages was determined by RP-HPLC-DAD using a ThermoFisher Scientific Vanquish Core HPLC system (Waltham, MA, USA) and the experimental conditions described by Silva *et al.*[5] Salts and reagents were acquired from Sigma-Aldrich/Merk (Algés, Portugal).

Phenylalanine ammonia lyase, PPO, and POD activity

The extract for determination of the enzymatic activity was prepared using fresh material (~ 300 mg) extracted in 15 mL of potassium phosphate buffer (0.01 M, pH 6.0), 100 mg of Triton X-100 and 500 mg polyvinylpolypyrrolidone (PVPP). The supernatant obtained by centrifugation (30 min, 10 000 *g*, 4 °C) was used to measure PAL, PPO, and POD activity as described by Sun *et al.*[12] Salts and reagents were acquired from Sigma-Aldrich/Merk (Algés, Portugal).

Climatic data, bioclimatic indexes and soil characteristics

Climatic data for the weather station located in Trancoso for the harvesting years 2013 and 2014 was supplied by the Direção Regional de Agricultura do Centro (DRAP-Centro), and it included the maximum (T_{max}), minimum (T_{min}), and mean (T_{mean}) daily temperatures along with the daily precipitation (P). To simplify the

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global description of weather conditions during the growing season in the two harvesting years, the Winkler Index (WI),[13] Huglin Heliothermal Index (HI),[14] Cool Night Index (CI),[15] and Dryness Index[16] were calculated, which quantify the impact of weather in single aggregate values.

Statistical analysis

The data were expressed as means ± standard deviations, and significant differences ($P < 0.05$) between harvesting years and cultivars, and their interaction were identified using a three-way ANOVA and the unequal N Tukey post-hoc test. All statistical treatments were performed using Statistica 7 (StatSoft, Inc., Tulsa, OK, USA) statistical software.

RESULTS AND DISCUSSION

Water content, dry berry weight, soluble solids content, and titratable acidity evolution during maturation

Figure 1(a) and Table S1 in the supporting information show the berry weight variation on a dry basis for the three cultivars, 'Sabugueiro', 'Sabugueira', and 'Bastardeira', for the five maturation stages, for the two harvesting years studied, 2013 and 2014. During the ripening period, a significant increase in the dry berry weight from fruit settling to harvesting was observed, ranging between 3.45–29.98 g (DW) and 6.71–43.98 g (DW), for the first and second harvesting years, respectively. Only for the two final maturation stages was a significant difference in dry berry weight not observed (post-hoc Tukey, $P < 0.9648$). There were no significant differences in the dry berry weight between the cultivars studied for the two harvesting years, although a significant interaction was observed between the dry berry weight for the different maturation stages and harvesting years. There were no differences between the 2 years studied for the two first maturation stages, but for the last three maturation stages, the dry berry weight for the 2013 harvesting year was significantly different from the 2014 harvesting year (Fig. 1(a)).

The water content of elderberries was significantly different for the 2014 harvesting year (72.73–79.76 g100 g^{-1}) when compared with the 2013 harvesting year (71.93–79.94 g100 g^{-1}, respectively) (Fig. 1(b) and Table S1 in the supporting information). The higher water content of elderberries in the first four stages of maturation in 2014, compared to 2013, can be explained by the higher amount of rainfall in July in 2014 (26.4 mm) compared with 2013 (5.4 mm). The water content of the Bastardeira cultivar was also significantly lower than that of the Sabugueiro and Sabugueira cultivars (ANOVA, $P < 0.00001$) for the 2013 harvesting year, with no differences observed between cultivars for the 2014 harvesting year. For the first, second and third maturation stages, the water content of elderberries was significantly higher than that observed for the fourth and fifth maturation stages (ANOVA, $P < 0.00135$).

The TSS content of the elderberries also increased significantly with the maturation stage. However, there was a significant interaction with the harvesting year, so that the actual development of the TSS content was dependent on the harvesting year. It ranged between 6.37–16.80 °Brix and 6.04 to 16.60 °Brix, for the first and second harvesting years, respectively (Fig. 1(c) and Table S1 in the supporting information). The TSS content of elderberries for the different maturation stages was significantly higher for the second, third, and fourth maturation stages for the 2013 harvesting year compared to the 2014 harvesting year. However, there were no significant differences between the 2013 and 2014 harvesting years for the fifth maturation stage (Fig. 1(c) and Table S1 in the supporting information). The higher TSS observed in the 2013 harvesting year can also be explained by the lower water content of elderberries in this harvesting year, probably caused by the lower rainfall observed in July of this harvesting year compared to the 2014 harvesting year, as previously discussed. Bastardeira cultivar showed a significantly higher TSS when compared with Sabugueiro and Sabugueira (Fig. 1(c)).

Figure 1(d) shows a significant difference between the cultivars in titratable acidity. A significant interaction was observed between the cultivar and harvesting year and maturity stage (ANOVA, $P < 0.0235$), with the Sabugueira cultivar presenting the highest titratable acidity when compared to the Sabugueiro and Bastardeira cultivars for the 2013 harvesting year, no differences being observed between cultivars for the 2014 harvesting year. With increasing maturation stage, a significant decrease in the titratable acidity of elderberries was observed, with the first and second maturation stage presenting significantly higher titratable acidity when compared with the third, fourth and fifth maturation stages and with the fourth and fifth maturation stages presenting the lowest titratable acidity, and not being significantly different from each other (Fig. 1(d) and supporting information Table S1).

The influence of the harvesting year on the amount of each physicochemical parameter evaluated is evident, although the profile along the maturation stages was similar. The results showed an increase in the weight of berries, dry matter, and TSS content. In contrast, the titratable acidity decreased along with the maturation. As far as we know, this study is the first study that evaluated different physicochemical parameters of elderberries in different maturation stages. However, a study by Salvador *et al.*[3] evaluated only the lipophilic profile with maturation in Portuguese elderberries.

Nevertheless, some studies evaluated the same physiochemical parameters along maturation stages in other berries for one harvesting year. For instance, dry matter of blueberries increased along the maturation stages for Birgitta (10.1–12.9%), Bluegold (12.3–15.2%) and Nelson (12.2–14.3%) cultivars.[17] Four cultivars of black currant harvested at the beginning of ripening, 50% ripened berries, technical maturity, and overripe berries also showed an increase in the dry matter, which depending on the cultivar, varied between 15.82% and 18.71% for technical maturity.[18] The total soluble content increased along with the maturation for all cultivars, ranging from 10.70 to 14.55 °Brix at technical maturity Moreover, the titratable acidity of black currant decreased along the maturation stages 3.37 to 2.23%.[18] Blackberries from Marion and Evergreen cultivars also increased the total soluble content (9.46–13.5 °Brix and 11.0–15.7 °Brix, Marion and Evergreen, respectively). At the same time, titratable acidity decreased from 2.32 to 1.28 g 100 g^{-1} in Marion and 2.38 to 1.12 g 100 g^{-1} in the Evergreen cultivar.[19] Mulberries showed the same behavior as the other fruits mentioned, along seven maturation stages, increasing the TSS from 5.6 to 13.7 °Brix.[20] The titratable acidity of mulberries also decreased along with the maturation (2.4 to 0.3 g 100 mL^{-1}).

