

Original article

## Characterization of blond and Star Ruby (red) Jaffa grapefruits using antioxidant and electrophoretic methods

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**Summary** Antioxidant and electrophoretic methods were used to characterize the quality differences between blond and Star Ruby (red) grapefruits. Dietary fibre, minerals and trace elements, total polyphenols, anthocyanins, flavonoids, phenolic and ascorbic acids were also determined. The antioxidant potential of red grapefruit was significantly higher than that of the blond fruit ( $P < 0.05$ ) and correlated well with the total polyphenols ( $R^2$  from 0.8456 to 0.9711). In both the cultivars studied, thirty-two electrophoretic bands were detected [sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE)]. The main electrophoretic bands occurred between 20 and 43 kDa in both grapefruits with few minor differences between the varieties. Our findings indicate the following (i) red grapefruit is preferable: it has a higher concentration of bioactive compounds and antioxidant potential than the blond; (ii) 1, 1-diphenyl-2-picrylhydrazyl (DPPH) test is a more sensitive method for the determination of antioxidant potential; (iii) there are some minor differences in electrophoretic patterns; (iv) antioxidant and electrophoretic methods are a good combination for characterization of differences of the same citrus fruits.

**Keywords**  $\beta$ -carotene-linoleate model system, citrus fruits, dietary fibre, gel electrophoresis, radical scavenging activity, total polyphenols.

### Introduction

The bioactive compounds of citrus fruits are relatively well studied (Stohr & Hermann, 1975; Peleg *et al.*, 1991; Rapisarda *et al.*, 1999; Gorinstein *et al.*, 2001a, 2003; Sass-Kiss *et al.*, 2004). The differences between cultivars of the same

citrus fruit are less known (Gorinstein *et al.*, 2001a). It is claimed that investigations of bioactive substances and proteins might be specific enough to characterize the various citrus fruits (Clements, 1966). Differences in protein composition of the exo-, meso-, and endocarp of citrus fruits (Clements, 1966) and species-specific immunogenicity of citrus juice have been reported (Cantagalli *et al.*, 1972). Gorinstein *et al.* (2001a) suggested that investigation of antioxidant compounds could reveal possible differences between cultivars of the same citrus fruit. However, assessment of the bioactivity of individual

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antioxidant compounds do not reflect the true antioxidant value of fruits (Lotito & Frei, 2004). Therefore, studies on the total antioxidant potential of fruits are preferable (Vinson *et al.*, 2001). There are many methods for total antioxidant determination and each one has its limitations (Yu *et al.*, 2002). Some of these antioxidant assays give different antioxidant activity trends (Ou *et al.*, 2002). Radical scavenging activity and determination of polyphenols are the most popular methods (Katsube *et al.*, 2004). For the determination of phenolics the Folin-Ciocalteu assay is preferable (Katsube *et al.*, 2004), and for radical scavenging activity the antioxidant potential of varying radical species is used (Parejo *et al.*, 2002). 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay using discoloration of DPPH radicals is applied because of its reproducibility (Kitts *et al.*, 2000). However, evaluation of antioxidant potential is complicated. It has been suggested that the  $\beta$ -carotene oxidation assay is more suitable and more informative for screening antioxidant activity than other methods such as DPPH radical scavenging assay (Singh *et al.*, 2002). Determination of peptides and proteins as well as antioxidant capacity is one of the major methods for quality control of citrus fruits.

Entirely new possibilities appeared with the development of electrophoretic techniques (Firon *et al.*, 1979; Sass-Kiss & Sass, 2000, 2002). As mentioned, interest in the characterization of citrus fruits is growing, however investigations of the cultivars of the same citrus fruits are practically non-existent (Gorinstein *et al.*, 2001a; Proteggente *et al.*, 2002; Sun *et al.*, 2002; Gorinstein *et al.*, 2003; Sass-Kiss *et al.*, 2004). Therefore, we decided to use an advanced analytical method, i.e. sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), to determine the total antioxidant potential and to compare the essential bioactive compounds, the antioxidant potential and the protein profile of blond and Star Ruby (red) grapefruits.

