JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Climate Effects on Anthocyanin Accumulation and Composition in the Pomegranate (*Punica granatum* L.) Fruit Arils

Hamutal Borochov-Neori,[*,†,‡,§] Sylvie Judeinstein,[†] Moti Harari,[†] Irit Bar-Ya'akov,[∥] Bhimanagouda S. Patil,[‡] Susan Lurie,[⊥] and Doron Holland[∥]

[†]Southern Arava Research and Development, M. P. Hevel Eilot, 88820, Israel

[∥]Unit of Deciduous Fruit Trees Sciences, Newe Ya'ar Research Center, Agricultural Research Organization, P.O. Box 1021, Ramat Yishay, 30095, Israel

[‡]Vegetable and Fruit Improvement Center, Department of Horticultural Science, Texas A&M University, College Station, Texas 77843-2119, United States

[⊥]Department of Postharvest Science, Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel

ABSTRACT: Worldwide pomegranate (*Punica granatum* L.) production has expanded greatly due to recent evidence on the fruit health attributes. The fruit's unique red color, conferred by anthocyanins, is an imperative sensory quality. Climate effects on the fruit's internal color were reported earlier. The present study investigated the influence of a wide range of temperature regimes (~7−40 °C) on pomegranates' aril anthocyanins. The study included two deciduous and two evergreen accessions as well as desert and Mediterranean orchards. RP-HPLC analysis of the arils' anthocyanins revealed mono- and diglucosylated delphinidins and cyanidins as the major anthocyanins and pelargonidins as minor components. Anthocyanin accumulation changed inversely to the season's temperatures. Cyanidins were generally more abundant but delphinidin accumulation was enhanced in cooler season. Monoglucosylated anthocyanins prevailed at cooler temperatures and subsided during seasonal warming with a concomitant increase in diglucoside proportion. The findings can benefit breeding and agricultural efforts to enhance pomegranate quality, especially in the face of "global warming".

KEYWORDS: anthocyanin accumulation, anthocyanin composition, anthocyanin glucosides, climatic conditions, cyanidin, delphinidin, pomegranate, *Punica granatum* L.

INTRODUCTION

The pomegranate (*Punica granatum* L.) fruit is highly valued for its potential health benefits. The health promoting effects of the fruit and its products were supported by numerous *in vivo* and *in vitro* studies (extensively reviewed in refs 1 and 2).[3,4] The recent growing awareness of consumers to health aspects of fresh and processed produce greatly increased the interest in consumption of pomegranate fruit and its products;[5] consequently, worldwide pomegranate production expanded considerably.[6]

The beneficial health qualities of pomegranate were attributed to the exceptionally high antioxidative capacity of the fruit[7] seemingly effected by the remarkably high content and unique composition of soluble polyphenolic compounds, especially in the fruit peel.[7−11] Pomegranate fruit is usually consumed fresh, the edible part being the fruit arils, or as processed products, mostly juice. During industrial processing, significant amounts of polyphenols from the fruit peel are extracted into the pomegranate juice (PJ). Indeed, the most abundant polyphenols in PJ are the ellagitannins from the fruit peel, including punicalagin.[7,9] PJ also contains anthocyanins that originate mostly from the arils and impart its color.[12−14] Anthocyanins exhibit low bioavailability and thus their role in PJ bioactivity is yet to be established.[15,16] However, consumers tend to associate the pomegranate fruit and its products with the distinctive intense red color. Therefore, the industry stipulates red-color rich pomegranates to enhance marketing.

Six anthocyanin pigments were identified in the pomegranate fruit, 3-mono- and 3,5-diglucosides of cyanidin, delphinidin and pelargonidin.[12,17,18] All six anthocyanin pigments were detected in fruit from Spanish, Californian, Tunisian, Italian, Iranian and Uzbekistanian accessions[12,13,19,20] in different amounts and proportions, depending on variety as well as geographical and cultural variables. Different contents and relative proportions of the three groups of anthocyanins determine the intensity and hue of the fruit color, and, indeed, pomegranates from different accessions range widely in their external and internal color, from white-yellow through orange-pink to intense red.[6,21−24]

Anthocyanin accumulation in plants is sensitive to environmental conditions.[25] Low temperatures enhance anthocyanin accumulation,[25,26] whereas at high temperatures the pigment concentration is reduced.[26,27] This reduction may reflect concurrent decrease in synthesis rate and accelerated degradation.[25] Our previous studies demonstrated the effects of seasonal[23] and geographical[24] variations on color and anthocyanin accumulation in pomegranate fruit in a diverse collection of accessions grown in Israel. Fruit that matured and ripened under extremely hot temperatures had lower external and internal color and accumulated less anthocyanins compared to moderate climate conditions.

Received: January 26, 2011
Accepted: April 20, 2011
Revised: April 15, 2011
Published: April 20, 2011

dx.doi.org/10.1021/jf2003688 | *J. Agric. Food Chem.* 2011, 59, 5325–5334

Table 1. Selected Fruit Quality Measures of Summer and Winter 'PG 128-29' Pomegranate Fruit[a]

harvest date	fruit weight (g)	juice content (%)	TSS (%)	TA (mequiv dL^{-1})	total phenolics (pyrogallol, mg L^{-1})
mid-July	225 ± 45	42 ± 1	14.3 ± 0.6	6.9 ± 0.6	1,501 ± 172
mid-December	210 ± 61	38 ± 2*	16.2 ± 1.0*	7.6 ± 0.7	1,689 ± 152*

[a] Values are average ± SD on ten replicates collected in the southern Arava during the years 2005 and 2007. Significant differences between seasons ($p < 0.05$, Student's *t*- test) are identified with an asterisk.

The effects were particularly large in the arils. Considering the increasing demand for intensely red pomegranates, further understanding of climate effects on the fruit coloration is needed, especially in face of "global warming" that already affects the climate in traditional cultivation regions of pomegranates. Knowledge of the factors and processes involved in anthocyanin accumulation can benefit the current breeding and agricultural efforts to enhance pomegranate fruit quality.

The present study is an extensive examination of the influence of climatic conditions during pomegranate fruit development and ripening on anthocyanin accumulation and composition in the arils, i.e. the edible part of the ripe fruit. The study employed deciduous and evergreen pomegranate accessions as well as Mediterranean and desert orchards. Ripe fruit was harvested throughout the entire year thus being exposed to diverse temperature regimes during development, maturation and ripening, ranging from cool to extreme conditions. Arils of the various accessions were analyzed to establish climatic effects on (1) anthocyanin accumulation, (2) ratio of delphinidins to cyanidins, and (3) level of pigment glucosylation.

MATERIALS AND METHODS

Plant Material. Fresh ripe pomegranate (*Punica granatum* L.) fruit was collected from two Israeli orchards located in the experimental farms of the southern Arava Valley (latitude 29°53′N; longitude 35°3′E, desert climate), and Newe Ya'ar Research Center, Yizre'el Valley (latitude 32°42′N, longitude 35°11′E, Mediterranean temperate to subtropical climate).[23,24] Year-round climatic data for the Israeli Southern Arava Valley, including temperature, humidity, daily evaporation and rainfall, as well as climatic data for Newe Ya'ar from March through October, including temperature, humidity and global radiation, were reported earlier.[23,24] The southern Arava orchard was irrigated year-round with a total of ~12,000 m^3 per hectare. Irrigation in Newe Ya'ar was applied from March through October, amounting to ~6,000 m^3 per hectare for the entire season. Daily irrigation was practiced in both orchards, calculated to compensate for daily water loss measured by evaporation from Class A evaporation pan. Four pomegranate accessions were studied: two deciduous accessions, 'PG 128-29' and 'PG 130-31' ('Shani-Yonay',[28]) [both registered in the Israel Gene Bank for Agricultural Crops (IGB, Web site: http://igb.agri.gov.il)], and two evergreen accessions, 'EG 1' and 'EG 2'. The manuscript summarizes studies conducted during the years 2005−2008. Fruit from each accession was harvested on selected dates during two or three years. Both harvest dates and sampling years are specified in the corresponding sections under Results. On each sampling date, ripe fruit was selected by external criteria according to customary grower practices. The latter included the attainment of final size, peel color intensity and distribution, skin shine and fruit shape with respect to length to diameter ratio and walls' depression. Five fruits of similar size, external color and shape were chosen from different trees and locations in the orchard and studied within 24 h.