Evolution of simple sugars during maturation. Effect of cultivar and harvesting year

The content of total simple sugars, glucose, fructose, and sucrose (Fig. 2(a) and Table S2 in the supporting information), increased significantly during maturation, with the total simple sugars in

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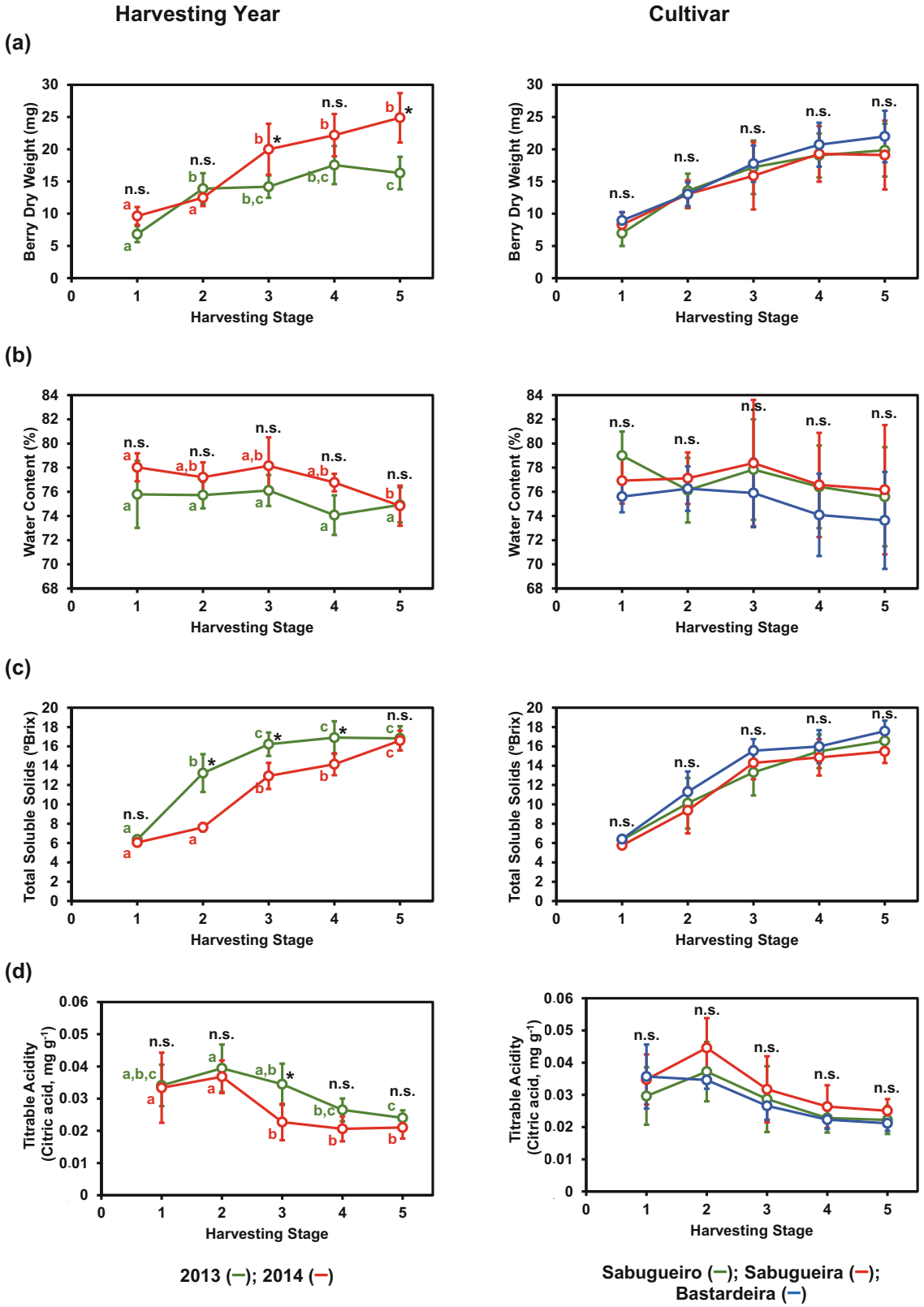

Figure 1. Physiochemical parameters of elderberries during different maturation stages (1 – 5). Variations observed for the two harvesting years (left panels) and three elderberry cultivars (right panels). (a) Berry dry weight (mg); (b) water content (%); (c) total soluble solids content (°Brix); (d) titratable acidity (TA) (citric acid mg g^{-1}). Data are presented as means ± standard deviations (n = 5 for Sabugueiro and Sabugueira cultivars and n = 8 for Bastardeira cultivar). For the left panels, for the different maturation stages within each year, means with different letters are significantly different ($P < 0.05$). For the different harvesting years and for the same maturation stage, means with n.s. are not significantly different ($P > 0.05$) and those with * are significantly different ($P < 0.05$). For the right panels, within the same maturity stage, means with n.s. are not significantly different ($P > 0.05$) and means with different symbols are significantly different ($P < 0.05$).

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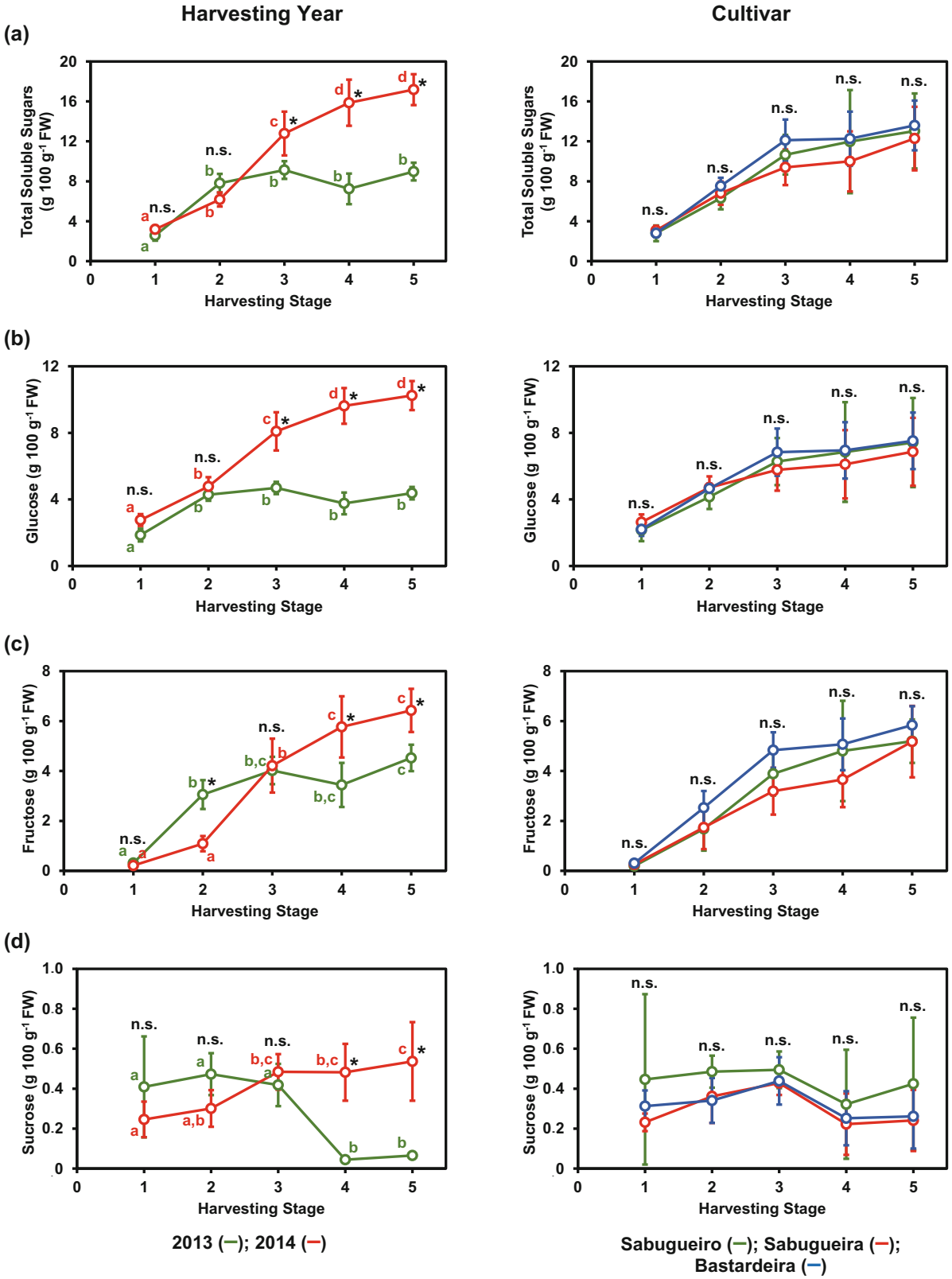