## Materials and methods

### Chemicals

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid),  $\beta$ -carotene, butylated hydroxy-

anisole (BHA), sodium dodecyl sulphate (SDS), DPPH and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St Louis, MO, USA). All reagents were of analytical grade.

### Samples

Israeli blond and Star Ruby (red) Jaffa grapefruits (*Citrus paradisi*), of the same ripeness, harvested in the period of 2003–2004, were purchased from the same farmer. Samples (twelve of each grapefruits) were obtained from twenty-four randomly selected fruits for the determination of all variables mentioned above.

### Type of sample used for the different determinations

The fruits were washed with tap water and manually separated into peels and peeled fruits. Peels and pulps were separately weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model, Hamilton Beach/Proctor Silex, Inc. of Glen Allen, VA, USA) for 1 min. Then weighed portions (50–100 g) were lyophilized (Virtis 10-324 lyophilizer, VirTis Industries, Gardiner, NY, USA; 48 h, 1mm Hg,  $-3^{\circ}\text{C}$ ), and the dry weight was determined. The samples were ground to pass through a 0.5 mm sieve and stored at  $-20^{\circ}\text{C}$  until further analysis.

### Preparation of extracts

Determinations were carried out on pulp, peel and their extracts in methanol/water and methanol. Total phenols were extracted with 5 mL of 1.2 M HCl in methanol/water (1:1 v/v); the sample was vortexed for 1 min and heated at  $90^{\circ}\text{C}$  for 3 h with vortexing every 30 min. After cooling, the sample was diluted to 10 mL with methanol and centrifuged for 5 min at  $4000 \times g$  in a bench top centrifuge to remove solids (Vinson *et al.*, 2001). Assays were repeated five times.

### Total polyphenols

Total polyphenol content was measured at 750 nm using the Folin-Ciocalteu reagent diluted fivefold before use after reaction for 10 min, with gallic

acid as the standard (Singleton & Rossi, 1965; Singleton *et al.*, 1999; Vinson *et al.*, 2001). Total polyphenols were expressed in milligram per 100 g fresh weight of gallic acid equivalents.

### Determination of the total antioxidant potential

As cited, despite the high antioxidant potential of individual compounds, the antioxidant effect of a whole fruit can be low (Lotito & Frei, 2004). Therefore, in addition to the study of individual bioactive compounds two different total antioxidant tests were used for the determination of the total antioxidant potential and compared with the Folin-Ciocalteu assay.

**1** Radical scavenging activity using DPPH was expressed as inhibition percentage:

$$\text{Per cent radical scavenging activity} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100,$$

where OD is optical density. Changes in the absorbance of the samples were measured at 517 nm (Singh *et al.*, 2002).

**2** Antioxidant activity (AA) using  $\beta$ -carotene Linoleate Model System was evaluated in terms of bleaching of the  $\beta$ -carotene, measuring the absorbance at 470 nm:

$$\text{AA} = 100 \frac{1 - (A_0 - A_t)}{A_0 - A_t^{\circ}},$$

where  $A_0$  and  $A_0^{\circ}$  were the absorbance values measured at zero time of the incubation for test sample and control, respectively, and  $A_t$  and  $A_t^{\circ}$  were the absorbance measured in the test sample and control, respectively, after incubation for 180 min. The results were expressed in percentage of inhibition. BHA was used for comparison in both methods (Jayaprakasha *et al.*, 2001; Singh *et al.*, 2002).

### Separation of proteins

Approximately 30  $\mu\text{L}$  of juice was filtered through filter paper and the filtrate was centrifuged at  $15\,000 \times g$  for 20 min. The sediment was resuspended in 550  $\mu\text{L}$  of 0.5 M Tris-HCl (pH 8.3) buffer and mixed with an equal volume of sample buffer. The solution was boiled for 3 min. After centrifugation at  $15\,000 \times g$  for 15 min the super-

natant was used for SDS-PAGE. SDS-PAGE was carried out according to Laemmli (1970), using a Hoeffer SE-600 apparatus (Hoeffer Pharmacia Biotech Inc., San Francisco, CA, USA).

