Comparison of the polyphenolic composition and antioxidant activity of European commercial fruit juices

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Thirty six commercial European fruit juices were tested to ascertain their antioxidant capacity and polyphenolic composition. Six of the products were labelled 100% pomegranate juice, the others included 20 brands of diluted pomegranate juice or pomegranate blended with other fruit juices and 10 different non-pomegranate fruit juices. The antioxidant capacity of all the juices was determined while anthocyanin, ellagitannin and ellagic acid profiles of the 26 pomegranate juices and pomegranate juice blends were obtained using HPLC-PDA-MS^2. Additional analysis was conducted on seven of the juices using HPLC with an on-line antioxidant detection system. Three of the "pure" pomegranate juices had the highest ellagitannin content and the highest antioxidant capacity. Only one of these three juices was rich in anthocyanins. The other "pure juices" had differences in their HPLC "pomegranate" fingerprint and also had a lower antioxidant capacity, in some cases lower than that of some of the blended juices. Vitamin C rather than phenolic compounds was the major contributor to the antioxidant capacity for some of the juices. Statistical analysis of both the antioxidant assay and the HPLC on-line antioxidant data demonstrated that the ellagitannins were the major antioxidants in the pomegranate juices. The complexity of the polyphenolic profile of pomegranates necessitates the use of HPLC-PDA-MS^2 for a thorough evaluation of juice composition and authenticity.

1 Introduction

The evidence that diets rich in fruits and vegetables provide a reduced risk of chronic diseases is compelling.[1] Flavonoids and related phenolic compounds that occur in plant-derived foods have been associated with these protective effects. As a consequence of the substantial research in this area, fruit juices are being used increasingly by people who are looking for healthy options as part of the WHO 5-a-day dietary recommendations. This is reflected in a steady global rise in fruit juice consumption. Western Europe is the second largest regional market.[2] Of the ten countries with the highest per capita consumption, six are found within this region with a consumption of more that 28 liters/person/year. Interest in pomegranate (*Punica granatum* L.) juice and its products has also increased markedly in recent years with a growing number of reports on their potential health benefits. These include pomegranate juice consumption being associated with inhibition of prostrate cancer in men,[3] a reduction in serum oxidative stress in plasma of type-2 diabetes mellitus patients,[4] reduced atherosclerosis in diabetic patients,[5] and potential protection against colon cancer.[6]

There is enormous variability in antioxidant (AOX) activity and phenolic compounds present in different commercial fruit juices.[7,8] Some products were of questionable authenticity with the actual ingredients not matching what was claimed on the label. Pomegranates are characterized by the presence of ellagitannins and anthocyanins. However, the levels vary in juices prepared from different pomegranate cultivars,[9] maturity stage,[9,10] and they are even absent in some commercial products.[7,11] Zhang *et al.*[12] used a combination of analytical procedures to develop an "International Multidimensional Authenticity Specification" (IMAS) algorithm to detect a diversity of adulterants of pomegranate juices and drinks.

This paper compares 36 European commercial juices derived from pomegranates, and in some instances other fruits, by measuring their total AOX capacity. HPLC-PDA-MS^2 was used to obtain fingerprints of pure pomegranate juices and blended pomegranate products to ascertain their composition. In addition, HPLC with on-line AOX detection was used to assess the relationship between the ellagitannin, ellagic acid and anthocyanin content of pomegranate juices and their AOX capacity.

2 Results and discussion

2.1 AOX capacity and vitamin C levels

Thirty six juices (Table 1) were investigated initially using the Folin-Ciocalteu assay for total phenols (TP)[13] and three different AOX assays. The FRAP[14] and TEAC[15] AOX assays are simple colorimetric methods based on a single electron transfer reaction and it is assumed that the antioxidant activity is equal to the reducing capacity. The ORAC assay quantifies the peroxyl radical scavenging capacity.[16] In terms of overall ranking of the AOX capacity and TP content of the individual juices the four assays yielded very similar results (Table 2). However, for comparative purposes an AOX index was calculated using procedures described by Seeram *et al.*[8] (AOX index = [(sample score/best score) × 100]) which gives a composite score taking into account the results obtained with the different methods. The

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Table 1 List of commercial juices analyzed with the ingredients shown in the labels[a]

Code	Name	Ingredients (as per label)
PG01[+]	BIONA Organic Pomegranate	Pomegranate (100%)
PG02[+]	POM Wonderful	Pomegranate (100%)
PG03[+]	Rabenshorst Granatapfel	Pomegranate (100%)
PG04[+]	Pomegreat Pure	Pomegranate (100%)
PG05[+]	Marks & Spencer Pure Pomegranate Juice	Pomegranate (100%)
PG06[+]	gn & r, Pur Jus de Grenade	Pomegranate (100%)
PG07*	Sainsbury's Pomegranate & Blueberry	Pomegranate (25%), blueberry (5%)
PG08*	Pomegreat Ruby	Pomegranate (32%), aronia (5%)
PG09*	Pomegreat de Originale	Pomegranate (30%), grapes (2%), fruit extract, vitamins C & E
PG10*	Pomegreat Sapphire	Pomegranate (28%), blueberry (4%), aronia (4%)
PG11*	Chiquita	Pomegranate (7%), raspberry (18.5%), banana, orange, lemon, grapes
PG12[#]	Welch's Purple Grape	Purple grape
PG13*	Breaking Wave Pomegranate Juice (Aldi)	Pomegranate, grape juice, aonia, berry juice
PG14*	Rubicon Pomegranate	Pomegranate (29%), aronia (7%)
PG15*	Pomegreat	Pomegranate (21%), white grapes (3%), elderberry (3%), acai (1.9%), grapefruit (0.5%), lime, vitamins C & E
PG16*	Healthy People	Pomegranate (30%), aronia (7%), vitamins C and E
PG17*	Pomegreat	Pomegranate (30%), red grape (7%), vitamins A, C and E, folic acid
PG18*	Pomegreat Granatapfel and Orange	Pomegranate (20%), mandarin (5%), orange juice (2%), elderberry (3.6%), red grape (0.5%), vitamins C & E
PG19[#]	Becker's Bester Roter Traubensaft	Red grape
PG20*	Sainsbury's Pomegranate Juice	Pomegranate (37%), vitamin C
PG21*	Amecke	Pomegranate, red and white grape, apple, red currant, cranberry, lemon
PG22[#]	Healthy People	Apple, acai, raspberries, red grapes, lemon
PG23[#]	Innocent Smoothie	Cranberry, yumberry, blackcurrant, orange
PG24*	Innocent Smoothie	Pomegranate (15%), blueberry (4%), acai (3%), banana, orange, grapes, lemon
PG25[#]	Eckes Roter Traubensaft	Red grape
PG26*	Ocean Spray Cranberry and Pomegranate	Pomegranate (14%), cranberry (10.5%), apple (6.5%), vitamin C
PG27*	Rauch Happy Day	Pomegranate (22%), aronia, apple, elderberry, vitamin C
PG28[#]	Innocent Smoothie	Guava, mango, goji, orange, apple
PG29*	Fruity King	Pomegranate (5%), grapes (55%)
PG30*	Applesientje Super Fruit	Pomegranate (9%), raspberries (3%), black currant (1.7%) cranberry (1%), strawberry (0.7%), apple, white grapes, vitamins C & E
PG31*	Coolbest Pomegranate	Pomegranate, raspberry, apple, lemon, blackcurrant
PG32[#]	Coolbest SeaBuckthorn	Kiwi, goji, orange
PG33*	Ribena (Really Light) Raspberry & Pomegranate	Pomegranate/raspberry (8%), vitamin C
PG34[#]	Healthy People	Goji, passionfruit, white grapes, pineapple
PG35[#]	Guanabana and appel	Soursop, apple, soy
PG36[#]	VIFIT yogurt	Passionfruit, goji

[a] [+] 100% Pomegranate juices, * reconstituted or blended pomegranate juices, [#] non-pomegranate fruit juices.