Figure 2. The simple sugar content of elderberries during different maturation stages (1 – 5). (a) Total sugar content (total soluble sugars, g 100 g^{-1} FW); (b) glucose content (g 100 g^{-1} FW); (c) fructose content (g 100 g^{-1} FW) along the maturation stage for the two harvesting years (2013 (green) and 2014 (red)); (d) fructose content (g 100 g^{-1} FW) in the different cultivars ('Sabugueiro' (So), 'Sabugueira' (Sa) and 'Bastardeira' (Ba), green, red and blue lines, respectively). Data are presented as mean ± standard deviation (n = 5 for Sabugueiro and Sabugueira cultivars and n = 8 for Bastardeira cultivar). For the left panels, for the different maturation stages within each year, means with different letters are significantly different ($P < 0.05$). For the different harvesting years and for the same maturation stage, means with n.s. are not significantly different ($P > 0.05$) and those with * are significantly different ($P < 0.05$). For the right panels, within the same maturity stage, means with n.s. are not significantly different ($P > 0.05$) and means with different symbols are significantly different ($P < 0.05$).

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the different maturity stages being significantly different, except for the third and fourth maturity stages. Nevertheless, significant differences in the total simple sugar content of elderberries were observed during ripening for the two harvesting years, with the 2014 harvesting year presenting significantly higher content of total simple soluble sugars (3.02–17.33 g/100 g FW) when compared with the 2013 harvesting year (2.38–9.34 g/100 g FW). On the other hand, as described previously, elderberries in the 2013 harvesting year showed higher TSS when compared with the 2014 harvesting year and this can be explained by the lower water content of elderberries in the 2013 harvesting year; nevertheless the accumulation of other compounds in elderberries, as for example soluble polysaccharides, also contributes to the measured °Brix. 'Bastardeira' cultivar showed significantly higher total simple sugars when compared with 'Sabugueira' and was not significantly different from 'Sabugueiro'. A significant interaction between the maturation stages and harvesting years was observed, showing that the total simple sugars increase pattern was different from the two harvesting years (Fig. 2(a)). As shown in Fig. 2(a), the differences observed in total simple sugar content for the 2014 harvesting year started at the third maturation stage, and from that maturation stage, the levels of total simple sugars remained higher than that of the 2013 harvesting year.

For glucose (Fig. 2(b)), effects for harvesting year, maturation stages, and the interaction between harvesting year and maturation stages were significant. Unlike total simple sugars, no significant differences were observed between cultivars (glucose content ranged between 1.62–4.51 g 100 g^{-1} DW, and 2.60–10.50 g 100 g^{-1} FW, in the first and second harvesting year, respectively – Table S2 in the supporting information). For fructose (Fig. 2(c)), the same trend was observed as was described for glucose: a significant effect for harvesting year, maturation stages, and the interaction between harvesting year and maturation stage, was observed. A significant effect of cultivar was also observed, with Bastardeira cultivar showing a significantly higher level of fructose than Sabugueira and Sabugueiro cultivars (Fig. 2 (d)). The levels of sucrose (Table S2 in the supporting information) in elderberries during ripening were highly variable between harvesting years, decreasing with increasing ripening stage for the 2013 harvesting year (0.531–0.071 g 100 g^{-1} FW; 0.275–0.072 g 100 g^{-1} FW; 0.416–0.058 g 100 g^{-1} FW, for So, Sa and Ba, respectively), and increasing with increasing maturation stage for the 2014 harvesting year (0.362–0.491 g 100 g^{-1} FW; 0.188–0.430 g 100 g^{-1} FW; 0.210–0.476 g 100 g^{-1} FW, for So, Sa, and Ba, respectively).

The same trend described in this work for elderberries concerning the evolution of total simple sugars during maturation was also described for mulberries, where the amount of the major sugars, glucose (0–10.6 g 100 g^{-1} DW) and fructose (1.2–10.2 g 100 g^{-1} DW), increased with the maturation stage.[20] While sucrose levels decreased in the early stages of maturation (2.0–1.1 g 100 g^{-1} DW), they increased again until the end of the maturation (1.1–5.1 g 100 g^{-1} DW).[20]

Evolution of phenolic compounds during elderberries maturation: effect of cultivar and harvesting year

The phenolic compound profile of elderberries obtained by high-performance liquid chromatography (RP-HPLC-DAD) for the different maturation stages is shown in Fig. 3 and Supporting Information Tables S3–S5. The phenolic profile was identical for the three cultivars, for the different maturation stages, and for the two harvesting years studied. Nevertheless, the mean content varied. For all samples analyzed, a total of 17 phenolic compounds were detected by HPLC, seven compounds were detected but not identified (peak 1, 2, 3, 4, 5, 6 and 9), and 10 compounds were unequivocally identified, as cryptochlorogenic acid (7), chlorogenic acid (8), quercetin-3-glucoside (10), quercetin-3-rutinoside (11), quercetin (12), isorhamnetin-3-glucoside (13), cyanidin-3,-5-diglucoside (14), cyanidin-3-sambubioside-5-glucoside (15), cyanidin-3-glucoside (16) and cyanidin-3-sambubioside (17), as previously described.[5] As reported by Jarzycka *et al.*,[21] Lee and Finn,[1] and Mikulic-Petkovsek *et al.*,[22] elderberries might have neochlorogenic acid in their composition; however in our extracts sometimes (without any trend) a small peak with a UV-spectra not completely clear was present at the same retention time of neochlorogenic acid, but as the identification was not certain this peak was not taken into account.

Anthocyanins

The development of the deep red color of elderberries is the most visible change during their ripening. This change is due to the accumulation of the four most abundant anthocyanins present in elderberries: cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside, and cyanidin-3-sambubioside-5-glucoside. The accumulation of total anthocyanins was significantly different between maturation stages, and a significant interaction between the maturation stage and harvesting year was observed (Fig. 4(a)). The level of total anthocyanins (the sum of the four anthocyanins detected) during ripening was significantly higher in 2014 than in the 2013 harvesting year. The level of total anthocyanins for the 'Bastardeira' cultivar was not significantly different from the level of the 'Sabugueira' cultivar, although it was significantly different from the total anthocyanins level in 'Sabugueiro' cultivar.