The Laemmli (1970) method was modified for juice proteins: the resolving gel was 13.7% total acrylamide (T) and 1.7% cross linker (C) and the stacking gel was 3.8% T and 1.8% C. The gel size was  $140 \times 160 \times 1.5$  mm. Juice supernatant (5  $\mu\text{L}$ ) was loaded on each of the lanes of the gel. The run was carried out at constant current 25 mA per gel. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 in methanol/water/glacial acetic acid solution (5:5:1 v/v) and destained in methanol/water/glacial acetic acid solution. The following molecular weight markers (Sigma) were used: myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and  $\alpha$ -lactalbumin (14 kDa).

### Determination of additional bioactive compounds

Dietary fibre was analysed by the modified AOAC method (Mañas *et al.*, 1994). The flame method was applied for minerals (Na, K, Mg, Ca) and trace elements (Fe, Cu, Zn) and the flameless method for Mn, using atomic absorption spectrophotometry (Gorinstein *et al.*, 2001a). Phenolic (Gorinstein *et al.*, 2004) and ascorbic (Perez *et al.*, 1997) acids were determined by high-performance liquid chromatography (HPLC). The total anthocyanins were estimated by a pH differential method (Cheng & Breen, 1991). Flavonoids were measured at 510 nm with known (+)-catechin concentration as a standard (Singleton *et al.*, 1999).

### Statistical analysis

To verify the statistical significance of the studied parameters, mean  $\pm$  SD of five measurements were determined. Where it was appropriate, differences between groups were tested by two-way ANOVA. To assess the relationship between the activities of the two different assays with the total polyphenols, analysis of linear regression was also used. The *P*-values of  $<0.05$  were considered significant.

## Results

### Phenolic and ascorbic acids

Ferulic, sinapic, *p*-coumaric, caffeic and ascorbic acids in peeled red and blond grapefruits and peels were comparable (Table 1) and were significantly higher in peels than in the peeled grapefruits ( $P < 0.05$ ). Among the phenolic acids the highest concentration was of ferulic and the lowest of caffeic acid. The content of ascorbic acid was significantly higher than of any of the other phenolic acids ( $P < 0.05$ ).

### Total polyphenols, anthocyanins and flavonoids

The total polyphenol content, anthocyanins and flavonoids in peeled red and blond grapefruits and peels are summarized in Table 2. The contents of the compounds in peeled red and blond grapefruits and peels are comparable ( $P > 0.05$ ). The content of total polyphenols, anthocyanins and flavonoids in peels was significantly higher than in peeled grapefruits ( $P < 0.05$ ).

### Antioxidant potential

The antioxidant assays showed that the antioxidant potential of the red grapefruit was significantly

higher than that of the blond grapefruit ( $P < 0.05$ ) and was significantly higher in peels than in the peeled fruits (Fig. 1).

### Proteins

During separation with SDS-PAGE thirty-two bands were detected (Fig. 2, lanes 2 and 3). Resolution of the bands was satisfactory in the molecular weight region ( $> 36$  kDa), but poor in the low molecular weight region ( $< 20$  kDa). Nevertheless, we detected more bands in the  $< 28$  kDa zone than Sass-Kiss & Sass, 2000, 2002. The use of a longer gel (14 cm instead of 7 cm) gave more distinct and sharp bands, especially in the  $> 36$  kDa zone.

### Additional bioactive compounds

The contents of total, soluble and insoluble dietary fibres (Fig. 3) in peeled blond and red grapefruits and their peels were comparable and were significantly higher in peels than in peeled fruits ( $P < 0.05$ ).

Minerals (Na, K, Mg and Ca) and trace elements (Fe, Mn, Zn and Cu) in blond and red peeled grapefruits and their peels (Figs 4 and 5) were comparable and were significantly higher in peels than in peeled fruits ( $P < 0.05$ ).