Fruit Processing. Each fruit was weighed, and intact arils were separated by hand from the pith and carpellary membranes. Ripeness was further established by lack of astringency as reported by a panel of 8 regular consumers of fresh pomegranates who tasted the freshly separated arils; only nonastringent edible fruits were analyzed. Juice was prepared from the isolated arils by a solid fruit juice extractor (Juice Extractor, Model Le Duo, Magimix, France), weighed and analyzed. Analytical assays were carried out in triplicate.

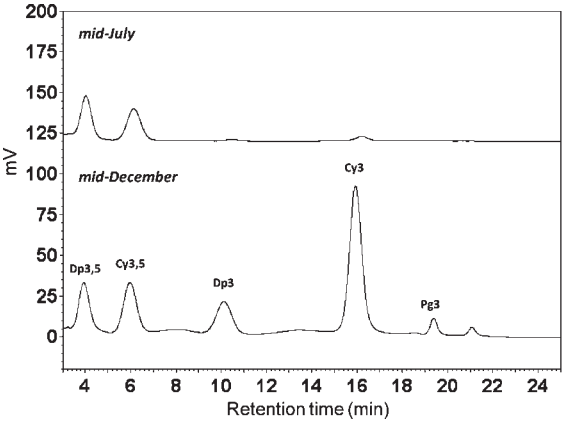

Figure 1. HPLC chromatograms at 520 nm of arils' methanolic extracts from 'PG 128-29' pomegranate fruit harvested in mid-July (top) and mid-December (bottom). Identified anthocyanin peaks are marked by names.

Total Soluble Solids (TSS) and Titratable Acidity (TA). TSS (in %) was measured with a hand refractometer (ATAGO, ATC-1E, Brix 0−32%, Japan, calibrated with distilled water). TA (in mequiv of acid dL^{-1}) was measured colorimetrically by titration with 0.1 N NaOH using the pH indicator phenolphthalein.

Total Soluble Phenolic Content. Pomegranate aril juice was extracted with 80% methanol supplemented with 2 mM NaF (1:2, v/v) and centrifuged (10,000 rpm for 10 min at 4 °C, Sorvall Instruments RC5C, rotor no. SS-34). The supernatant was diluted 10-fold with double distilled water (DDW). Concentration of total soluble phenolics was measured colorimetrically with Folin−Ciocalteau 2 N phenol reagent (SIGMA Chemical Co., USA) according to Singleton and Rossi.[29] Aliquots of 100 μL were added to 900 μL of reaction solution consisting of 200 μL of freshly prepared 10-fold diluted Folin−Ciocalteau reagent, 100 μL of 20% Na_2CO_3 and 600 μL of DDW. Pyrogallol (SIGMA Chemical Co., USA) was used for the calibration curve (0−100 μg mL^{-1}). The absorbance at 765 nm was measured with a spectrophotometer (SHIMADZU Corporation, UV-1650PC, Kyoto, Japan) after 1 h incubation, and the content of phenolics in the juice was expressed in pyrogallol equivalents, mg L^{-1}.

RP-HPLC Analysis of Anthocyanins. Methanolic extracts of pomegranate aril juice were prepared as described above. The supernatant was filtered through a 0.45 μm PTFE filter before injection. Samples were analyzed with LaChrom Merck Hitachi HPLC system, consisting of Pump L7100, column oven L7350 and mixer−degasser L-7614, coupled with a diode array detector with 3D feature (multiwavelength detector, Jasco MD-2010 Plus), interface (Jasco LC-Net II/ADC) and scientific software (EZChrom *Elite* Client/Server version 3.1.6 build 3.1.6.2433). Twenty μL of extract were injected using a

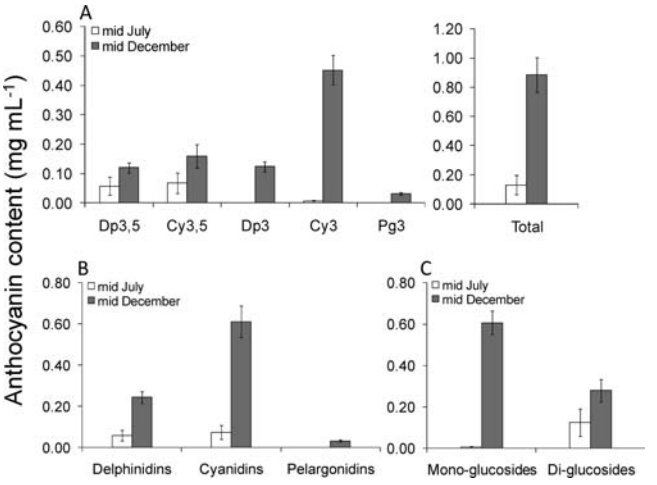

Figure 2. Anthocyanin composition in the arils of 'PG 128-29' pomegranate fruit harvested in mid-July and mid-December. Concentration of total and individual anthocyanins (A); delphinidins, cyanidins and pelargonidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of six (mid-December) and seven (mid-July) replicates collected during the years 2005 and 2007.

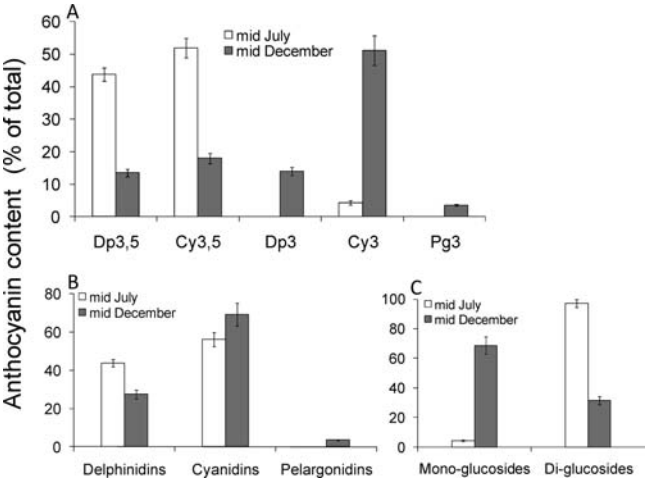

Figure 3. Anthocyanin relative abundance in the arils of 'PG 128-29' pomegranate fruit harvested in mid-July and mid-December. Individual anthocyanins (A); delphinidins, cyanidins and pelargonidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of six (mid-December) and seven (mid-July) replicates collected during the years 2005 and 2007.

manual injector (Rheodyne, Rohnert Park, CA) and loaded onto a column of Lichrospher100 RP-18 (5 μm particle size in 250 × 4 mm LichroCART cartridge) with a guard column of the same packing material (4 × 4 mm LichroCART cartridge). Column temperature was maintained at 40 °C. The binary mobile phase consisted of phosphoric acid in DDW (0.1%, pH 2.4) (A) and acetonitrile (B). Elution was carried out with the following scheme: 10% B at 1 mL/min for the first 10 min; 10% B to reach 20% by 15 min, at 1 mL/min; 20% B at 1 mL/min to reach 0.6 mL/min at 16 min; 20% B at 0.6 mL/min for 10 min. Following anthocyanin elution, the column was washed and equilibrated by 10 min post runs with 80% and 10% B, respectively.

Acetonitrile was HPLC grade (LiChrosolv Merck); DDW was passed through a 0.20 μm nylon membrane. Phosphoric acid and NaF were of analytical grade.