For cyanidin-3,5-diglucoside (Fig. 4(b)), a significant effect of harvesting year, cultivar, maturation stage and a significant interaction between the maturation stage and cultivar were observed. The amount of cyanidin-3,5-diglucoside was significantly lower for the 'Sabugueiro' cultivar, with differences being observed only for the fourth and fifth maturation stages. For cyanidin-3-sambubioside-5-glucoside (Fig. 4(c)), a significant effect of harvesting year, maturation stage, cultivar, and a significant interaction between all of these factors were observed. 'Bastardeira' cultivar showed a higher cyanidin-3-sambubioside-5-glucoside concentration for the third, fourth, and fifth maturity stage compared to the 'Sabugueiro' cultivar and 'Sabugueira' cultivar showed a significantly higher concentration of this anthocyanins when compared with 'Sabugueiro' cultivar only for the fifth maturity stage (Fig. 4(c)).

For cyanidin-3-glucoside (Fig. 4(d)), the same trend was observed as previously described for the other anthocyanins, concerning the effect of harvesting year, maturation stage, cultivar and the interaction between these factors. For the harvesting year, differences between harvesting years start from the second maturity stage to the fifth. Unlike the other anthocyanins, the 'Bastardeira' cultivar did not present significantly higher levels of cyanidin-3-glucoside when compared the other two cultivars.

For cyanidin-3-sambubioside (Fig. 4(e)), a significant interaction between harvesting year and maturation stage was observed, although the interaction between cultivar and maturation stage was not significant. The amount of cyanidin-3-sambubioside was significantly higher at the third, fourth, and fifth maturation stages for the 2014 harvesting year compared to the 2013 harvesting year. 'Bastardeira' cultivar presented a significantly higher

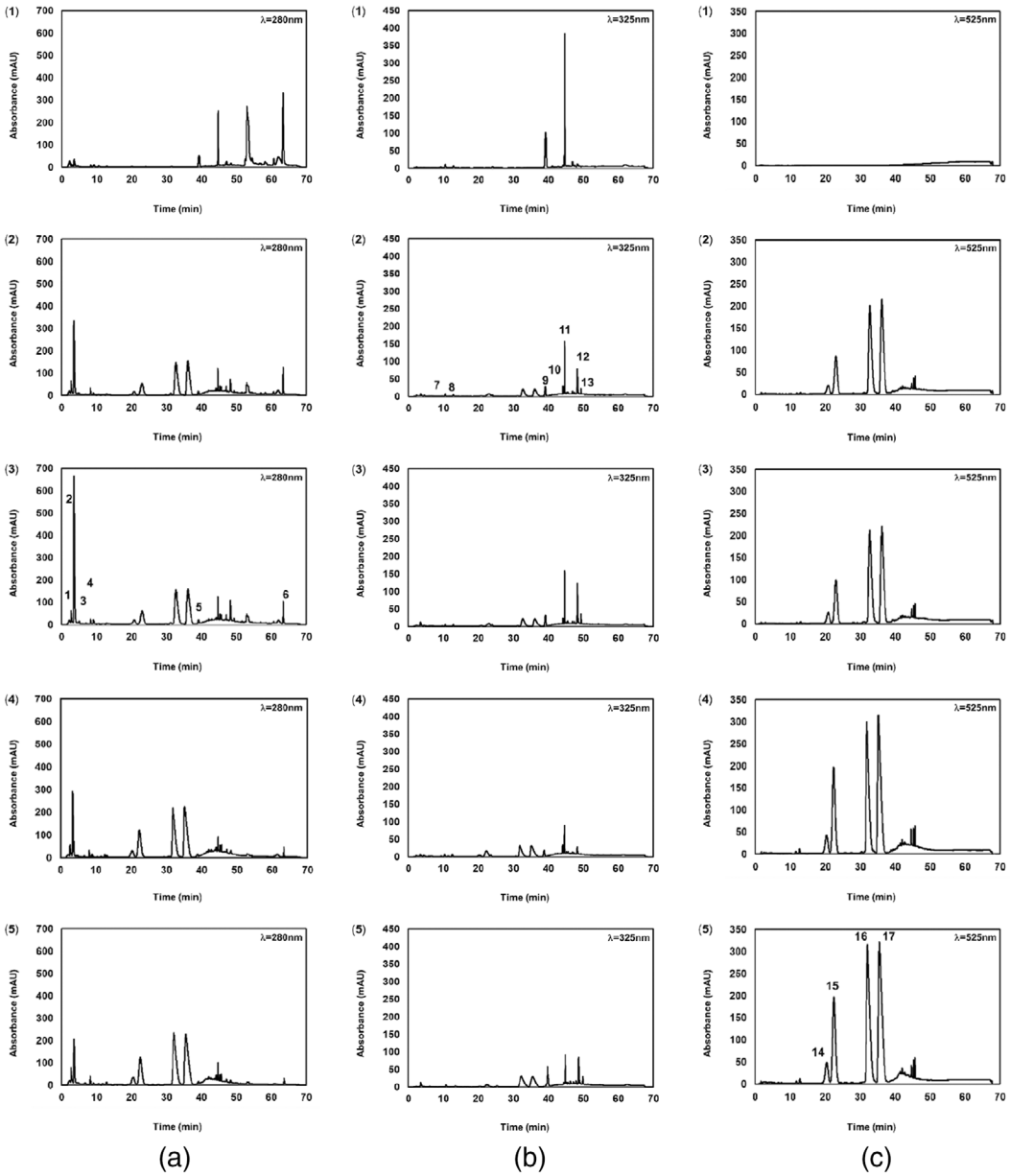

Figure 3. Phenolic profile of elderberries during different maturation stages (1–5). Chromatograms at 280 nm (a); 325 nm (b) and 525 nm (c). Peak identification: 7 – cryptochlorogenic acid; 8 – chlorogenic acid; 10 – quercetin-3-glucoside; 11 – quercetin-3-rutinoside; 12 – quercetin; 13 – isorhamnetin-3-glucoside; 14 – cyanidin-3,5-diglucoside; 15 – cyanidin-3-sambubioside-5-glucoside; 16 – cyanidin-3-glucoside; 17 – cyanidin-3-sambubioside; 1, 2, 3, 4, 5, 6 and 9 - unknown compound. These chromatograms refer to the 'Bastardeira' cultivar, and the harvesting year of 2013, and are representative chromatograms as the phenolic profile was identical between cultivars and harvesting years.

amount of cyanidin-3-sambubioside when compared to 'Sabugueiro', although not significantly higher than 'Sabugueira' cultivar.

Flavonoids

The effect of harvesting year and maturation stage for the total flavonoids (quercetin-3-glucoside, quercetin-3-rutinoside, quercetin and isorhamnetin-3-glucoside) was significant for harvesting year and maturation stage and also a significant interaction between harvesting year and cultivar was observed (Fig. 5(a)). The level of total flavonoids was significantly higher in the 2014 harvesting year. A decrease in the levels of total flavonoids was observed during maturation, although their levels for the third, fourth, and fifth maturation stages were not significantly different. Total flavonoid content between the cultivars was not significantly different, although on average 'Sabugueiro' cultivar presented higher levels of total flavonoids in the 2014 harvesting year (Fig. 5(a)).