AOX index was very high (>95) for three of the 'pure' juices, PG01, PG02, PG03 while values of <54 were obtained for the remainder of the juices including the other three 'pure' pomegranate (PG04, PG05, and PG06) (Table 2).

The AOX capacity showed great variability not only among the "pure" pomegranate juices but also the "blended" group of samples. Two of the blended juices, PG07 and PG08, containing 25% and 32% of pomegranate, respectively, scored slightly higher than three of the 100% pomegranate juices (PG04, PG05, and PG06). Also of interest was PG20 which contained 37% pomegranate and had an AOX index of 27 which was lower than that of several juices including PG09 and PG10 which contained less pomegranate (Table 2). This may reflect dilution, adulteration and/or reconstitution factors associated with manufacture. It is, however, more difficult to explain in the context of juices from the same label company like PG04 (100% pomegranate; AOX index 49) *vs.* PG08 (32% pomegranate, 5% aronia; AOX index 51%) both from Pomegreat. The same applies for PG07 (25% pomegranate and 5% blueberry; AOX index 54) and PG20 (37% pomegranate; AOX index 27) from Sainsbury's. The levels of vitamin C in the blended products ranged from zero to 58 mg/100 ml in PG33. Vitamin C can influence the AOX activity as observed in the FRAP assay where removal of vitamin C with ascorbate oxidase resulted in a marked reduction in the AOX capacity of some of the blended products (Table 2). Most notable were PG33 where there was a 63% decline following treatment with ascorbate oxidase and PG14 where vitamin C made a 21.5% contribution to the FRAP AOX capacity (Table 2). It would appear that vitamin C is added to several of the juices during processing after pasteurisation and it is this supplementation, rather than the polyphenolic constituents of the fruit that boost the AOX capacity of the juice.

Very similar AOX profiles were detected with all four assays and when the data were analyzed statistically highly significant correlation values were obtained with the TP, FRAP, TEAC and ORAC assays (Table 3).

The analysis of the AOX capacity of juices for comparative purposes using simple colorimetric assays such as FRAP, TP and TEAC, as well as the more complex ORAC method, is of value as the data are well correlated (Table 3). However, more detailed HPLC-PDA-MS^2 analysis is required to investigate quality issues and the great variability shown between supposedly similar juices.

Table 2 Results for the AOX assays and vitamin C content in the 36 commercial European juices[a]

Code	Vit C (mg/100ml)	TP (mmol/L)	FRAP (mmol/L)	FRAP-VitC (mmol/L)	Vit C (%) Contribution	ORAC (mmol/L)	TEAC (mmol/L)	AOX index	Labelled pomegranate content
PG01^{+}	n.d.	20.1 ± 0.1	55.3 ± 0.2	n.a.	n.a.	83.7 ± 0.5	40.5 ± 1.7	98	100%
PG02^{+}	n.d.	20.7 ± 0.1	52.4 ± 0.6	n.a.	n.a.	85.8 ± 1.3	39.7 ± 1.8	98	100%
PG03^{+}	n.d.	19.9 ± 0.6	51.8 ± 0.3	n.a.	n.a.	82.7 ± 0.4	41.3 ± 2.7	96	100%
PG04^{+}	n.d.	10.8 ± 0.4	25.4 ± 0.3	n.a.	n.a.	40.7 ± 0.5	19.5 ± 0.4	49	100%
PG05^{+}	2.0 ± 0.0	10.7 ± 0.1	25.7 ± 0.3	24.5 ± 0.1	4.3 ± 2.1	34.5 ± 4.4	21.4 ± 2.3	47	100%
PG06^{+}	n.d.	10.3 ± 0.2	24.1 ± 1.1	n.a.	n.a.	35.2 ± 1.6	17.9 ± 2.1	44	100%
PG07*	2.0 ± 0.0	13.3 ± 0.2	34.4 ± 0.5	30.7 ± 0.0	10.6 ± 0.1	30.6 ± 1.2	25.9 ± 1.8	54	25%
PG08*	n.d.	11.9 ± 0.0	27.8 ± 0.7	n.a.	n.a.	34.2 ± 1.7	23.6 ± 1.6	51	32%
PG09*	6.1 ± 0.0	9.7 ± 0.1	24.7 ± 0.2	23.4 ± 0.1	5.4 ± 2.3	18.2 ± 2.2	20.5 ± 1.7	40	30%
PG10*	n.d.	9.1 ± 0.2	21.9 ± 0.3	n.a.	n.a.	28.2 ± 0.7	18.5 ± 2.0	40	28%
PG11*	1.4 ± 0.0	8.0 ± 0.2	18.3 ± 0.1	16.6 ± 0.4	9.3 ± 0.0	46.5 ± 4.3	13.2 ± 2.0	39	7%
PG12$^{\#}$	n.d.	8.8 ± 0.0	14.1 ± 0.4	14.1 ± 0.4	0	33.2 ± 4.9	10.6 ± 1.0	38	0%
PG13*	1.1 ± 0.1	6.7 ± 0.1	16.5 ± 0.1	16.0 ± 0.1	3.0 ± 0.3	37.8 ± 6.4	10.9 ± 1.4	33	n.s
PG14*	38.0 ± 0.1	9.3 ± 0.1	19.7 ± 0.2	15.5 ± 0.1	21.5 ± 1.3	17.6 ± 0.4	14.1 ± 1.3	32	29%
PG15*	4.9 ± 0.1	7.7 ± 0.0	18.5 ± 0.3	17.3 ± 0.1	6.7 ± 1.6	16.2 ± 3.6	13.5 ± 1.6	30	21%
PG16*	11.9 ± 0.1	7.5 ± 0.0	15.8 ± 0.4	14.2 ± 0.2	10.0 ± 0.7	22.5 ± 2.4	12.8 ± 1.7	30	30%
PG17*	1.5 ± 0.0	5.6 ± 0.1	13.4 ± 0.4	12.7 ± 0.0	5.7 ± 2.3	41.9 ± 1.0	8.5 ± 0.4	30	30%
PG18*	1.6 ± 0.0	7.5 ± 0.2	18.7 ± 0.2	18.0 ± 0.1	3.7 ± 0.0	15.5 ± 0.4	13.4 ± 1.5	30	20%
PG19$^{\#}$	n.d.	6.3 ± 0.0	9.9 ± 0.2	9.9 ± 0.2	0	26.5 ± 1.2	7.0 ± 0.4	28	0%
PG20*	7.1 ± 0.1	6.7 ± 0.0	17.4 ± 0.1	16.1 ± 0.6	7.9 ± 0.6	17.8 ± 1.1	11.5 ± 1.5	27	37%
PG21*	n.d.	7.7 ± 0.0	12.8 ± 0.2	n.a.	n.a.	20.6 ± 1.6	9.4 ± 0.8	27	n.s.
PG22$^{\#}$	n.d.	6.3 ± 0.0	11.6 ± 0.1	11.6 ± 0.1	0	20.1 ± 0.6	9.0 ± 0.5	27	0%
PG23$^{\#}$	26.4 ± 0.3	7.6 ± 0.1	12.7 ± 0.2	9.9 ± 0.1	22.4 ± 1.5	16.0 ± 3.4	8.6 ± 1.1	26	0%
PG24*	n.d.	6.8 ± 0.1	10.7 ± 0.4	n.a.	n.a.	22.5 ± 8.0	7.9 ± 0.8	24	15%
PG25$^{\#}$	n.d.	5.9 ± 0.0	9.5 ± 0.1	9.5 ± 0.1	0	19.1 ± 0.2	7.6 ± 0.7	24	0%
PG26*	48.2 ± 0.6	6.2 ± 0.0	14.8 ± 0.1	10.9 ± 0.0	26.7 ± 0.8	16.9 ± 0.4	8.4 ± 0.4	22	14%
PG27*	24.6 ± 0.2	6.0 ± 0.1	14.7 ± 0.1	11.6 ± 0.2	21.3 ± 0.7	16.7 ± 0.9	7.4 ± 0.3	22	22%
PG28$^{\#}$	31.2 ± 0.1	5.7 ± 0.2	10.3 ± 0.1	7.0 ± 0.0	32.1 ± 1.4	20.0 ± 0.5	6.3 ± 0.1	22	0%
PG29*	n.d	5.6 ± 0.0	8.7 ± 0.1	n.a.	n.a.	21.8 ± 0.8	7.2 ± 0.4	21	5%
PG30*	38.5 ± 0.2	5.7 ± 0.0	12.5 ± 0.1	7.9 ± 0.0	36.7 ± 1.4	16.9 ± 2.0	7.2 ± 0.5	20	9%
PG31*	21.4 ± 0.1	5.9 ± 0.0	13.3 ± 0.3	11.1 ± 0.2	16.6 ± 2.8	10.3 ± 1.6	8.1 ± 0.5	20	n.s.
PG32$^{\#}$	21.6 ± 0.1	3.3 ± 0.1	4.8 ± 0.0	2.8 ± 0.1	41.4 ± 0.2	16.0 ± 0.4	3.0 ± 0.2	14	0%
PG33*	58.0 ± 0.2	3.8 ± 0.0	10.5 ± 0.0	3.9 ± 0.0	63.0 ± 1.0	7.4 ± 0.8	5.3 ± 0.1	12	8%
PG34$^{\#}$	14.7 ± 0.2	2.4 ± 0.0	3.4 ± 0.0	1.7 ± 0.0	42.3 ± 1.0	14.2 ± 0.2	2.5 ± 0.2	11	0%
PG35$^{\#}$	16.9 ± 0.1	1.8 ± 0.0	2.8 ± 0.0	1.0 ± 0.1	65.5 ± 0.4	6.0 ± 0.5	1.6 ± 0.1	6	0%
PG36$^{\#}$	12.8 ± 0.0	1.2 ± 0.0	1.8 ± 0.0	0.5 ± 0.0	67.7 ± 0.0	4.4 ± 0.2	0.9 ± 0.2	4	0%