**Table 1** The contents of ferulic, sinapic, *p*-coumaric, caffeic and ascorbic acids (mg/100 g fresh weight) in peeled red and blond grapefruits and their peels

Fruits	Ferulic	Sinapic	<i>p</i> -Coumaric	Caffeic	Ascorbic
Red peeled grapefruits	29.3 ± 2.5 <sup>a</sup>	22.1 ± 1.8 <sup>a</sup>	10.1 ± 0.2 <sup>a</sup>	5.9 ± 0.8 <sup>a</sup>	35.9 ± 3.1 <sup>a</sup>
Blond peeled grapefruits	28.1 ± 2.4 <sup>a</sup>	20.9 ± 1.7 <sup>a</sup>	9.3 ± 0.8 <sup>a</sup>	5.7 ± 0.8 <sup>a</sup>	33.7 ± 3.0 <sup>a</sup>
Peel of red grapefruits	39.9 ± 3.1 <sup>b</sup>	30.1 ± 2.6 <sup>b</sup>	14.1 ± 1.2 <sup>b</sup>	8.2 ± 0.8 <sup>b</sup>	49.9 ± 4.1 <sup>b</sup>
Peel of blond grapefruits	37.8 ± 3.0 <sup>b</sup>	28.9 ± 2.4 <sup>b</sup>	13.8 ± 1.2 <sup>b</sup>	7.9 ± 0.8 <sup>b</sup>	47.8 ± 4.0 <sup>b</sup>

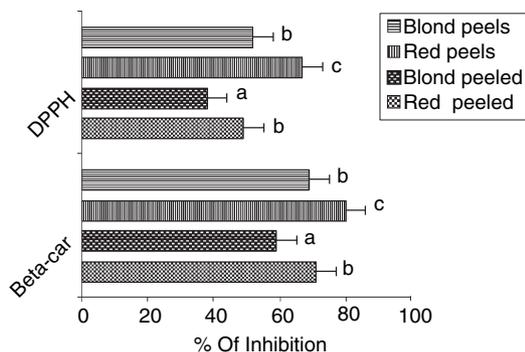
Values are mean ± SD of five measurements. Mean values in same columns with different superscript letters differ significantly ( $P < 0.05$ ).

**Table 2** The contents of total polyphenols, anthocyanins and flavonoids (100 g fresh weight) in peeled red and blond grapefruits and their peels

Fruits	Total polyphenols (mg)	Anthocyanins (µg)	Flavonoids (mg)
Red peeled grapefruits	158.3 ± 7.1 <sup>a</sup>	51.5 ± 4.6 <sup>a</sup>	21.61 ± 1.3 <sup>a</sup>
Blond peeled grapefruits	149.1 ± 6.3 <sup>a</sup>	49.3 ± 4.5 <sup>a</sup>	19.53 ± 1.2 <sup>a</sup>
Peel of red grapefruits	185.1 ± 7.3 <sup>b</sup>	87.6 ± 7.3 <sup>b</sup>	71.61 ± 6.3 <sup>b</sup>
Peel of blond grapefruits	168.2 ± 7.0 <sup>b</sup>	85.3 ± 7.2 <sup>b</sup>	69.53 ± 6.2 <sup>b</sup>

Values are mean ± SD of five measurements.

Mean values in columns with different letters differ significantly ( $P < 0.05$ ).