The same trend was observed for the evolution of quercetin-3-rutinoside (Fig. 5(b)). A significant effect of the harvesting year was observed, with the 2014 harvesting year presenting a significantly higher level of quercetin-3-rutinoside compared to the 2013 harvesting year. Again, no significant differences were observed between the cultivars, although on average, the levels presented by the Sabugueiro cultivar were higher ('Sabugueiro' > 'Sabugueira' > 'Bastardeira').

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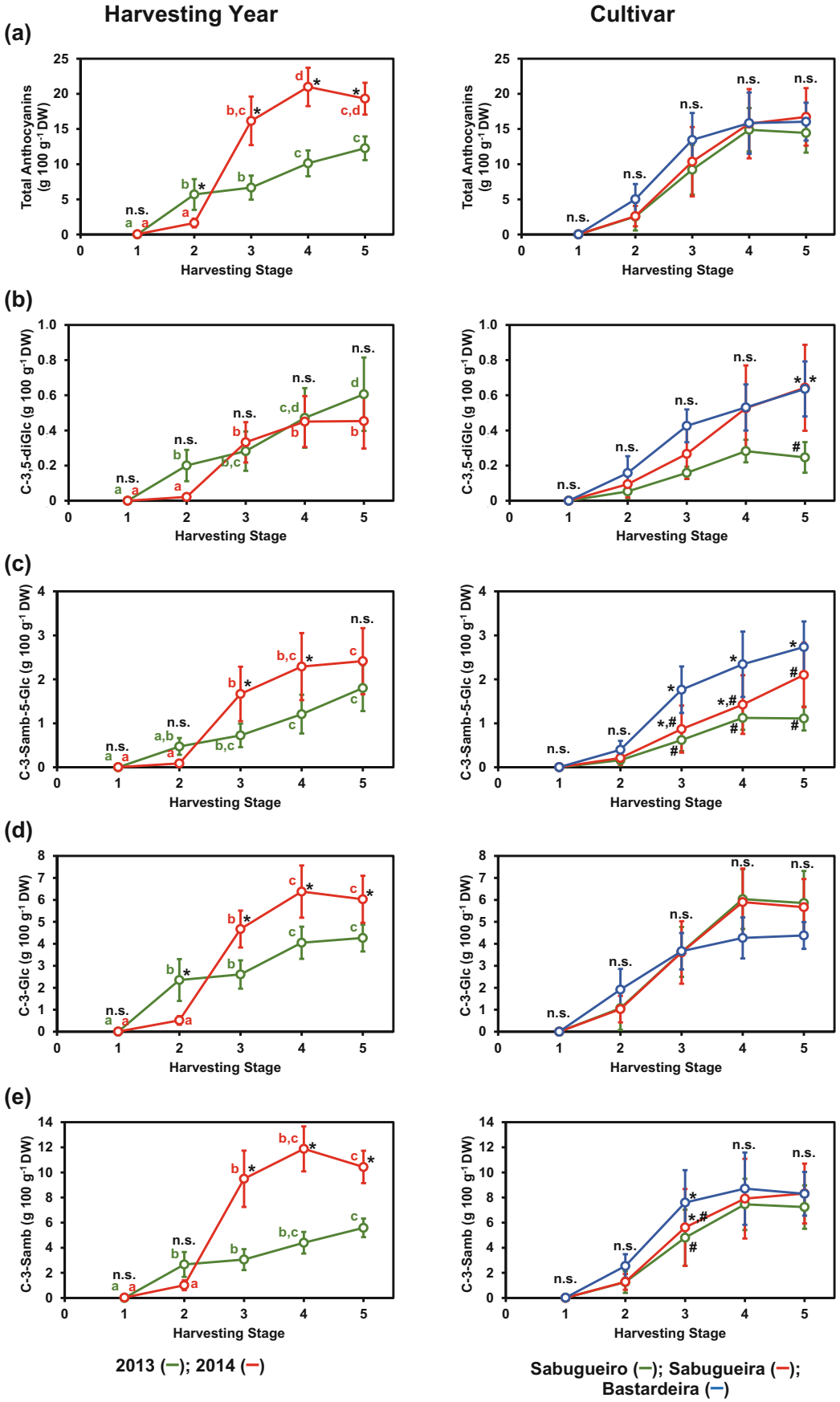

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For quercetin-3-glucoside, a different pattern was observed (Fig. 5(c)). There was a significant effect for the harvesting year, with the 2013 harvesting year presenting a significantly higher level of quercetin-3-glucoside than the 2014 harvesting year. There was also a significant interaction between cultivar and maturation stage, an increase of querecetin-3-glucoside being observed with increasing maturation stage. This was not significantly different between cultivars. For the last maturation stage, the content of quercetin-3-glucoside was significantly higher for the 'Sabugueiro' cultivar than 'Sabugueira' and 'Bastardeira'. Nevertheless, there was also a significant interaction between cultivar and harvesting year, with the levels of quercetin-3-glucoside being significantly higher for 'Sabugueiro' only for the 2014 harvesting year. It was similar to the other cultivars for the 2013 harvesting year.

For isorhamnetin-3-glucoside and quercetin, different trends were observed (Fig. 5(d), (e), respectively). For quercetin, a significant effect of harvesting year was observed, with the levels obtained for the 2014 harvesting year significantly higher than those of 2013 harvesting year. There was also a significant interaction between harvesting year and maturation stage: for the 2014 harvesting year, the levels of quercetin remained constant during ripening; for the 2013 harvesting year, there was a decrease in the levels of quercetin with increasing maturation stage until the third maturation stage and it increased in the fourth maturation stage to the levels of the first maturation stage (Fig. 5(e)). Concerning isorhamnetin-3-glucoside, differences in the variation trend during ripening were observed between harvesting years: for the 2014 harvesting year, the levels of isorhamnetin-3-glucoside remained almost constant during ripening; for the 2013 harvesting year they increased significantly until the third harvesting stage but decreased after that until the fifth maturation stage (Fig. 5(d)). No significant differences were observed between cultivars (Fig. 5(d)).

Cinnamic acids

During ripening, only two cinnamic acids were consistently identified in elderberries: cryptoclorogenic acid (CAA) and chlorogenic acid (CA). Their levels were low during all the maturation stages, although a significant effect was observed for harvesting year and there was a significant interaction between the harvesting year and maturation stage. The levels of cinnamic acids for the 2014 harvesting year were significantly higher than those obtained for 2013. The same trend was observed for the CA and CCA. These were the phenolic compounds present in lower amounts in each maturation stage (results not shown).