[a] The AOX index was calculated according to Seeram *et al.*[8] TP in gallic acid equivalents, FRAP in Fe^{+2} eq., ORAC and TEAC in trolox equivalents. n.d., - not detected; n.a. - not analysed; n.s. - not stated. $^{+}$ 100% Pomegranate juices, * reconstituted or blended pomegranate juices, $^{\#}$ non-pomegranate fruit juices.

Table 3 Pearson's correlation factor for the AOX activity of different assays[a]

	TP	FRAP	ORAC	TEAC
TP	1			
FRAP	0.986(***)	1		
ORAC	0.882(***)	0.876(***)	1	
TEAC	0.988(***)	0.989(***)	0.863(***)	1

[a] *** Correlation is significant at $p < 0.001$.

2.2 Qualitative HPLC-PDA-MS²-on-line AOX analysis of pomegranate juices

Polyphenolic compounds in the 100% pomegranate juices as well as the other pomegranate juices were analysed by HPLC-PDA-MS² and the identifications used to compile a "pomegranate fingerprint" to compare to those obtained from the blended drinks. At the same time, the AOX capacity contribution of the peaks were measured by an on-line ABTS system.[17] Fig. 1–7 show the 520, 280 and 720 nm traces for PG01- PG04, PG06, PG14 and PG33. The anthocyanin profile can be seen at 520 nm and the ellagitannins/ellagic acids at 280 nm while the 720 nm trace depicts the AOX activity associated with each peak.

A total of 17 compounds were identified in all 100% juices. The identities of the peaks numbered in the traces (Fig. 1–7) are summarised in Table 4, and their contribution to the ABTS AOX content is evaluated in Table 5.

Peak 1 (retention time [Rt] - 6.6 min) had a $[M - H]^-$ at *m/z* 1101 and, like peaks 6 and 7, produced MS² ions at *m/z* 781, 721, 601 and 301. On the basis of this fragmentation pattern, peak 1 is identified as a punicalagin-like compound. This type of compound has not been described before in pomegranate. This peak was the second major contributor to the ABTS AOX of the 'pure' juices ranging between 10.8% and 24.7% in PG03 and PG06 respectively.

Peaks 2 and 3 (Rts - 7.2 and 7.5 min) had a negatively charged molecular ion ($[M - H]^-$) at *m/z* 781 which fragmented, yielding a base peak at *m/z* 721 and other ions at *m/z* 601 and *m/z* 301, which are from gallagic acid and ellagic acid moieties. Based on the report of Tanaka *et al.*,[18] the fragmentation pattern and elution order identified these compounds as punicalins A and B. This is one of the typical ellagitannins in pomegranate and one of the major contributors to the AOX capacity with values between 11.7% to 20.9% (Table 5).

Peak 4 (Rt - 9.9 min, λ_{max} - 520 nm) was characterized by a positively charged molecular ion ($[M - H]^+$) at *m/z* 627 which produced two MS² fragment ions at *m/z* 465 and 303. This fragmentation pattern and the absorbance spectrum identified this compound as delphinidin-3,5-*O*-diglucoside, a known pomegranate component.[19]

Peak 5 (Rt - 10.5 min) had a $[M - H]^-$ at *m/z* 933 which yielded daughter ions at *m/z* 781, 721, 601 and 301. This fragmentation pattern in keeping with published data[19] identified this compound as 2-*O*-galloylpunicalagin. Its contribution to the AOX capacity is minor (2.5% and 3.8%) (Table 5).