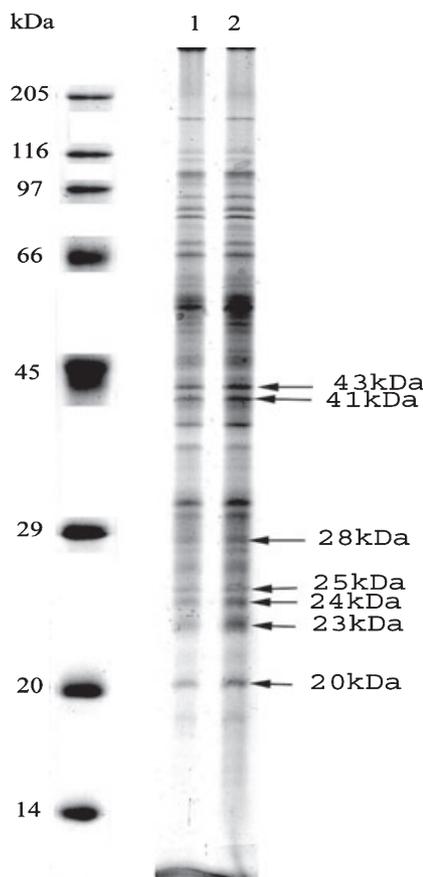
**Figure 1** Antioxidant potential of the red and blond peeled grapefruits and their peels as determined by both used assays. Mean + SD (horizontal lines). Mean values with different letters are significantly different. Beta-car, beta-carotene.

The correlations showed that the contribution of dietary fibres to the antioxidant potential of both red and blond grapefruits was minimal ( $R^2$  from 0.35 to 0.39), of ascorbic acid – moderate ( $R^2$  from 0.5563 to 0.7365) and of total phenols – decisive ( $R^2$  from 0.8456 to 0.9711; Fig. 6a,b).

## Discussion

Expanded production and consumption of citrus fruits and increased demand for healthy nutrition needs knowledge of the chemical constituents of citrus fruits and their products (Frankel *et al.*, 1993; Hertog *et al.*, 1995; Rimm *et al.*, 1996).

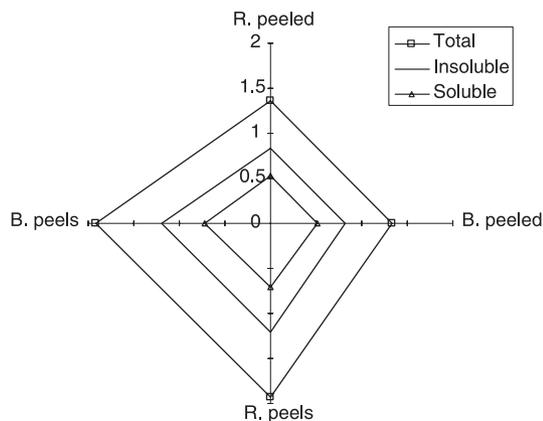
It was shown by many authors that these fruits contain high concentrations of bioactive compounds including phenolics and dietary fibre (Stohr & Hermann, 1975; Peleg *et al.*, 1991; Gorinstein *et al.*, 2003, 2004). However, the differences between cultivars of the same citrus fruit have been less studied (Gorinstein *et al.*, 2001a). Therefore, we decided to investigate the popular red and blond Jaffa grapefruits using antioxidant and electrophoretic methods. Antioxidant potential of fruits and vegetables as estimated by some authors differ significantly (Wang *et al.*, 1996; Proteggente *et al.*, 2002). In our opinion, the discrepancy in their estimation is connected with the samples used: they were not from the same geographical region and most probably not of the same ripeness. Therefore, in this investigation grapefruits of the same ripeness purchased from the same farmer were used.



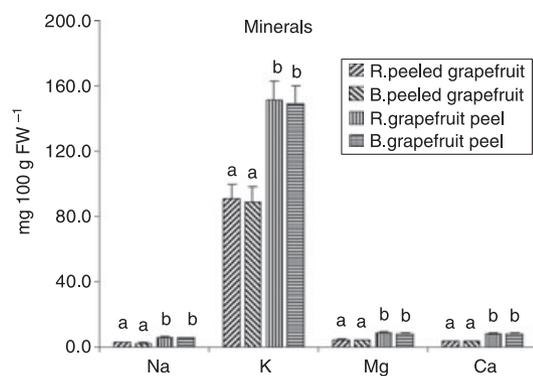
**Figure 2** Comparison of the band intensity of proteins extracted from grapefruits and separated by SDS-PAGE: lanes 1, 2, red and blond grapefruits, respectively. Molecular marker: myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa),  $\alpha$ -lactalbumin (14 kDa).