The evolution of anthocyanins and phenolic compounds during the maturation of elderberries is similar to that observed for other small red fruits. For example, the major anthocyanins of blackberries, cyanidin-3-glucoside and cyanidin-3-rutinoside, increased with maturation,[19] whereas other anthocyanins, such as cyanidin-3-dioxaly-glucoside, decreased with maturation.[19] In mulberries, it was observed that cyanidin-3-glucoside and cyanidin-3-rutinoside also increased with maturation (from 33.3–6262.3 mg kg^{-1} DW and 23.5–3031.3 mg kg^{-1} DW, respectively).[20] In general, phenolic acids and flavonoids tended to decrease along with maturation, although some present the opposite trend. In mulberries, protocatechuic acid and ferulic acid increased along with maturation, from 6.1 to 43.4 mg kg^{-1} DW and 2.1 to 10 mg kg^{-1} DW, respectively. Whereas others phenolic compounds decreased along with the maturation, such as chlorogenic acid (3923.9–582.4 mg kg^{-1} DW), caffeic acid (471.7–90.9 mg kg^{-1} DW), ellagic acid (539.8–122.9 mg kg^{-1} DW), and quercetin-3-rutinoside (699–592 mg kg^{-1} DW) (20). Thus, the results obtained for elderberries were in accordance with other berries, showing an increase in the anthocyanin content, more markedly with the sugar accumulation, as sugars are a pivotal substrate for anthocyanin synthesis.[23] Whereas, some phenolic acids and flavonoids decreased along with the maturation, as they are precursors for the biosynthesis of other compounds, as well as their role at cellular levels may lead to their reduction.[23]

Since our experimental design included the same field, the same shrub, and the same agricultural practices along the different harvesting years and maturation stages, the difference in the chemical composition of elderberries between the two harvesting years analyzed might be explained by the different climatic conditions to which the elderberries were exposed in the three harvesting years. To simplify the global description of weather conditions for each harvesting year, Table 1 shows the bioclimatic indices of the 2013 and 2014 harvesting years. The Winkler index (WI) measures the heat accumulation during the growing season.[13] The year 2014 is included in Winkler's Region I and classified as cold, and the year 2013 was included in Winkler's Region II and classified as moderately cold. When considering the Huglin Heliothermic Index (HI), all years correspond to temperate years. The Dryness Index (DI) allows accessing the availability of soil water content for the plant in the growing season, considering precipitation and reference evapotranspiration.[16] According to the values between April and October, in the 2014 harvesting year, they were sub-humid, and in 2013 it was a humid year. The cool night index (CI)[15] considers the minimum temperatures during the maturation period, providing complementary information about the thermal regime in this important period. The total number of days with a temperature below 10 °C during the maturation months (August and September) were similar in the 2013 and 2014 harvesting years (4 and 6 days, respectively).

On the other hand, the number of days with a maximum temperature above 30 °C was much higher in 2013 (20 days) when compared to 2014 (5 days). This different temperature profile between these two harvesting years might explain the differences observed in the anthocyanin content of elderberries in the two harvesting years. In warmer climates, higher temperatures may result in adverse changes in fruit composition. For example, a significantly lower anthocyanins concentration at maturity was observed in grapevines exposed to 30 °C rather than 20 °C temperature treatments.[24] Another study found that high

FIGURE 4. Anthocyanin content (g 100 g^{-1} DW) of elderberries during different maturation stages (1 – 5), obtained by HPLC. Variations observed for the two harvesting years (left panels: 2013 (green), 2014 (red)) and three elderberry cultivars (right panels: Sabugueiro (green); Sabugueira (red); Bastardeira (blue)). (a) Total anthocyanins; (b) cyanidin-3,5-diglucoside; (c) cyanidin-3-sambubioside-5-glucoside; (d) cyanidin-3-glucoside (C3G); (e) cyanidin-3-sambubioside (C3S). Data are presented as means ± standard deviations (n = 5 for Sabugueiro and Sabugueira cultivars and n = 8 for Bastardeira cultivar). For the left panels, for the different maturation stages within each year, means with different letters are significantly different ($P < 0.05$). For the different harvesting years and for the same maturation stage, means with n.s. are not significantly different ($P > 0.05$) and those with * are significantly different ($P < 0.05$). For the right panels, within the same maturity stage, means with n.s. are not significantly different ($P > 0.05$) and means with different symbols are significantly different ($P < 0.05$).

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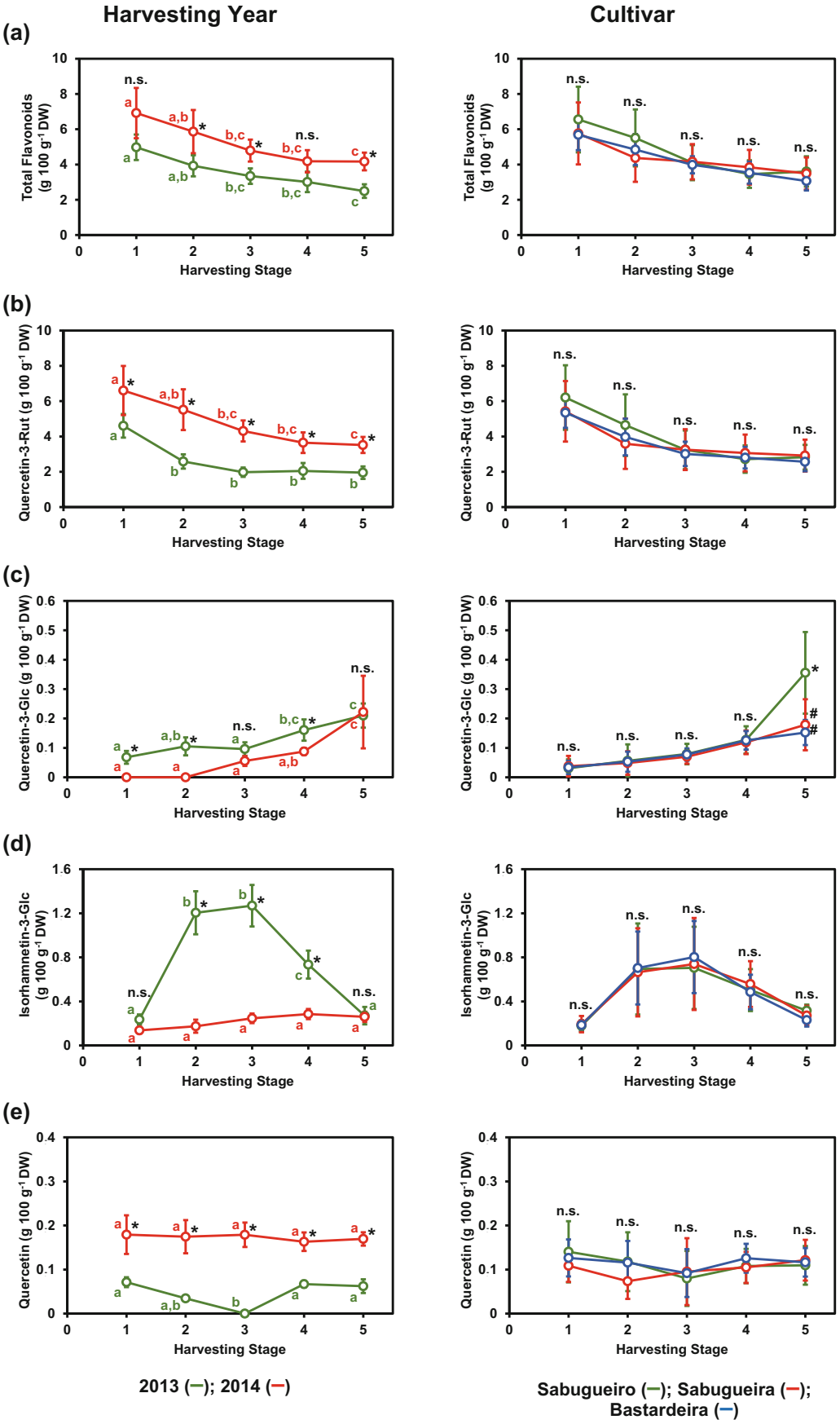

Figure 5. Legend on next page.