Peaks 6 and 7 (Rts - 11.1 and 12.1 min) both had a $[M - H]^-$ at *m/z* 1083 which produced identical MS² fragments at *m/z* 781, 721, and 601. This fragmentation pattern identifies these compounds as punicalagins.[19]

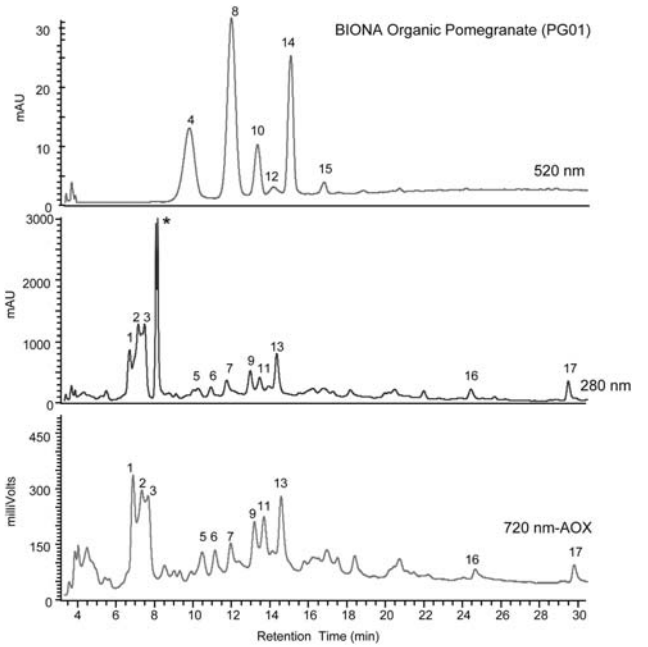

Fig. 1 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG01 [BIONA Organic Pomegranate] (see Table 1) with detection at 520 nm (anthocyanins), 280 nm (ellagitannins and ellagic acid derivatives) and 720 nm (AOX activity). *Peak 1* - punicalagin-like, *peak 2* - punicalin A, *peak 3* - punicalin B, *peak 4* - delphinidin-3,5-*O*-diglucoside, *peak 5* - 2-*O*-galloylpunicalagin, *peak 6* - punicalagin A, *peak 7* - punicalagin B, *peak 8* - cyanidin-3,5-*O*-diglucoside, *peak 9* - granatin A, *peak 10* - pelargonidin-3,5-*O*-diglucoside, *peak 11* - granatin B, *peak 12* - pelargonidin-3,5-*O*-diglucoside, *peak 13* - punicalagin isomer, *peak 14* - cyanidin-3-*O*-glucoside, *peak 15* - pelargonidin-3-*O*-glucoside, *peak 16* - ellagic acid-*O*-hexoside and *peak 17* - ellagic acid. For identification of peaks see Table 4.

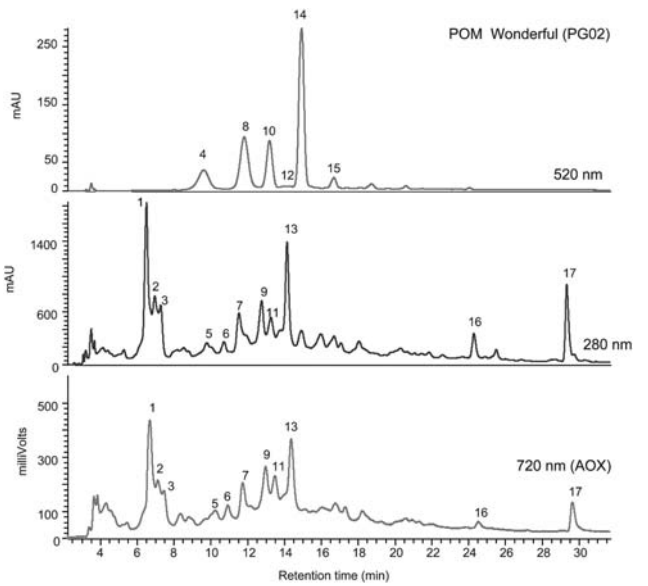

Fig. 2 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG02 [POM Wonderful] (see Table 1). For peak identification see legend to Fig. 1 and Table 4.

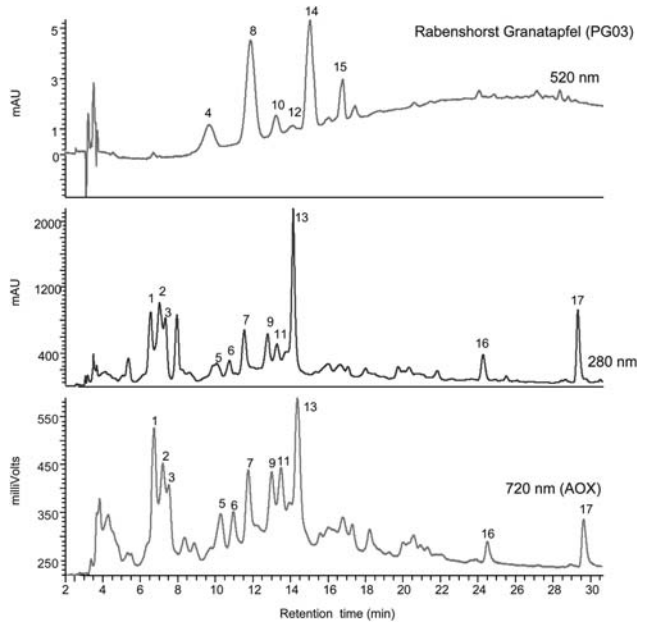

Fig. 3 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG03 [Rabenshorst Granatapfel] (see Table 1). For details and peak identification see legend to Fig. 1 and Table 4.

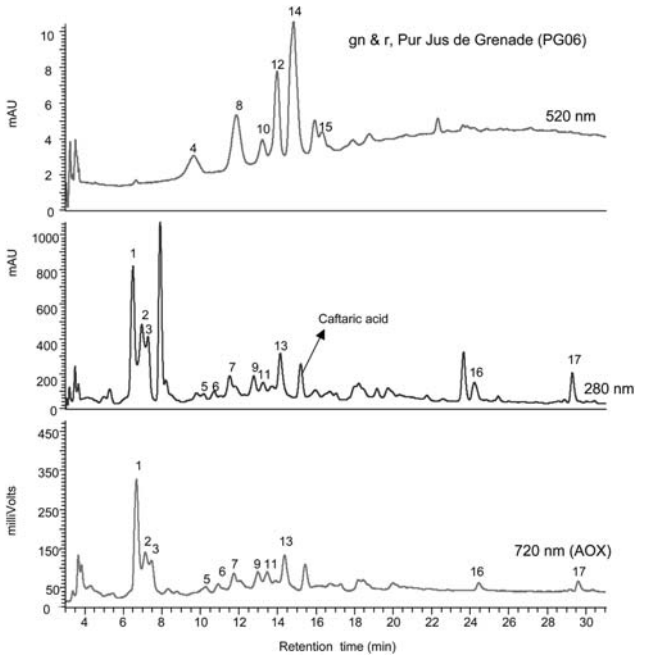

Fig. 5 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG06 [gn & r 100% Pur, Jus de Grenade (see Table 1). For details and peak identification see legend to Fig. 1 and Table 4.

