Targeting excessive free radicals with peels and juices of citrus fruits: grapefruit, lemon, lime and orange

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ABSTRACT

A comparative study between the antioxidant properties of peel (flavedo and albedo) and juice of some commercially grown citrus fruit (Rutaceae), grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), lime (*Citrus x aurantiifolia*) and sweet orange (*Citrus sinensis*) was performed. Different in vitro assays were applied to the volatile and polar fractions of peels and to crude and polar fraction of juices: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β-carotene-linoleate model system in lipossomes and thiobarbituric acid reactive substances (TBARS) assay in brain homogenates. Reducing sugars and phenolics were the main antioxidant compounds found in all the extracts. Peels polar fractions revealed the highest contents in phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars, which certainly contribute to the highest antioxidant potential found in these fractions. Peels volatile fractions were clearly separated using discriminat analysis, which is in agreement with their lowest antioxidant potential.

KEYWORDS: Citrus fruits; Antioxidants; Scavenging activity; Peroxidation inhibition.

1. Introduction

During the past years, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer (Halliwell, 1996). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in food or pharmaceutical applications, which can protect the human body from free radicals and retard the progress of many chronical diseases as well as retard lipid oxidative rancidity in food (Prior, 2003). In fact, many antioxidant compounds extracted from plant sources (phytochemicals) have been identified as free radical or active oxygen scavengers (Ramarathnam et al., 1995).

Citrus (*Citrus* L. from Rutaceae) is one of the most important world fruit crops and is consumed mostly as fresh produce or juice because of its nutritional value and special flavour. Most popular within European and North American consumers are grapefruits (*Citrus paradisi*), lemons (*Citrus limon*), limes (*Citrus* × *aurantiifolia*) and sweet oranges (*Citrus sinensis*) (Mabberley, 1997; Citrus Pages, 2009). Consumption of citrus fruit or juice is found to be inversely associated with several diseases (Joshipura et al., 2001). The health benefits of citrus fruit have mainly been attributed to the presence of bioactive compounds, such as phenolics (e.g. flavanone glycosides, hydroxycinnamic acids) (Marchand, 2002), vitamin C (Halliwell, 1996), and carotenoids (Rao and Rao, 2007). Although, the fruits are mainly used for dessert, they are also sources of essential oils due to their aromatic compounds (Minh Tu et al., 2002; Chutia et al., 2009). For instance, lime flavours are used in beverage, confectionary, cookies and desserts (Dharmawan et al., 2007; Chutia et al., 2009). Many authors have reported antioxidant

and radical-scavenging properties of essential oils (Sacchetti et al., 2005) and in some cases, a direct food-related application also (Madsen and Bertelsen, 1995).

So far, studies on bioactive compounds and antioxidant activity of citrus have mainly focused on the fruits (peels, pulps and juices) polar fractions (Abeysinghe et al., 2007; Gorinstein et al., 2001). Herein we developed a comparative study between four citrus fruits (peels and juices) in order to understand which of them are preferable for dietary prevention of cardiovascular and other diseases related to oxidative stress. Volatile and polar fractions of grapefruits, lemons, limes and oranges studied and compared considering free radical scavenging properties, reducing power, and inhibition of lipid peroxidation capacity (in lipossomes and in brain homogenates). Antioxidant molecules such as phenolics, sugars, ascorbic acid and carotenoids were also quantified in order to understand their contribution to the overall bioactive properties.

2. Materials and methods

2.1. Samples

Commercially grown grapefruit (*Citrus paradisi* 'Star Ruby'), lime (*Citrus* × *aurantiifolia* (Christm.) Swingle) were purchased from a local supermarket, and lemon (*Citrus limon* (L.) Burm.f.) and sweet orange (*Citrus sinensis* (L.) Osbeck, 'Valencia' group) from a rural market, in February 2009. The citrus *taxa* studied were botanically classified using the synthetic proposal of Mabberley (1997) and the information published in Citrus Pages (http://users.kymp.net/citruspages/introduction.html, last update April 2009). Morphological characterization of the samples (8 fruits analysed per sample and species) was performed (**Table 1**) for botanical description and comparison in future research. Size, shape, form of the basal (stem) and apical (stylar) ends, and

other distinctive general characters (**Figure 1**), such as peel (flavedo and albedo) thickness and the smoothness of the surface, number of segments of the endocarp, central axis or medulla, some special structures that are or may be present in the apex (areole, mammilla, navel) and seed presence were described according to horticultural criteria defined by Hodgson (1986). Fruits range in size is expressed by the average D/H index (**Table 1**). The D/H index is obtained by dividing the diameter of each fruit measured by its height (distance from stem to apex).