Table 1. Bioclimatic index of 2013 and 2014 harvesting years

Bioclimatic index	2013	2014
Wrinkler	1430	1356
	Moderately cold	Cold
Huglin Heliothermic	1988	1928
	Temperate	Temperate
Dryness	168	137
	Humid	Sub-humid
Cool night	12.7	12.2
	Cool nights	Cool nights
Number of hot days (> 30 °C)	20	5
Number of cool nights (< 10 °C)	4	6

temperatures (35 °C) inhibited anthocyanin production and degraded the anthocyanins produced.[25] A day temperature of 35 °C completely inhibited anthocyanin synthesis in 'Tokay' berries, regardless of night temperature.[26]

Evolution of PAL, PPO, and POD activity during maturation: Effect of cultivar and harvesting year

To understand the pattern of evolution of phenolic compounds during maturation and the differences observed between harvesting years, the activity of PAL, which is a key enzyme in the biosynthesis of phenolic compounds, and the activity of the two enzymes responsible for the degradation of phenolic compounds (PPO and POD) were determined in elderberries in the 2013 and 2014 harvesting years and the results are shown in Fig. 6 and Table S6 in the supporting information.

A significant difference between the two harvesting years, maturation stage, and interaction between the maturation stage and harvesting year was observed for PAL activity (Fig. 6(a)). For the 2013 harvesting year, a steady decrease in PAL activity with an increasing maturation stage was observed, but the decrease was only significant until the third maturation stage. Fruits harvested in 2014 showed higher PAL activity than those harvested in 2013 and presented a different pattern during maturation. The PAL activity for the first and second maturation stages was not significantly different, decreasing for the third maturation stage and again for the fourth and fifth maturation stages. There was no significant interaction between the cultivar and maturation stage, although a significant interaction between cultivar and harvesting year was observed. PAL activity was identical for the three cultivars harvested in 2013. Nevertheless, for the 2014 harvesting year, 'Sabugueira' cultivar presented a significantly higher PAL activity than 'Bastardeira', but 'Sabugueir'o cultivar did not show significant differences with the other two cultivars.

Concerning PPO activity, a significant effect of harvesting year was observed, but unlike PAL activity, PPO activity was significantly higher in 2013 than in 2014 harvesting year (Fig. 6(b)). A significant effect between cultivars and between cultivars and harvesting years was observed. The PPO activity was not significantly different between cultivars in the 2014 harvesting year, but, for the 2013 harvesting year, 'Sabugueiro' presented a significantly higher PPO activity than 'Sabugueira' and 'Bastardeira' cultivars. A significant effect of the maturation stage and the interaction between the maturation stage and harvesting year was observed, meaning that the evolution pattern of PPO activity during the maturation stages was significantly different for the two harvesting years. In 2014, PPO activity remained almost unchanged during the five maturation stages, whereas for the 2013 harvesting year, a significant decrease in PPO activity from the first to the second and third maturation stages was observed. For the last two maturation stages, the PPO activity remained low and similar to the PPO activity observed for the 2014 harvesting year.

For POD activity, significant effects for the harvesting year, the cultivar, and a significant interaction between the cultivar and harvesting year were observed, showing a similar trend as described for PPO activity (Fig. 6(c)). Peroxidase activity was identical for the different cultivars for the 2014 harvesting year, whereas in the 2013 harvesting year the cultivar 'Sabugueiro' displayed significant higher POD activity than the other two cultivars (Fig. 6(c)). Regarding POD activity along the maturation stages, as well as the interaction between the maturation stage and harvesting year (Fig. 6(c)), results show that, in the 2014 harvesting year, its activity remained almost constant, while, in the 2013 harvesting year, the first maturation stage presented a significantly higher POD activity which decreased (second maturation stage) and remained almost constant until the end of the maturation. Except for the first maturation stage for the 2013 harvesting year, no significant differences in the POD activity were found for all the other maturation stages for both harvesting years.

The higher levels of phenolic compounds in the 2014 harvesting year agree with the higher PAL activity in elderberries in all but the fifth maturation stages compared to the 2013 harvesting year. The higher PPO and POD activity in elderberries harvested in 2013 might also be related to the lower phenolic content in elderberries harvested in 2013.

In some fruits, such as sweet cherry,[27] strawberry,[28,29] grape berries[30] and loquat fruit,[31] PAL activity presented two peaks along the maturation stages. The first peak occurred after the end of the flowering (green-immature stage), decreasing considerably along the maturation stages, and then in the last stage of maturation, PAL activity rose, reaching a new peak but much lower than the first one. In most cases, the second peak is correlated with flavonoid content increase, mainly anthocyanins. Nevertheless, depending on the fruit and their phenolic composition, PAL activity can be correlated with anthocyanin content or other phenolic compounds. The increase in the anthocyanin content along maturation was followed by an increase in the PAL activity in strawberries.[28,29] However, in other red fruits, such as sweet cherry,[27] the peaks of PAL activity were correlated with other phenolic compounds. In grape berries, two peaks were found, at 30 days after full bloom (upper than 2 μmol cinnamic acid h mg protein^{-1}) and other 70 days after full bloom (upper

FIGURE 5. Flavonoid content (g 100 g-1 DW) of elderberries during different maturation stages (1 – 5), obtained by HPLC. Variations observed for the two harvesting years (left panels) and three elderberry cultivars (right panels). (a) Total flavonoids; (b) quercetin-3-rutinoside (Q3R); (c) quercetin-3-glucoside (Q3G); (d) isorhamnetin-3-glucoside (I3G); (e) quercetin (Q). Data are presented as means ± standard deviations (n = 5 for Sabugueiro and Sabugueira cultivars and n = 8 for Bastardeira cultivar). For the left panels, for the different maturation stages within each year, means with different letters are significantly different ($P < 0.05$). For the different harvesting years and for the same maturation stage, means with n.s. are not significantly different ($P > 0.05$) and those with * are significantly different ($P < 0.05$). For the right panels, within the same maturity stage, means with n.s. are not significantly different ($P > 0.05$) and means with different symbols are significantly different ($P < 0.05$).