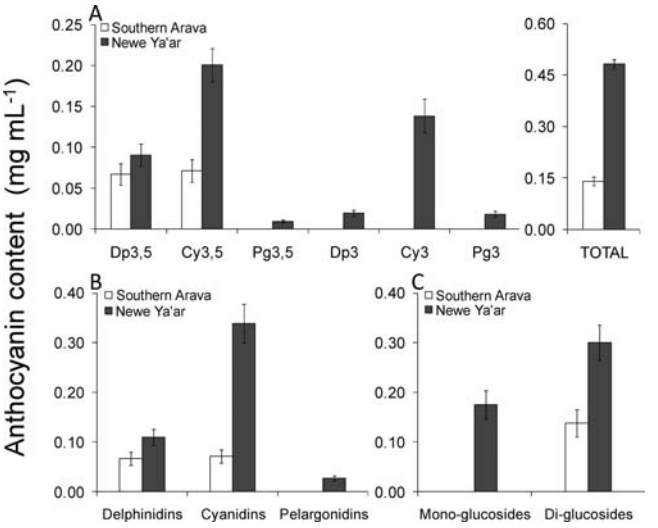

Figure 4. Anthocyanin composition in the arils of 'PG 130-31' summer fruit from the southern Arava and Newe Ya'ar orchards. Concentration of total and individual anthocyanins (A); delphinidins, cyanidins and pelargonidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of six replicates collected during the years 2005 (both sites), 2007 (Newe Ya'ar) and 2008 (southern Arava).

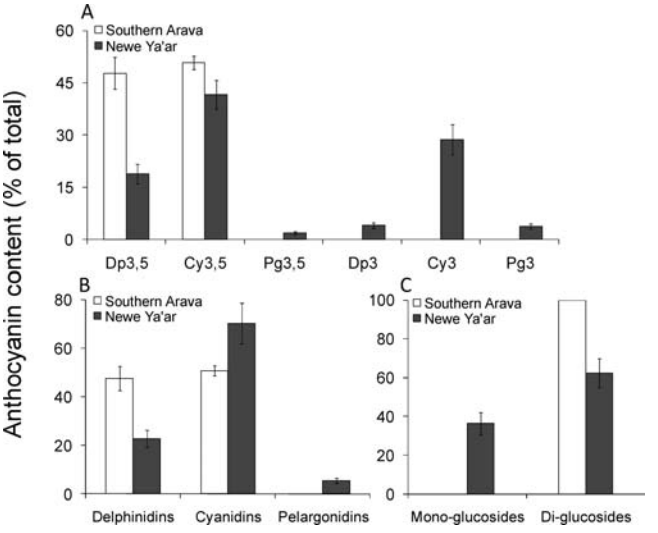

Figure 5. Anthocyanin relative abundance in the arils of 'PG 130-31' summer fruit from the southern Arava and Newe Ya'ar orchards. Individual anthocyanins (A); delphinidins, cyanidins and pelargonidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of six replicates collected during the years 2005 (both sites), 2007 (Newe Ya'ar) and 2008 (southern Arava).

Anthocyanin Identification and Quantification. Peak assignment was performed by the software on the basis of UV/vis absorbance spectra and the retention times of anthocyanin standards. The standard library was constructed from delphinidin 3,5-diglucoside (Dp3,5), cyanidin 3,5-diglucoside (Cy3,5), pelargonidin 3,5-diglucoside (Pg3,5), malvidin 3-glucoside chloride (Apin Chemicals), delphinidin 3-glucoside (Dp3), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) (Polyphenols Laboratories AS). Each standard (50−100 μg/mL methanol) was injected separately, and the data acquired by the photodiode array detector with the 3D feature were incorporated into the

Table 2. Selected Fruit Quality Measures of 'EG 2' Pomegranate Fruit on Several Harvest Dates along Season Cooling[a]

harvest date	fruit weight (g)	juice content (%)	TSS (%)	TA (mequiv DL^{-1})	total phenolics (pyrogallol, mg L^{-1})
7/11	281 ± 17 c	55 ± 3 a	15.2 ± 0.4 a	7.2 ± 0.6 a	1,167 ± 48 c
8/30	379 ± 29 b	39 ± 2 b	15.8 ± 0.2 a	5.5 ± 0.3 cd	1,195 ± 56 bc
10/24	442 ± 32 a	41 ± 4 b	14.7 ± 0.7 a	5.7 ± 0.2 c	1,289 ± 63 b
12/4	239 ± 35 d	39 ± 4 b	15.0 ± 0.6 a	6.5 ± 0.1 b	1,264 ± 94 bc
1/22	221 ± 2 d	34 ± 4 c	14.7 ± 1.0 a	5.0 ± 0.6 d	1,740 ± 103 a

[a] Values are average ± SD on eight replicates collected in the southern Arava during the years 2005, 2007 and 2008. Different letters (a, b, c, d) within a column represent significant differences ($p < 0.05$, Student's t test) between sampling dates.

system anthocyanin standard library. Under the conditions employed in this study, the retention time (in min) and wavelength of maximal absorbance (in nm) for Dp3,5, Cy3,5, Pg3,5, Dp3, Cy3 and Pg3 were 4.1 and 520, 6.2 and 512, 9.4 and 497, 11.0 and 522, 16.3 and 515, and 19.7 and 501, respectively. Relative standard deviation (RSD) for the retention times in repetitive runs was in the range of 0.4−1.9%.

Individual anthocyanins were quantified from the corresponding chromatogram peak area calculated by the software. Calibration curves (linear, $R^2 = 0.9999$) were constructed with standards for each of the above six anthocyanins at four concentrations (0.01, 0.10, 0.25, and 0.50 mg mL^{-1}). RSD for peak areas in multiple runs was within 0.4−3.2%. A detection limit (minimal peak area) was set at 50,000. Total anthocyanin concentration was calculated as the sum of concentrations of the individual anthocyanins.

Statistical Analysis. All data are reported as means ± their respective standard deviations. Variables were analyzed with Student's t test (SPSS for Windows version 14.02, SPSS Inc., 1989−2005) to check for significant differences ($p < 0.05$) between harvest dates.

RESULTS

Arils' Anthocyanin Content and Profile in Summer and Winter Fruit of the Deciduous 'PG 128-29' Pomegranate Accession. 'PG 128-29' is an early ripening deciduous pomegranate accession with sweet and intense red-colored arils. The harvest season of 'PG 128-29' in the Israeli southern Arava Valley starts at the beginning of July; under appropriate cultivation management the trees continue to produce newly ripened fruit until winter.[23] Certain physical and chemical properties of 'PG 128-29' fruit, such as external and internal color, fruit weight and juice content, total phenolic content and antioxidative capacity, sugar content and organic acid composition, are sensitive to seasonal[23] and geographical[24] variations.

Ripe fruit were collected during the second and third weeks of July (mid-July) and December (mid-December). Sample collection and analysis was repeated in the years 2005 and 2007. Selected quality measurements and the analysis of arils' anthocyanin composition are presented in Table 1 and Figures 1−3. The values are the average ± standard deviation of measurements taken on fruit replicates from the two years of sampling. The average maximal and minimal temperatures during ripening were ∼38 and 23 °C and ∼24 and 12 °C for fruit harvested in mid-July and mid-December, respectively.

Ripening season did not influence fruit weight and arils' juice TA (Table 1); small yet significant ($p < 0.05$, Student's t test) decrease in fruit juice content and increase in TSS and total phenolics concentration were measured in winter fruit.

Anthocyanin composition in the arils of mid-July and mid-December fruit, as determined by RP-HPLC, showed strikingly different peak profiles, suggesting distinct composition and accumulation of pigment in ripe fruit from the two seasons

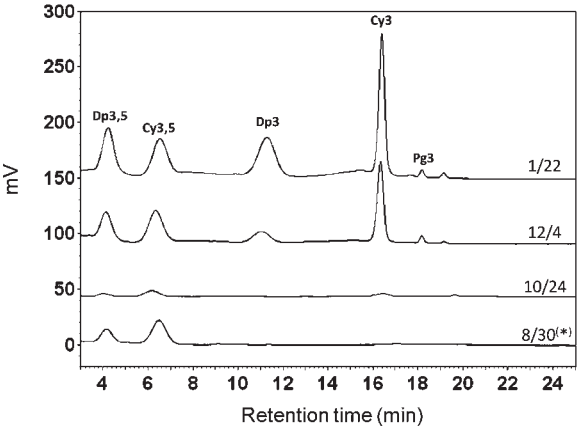

Figure 6. HPLC chromatograms at 520 nm of methanolic extracts of 'EG 2' fruit arils from summer, autumn and winter harvests. Identified anthocyanin peaks are marked by names. (*) Extract from selected colored arils.