2.2. Standards and reagents

All the solvents were of analytical grade purity; methanol was supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), L-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standard butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Volatile fraction

The essential oils were isolated from the fresh material (~150 g peels plus 350 mL of distilled ultra pure water) by hydro-distillation for 3 h, using a Clevenger-type apparatus. The extracts were dried with anhydrous sodium sulphate and concentrated under reduced pressure by rotary evaporator (Büchi R-210). The extraction yield was calculated in g of oil/100 g of fresh material. The collected oil was weighed, dissolved

in methanol at a concentration of 500 mg/mL, and stored in sealed vials at -20 ◦C for further use.

2.4. Polar fraction

Lyophilized (Ly-8-FM-ULE, Snijders, HOLLAND) powdered samples (peels and juice; ~3 g) were extracted by stirring with 50 mL of methanol at 25 ºC at 150 rpm for 12h and filtered through Whatman nº 4 paper. The residue was then extracted with one additional 50 mL portion of the methanol. The extracts were evaporated to dryness and redissolved in methanol at a concentration of 20 mg/mL, and stored at 4 ºC for further use. Also, the lyophilized juices were directly dissolved in water at a concentration of 20 mg/mL (Crude juices), and stored at 4 ºC for further use.

Total phenolics were estimated by a colorimetric assay, based on procedures described by (Wolfe et al., 2003) with some modifications. An aliquot of the extract solution was mixed with *Folin-Ciocalteu* reagent (5 ml, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/l, 4 ml). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (0.05-0.8 mM; $y = 1.9799x + 0.0299$; $R^2 = 0.9997$), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Total flavonoids contents were determined spectrophotometrically using the method of Jia et al. (1999) based on the formation of a complex flavonoid-aluminum, with some modifications. An aliquot (0.5 ml) of the extract solution was mixed with distilled water (2 ml) and subsequently with $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 ml) was added to the mixture. Immediately, distilled water was

added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.0156-1.0 mM; $y = 0.9186x - 0.0003$; $R^2 = 0.9999$) and the results were expressed as mg of (+)-chatequin equivalents (CEs) per g of extract.

Ascorbic acid was determined according to the method of Klein and Perry (1982). A fine powder (20 mesh) of sample (150 mg) was extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman Nº 4 filter paper. The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml; $y = 3.0062x + 0.007$; $R^2 = 0.9999$), and the results were expressed as µg of ascorbic acid per g of extract.

For β-carotene and lycopene determination a fine dried powder (150 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm (Barros et al., 2008). Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 mL) = - 0.0458 × A_{663} + 0.204 × A_{645} + 0.372 × A_{505} - 0.0806 × A_{453}; β-carotene (mg/100 mL) = 0.216 × A_{663} – 1.220 × A_{645} - 0.304 × A_{505} + 0.452 × A_{453}. The results were expressed as µg of carotenoid per g of extract.

Reducing sugars were determined by the DNS (dinitrosalicylic acid) method and glucose was used to calculate the standard curve (250-1500 µg/mL; Y=0.0007X-0.0567; R^2=0.9997); the results were expressed as g of reducing sugars per g of extract.

2.5. Radical scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of extract solution (30 μL) and aqueous methanolic solution (80:20 v/v, 270 μL) containing DPPH radicals ($6x10^{-5}$ mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

2.6. Reducing power

This methodology was performed using the Microplate Reader described above. The extract solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. BHA and α-tocopherol were used as standards.

2.7. Inhibition of lipid peroxidation

β-carotene bleaching inhibition. The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model system, as described previously by us (Barros et al., 2008). A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. A blank, devoid of β-carotene, was prepared for background subtraction. β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. TBHQ was used as standard.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS). Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with the extracts solutions (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 ml) and ascorbic acid (0.1 mM; 0.1 ml) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL),

followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract concentration (Barros et al., 2008). BHA was used as standard.