The results of the present study show that dietary fibres, minerals, trace elements, phenolic and ascorbic acids in both grapefruits are comparable and correspond with those of others and our previous data (Peleg *et al.*, 1991; Bartnikowska, 1999; Gorinstein *et al.*, 2001a,b, 2003, 2004).

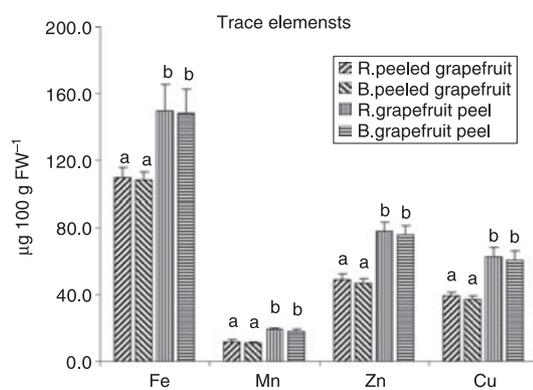
Some authors reported that the contribution of ascorbic acid to the total antioxidant potential of fruits is minimal (Rapisarda *et al.*, 1999). Others claim that the total antioxidant potential correlates well with the content of vitamin C (Proteggente *et al.*, 2002). The results of the present investigation show that the contribution of



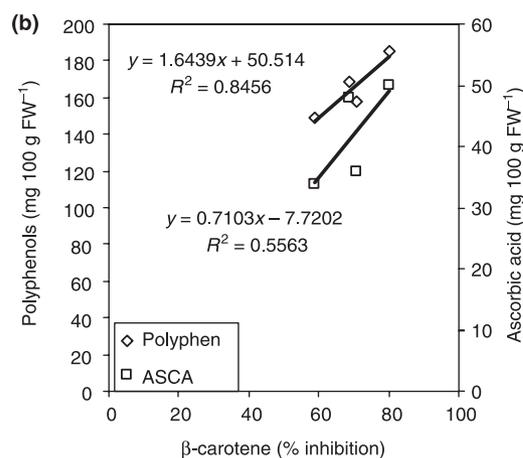
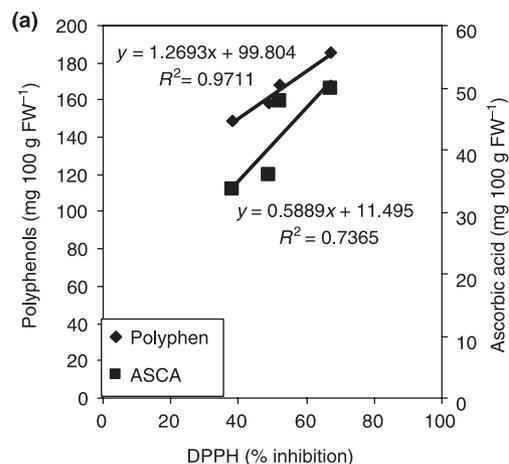
**Figure 3** Total, insoluble and soluble fibres in peels and peeled red and blond grapefruits. B, blond; R, red.



**Figure 4** Main minerals in peels and peeled red and blond grapefruits. Mean values with different letters are significantly different. B, blond; R, red.



**Figure 5** Essential trace elements in peels and peeled red and white grapefruits. Mean values with different letters are significantly different. B, blond; R, red.



**Figure 6** (a) correlation between DPPH (% inhibition) and the content of polyphenols [mg/100 g fresh weight (FW)] and DPPH and the content of ascorbic acid (mg/100 g FW) and (b)  $\beta$ -carotene bleaching effect (% inhibition) and the content of polyphenols (mg/100 g FW) and  $\beta$ -carotene bleaching effect and the content of ascorbic acid (mg/100 g FW) in peels and peeled red and blond grapefruits. Polyphen, polyphenols; ASCA, ascorbic acid.

ascorbic acid to the total antioxidant potential is moderate ( $R^2$  from 0.5563 to 0.7365).