(Figure 1). The anthocyanin profile in arils of mid-July fruit contained peaks corresponding to Dp3,5, Cy3,5 and Cy3. Arils from mid-December fruit contained in addition to the latter also Dp3 and Pg3, resembling the anthocyanin profile reported for pomegranates grown in the Mediterranean, Iran and Uzbekistan.[12,13,19,20]

The concentrations of the individual anthocyanins were evaluated from the corresponding chromatograms' peak areas and the relevant calibration curve, as previously described. Total anthocyanin level was calculated by their summation. Figures 2 and 3 depict the contents of total and individual anthocyanins as well as the classes of anthocyanins in units of weight concentration (mg mL^{-1}, Figure 2) and relative abundance (% of the total anthocyanin content, Figure 3). The values of weight concentration varied considerably within fruit replicates from the same harvest season and different sampling years, as manifested by the sizable error bars, especially in the pale fruit of mid-July (Figure 2). This variability was expected with replicate fruit from different trees and/or locations in the orchard since the fruit color is sensitive to the microenvironment.[12,13,23,24,30] Moreover, natural climate fluctuations prevailed between the two sampling years. Despite the substantial deviations in concentrations, the anthocyanin profile in the different replicates was largely consistent, as reflected in the much smaller error bars in Figure 3 compared to Figure 2. Presentation of the anthocyanin composition in terms of relative abundance was thus exceedingly valuable in substantiating the interpretation of the concentration measurements.

The total anthocyanin concentration in the arils of mid-July fruit was notably lower than that measured in mid-December (Figure 2A) and consisted of mostly Dp3,5 and Cy3,5 at comparable levels (Figures 2A and 3A). A minor amount of Cy3 was

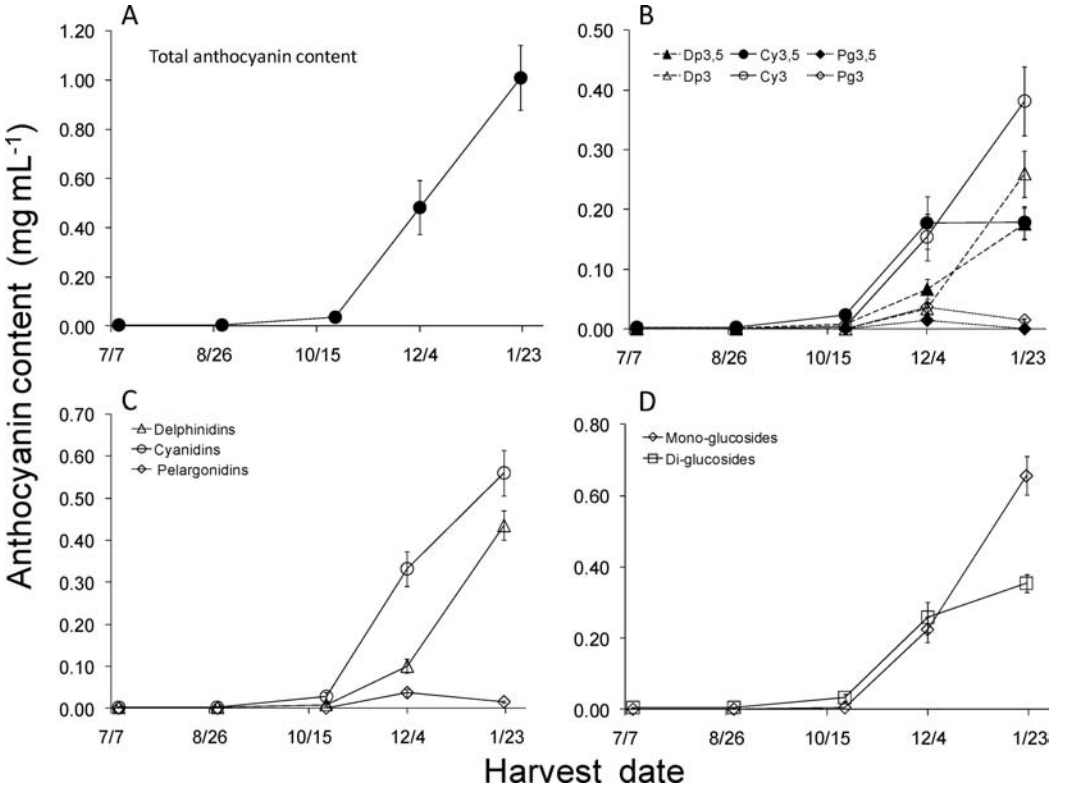

Figure 7. Anthocyanin composition in the arils of 'EG 2' pomegranate fruit harvested from early summer to midwinter. Concentration of total (A) and individual (B) anthocyanins; delphinidins, cyanidins and pelargonidins (C); mono- and diglucosides (D). Each data point represents the average and standard deviation of five to seven replicates collected during the years 2005, 2007 and 2008.

also detected (<0.010 mg mL^{-1}, never to exceed 6% of the total anthocyanin content). The arils of mid-December fruit contained significant amounts of Dp3,5, Cy3,5, Dp3, Cy3 and Pg3.

Cyanidins were slightly more abundant than delphinidins in arils of mid-July fruit and in a significantly higher proportion than delphinidins and pelargonidins in arils of mid-December fruit (Figures 2B and 3B).

Nearly all the anthocyanins were in the form of diglucosides in arils of mid-July fruit whereas most of the anthocyanins of mid-December fruit arils (~69%) were in the form of monoglucosides (Figures 2C and 3C).

In summary, the anthocyanins in arils of mid-July and mid-December fruit markedly differed in content, profile and level of glucosylation. Mid-July arils contained a much lower concentration of anthocyanins, mostly diglucosides of delphinidin and cyanidin in comparable amounts. Mid-December arils, on the other hand, contained high proportions of monoglucosides of delphinidin and cyanidin. The level of cyanidins significantly exceeded that of delphinidins, and a small amount of Pg3 was detected as well.

Arils' Anthocyanin Composition in Summer Fruit of the Deciduous Pomegranate Accession 'PG 130-31' from Desert and Mediterranean Orchards. 'PG 130-31' ('Shani-Yonay'[28]) is an early ripening deciduous pomegranate accession with sweet red colored arils. Certain physical and chemical properties of 'PG 130-31' fruit are sensitive to orchard geographical location.[24]

'PG 130-31' fruit from summer harvests in the southern Arava Valley (July, desert climate) and Newe Ya'ar Research Center (September, Mediterranean climate) were sampled repeatedly in the years 2005 (both sites), 2007 (Newe Ya'ar) and 2008 (southern Arava). Anthocyanin composition in the arils is presented in Figures 4 and 5. The average maximal and minimal temperatures during ripening of fruit collected from Newe Ya'ar and the southern Arava were approximately 33 and 21 °C and 38 and 23 °C, respectively.

The concentration and relative abundance of individual anthocyanins as well as the classes of anthocyanins are depicted in Figures 4 and 5, respectively. As noted earlier for 'PG 128-29', anthocyanin concentration varied considerably among fruit replicates but the pigment profile was consistently uniform. The total anthocyanin concentration was significantly higher (~3.4-fold) in the arils of Newe Ya'ar fruit compared to that measured in the southern Arava fruit (Figure 4A). The concentrations and relative abundance of the individual anthocyanins in Newe Ya'ar fruit arils were in the order of Cy3,5 > Cy3 > Dp3,5 ≫ Pg3 ~ Dp3 > Pg3,5 (Figures 4A and 5A). Cy3,5 and Dp3,5 were the only anthocyanins present in measurable amounts in the southern Arava fruit arils; their concentrations were comparable.