2.8. Statistical analysis

For each one of the fruits three samples were analysed and also all the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) or standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic.

Discriminant function analysis was done following stepwise method, aiming to determine which variables discriminate between the four naturally occurring groups. The values of *F* to enter and *F* to remove are the guidelines of the stepwise procedure. The *F*-value for a variable indicates its statistical significance in the discrimination between groups. Discriminant analysis defines an optimal combination of varieties in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on (Benitez et al., 2006).

These treatments were carried out using SPSS v. 16.0 program.

3. Results and discussion

The yields obtained in the extraction of volatiles and polar compounds of citrus peels and in the extraction of juice polar compounds are presented in **Table 2**. Juices polar fractions gave extraction yields higher than 95% (measured as ratio between the extract weight and the dry weight of each sample), followed by the peels polar fraction. As expected, the yields obtained for the peels volatile fractions (calculated as ratio between the oil weight and the fresh weight of each sample) were significantly lower (less than 1.3%).

The antioxidant properties of citrus fruits were evaluated considering the separate contribution of peels volatile fraction (including essential oils) and polar fraction (including antioxidants such as phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars). The corresponding juices were also evaluated considering the polar fraction and the crude juice. Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively. Clearly, matching radical source and system characteristics to antioxidant reaction mechanisms is critical in the selection of appropriate assessing antioxidant capacity assay methods, as is consideration of the end use of the results (Prior et al., 2005). In this way, to screen the antioxidant properties of the samples, four different in vitro assays were performed: DPPH radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β-carotene-linoleate model system in lipossomes and TBARS assay in brain homogenates.

The peels polar fractions revealed the highest antioxidant properties (significantly lower EC_{50} values; $p<0.05$), while the peels volatile fractions gave the lowest antioxidant

potential (**Table 3**). For all citrus fruits, peels gave better results than the corresponding juices. Orange revealed the highest antioxidant potential in all the studied fractions, with the exception of peels polar fraction, in which lime gave the best results. Grapefruits peels and lime juices gave the lowest antioxidant properties.

Using peels volatile fractions, the best results of antioxidant activity were obtained in the reducing power assay (**Table 3**). In this assay, the transformation of Fe^{3+} into Fe^{2+} in the presence of various fractions was measured. The antioxidants present cause the reduction of Fe^{3+}/ferricyanide complex ($FeCl_3/K_3Fe(CN)_6$) to the ferrous form (Fe^{2+}). Therefore, depending on the reducing power of the samples, the yellow colour of the test solution changes to various shades of green or blue (Amarowicz et al., 2004); this can be measured spectrophotometrically at 700 nm determining the reducing power (redox cycle) of the tested substances faced with a certain metal responsible for free radicals production and in some cases for antioxidants regeneration.

The chemistry of iron-based assays may be summarized with the following reaction equation:

$$Fe^{3+}\text{–L} + \text{antioxidant} \longrightarrow Fe^{2+}\text{–L} + \text{oxidized antioxidant}$$

where L is the ferrous-selective chromogenic ligand producing the colored species Fe^{2+} –L as a result of the concerned redox reaction. Either the oxidant species is Fe^{3+} –L or $Fe(CN)_6^{3-}$ (in the composite ferricyanide reagent), the reduction product with the antioxidant, either Fe^{2+} –L or $Fe(CN)_6^{4-}$, respectively, combines with the other reagent component to produce Prussian blue, $KFe[Fe(CN)_6]$, as the coloured product (Berker et al., 2007).

For peels polar fractions and crude juices, the best results of antioxidant activity were obtained in the TBARS assay (**Table 3**). This procedure measures the malondialdehyde (MDA) formed as the split product of an endoperoxide of unsaturated fatty acids

resulting from oxidation of a lipid substrate. It is postulated that the formation of MDA from fatty acids with less than three double bonds (*e.g.*, linoleic acid) occurs *via* the secondary oxidation of primary carbonyl compounds (*e.g.*, non-2-enal) (Fernández et al., 1997). The MDA is reacted with thiobarbituric acid (TBA) to form a pink pigment (TBARS) that is measured spectrophotometrically at 532 nm (Ng et al., 2000).