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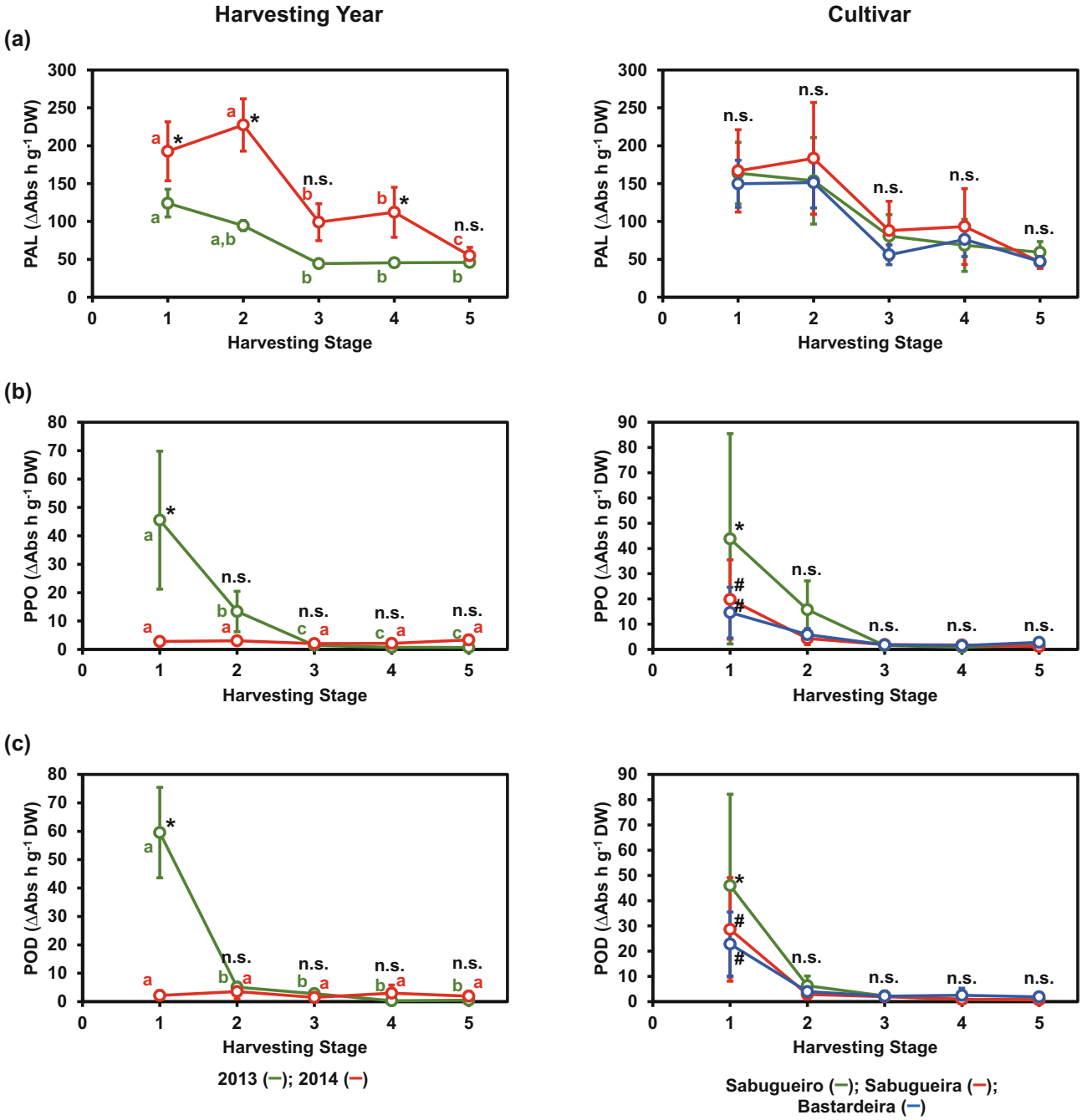

Figure 6. Phenylalanine ammonia lyase (PAL) (a), polyphenol oxidase (PPO) (b) and peroxidase (POD) activity (c) during different elderberry maturation stages (1–5) Variations observed for the two harvesting years (left panels: 2013 (green line) and 2014 (red line)) and three elderberry cultivars (right panels: 'Sabugueiro' (green); 'Sabugueira' (red); 'Bastardeira' (blue)). Data are presented as means ± standard deviations (n = 5 for 'Sabugueiro' and 'Sabugueira' cultivars, and n = 8 for 'Bastardeira' cultivar). For the left panels, for the different maturation stages within each year, means with different letters are significantly different ($P < 0.05$). For the different harvesting years and for the same maturation stage, means with n.s. are not significantly different ($P > 0.05$) and those with * are significantly different ($P < 0.05$). For the right panels, within the same maturity stage, means with n.s. are not significantly different ($P > 0.05$) and means with different symbols are significantly different ($P < 0.05$).

than 6 μmol cinnamic acid h mg protein^{-1}), which was correlated with the total phenolic acids content.[30] In loquat fruit, PAL presented the highest activity at the early stage of maturation (upper than 1.34 units g^{-1} of FW), then decreased, reaching a value of 0.67 units g^{-1} of FW. At the middle of the maturation stage, PAL activity rose to a new peak of 1.34 units g^{-1} of FW, then decreased again until the end of the maturation, which was associated with the variation in chlorogenic acid.[31] In nine red and green cultivars of apples, Lister *et al.*[32] also found the highest PAL activity at the immature stage, which then decreased during maturation, and then rose in the final maturation stage but to a lower level than the initial PAL activity, and depending on cultivar the second peak of activity was slightly or more pronounced. They also demonstrated that PAL activity was not correlated with the concentration of anthocyanins but correlated with the total flavonoids content. However, different patterns of PAL activity in apples were

reported by Ju *et al.*,[33] who observed an increase in PAL activity during 30 days after full bloom (first peak), followed by a rapid decrease in PAL activity to values that were maintained constant along with the maturation. The PPO activity was also reported to decrease along the maturation stages in four cultivars of apples, with different concentrations of phenolic compounds,[34] as we observed for elderberries. Fuji cultivar showed a decrease in the PPO activity from 22.0 to 13.1 units g^{-1} of FW. The PPO activity decreased from 13.1 to 5.7 units g^{-1} of FW for Mellow cultivar, from 21.9 to 11.6 units g^{-1} of FW and 5.4 to 0.69 units g^{-1} of FW, for Elstar and Aori cultivars,[27] respectively.

The regulation of PAL has been described as complex and dependent on many intrinsic and extrinsic factors; thus, extrinsic stress factors, such as light and drought, among others, can influence the plant response and consequently gene expression.[35-37] As mentioned, different fruits demonstrated different patterns in PAL activity, not always associated whit the accumulation of anthocyanins, which might make difficult comparisons between studies and enzymatic activity between different fruits. In the case of elderberries, the PAL activity was significantly correlated with the levels of chlorogenic acid ($r = 0.325$, $P < 0.00001$) and quercetin-3-rutinoside ($r = 0.640$, $P < 0.00001$) and quercetin ($r = 0.367$, $P < 0.00001$).

CONCLUSION

This is the first study describing the evolution of simple sugars and phenolic compounds during the maturation of elderberries. Over 2 years, fruits from the same selected plants belonging to the three most important Portuguese cultivars, Sabugueiro, Sabugueira, and Bastardeira, grown at the same location, were studied from green immaturity to commercial maturity, with sampling in five stages of maturation. It was observed that, although the levels of simple sugars (glucose, fructose, and sucrose), and phenolic compounds were dependent on the harvesting year, the simple sugar and anthocyanin content increased significantly with the maturation stage ($P < 0.00001$). The levels of total flavonoids, especially quercetin-3-rutinoside, decreased with increasing maturation stage ($P < 0.00001$), and the level of total cinnamic acids did not show a clear trend during maturation. The differences observed for the accumulation of anthocyanins between the two different harvesting years might be explained by the different climatic conditions of each harvesting year, namely the total number of hot (>30 °C) days, which were higher in the 2013 harvesting year than in the 2014 harvesting year, resulting in a lower accumulation of anthocyanins, probably due to the inhibition of their synthesis and also due to their degradation. The higher levels of phenolic compounds in the 2014 harvesting year agree with the highest PAL activity in elderberries compared with the 2013 harvesting year. The higher PPO and POD activity in elderberries harvested in the 2013 harvesting year might also explain the lower phenolic compound content in elderberries harvested in 2013. These results highlighted the influence of climatic conditions and cultivars in fruit development and the quality of each harvesting season.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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