Cyanidins' concentration and abundance in NeweYa'ar fruit arils were much higher compared to delphinidins, in contrast to the practically equal concentration of the two anthocyanin types in the southern Arava fruit arils (Figures 4B and 5B).

Newe Ya'ar fruit arils contained both mono- and diglucosylated anthocyanins (at a ratio of approximately 1:2) whereas practically all the anthocyanins in the southern Arava fruit were in the form of diglucosides (Figures 4C and 5C).

In summary, anthocyanin content in Newe Ya'ar summer fruit arils was much higher than in the southern Arava fruit and included Dp3,5, Cy3,5, Pg3,5, Dp3, Cy3 and Pg3 while the southern Arava fruit contained only Dp3,5 and Cy3,5. Cyanidins

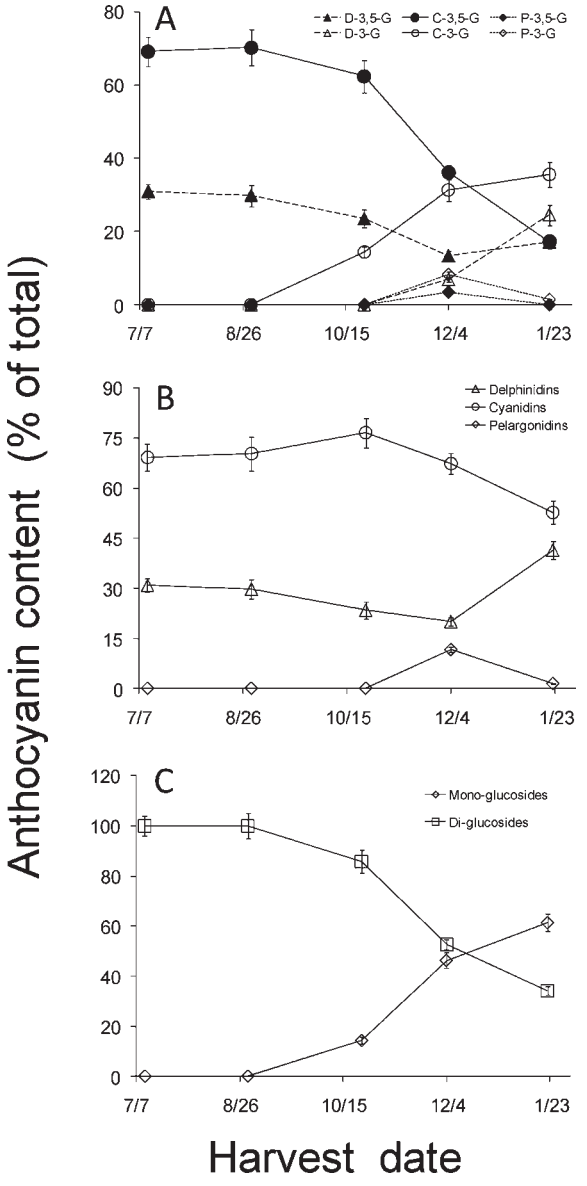

Figure 8. Anthocyanin relative abundance in the arils of 'EG 2' pomegranate fruit harvested from early summer to midwinter. Individual anthocyanins (A); delphinidins, cyanidins and pelargonidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of five to seven replicates collected during the years 2005, 2007 and 2008.

were the predominant anthocyanin group in Newe Ya'ar fruit, and over one-third of the anthocyanins were in the form of monoglucosides. In the southern Arava fruit comparable levels of diglucosylated cyanidins and delphinidins prevailed.

Anthocyanin Composition of 'EG 2' Pomegranate Fruit Arils with Progression from Hot to Cool Season. 'EG 2' is an evergreen pomegranate accession with sweet and intense red colored arils. In the climate of the Israeli southern Arava Valley the plant flowers and produces fruit during most of the year (M. Harari, unpublished results).

'EG 2' fruit quality and arils' anthocyanin composition were monitored from mid-July to the end of January, i.e. parallel to season cooling from ~40 to 7 °C. Table 2 presents selected quality measures of the fruit harvested on 7/11 (early summer), 8/30 (late summer), 10/25 (autumn), 12/4 (early winter), and 1/22 (midwinter) during the years 2005, 2007, and 2008. It should be noted that in the southern Arava the average maximal and minimal temperatures in October (autumn) are still relatively high, ~32.2 and 18.7 °C, respectively.[23] Fruit that ripened in early summer and during the winter were significantly smaller ($p < 0.05$, Student's t test) than late summer and autumn ripened fruit. Early summer fruit had the highest juice content and TA whereas midwinter fruit contained the highest level of total phenolics. TSS was not significantly affected by the ripening season.

Figure 6 portrays representative HPLC chromatograms at 520 nm of methanolic extracts from arils of 'EG 2' fruit harvested on selected dates from summer to midwinter. Marked differences in peak profile and size were detected between chromatograms of fruit from the different seasons, indicative of the significant effect of climate on anthocyanin composition in 'EG 2' fruit arils. The anthocyanin profile in arils of summer fruit consisted of virtually only the diglucosides Dp3,5 and Cy3,5. Autumn fruit's profile included also a low level of Cy3, and winter fruit contained significant amounts of Dp3,5, Cy3,5, Dp3 and Cy3, a small quantity of Pg3 and a minute quantity of Pg3,5 that is discernible at higher chromatogram amplifications (not shown).

Anthocyanin composition and relative abundance are presented in Figures 7 and 8, respectively. Similarly to the previous accessions, anthocyanins' concentration varied considerably within fruit replicates but their profile was highly consistent.

The total anthocyanin concentration (Figure 7A) was very low in arils of summer fruit (~0.005 mg mL^{-1}) and slightly higher in autumn fruit. Anthocyanin accumulation was exceedingly higher in winter harvests, reaching levels of around 1 mg mL^{-1} in midwinter (over 200-fold increase compared to summer fruit).

The content and relative abundance of individual anthocyanins in the fruit arils' juice are presented in Figures 7B and 8A. Minute amounts of Dp3,5 and Cy3,5 were detected in summer fruit arils (7/11 and 8/30). To obtain workable chromatograms for the analysis of the relative abundance of different anthocyanins in the arils of summer fruit, more concentrated extracts were prepared from juice made of selected colored arils. Cy3,5 was ~2.2-fold more abundant than Dp3,5. Autumn fruit arils contained Dp3,5 and Cy3,5 (at a ratio approximately 1:2.7), and a small amount of Cy3 which amounted to approximately 14% of the total anthocyanins. In winter, the contents of Dp3,5, Cy3,5 and Cy3 markedly increased and additional anthocyanins, Dp3, Pg3 and Pg3,5, accumulated as well. Progressing from early winter to midwinter was accompanied by marked increases in the contents of Dp3,5, Dp3 and Cy3.

Figures 7C and 8B portray the concentration and relative abundance of delphinidins, cyanidins and pelargonidins in fruit arils along the sampling period. Accumulation of both cyanidins and delphinidins increased while progressing from summer to early winter harvests, with cyanidins being considerably in excess (67−76% of the total anthocyanins). On the other hand, proceeding from early to midwinter, the rate of accumulation of delphinidins was faster than that of cyanidins and the two groups reached comparable concentrations. Pelargonidins accumulated only in winter fruit arils and only in small amounts.