Using juice polar fractions, the best results of antioxidant activity were obtained in the β-carotene-linoleate assay (**Table 3**). Decolourization of β-carotene can be monitored by spectrophotometry at 470 nm and can be employed as an assay of antioxidant activity. The β-carotene undergoes a rapid discoloration in the absence of an antioxidant since the free linoleic acid radical attacks the β-carotene molecule, which loses the double bonds and, consequently, loses its characteristic orange colour. β-carotene is extremely sensitive to free radical mediated oxidation of linoleic acid (Gutierrez et al., 2006). Classical antioxidants can donate hydrogen atoms to quench radicals and prevent decolourization of carotenoids:

β-carotene – H (orange) + $ROO^{\bullet}$ ⟶ β-carotene• (bleached) + ROOH

β-carotene – H (orange) + $ROO^{\bullet}$ + AH ⟶ β-carotene – H (orange) + ROOH + $A^{\bullet}$

Antioxidants can neutralize any free radicals formed within the system (*e.g.*, the linoleate free radical) and, consequently, may delay decolourization of β-carotene (Jayaprakasha et al., 2001; Amarowicz et al., 2004). Accordingly, the absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time.

DPPH assay gave the highest EC_{50} values in all the fractions (**Table 3**). The 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) is a stable organic nitrogen radical, is commercially available and has a deep purple colour. The radical scavenging activity (RSA) assay measures the reducing capacity of antioxidants toward $DPPH^{\bullet}$. Upon

reduction, the colour of DPPH$^{\bullet}$ solution fades and this colour change is conveniently monitored spectrophotometrically at 517 nm. Therefore, test compounds with high antioxidant activity result in a rapid decline in the absorbance of the DPPH$^{\bullet}$ (Antolovich et al., 2002; Amarowicz et al., 2004). When a solution of DPPH$^{\bullet}$ is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour (Ali et al., 2008). Representing the DPPH$^{\bullet}$ by X$^{\bullet}$ and the donor molecule by AH, the primary reaction is:

$$X^{\bullet} + AH \longrightarrow XH + A^{\bullet}$$

As the polar fractions gave better antioxidant activity results than volatile fractions, it was investigated the composition of the polar extracts (peels and juices) in antioxidant compounds, including phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars (**Table 4**). Reducing sugars and phenolics were the main antioxidant compounds found in all the extracts. Although, it should be pointed out that ascorbic acid (vitamin C) is very fragile in polar solutions and might be destroyed upon exposure to air, light or heat. Peels polar fractions revealed the highest contents in phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars, which certainly contribute to the highest antioxidant potential found in these fractions (**Table 3**). The better scavenging activity, reducing power and lipid peroxidation inhibition showed by lime peels polar fraction (**Table 3**) might be due to the highest levels and synergy between phenolics, ascorbic acid and lycopene found in this sample (**Table 4**). Orange peels polar fraction also revealed high antioxidant potential (**Table 3**) with the contribution of β-carotene and reducing sugars, found in this sample in high levels (**Table 4**). Considering flavonoids, lemon gave the highest concentrations, which is in agreement with reports available in literature (Del Rio et al., 2004).

Statistical Analysis

In the discriminant analysis (DA) several combinations of the obtained results were used (**Table 5**). As it can be seen in **Figures 2** and **3**, only the first two functions defined in each one of the DA studies were plotted.

Regarding antioxidant activity assays, the DA defined four functions, with 96.4% of the observed variance explained by the first two (**Figure 2**). The first function separates primarily peels volatile fraction samples from the other citric components (means of the canonical variance, MCV: orange peels volatile fraction (9) = 27.256, lemon peels volatile fraction (10) = 40.305, lime peels volatile fraction (11) = 50.280 and grapefruit peels volatile fraction (12) = 108.12), and revealed to be more powerfully correlated with TBARS and β-carotene bleaching inhibition assays. The second dimension confirmed the separation of 9, 10, 11 and 12, separating also lime juice polar fraction (3) (MCV: (3) = 5.699, (9) = -10.238, (10) = -15.394, (11) = -17.293 and (12) = 19.978) and showed to be more correlated with β-carotene bleaching and TBARS inhibition assays. Neither function 1 nor function 2 were able to separate the remaining samples.