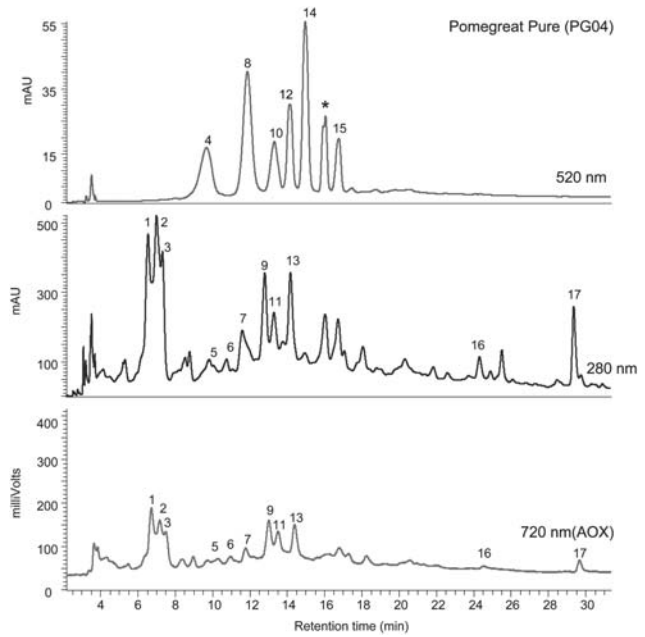

Fig. 4 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG04 [100% Pomegreat] (see Table 1). For details and peak identification see legend to Fig. 1 and Table 4.

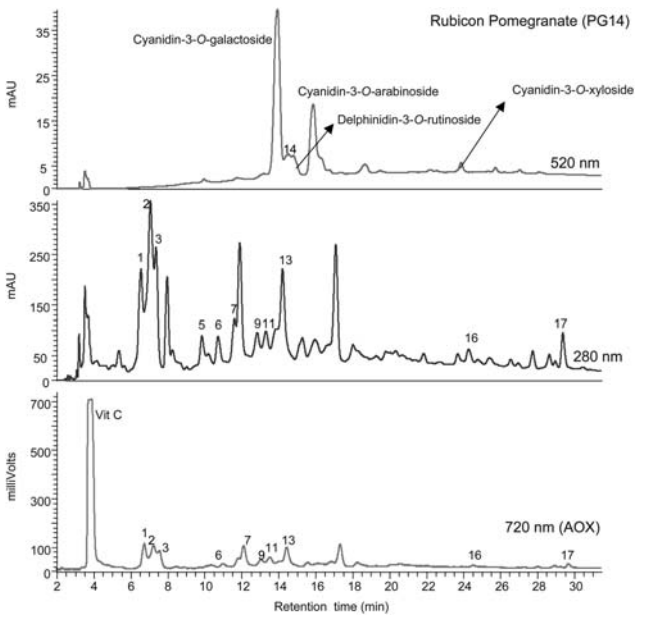

Fig. 6 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG14 [Rubicon Pomegranate] (see Table 1). For details and peak identification see legend to Fig. 1 and Table 4.

Peak 8 (Rt - 12.2 min, λ_{max} - 520 nm) produced a $[M - H]^+$ at *m*/*z* 611 and daughter ions at *m*/*z* 449 and 287. This fragmentation pattern and the absorbance spectrum identified this compound as cyanidin-3,5-*O*-diglucoside, another known pomegranate anthocyanin.[19]

Peaks 9 and *11* (Rts - 13.2 and 13.7 min) had a $[M - H]^-$ at *m*/*z* 783 and *m*/*z* 951 respectively. No fragmentation information was obtained. This is in keeping with the presence of granatin A and B, known constituents of pomegranate.[20]

Peak 10 (Rt - 13.4 min, λ_{max} - 520 nm) produced a $[M - H]^+$ at *m*/*z* 465 and a single MS^2 fragment ion at *m*/*z* 303. This fragmentation pattern, absorbance spectrum and co-chromatography identified this compound as delphinidin-3-*O*-glucoside, a known constituent of pomegranates.[19]

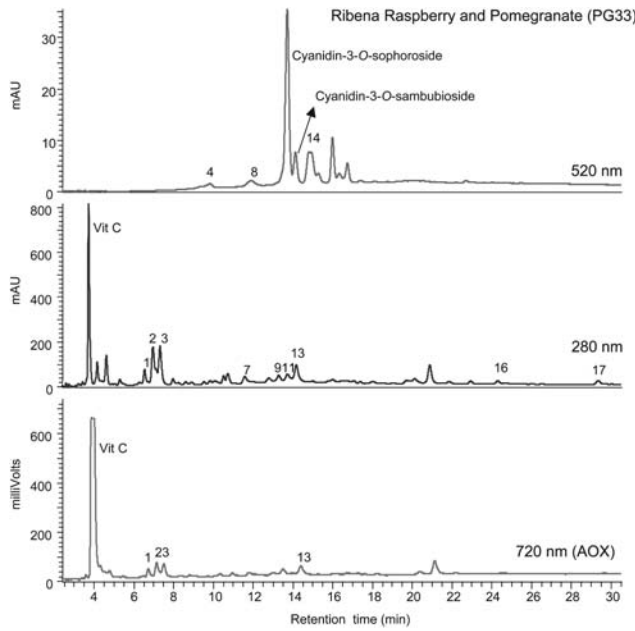

Fig. 7 Gradient reversed phase HPLC-PDA-AOX analysis of juice PG33 [Ribena Raspberry and Pomegranate] (see Table 1). For details and peak identification see legend to Fig. 1 and Table 4.

Peak 12 (Rt - 14.2 min, λ_{max} - 520 nm) had a $[M - H]^+$ at *m/z* 595 and MS^2 ions at *m/z* 433 and 271. This fragmentation pattern and the absorbance spectrum identified this compound as pelargonidin-3,5-*O*-diglucoside, a minor pomegranate anthocyanin.[19]

Peaks 13 (Rt - 14.7 min) had a $[M - H]^-$ at *m/z* 1083 and, like peaks 6 and 7, produced MS^2 ions at *m/z* 781, 721, 601 and 301. On the basis of this fragmentation pattern, peak 13 is identified as a punicalagin-like compound. There are two known punicalagins, A and B, however, additional isomers occur in pomegranate.[18]

Peak 14 (Rt - 15.2 min, λ_{max} - 520 nm) yielded a $[M - H]^+$ at *m/z* 449 and a single MS^2 fragment at *m/z* 287. This fragmentation pattern, absorbance spectrum and co-chromatography identified this compound as cyanidin-3-*O*-glucoside.[19]

Peak 15 (Rt - 17.0 min, λ_{max} - 520 nm) was characterised by a $[M - H]^+$ at *m/z* 433 which yields a daughter ion at *m/z* 271. This fragmentation pattern, absorbance spectrum and co-chromatography identified this compound as pelargonidin-3-*O*-glucoside.[19] None of the anthocyanins seems to have any effect on the on-line AOX activity, as they are not reflected in the 720 nm ABTS profile.