The level of anthocyanin glucosylation was highly dependent on harvest date (Figures 7D and 8C). With the advancement in season the contents of both mono- and diglucosylated anthocyanins increased, however, while all the anthocyanins in summer arils were in the form of diglucosides, the proportion of diglucosides decreased with season cooling and by midwinter the

Table 3. Selected Fruit Quality Measures of 'EG 1' Pomegranate Fruit on Several Harvest Dates along Season Warming[a]

harvest date	fruit weight (g)	juice content (%)	TSS (%)	TA (mequiv dL^{-1})	total phenolics (pyrogallol, mg L^{-1})
12/12	390 ± 48 c	37 ± 4 b	15.0 ± 0.5 ab	6.0 ± 0.2 a	1,160 ± 112 ab
3/26	495 ± 35 b	40 ± 3 b	14.4 ± 0.8 b	4.9 ± 0.3 c	1,324 ± 153 b
4/18	626 ± 109 a	40 ± 5 ab	14.6 ± 0.4 b	5.4 ± 0.2 b	1,232 ± 58 ab
4/25	581 ± 34 a	37 ± 3 b	14.9 ± 0.4 ab	5.3 ± 0.4 bc	1,014 ± 98 bc
5/6	544 ± 66 ab	39 ± 1 b	14.3 ± 0.9 ab	4.8 ± 0.2 b	1,165 ± 332 ab
5/16	627 ± 150 a	45 ± 4 a	15.1 ± 0.2 a	4.1 ± 0.5 d	957 ± 102 bc
5/28	597 ± 107 a	46 ± 6 ab	14.8 ± 0.7 ab	4.6 ± 0.9 cd	948 ± 112 c
8/30	612 ± 85 a	42 ± 4 ab	15.1 ± 0.6 ab	4.5 ± 0.4 cd	986 ± 95 bc

[a] Values are average ± SD on eight replicates collected in the southern Arava during the years 2006 and 2007. Different letters (a, b, c, d) within a column represent significant differences ($p < 0.05$, Student's t test) between sampling dates.

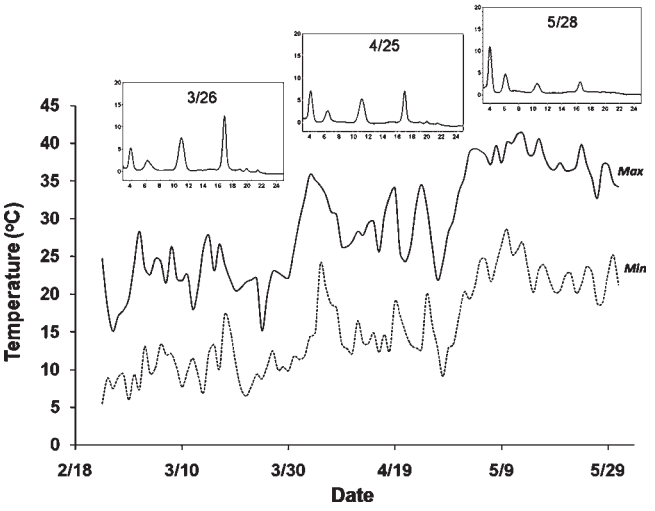

Figure 9. Spring three-day-average thermograms and HPLC chromatograms of 'EG 1' fruit arils' extracts at 520 nm. Chromatograms are marked by the corresponding harvest dates.

majority of the pigment (>60%) was in the form of monoglucosides.

In summary, anthocyanin concentration was very low in the summer, somewhat higher in autumn and substantially higher in winter fruit arils. Delphinidins and cyanidins were present throughout the whole study period; small levels of pelargonidins were detected only in winter fruit. Cyanidins were always the most abundant anthocyanins; however, during winter the accumulation rate of delphinidins superseded that of cyanidins. Summer arils' anthocyanins were all diglucosylated. The content of monoglucosides increased with climate cooling reaching over 60% by midwinter.

Anthocyanin Composition of 'EG 1' Pomegranate Fruit Arils with the Progression from Cool to Hot Season. 'EG 1' is an evergreen pomegranate accession with sweet and light red colored arils. Similarly to 'EG 2', accession 'EG 1' flowers and produces fruit year round in the Israeli southern Arava (M. Harari, unpublished results). 'EG-1' fruit quality and arils' anthocyanin composition were studied during the years 2006 and 2007 from early winter, during spring and in late summer, i.e. parallel to season warming from ~7 to 40 °C. The results are summarized in Table 3 and Figures 9−11.

Table 3 presents selected quality measures of the fruit harvested in early winter (12/12), early to late spring (3/23−5/28), and late summer (8/30). Early winter fruits were significantly smaller and with the highest TA ($p < 0.05$, Student's t test). No significant effects of ripening season on the fruit juice content, TSS and total phenolics were found.

The anthocyanin HPLC chromatogram profiles changed significantly along the sampling period. Figure 9 portrays the changes in temperature in the southern Arava during spring, from the beginning of March through the end of May, with representative chromatograms from three sampling dates. With the progression from early to late spring, peaks corresponding to the diglucosides Dp3,5 and Cy3,5 increased with a concomitant decrease in the corresponding monoglucosides peaks.

Figures 10 and 11 display anthocyanin composition and relative abundance for all the sampling dates.

The highest level of total anthocyanins was measured in arils of winter fruit (Figure 10A). In early spring it was approximately 35% lower and continued to decrease stepwise with spring progression. The level of anthocyanins in summer arils was very low, similar to that measured in 'EG 2' fruit.

The content and relative abundance of individual anthocyanins are presented in Figures 10B and 11A. Winter arils contained significant amounts of Dp3 and Cy3, at 5- and 8-fold excess to the corresponding diglucosides. With season advancement toward and during spring the concentration of the diglucosides increased, especially that of Dp3,5, with a concomitant decrease in the concentration of the monoglucosides. In summer fruit only minute amounts of Dp3,5, Cy3,5 and Cy3 were detected in the arils. Cy3,5 was the most abundant anthocyanin, ~1.6- and 2.0-fold that of Dp3,5 and Cy3, respectively. The concentration of Pg3 was below resolution on all harvest dates.

The concentration and relative abundance of delphinidins and cyanidins along the sampling period are portrayed in Figures 10C and 11B. The concentrations of both anthocyanin groups decreased when proceeding from winter, through spring and to summer harvests. Cyanidins were in excess in early winter and summer fruit (~1.4- and ~2.5-fold, respectively). In contrast, during spring, fruit arils contained more delphinidins than cyanidins (from 1.3- to 1.9-fold in early to late spring, respectively).

The level of anthocyanin glucosylation was highly dependent on the harvest date (Figures 10D and 11C). With season advancement from winter throughout spring, the concentration of monoglucosides decreased whereas that of diglucosides gradually increased; the relative abundance of monoglucosides decreased during this period from ~87% to ~24%. In summer the concentrations of both mono- and diglucosides were very