Concerning bioactive compounds content, DA defined five dimensions, being 99.3% of the observed variance explained by the first two (**Figure 3**). The first function separates primarily orange peels polar fraction (5) (MCV: (5) = 454.91), and revealed to be more effectively correlated with carotenoids. The second function separates the polar fractions of lemon (6), lime (7) and grapefruit (8) from the other samples (MCV: (6) = 91.705, (7) = 134.83 and (8) = 16.921) and showed to be more correlated with phenolics. Neither function 1 nor function 2 separated clearly crude juice and juice polar fractions samples.

From the seven functions defined when the antioxidant activity assays results were considered together with bioactive compounds content, using only crude juice and juice

polar fraction samples, the first two explained 96.4% of the observed variance (**Figure 4**). The first function separates mostly orange (1) and grapefruit (4) juice polar extractions and lime (15) and grapefruit (16) crude juices (MCV: (1) = 53.212, (4) = 44.064, (15) = -48.973 and (16) = -59.795), and revealed to be more strongly correlated with ascorbic acid and β-carotene bleaching inhibition assay. The second function separated the remaining samples (MCV: lemon juice polar fraction (2) = -11.113, lime juice polar extraction (3) = -4.3283, orange crude juice (13) = 28.566 and lemon crude juice (14) = -2.0461) and showed to be more correlated with reducing sugars and carotenoids.

In summary, the different samples could only be clustered in individual groups, when the algorithm was applied for selecting variables according with different parameters. Peels volatile fractions were separated with high clearness according with antioxidant activity assays. In the other hand, only bioactive compounds contents made the individualization of peels polar fractions possible. The DA revealed also very close proximity among the results obtained for juices, either in the crude form, as well as the corresponding polar fractions. Actually, the results for juices samples were only clustered individually when data regarding peels where removed.

Overall it was found that peels of fruits are major sources of different antioxidants and these by-products of the juice extraction industry could be used as natural antioxidants. Otherwise, the use of the whole extract instead of individual antioxidants allows taking advantage of additive and synergistic effects of different phenolics, flavonoids, ascorbic acid, carotenoids and reducing sugars present in the samples.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (Portugal) for financial support to the research centre CIMO and L. Barros grant (SFRH/BPD/4609/2008).

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Figure 1. 1 – Flavedo (the orange peripheral surface of the peel or epicarp); 2 – Albedo (the white soft fiber middle layer of the peel or mesocarp); 3 – The inside layer of the fruit or endocarp, divided in segments or carpels with juicy vesicles; 4 – central column or medulla.

Figure 2. Canonical analysis of citric fruits samples based on antioxidant activity results. Orange (1), lemon (2), lime (3) and grapefruit (4) juice polar fractions; orange (5), lemon (6), lime (7) and grapefruit (8) peels polar fractions; orange (9), lemon (10), lime (11) and grapefruit (12) peels volatile fractions; orange (13) = 28.566, lemon (14); lime (15) and grapefruit (16) crude juices.

Figure 3. Canonical analysis of crude juice, juice polar fractions and peels polar fractions samples based on bioactive compounds contents.
Orange (1), lemon (2), lime (3) and grapefruit (4) juice polar fractions; orange (5), lemon (6), lime (7) and grapefruit (8) peels polar fractions; orange (13) = 28.566, lemon (14); lime (15) and grapefruit (16) crude juices.

Figure 4. Canonical analysis of crude juice and juice polar fractions samples based on antioxidant activity results and bioactive compounds contents.
Orange (1), lemon (2), lime (3) and grapefruit (4) juice polar fractions; orange (13) = 28.566, lemon (14); lime (15) and grapefruit (16) crude juices.