Peak 16 (Rt - 24.8 min) had a $[M - H]^-$ at *m/z* 463 and a single MS^2 fragment at *m/z* 301. This is in keeping with the presence of an ellagic acid-*O*-hexoside conjugate which has previously been reported to occur in pomegranates.[19]

Peak 17 (Rt - 29.9 min) had a $[M - H]^-$ at *m/z* 301 that yielded no MS^2 fragment ions. Co-chromatography and the identical fragmentation of a reference compound identified this component as ellagic acid. Both ellagic acid and conjugate were minor contributors to the AOX capacity (Table 5).

In addition to these 17 peaks, the 280 nm trace of the PG01 juice (Fig. 1) had a peak with a retention time of 7.9 min that is also present in PG06 (Fig. 5) and appeared in smaller amounts in PG03 (Fig. 3) and PG14 (Fig. 6). This peak did not ionise, so no MS data were obtained to assist identification, nor did it exhibit on-line AOX activity.

Overall these results show that the two main groups of polyphenolic compounds in pomegranate juice are anthocyanins and ellagitannins. The spectrum of anthocyanins comprising principally of cyanidin-3,5-*O*-diglucoside, cyanidin-3-*O*-glucoside, delphinidin-3,5-*O*-diglucoside and delphinidin-3-*O*-glucoside together with smaller amounts of pelargonidin-3,5-*O*-diglucoside and pelargonidin-3-*O*-glucoside is in agreement with earlier reports.[10,21] This can be used as a convenient fingerprint of pomegranate authenticity. The other potential diagnostic components are the ellagitannins in the form of punicalagins and punicalagin-like (peaks 1, 2, 3 and 13), 2-*O*-galloylpunicalagin (peak 5), punicalin A and B (peaks 6 and 7) and granatin A and B (peaks 9 and 11) which are the main contributors to the AOX capacity. Ellagic acid and an ellagic acid-hexose conjugate also

Table 4 HPLC-MS^2-based identifications of flavonoids and phenolic compounds in pure pomegranate juices[a]

Peak No.	Rt (min)	$[M - H]^-$ (*m/z*)*	MS^2 daughter ions	Compound
1	6.6	1101	781, 601, 301	Punicalagin-like
2	7.2	781	601, 301	Punicalin A
3	7.5	781	601, 301	Punicalin B
4	9.9	627^+	465, 303	Delphinidin-3,5-*O*-diglucoside
5	10.5	933	781, 721, 601, 301	2-*O*-Galloylpunicalagin
6	11.1	1083	781, 721, 601, 301	Punicalagin A
7	12.1	1083	781, 721, 601, 301	Punicalagin B
8	12.2	611^+	449, 287	Cyanidin-3,5-*O*-diglucoside
9	13.2	783		Granatin A
10	13.4	465^+	303	Delphinidin-3-*O*-glucoside
11	13.7	951		Granatin B
12	14.2	595^+	433, 271	Pelargonidin-3,5-*O*-diglucoside
13	14.7	1083	781, 721, 601, 301	Punicalagin isomer
14	15.2	449^+	287	Cyanidin-3-*O*-glucoside
15	17.0	433^+	271	Pelargonidin-3-*O*-glucoside
16	24.8	463	301	Ellagic acid-*O*-hexoside
17	29.9	301		Ellagic acid

[a] $[M - H]^-$ negatively charged molecular ion; $^+$ indicates positively charged molecular ion.

Table 5 Percentage of contribution of the phenolic pomegranate markers on the HPLC-ABTS on-line AOX activity[a]

	PG01[+]	PG02[+]	PG03[+]	PG04[+]	PG06[+]	PG14*	PG33*
Punicalagin-like	12.8	18.1	10.8	15	24.7	5.6	2.8
Punicalins A–B	19.8	11.7	13.5	20.9	17.6	11.5	3.1
2-*O*-Galloylpunicalagin	3.2	2.6	3.8	2.9	2.5	0	0
Punicalagin A + B + isomer	12	19.4	20.4	13.2	14.3	7.4	3.2
Granatin A	5.5	7.6	5.1	9.8	3.9	1	0
Granatin B	4.9	4.9	4.7	6.8	3.4	1.9	0
Total ellagitannins	**58.2**	**64.3**	**58.3**	**68.6**	**66.4**	**27.4**	**9.1**
Ellagic acid-*O*-hexoside	1.6	1.3	1.6	1.2	2.1	0.5	0
Ellagic acid	2.1	4.8	3.7	2.9	2.4	0.8	0
Total ellagic acids	**3.7**	**6.1**	**5.3**	**4.1**	**4.5**	**1.3**	**0**
Anthocyanins	0	0	0	0	0	0	0
Vitamin C	0	0	0	0	0	62.3	81
Caftaric acid	0	0	0	0	5.2	0	0
Unidentified compounds	38.2	29.7	36.5	27.5	23.9	9.2	9.9

[a] + 100% Pomegranate juices, * reconstituted or blended pomegranate juices.

occur but their presence is not specific to pomegranate as they can be derived from raspberries and other sources.[22–24]

The 720 nm traces for all the juices tested in the on-line AOX detector is almost identical to the 280 nm fingerprint of ellagitannins/ellagic acids (Fig. 1–7). Thus, as outlined in Table 5, the ellagitannins are the main antioxidants in the five pure pomegranate juices, PGO1–PGO4 and PGO6. The major contributors were the punicalins, punicalagins, and galloylpunicalagin which accounted for 58% to 69% of the total AOX of the juices. Around 30% of the AOX activity was due to an increased background probably due to unresolved oligomeric ellagitannins or proanthocyanidins[25] with AOX activity. None of the anthocyanin peaks were associated with the 720 nm AOX peaks.

In Fig. 6 and 7, the profiles for juices PG14 and PG33, it can be seen that the predominant AOX is vitamin C, which is responsible for 62.3 and 81%, respectively, of the total AOX activity, with negligible contributions from pomegranate constituents. This confirms the observation made when the juices were analysed in the FRAP assay before and after treatment with ascorbate oxidase (Table 2).

2.3 Quantification of the phenolic pomegranate markers in the 26 juices analyzed

The results for the quantification of ellagitannins and anthocyanins and the overall HPLC total phenolics for the 26 pomegranate juices and pomegranate juice blends are presented in Table 6. For the anthocyanin quantification all the peaks appearing in 520 nm traces were quantified in cyanidin-3-*O*-glucoside equivalents. As expected, the levels of the total HPLC phenolics quantified for the three top samples (PG01, PG02, PG03) were of the order of 2 mmol/L, much higher than the other 23 samples (Table 6), in agreement with the AOX results in Table 2.

2.3.1 Anthocyanins. The levels of anthocyanins did not reflect the AOX indices of the juices. PG01–PG03 all had a high AOX content (Table 2) but PG01 and PG03 contained low amounts of total anthocyanins (68 and 11 μmol/L respectively) relative to PG02 (344 μmol/L). This was visually apparent when comparing the intense dark red colour of PG02 juice with the dark brownish colour of PG01 and PG03. Anthocyanins are located in the flesh of the arils of the pomegranate fruit and are positively correlated with the juice colour.[9] In the case of the blended pomegranate juices, anthocyanins were derived from other fruit in addition to pomegranate. Several, most notably PG11 which comprised 18.5% raspberry and had a 288 μmol/L anthocyanin content, contained substantial amounts of anthocyanins (Table 6) but did not possess high AOX activity (Table 2).