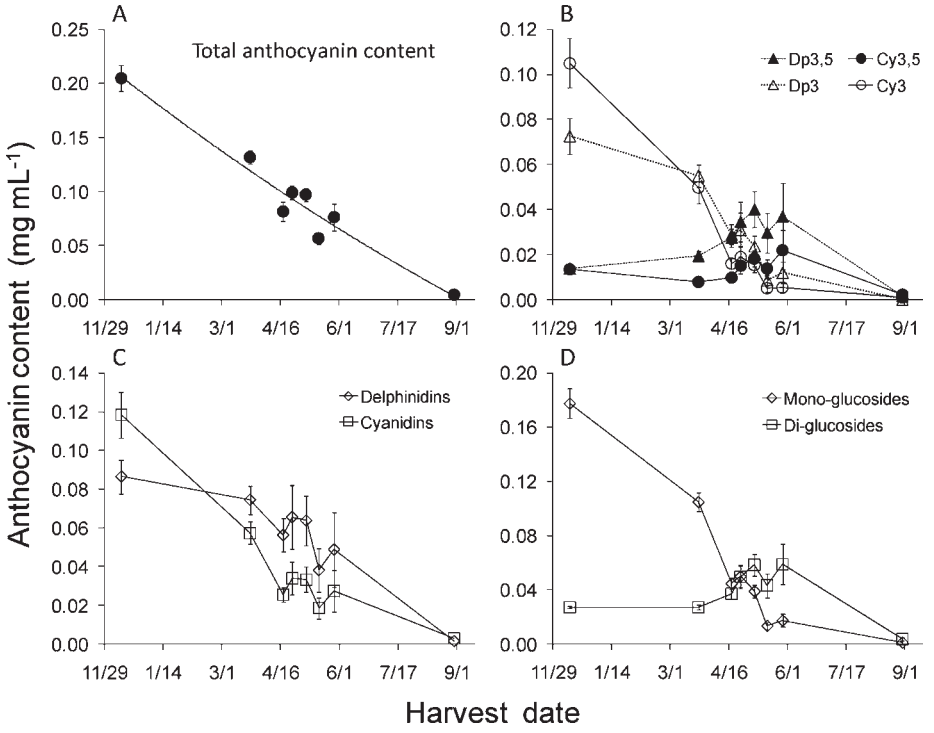

Figure 10. Anthocyanin composition in the arils of 'EG 1' pomegranate fruit harvested from early winter to late summer. Concentration of total (A) and individual (B) anthocyanins; delphinidins and cyanidins (C); mono- and diglucosides (D). Each data point represents the average and standard deviation of six replicates collected during the years 2006 and 2007.

low; their proportions remained similar to those measured at the end of spring.

To summarize, arils' anthocyanins during the entire studied period consisted of virtually only cyanidins and delphinidins. Total as well as monoglucosylated anthocyanin concentrations were the highest in the winter, decreased gradually during spring and almost diminished in late summer, whereas that of the diglucosides increased during spring and then decreased in summer. In winter fruit most of the anthocyanins were monoglucosylated. During spring the proportion of monoglucosides decreased gradually with a concomitant increase in that of diglucosides. In late spring and summer most of the anthocyanins were diglucosylated. Cyanidins were more abundant in early winter and summer fruit, however, during spring delphinidins were the most abundant anthocyanins.

DISCUSSION

The present report provides the first extensive account on the effect of climate, from cool to extreme, on the anthocyanins in the edible part of pomegranates, i.e. the fruit arils. A wide range of climate regimes (~7−40 °C) during fruit development, maturation and ripening was studied in both deciduous and evergreen pomegranate accessions, as well as orchards from Mediterranean and desert locations.

Climate effects on arils' anthocyanin accumulation and composition were substantial and consistent in the four pomegranate accessions studied. The level of anthocyanin accumulation varied inversely to the season's temperatures, similarly to the trend reported for other plant systems.[25−27] The detrimental effect of temperature on pomegranate anthocyanin accumulation over other seasonal variations, such as length of daylight period and radiation level, was implied by our previous studies.[23,24]

Delphinidins and cyanidins were the major anthocyanins in the arils of the four accessions. Pelargonidins were detected only in winter fruit and always in small concentrations. The relative proportions of delphinidins and cyanidins were accession and season dependent. Cyanidins were usually in excess to delphinidins except for in the arils of summer 'PG 129-28' fruit, where the two anthocyanin types were present in comparably low concentrations, and spring 'EG 1' fruit, where delphinidins were in excess. In that context, it is worth noting that the rate of accumulation of delphinidins in 'EG 2' fruit arils surpassed that of cyanidins when advancing from early winter to midwinter. The latter observation together with the results on spring 'EG 1' fruit arils suggests that cool temperatures during fruit development may enhance the accumulation of delphinidins, which are more temperature-labile than cyanidins.[31] Possible contribution of shorter daylight period cannot, however, be ruled out. Interestingly, a recent study on bilberries[32] reports on preferential accumulation of delphinidins over other anthocyanidins in plants grown at Northern latitudes or from Northern parental origin. Variations in the relative proportions of delphinidins and cyanidins influence the aril's hue; delphinidins contribute a deep purplish shade whereas cyanidins impart bright red tone. Moreover, delphinidin is a more potent antioxidant than cyanidin.[33]

The degree of arils' anthocyanin glucosylation was also highly dependent on the climate conditions. The majority of the anthocyanins were monoglucosylated in the winter. The proportion of diglucosylated anthocyanins increased with seasonal warming, and in summer fruit most or all of the pigment molecules were diglucosylated. The results in our study are in agreement with the temperature stabilizing effect attributed to anthocyanin glucosylation.[34,35]

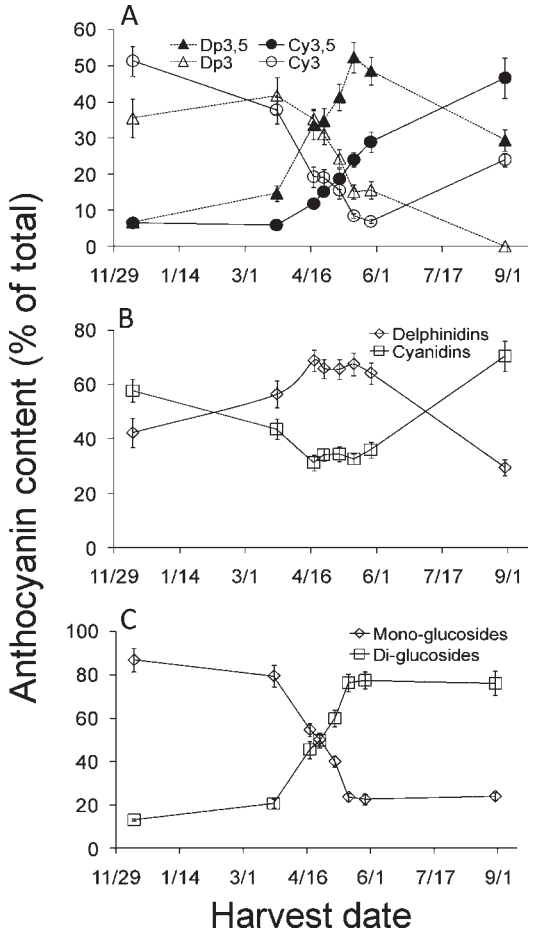

Figure 11. Anthocyanin relative abundance in the arils of 'EG 1' pomegranate fruit harvested from early winter to late summer. Individual anthocyanins (A); delphinidins and cyanidins (B); mono- and diglucosides (C). Each data point represents the average and standard deviation of six replicates collected during the years 2006 and 2007.

In view of the augmentation in "global warming" associated damages to fruit tree orchards, the findings of the current study on climate effects on anthocyanin accumulation and composition in the pomegranate fruit arils are especially important and can benefit the ongoing efforts to enhance the fruit quality by breeding and agricultural practices. The information obtained in this study supplements the recently acquired knowledge on the molecular biology of anthocyanin biosynthesis pathways in pomegranates (Holland et al., unpublished results) and may facilitate production of cultivars with improved color in warm climate. Moreover, cultivation approaches that influence the harvest season and include climate management during fruit development and ripening will assist growers in meeting market demands for fruit with intense internal red color.

AUTHOR INFORMATION

Corresponding Author

*Tel: +972-8-6355747. Fax: +972-8-6355730. E-mail: hamutalborochov@rd.ardom.co.il.

Notes

We thank the Jewish National Fund (KKL), the JCA Charitable Foundation and Israel Ministry of Agriculture for financing this study.

Present Addresses

§H.B.-N. is currently at the Vegetable and Fruit Improvement Center, Department of Horticultural Science, Texas A&M University, College Station, TX 77843-2119, USA. Tel: +1979-8451044. Fax: +1979-8624522.

ACKNOWLEDGMENT

The help of Efi Tripler with the statistical analysis is greatly appreciated. H.B.-N. also thanks the Vegetable and Fruit Improvement Center, Texas A&M University, for providing an opportunity to conduct studies during her sabbatical leave.