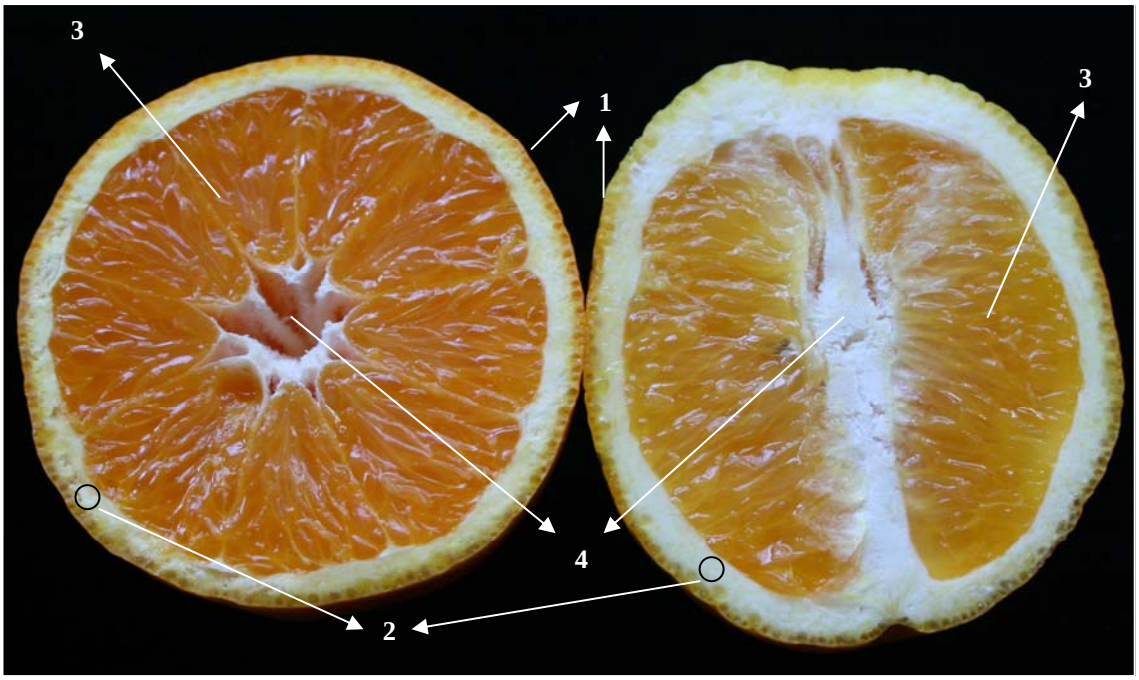
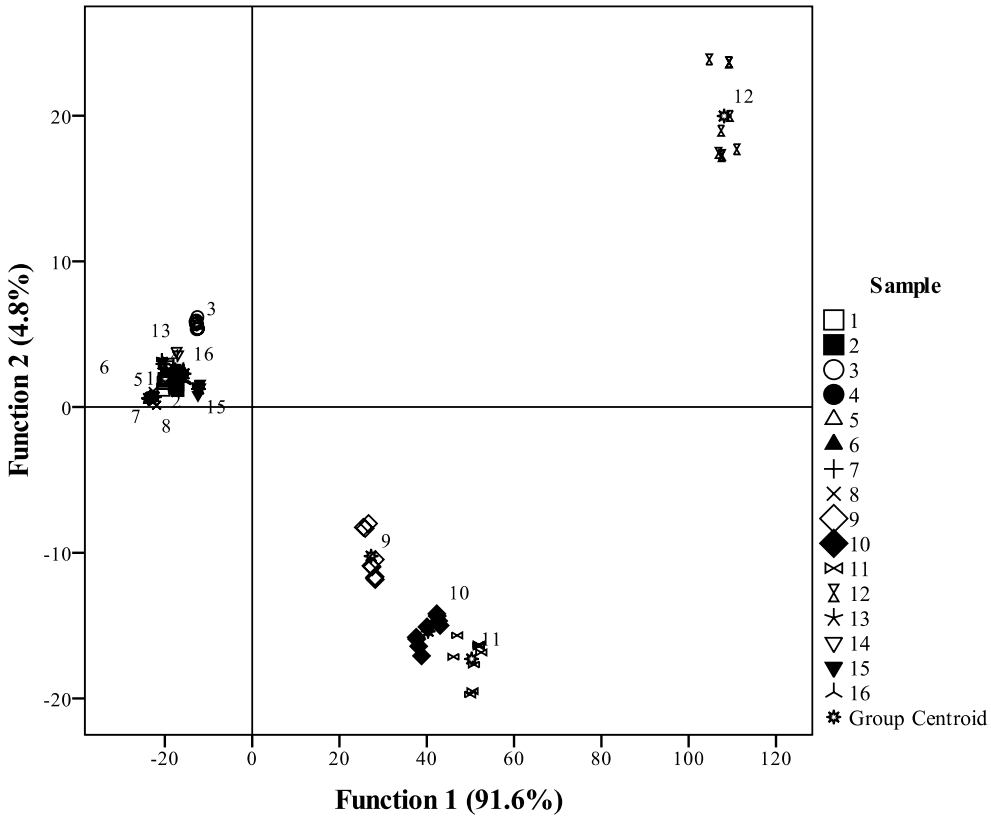