Although the total quantities of anthocyanins in the 100% pomegranate juices varied substantially, as discussed above, the 520 nm anthocyanin HPLC profiles were similar with only slight differences in the relative amounts of cyanidin-3,5-*O*-diglucoside (peak 8) and cyandin-3-*O*-glucoside (peak 14) (Fig. 1–3). Likewise, a similar profile was obtained with PG04 (Fig. 4) and also PG06 (Fig. 5). In both these juices, however, peak 12, pelargonidin-3,5-*O*-diglucoside, was much more prominent. PG04 also contained an anthocyanin peak (marked *) which was not detected in the other 100% pomegranate juices. This peak had a $[M - H]^+$ at *m*/*z* 949 which produced MS^2 fragments at *m*/*z* 611, 449 and 287 indicating a cyanidin-based compound. The unusual mass spectrum and the relatively late elution of this component suggest that it might be a cyanidin-*O*-feruloyl-triglucoside.

Among the blended pomegranate products, PG14, a 29% pomegranate, 7% aronia mixture, had an anthocyanin HPLC profile dominated by aronia anthocyanins[26] principally in the form of cyanidin-3-*O*-galatoside and cyanidin-3-*O*-arabinoside, rather than pomegranate anthocyanins (Fig. 6). PG14 did, however, contain ellagitannins, suggesting that the pomegranate components might be derived from rind rather than arils which, as noted earlier, are the principal source of pomegranate anthocyanins.[9] PG33, which is a 8% raspberry/pomegranate blend had a raspberry rather than a pomegranate anthocyanin fingerprint with cyanidin-3-*O*-sophoroside being the main component[24] (Fig. 7).

2.3.2 Ellagitannins. This group of hydrolysable tannins, comprising punicalins, punicalagins, and granatins, occurs mainly in the peel, piths and arils of pomegranate.[19,27] PG02, which was high in anthocyanins, contained less punicalins than

Table 6 Quantification of phenolic and polyphenolic compounds in 26 commercial pomegranate juices.[a]

	Punicalagin-like	Punicalins A and B	2-*O*-Galloyl punicalagin	Punicalagin A	Punicalagin B	Punicalagin isomer	Granatin A	Granatin B	Total ellagitannins	Ellagic acid hexose	Ellagic acid	Total ellagic Acid	Total anthocyanins	Total
PG01[+]	178	972	22	30	58	181	123	47	1611	68	214	282	68	1961
PG02[+]	261	345	14	18	63	196	136	35	1068	85	408	493	344	1905
PG03[+]	158	512	26	34	125	382	116	48	1401	116	515	631	11	2043
PG04[+]	62	236	5	7	14	48	68	20	460	19	104	123	115	698
PG05[+]	58	167	3	7	18	66	78	18	415	23	160	183	155	753
PG06[+]	123	230	8	9	21	61	34	11	497	33	96	129	9	635
PG07*	81	543	12	24	58	169	44	32	963	36	98	134	18	1115
PG08*	78	331	9	16	33	97	37	21	622	37	199	236	30	888
PG09*	53	375	12	14	23	70	33	21	601	28	65	93	62	756
PG10*	48	287	7	13	20	58	22	13	468	29	102	131	26	625
PG11*	0	8	0	0	0	2	0	1	11	3	21	24	288	323
PG13*	41	248	5	9	5	16	13	17	354	7	32	39	23	416
PG14*	32	172	2	5	5	33	15	9	273	9	39	48	34	355
PG15*	25	250	5	10	8	26	18	14	356	16	122	138	52	546
PG16*	14	63	3	5	20	55	28	10	198	17	34	51	159	408
PG17*	45	237	2	4	3	11	15	7	324	5	16	21	1	346
PG18*	29	263	4	9	10	28	16	12	371	17	42	59	46	476
PG20*	62	199	5	9	18	56	27	9	385	15	52	68	35	488
PG21*	7	5	0	0	0	0	0	0	12	10	10	20	222	254
PG24*	16	32	0	0	0	0	0	4	52	4	7	11	101	164
PG26*	22	121	3	4	6	15	13	7	191	13	26	39	3	233
PG27*	26	137	3	4	5	13	17	7	212	14	50	64	74	350
PG29*	25	18	0	0	0	1	13	8	65	4	20	24	151	240
PG30*	13	37	1	2	2	8	6	0	69	5	25	30	77	176
PG31*	11	39	2	3	7	19	13	7	101	8	28	36	112	249
PG33*	6	36	0	0	1	3	2	1	49	3	14	18	14	81

[a] Data expressed as mean values in μmol/L. The standard error ($n = 3$) values (not shown), were less than 10% of the mean values. [+] 100% pomegranate juices, * reconstituted or blended pomegranate juices.

PG01 and PG03 and, as a consequence, had lower total ellagitannin content (Table 6). This implies that with PG01 and PG03, proportionally more rind was extracted than with PG02. However, this does not explain the similar total AOX capacity of the three juices (Table 2). Further investigation is required, but this could be a consequence of polymeric ellagitannins and or other high molecular weight compounds with AOX activity in P02 being retained on the HPLC column and therefore not contributing to the on-line AOX measurements.

2.3.3 Ellagic acid. Ellagic acid and an ellagic acid hexose conjugate were detected in substantial amounts in the pure pomegranate juices and typically in smaller amounts in the blended pomegranate drinks. The concentration of ellagic acid and its hexose conjugate ranged from 11 μmol/L (PG24) to 631 μmol/L (PG03) (Table 6). Ellagic acid, which is a product of the hydrolysis of ellagitannins, has been used as a marker for assuring commercial pomegranate extracts are made from genuine pomegranate fruit.[11,12] However, this is not necessarily an accurate measure of authenticity as it does not distinguish between pomegranate ellagic acid and ellagic acid derived from other sources of ellagitannins including berries, such as blackberries,[22] raspberries,[24] and cheaper material such as chestnut bark.[23]

The Pearson's correlation coefficients (Table 7) confirmed the significant relationship between the total ellagitannin and ellagic acid contents and the *in vitro* AOX capacity of the juices measured by TP, FRAP, ORAC and TEAC of the juices. In contrast *in vitro* AOX capacity was not associated with anthocyanin levels. This in agreement with earlier observations[9,19] that anthocyanins make, at best, a very minor contribution to the AOX capacity of pomegranates.

Table 7 Pearson's correlations coefficient.[a]

Assay	Total ellagitannins	Total ellagic acid	Total anthocyanins
TP	0.879(***)	0.899(***)	NS
FRAP	0.918(***)	0.895(***)	NS
ORAC	0.730(***)	0.815(***)	NS
TEAC	0.919(***)	0.906(***)	NS

[a] *** Correlation is significant at $p < 0.001$. NS, not significant.

3 Experimental

3.1 Chemicals

5-*O*-Caffeoylquinic acid, potassium persulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ascorbate oxidase (EC 1.10.3.3) and 2′,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich (Poole, UK). Ellagic acid was obtained from AASC Ltd (Southampton, UK). Cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside were purchased from Extrasynthese (Genay, France), and methanol was obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid and acetic acid were supplied by Fisher Scientific (Loughborough, UK). Punicalagin was purchased from LGC Standards (Teddington, Middlesex, UK).