ABBREVIATIONS USED

Cy3, cyanidin 3-glucoside; Cy3,5, cyanidin 3,5-diglucoside; DDW, double distilled water; Dp3, delphinidin 3-glucoside; Dp3,5, delphinidin 3,5-diglucoside; Pg3, pelargonidin 3-glucoside; Pg3,5, pelargonidin 3,5-diglucoside; PJ, pomegranate juice; RSD, relative standard deviation; RP-HPLC, reverse phase high performance liquid chromatography; TA, titratable acidity; TSS, total soluble solids

REFERENCES

(1) *Pomegranates: Ancient Roots to Modern Medicine*, 1st ed.; Seeram, N. P., Schulman, R. N., Heber, D., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2006; 244 pp.

(2) Bell, C.; Hawthorne, S. Ellagic acid, pomegranate and prostate cancer - a mini review. *J. Pharm. Pharmacol.* **2008**, *60*, 139–144.

(3) de Nigris, F; Balestrieri, M. L.; Williams-Ignarro, S.; D'Armiento, F. P.; Fiorito, C.; Ignarro, L. J.; Napoli, C. The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats. *Nitric Oxide* **2007**, *17*, 50–54.

(4) Davidson, M. H.; Maki, K. C.; Dicklin, M. R.; Feinstein, S. B.; Witchger, M.; Bell, M.; McGuire, D. K.; Provost, J. C.; Liker, H.; Aviram, M. Effects of consumption of pomegranate juice on carotid intima-media thickness in men and women at moderate risk for coronary heart disease. *Am. J. Cardiol.* **2009**, *104*, 936–942.

(5) Seeram, N. P.; Zhang, Y.; Heber, D. Commercialization of pomegranates: fresh fruit, beverages, and botanical extracts. In *Pomegranates: Ancient Roots to Modern Medicine*, 1st ed.; Seeram, N. P., Schulman, R. N., Heber, D., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2006; pp 187−198.

(6) Holland, D.; Hatib, K.; Bar-Ya'akov, I. Pomegranate: Botany, Horticulture, Breeding. *Hortic. Rev.* **2009**, *35*, 127–191.

(7) Gil, M. I.; Tomas-Barberan, F. A.; Hess-Pierce, B.; Holcroft, D. M.; Kader, A. A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* **2000**, *48*, 4581–4589.

(8) Murthy, K. N. C.; Jayaprakasha, G. K.; Singh, R. P. Studies on Antioxidant Activity of Pomegranate (*Punica granatum*) Peel Extract Using in Vivo Models. *J. Agric. Food Chem.* **2002**, *50*, 4791–4795.

(9) Seeram, N. P.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68.

(10) Seeram, N. P.; Adams, L. S.; Henning, S. M.; Niu, Y.; Zhang, Y.; Nair, M. G.; Heber, D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J. Nutr. Biochem.* **2005**, *16*, 360–367.

(11) Seeram, N.; Lee, R.; Hardy, M.; Heber, D. Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. *Sep. Purif. Technol.* **2005**, *41*, 49–55.

(12) Gil, M. I.; Garcia-Viguera, C.; Artes, F.; Tomas-Barberan, F. A. Changes in pomegranate juice pigmentation during ripening. *J. Sci. Food Agric.* **1995**, *68*, 77–81.

(13) Gil, M. I.; Cherif, J.; Ayed, N.; Artes, F.; Tomas-Barberan, F. A. Influence of cultivar, maturity stage and geographical location on the juice pigmentation of Tunisian pomegranates. *Z. Lebensm.-Unters. Forsch.* **1995**, *201*, 361–364.

(14) Hernandez, F.; Melgarejo, P.; Tomas-Barberan, F. A.; Artes, F. Evolution of juice anthocyanins during ripening of new selected pomegranate (*Punica granatum*) clones. *Eur. Food Res. Technol.* **1999**, *210*, 39–42.

(15) Perez-Vincente, A.; Gil-Izquierdo, A.; Garcia-Viguera, C. In vitro gastro-intestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *J. Agric. Food Chem.* **2002**, *50*, 2308–2312.

(16) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.

(17) Du, C. T.; Wang, P. L.; Francis, F. J. Anthocyanins of pomegranate, *Punica granatum*. *J. Food Sci.* **1975**, *2*, 417–418.

(18) Noda, Y.; Kaneyuki, T.; Mori, A.; Packer, L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *J. Agric. Food Chem.* **2002**, *50*, 166–171.

(19) Mavlyanov, S. M.; Islambekov, S. Y.; Karimdzhanov, A. K.; Ismailov, A. I. Polyphenols of the fruits of some varieties of pomegranate growing in Uzbekistan. *Chem. Nat. Compd.* **1997**, *33*, 98–99.

(20) Alighourchi, H.; Barzegar, M.; Abbasi, S. Anthocyanins characterization of 15 Iranian pomegranate (*Punica granatum* L.) varieties and their variation after cold storage and pasteurization. *Eur. Food Res. Technol.* **2008**, *227*, 881–887.

(21) Tzulker, R.; Glazer, I.; Bar-Ilan, I.; Holland, D.; Aviram, M.; Amir, R. Antioxidant activity, polyphenol content and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. *J. Agric. Food Chem.* **2007**, *55*, 9559–9570.

(22) Holland, D.; Bar-Ya'akov, I. The pomegranate: new interest in an ancient fruit. *Chron. Hortic.* **2008**, *48*, 12–15.

(23) Borochov-Neori, H.; Judeinstein, S.; Tripler, E.; Harari, M.; Greenberg, A.; Shomer, I.; Holland, D. Seasonal and cultivar variations in antioxidant and sensory quality of pomegranate *(Punica granatum L.)* fruit. *J. Food Compos. Anal.* **2009**, *22*, 189–195.

(24) Schwartz, E.; Zulker, R.; Glazer, I.; Bar-Ya'akov, I.; Wiesman, Z.; Tripler, E.; Bar-Ilan, I.; Fromm, H.; Borochov-Neori, H.; Holland, D.; Amir, R. Environmental conditions affect the color, taste and antioxidant capacity of 11 pomegranate accessions fruits. *J. Agric. Food Chem.* **2009**, *57*, 9197–9209.

(25) Oren-Shamir, M. Does anthocyanin degradation play a significant role in determining pigment concentration in plants? *Plant Sci.* **2009**, *177*, 310–316.

(26) Tarara, M. J.; Lee, J.; Spayd, S. E.; Scagel, C. F. Berry temperature and solar radiation alter acylation, proportion and concentration of anthocyanins in Merlot grapes. *Am. J. Enol. Vitic.* **2008**, *59*, 235–247.

(27) Mori, K.; Goto-Yamamoto, N.; Kitayama, M.; Hashizume, K. Loss of anthocyanins in red-wine grapes under high temperature. *J. Exp. Bot.* **2007**, *58*, 1935–1945.

(28) Holland, D.; Hatib, K.; Bar-Ya'akov, I.; Yonay, E.; Abd El Hadi, F. 'Shani-Yonay' Pomegranate. *HortScience* **2007**, *42*, 710–711.

(29) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(30) Ben-Arie, R.; Segal, N.; Guelfat-Reich, S. The maturation and ripening of the 'wonderful' pomegranate. *J. Am. Soc. Hort Sci.* **1984**, *109*, 898–902.

(31) Hrazdina, G.; Borzell, A. J.; Robinson, W. B. Studies on the Stability of the Anthocyanidin-3,5-Digucosides. *Trends Food Sci. Technol.* **2010**, *21*, 3–11.

(32) Åkerstrom, A.; Jaakola, L.; Bång, U.; Jäderlund, A. Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *Vaccinium myrtillus* L. (Bilberries). *J. Agric. Food Chem.* **2010**, *58*, 11939–11945.

(33) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.

(34) Jackman, R. I.; Smith, J. I. Anthocyanins and betalains. In *Natural Food Colorants*, 2nd ed.; Hendry, G. A. F., Houghton, J. D., Eds.; Chapman & Hall: London, U.K., 1996; pp 244−309.

(35) Welch, C. R.; Wu, Q.; Simon, J. E. Recent advances in anthocyanin analysis and characterization. *Curr. Anal. Chem.* **2008**, *4*, 75–101.