Figure 1.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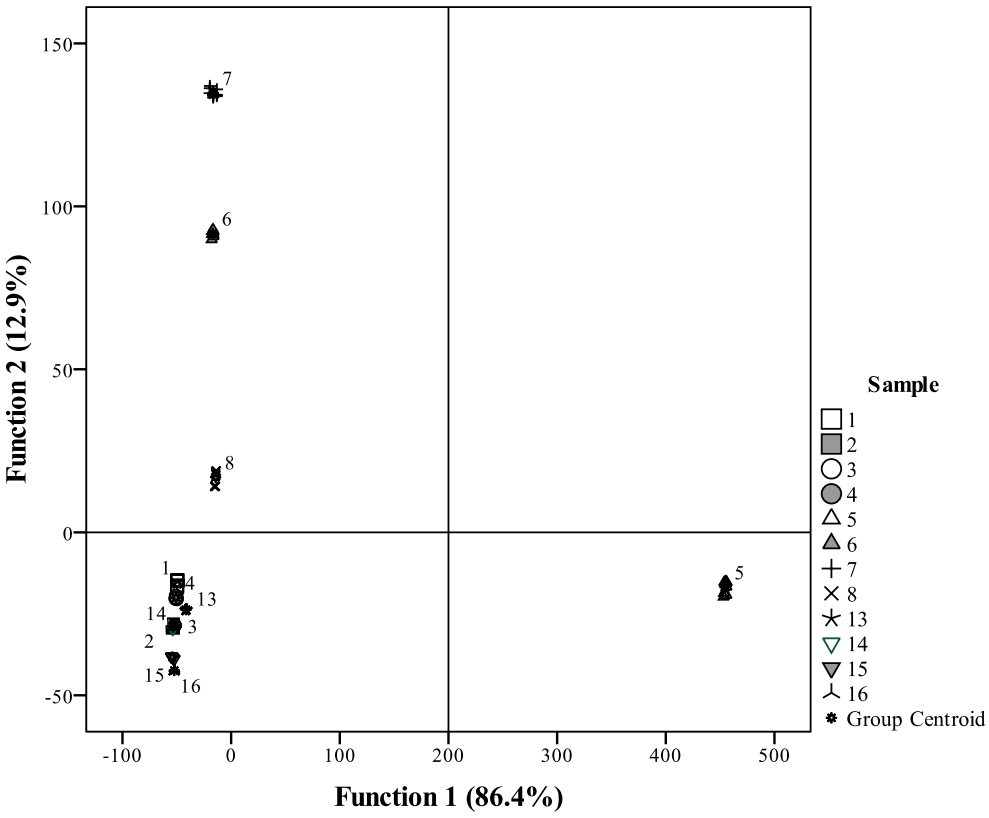
Function 1 (91.6%)
Sample
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Group Centroid

Figure 2.