The contents of polyphenols, anthocyanins and flavonoids were higher in the red grapefruit, but the differences were not significant. However, despite these data the antioxidant assays used have shown that the antioxidant potential of the red grapefruit is significantly higher than that of the blond fruit. The contents of all studied bioactive compounds and the total antioxidant potential of the peels of both grapefruits were

significantly higher than in peeled fruits. These results are in accordance with the data of others and our previously published results (Bocco *et al.*, 1998; Gorinstein *et al.*, 2001a).

It was found that the correlation between the total polyphenol content and the total antioxidant potential with DPPH and  $\beta$ -carotene assays are very high ( $R^2$  0.8456 and 0.9711, respectively). These data are in accordance with the data of others, who have shown that high total polyphenol content increases total antioxidant potential (Wang *et al.*, 1996; Eberhardt *et al.*, 2000; Vinson *et al.*, 2001) and there is a linear correlation between phenolic content and antioxidant potential (Gorinstein *et al.*, 2001a; Proteggente *et al.*, 2002).

According to our data, the correlation coefficient between the Folin-Ciocalteu assay and the DPPH radical scavenging assay is higher. These results correspond with the data of others (Parejo *et al.*, 2002; Katsube *et al.*, 2004) who reported that the correlation between DPPH radical scavenging activity and total phenol content as estimated by the Folin-Ciocalteu method was significantly high and varied from 0.70 to 0.90. Such results indicate that DPPH radical scavenging activity can be credibly predicted on the basis of the Folin-Ciocalteu assay and that these two methods depend on a similar mechanism: the propensity to donate hydrogen (Parejo *et al.*, 2002; Katsube *et al.*, 2004).

The purity of the isolated proteins was tested by SDS-PAGE and the characteristics of the grapefruits were identified after the extraction of the peptides with TRIS and sample buffers. The main bands displayed by SDS-PAGE were between 20 and 43 kDa. The results were in agreement with others (Sass-Kiss & Sass, 2000), but in their case the proteins were precipitated with trichloroacetic acid (TCA) and then separated with SDS-PAGE. In the present report the proteins were preliminary not precipitated, but the method was modified using different gel concentrations. The presence of four more characteristic proteins (85, 67, 50 and 35 kDa) was detected in the juice in the present study in comparison with Sass-Kiss & Sass (2002). According to Sass-Kiss & Sass (2002) another four bands of 65, 46, 82 and 117 kDa were characteristic for grapefruit juices. The antibody of the 82 kDa peptide gave a single positive band with

the proteins prepared from commercial grapefruit juice products according to Sass-Kiss & Sass (2002), which is shown in the present report as well. The protein bands were less intense in grapefruit juice (Sass-Kiss & Sass, 2002) than in the separated grapefruit samples in the present report, presumably because of different variables. It is known that fruit composition is influenced by a large number of natural factors, namely the variety of fruit, the geographical location, the climatic zone, the soil, and the degree of maturity as well as other factors. The composition of juice products may be influenced by technology used in processing and packaging (Sass-Kiss *et al.*, 2004). It may be supposed that the larger number of detected bands would be a factor in searching for species-specific proteins in both varieties of grapefruit juices. Proteins were separated into numerous components and showed low polymorphism among different genotypes. Both forms of grapefruits showed small differences in their protein patterns. Our results confirm the data of other investigators (Sass-Kiss & Sass, 2000, 2002).

In conclusion (i) the red grapefruit is preferable: it contains a higher concentration of bioactive compounds and has a higher antioxidant potential than the blond grapefruit; (ii) the DPPH test is more sensitive in determining of the antioxidant potential of the studied grapefruits; (iii) there are some minor differences in the SDS-electrophoretic patterns of the studied cultivars; (iii) the applied antioxidant and electrophoretic methods are a good combination for characterization of differences of the same citrus fruits.

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