3.2 Juices

Thirty six European juices were procured commercially. The brands and ingredients described on the label are shown in Table 1. The first six juices were labelled as "100% pomegranate juices", and the remainder included 20 brands of diluted pomegranate juice or pomegranate blended with other fruit juices and 10 different non-pomegranate fruit juices.

3.3 Extraction of juices

A 500 μL aliquot of juice was added to 500 μL of methanol and shaken for 3 min. The mixture was then centrifuged at 13000 *g* at 4 °C for 5 min and the supernatant stored at −80 °C prior to analysis.

3.4 Analysis of vitamin C

The vitamin C (ascorbic acid) content of the juices was assessed using HPLC-PDA as described by Ross[28] with a Surveyor HPLC system (Thermo-Fisher, Scientific, Waltham, MA). Separation was carried out using a 5 μm 250 × 4.6 mm i.d. Nucleosil C_{18} column (Phenomenex, Macclesfield, UK) fitted with a C_{18} guard cartridge. The column was eluted isocratically with a mobile phase comprising 0.05 mM sodium hydroxide, 25 mM myristyltrimethylammonium bromide, 0.06 M acetic acid, 7.5% acetonitrile mobile phase containing 100 mg/L homocysteine and 200 mg/L EDTA. The system was operated at 40 °C with a flow-rate of 0.6 mL/min and absorbance detection at 265 nm. The amount of ascorbic acid was calculated by reference to 0–200 μM vitamin C calibration curve.

3.5 Total phenol content

The TP content of the juices was determined in triplicate, in diluted samples, using the Folin-Ciocalteu assay.[13] The data were recorded in gallic acid equivalents (GAE).

3.6 Ferric-reducing antioxidant power assay

The FRAP assay was used to estimate the AOX capacity of the juices. It measures the ability of a solution to reduce a ferric-tripyridyl-triazine complex (Fe^{3+}-TPTZ) to the ferrous form, Fe^{2+}, producing a blue color with absorption at 593 nm. One and a half mL of freshly prepared FRAP reagent (containing the Fe^{3+}-TPTZ in excess at pH 3.6), was added to 50 μL of juice and 150 μL water. The absorbance at 593 nm, measured after a 4 min reaction period, was compared to a 0 to 1 mM Fe^{2+} standard curve.[14]

3.7 Contribution of vitamin C to FRAP antioxidant activity

Vitamin C reacts almost instantaneously in the FRAP assay. To determine the contribution of vitamin C to the antioxidant activity of the juices, it was selectively destroyed by the addition of ascorbate oxidase. Twenty μL of a 4 U/mL enzyme solution was added to one of a pair of juice aliquots before the FRAP

reaction. Standard solutions of ascorbic acid (0–0.5 mM), in the presence and absence of the enzyme, were tested.

3.8 Oxygen radical absorbance capacity assay

The principle of the ORAC assay is to monitor the capability of a test antioxidant to quench the fluorescent signal obtained when fluoroscein is exposed to an oxygen radical generator (2,2′-azo-bis-2-methyl-propionamide). The standard means of "normalising" the data requires comparison of the inhibitory effect of the test agent with that of Trolox, a water-soluble analogue of α-tocopherol. Comparing the area under the curve for 60 min incubations is the conventional means of analysing data obtained with this method.[16]

3.9 Trolox equivalent antioxidant capacity assay

After 12 h in darkness, a stock solution of 7 mM ABTS and 2.45 mM potassium persulfate was diluted with ethanol to an absorbance of 0.70 at 734 nm. Diluted juice samples were mixed with 1 mL of the ABTS solution and after 5 min, absorbance determined at 734 nm. TEAC values were calculated by reference to a Trolox standard curve.[15] The same basic procedure was applied with the HPLC on-line antioxidant detection system described below.

3.10 HPLC with PDA, MS^2 and AOX detection

Analysis was carried out on a Surveyor HPLC system comprising of an autosampler with sampler cooler maintained at 4 °C, a PDA detector (Thermo Fisher Scientific), scanning from 200–600 nm. Samples were analysed on a 250 × 4.6 mm Gemini C_6 phenyl column (Phenomenex, Macclesfield, UK), maintained at 40 °C using a 40 min mobile phase gradient of 5 to 60% methanol in 0.1% aqueous formic acid at a flow rate of 1 ml/min. After passing through the flow cell of the PDA detector, the eluate was split and 200 μL directed to a LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific). Capillary temperature was 300 °C, sheath gas and auxiliary gas were 60 and 20 units respectively, the source voltage was 4 kV. Samples were analysed using full scan in both positive and negative ionisation modes, the scan range was from 150–2000 *m*/*z* for negative ion and 190–1000 *m*/*z* for positive ion. Identifications are based on co-chromatography with authentic standards, where available. Absorbance spectra and mass spectra, using MS^2, were used to identify compounds reported previously in the literature.

For the detection of components with AOX activity, the remaining 800 μL/min of the HPLC eluate was mixed with an ABTS solution flowing at 0.5 mL/min and the resultant mixture passed through a holding coil before being directed to a P2000 absorbance detector (Nemphlar Bioscience, Lanark, UK) operating at 720 nm.[17]

3.11 Statistical analysis

The data were analyzed by SPSS software ver.14.0 to calculate Pearson correlation coefficients.

4 Conclusions

Although consumption of dietary flavonoids and polyphenolics has been increasingly implicated in health benefits it is remains unclear as to whether or not they function *in vivo* by directly modulating the body's AOX network.[29] There is growing evidence that in the body, they function in more subtle ways, at low concentrations, by regulating processes such as signal transduction pathways.[30] None-the-less, monitoring the AOX and/or total phenolic content of plant-derived foods, including fruit juices, provides a useful initial guide to their potential protective effects as polyphenol-rich products, such as PG01–PG03, are more likely to have a beneficial effects on health effects than the more dilute blended juices (Table 2). It is also necessary to identify at this stage, juices such PG14 and PG33, that contain relatively low levels of fruit but have their AOX capacity boosted by the presence of substantial amounts of vitamin C (Table 2, Fig. 1F and 1G).

The results of this study have provided an insight into the differences in both AOX activity and the concentrations of the main phenolic compounds in pure or blended pomegranate juices sold in Europe. While the ellagitannin profile can be used as a fingerprint for confirmation of the origin of the juice, it cannot on its own be used to judge purity or quality. It was, for instance, evident that the PG01 and PG03 juices, both of which had a very high AOX index, were authentic pomegranate juices from their ellagitannin profiles. However, the anthocyanin content of these juices was low compared to PG02 suggesting that juice from the arils had been diluted by more extensive extraction of the rind of the pomegranates. This may have resulted in PG01 and PG03 having an astringent taste which, arguably, may be masked by the addition of sweetener. The dark brown colour of these juices is in keeping with their low anthocyanin content.

The anthocyanins, although not associated with AOX activity, readily provide an additional specific fingerprint of pomegranate juice authenticity. The concentration of anthocyanins, along with the ellagitannin profile, can be used as indicators of both authenticity and quality of pomegranate juices. The HPLC-PDA-MS methodology utilised in this study provides a means of assessing, not just potential adulteration of pomegranate juices and drinks, but also that of a diversity of other fruit-based beverages.

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