Function 2 (12.9%)
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100
50
0
-50
-100
0
100
200
300
400
500
Function 1 (86.4%)
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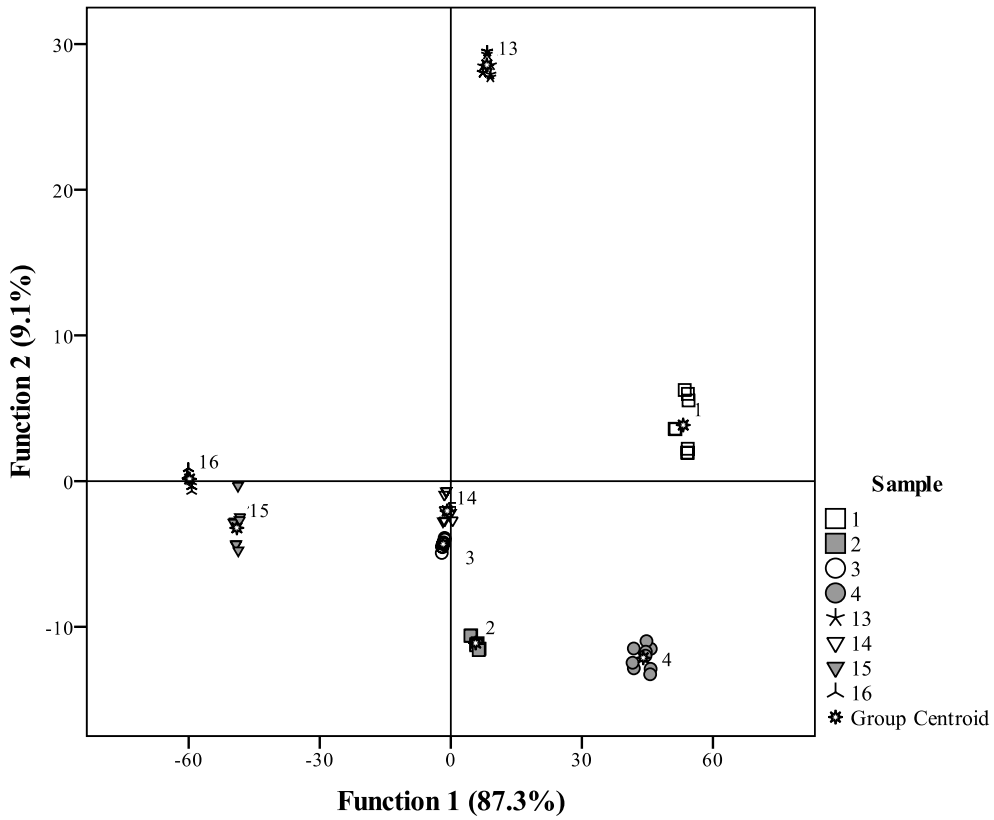
6
8
1
4
13
14
3
2
15
16
5
Sample
1
2
3
4
5
6
7
8
13
14
15
16
Group Centroid

Figure 3.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20
10
0
-10
13
1
16
15
14
3
2
4
-60
-30
0
30
60
Function 1 (87.3%)
Sample
1
2
3
4
13
14
15
16
Group Centroid

Figure 4.

Table 1. Morphological characterization of citrus fruits samples purchased in local markets. Grapefruit and lime collection date unknown; Lemon and orange collected in January 2009. Average values and patrons.

Samples	Origin	Weight (g)	H/D	Shape	Skin	Flavedo (mm)	Albedo (mm)	Segments number	Medulla (core)	Apex	Seed
Grapefruit	Spain	296.4	0.80	subglobose	smooth dotted	2.0	4.0	12.8	hollow	flattened slightly depressed	vestigial
Lemon	Portugal (Trás-os-Montes)	102.3	1.27	elliptical	rough	0.9	4.4	7.4	solid	nipple	seedless
Lime	Spain	76.6	1.22	ovate	smooth	1.0	1.3	9.5	solid	small papilla	seedless
Orange	Portugal (Trás-os-Montes	168.7	1.21	spherical	smooth	1.3	2.8	10.3	semi solid	navel	seedless

Table 2. Yields obtained in the extraction of volatiles and polar compounds of citrus fruits. In each column different letters mean significant differences ($p<0.05$).

	Samples	Yield (%)
Grapefruit	Peels volatile fraction	0.19 ± 0.05 i
	Peels polar fraction	50.13 ± 0.65 c
	Juice polar fraction	Quantitative
Lemon	Peels volatile fraction	0.18 ± 0.04 j
	Peels polar fraction	44.68 ± 0.59 d
	Juice polar fraction	Quantitative
Lime	Peels volatile fraction	1.26 ± 0.16 g
	Peels polar fraction	15.18 ± 0.20 f
	Juice polar fraction	99.37 ± 1.36 a
Orange	Peels volatile fraction	0.32 ± 0.09 h
	Peels polar fraction	37.27 ± 0.81 e
	Juice polar fraction	95.30 ± 2.02 b

Table 3. Antioxidant activity EC_{50} values (mg/mL) of different fractions obtained from citrus fruits. The results are expressed as mean ± SD (n=9). In each column different letters mean significant differences ($p<0.05$).

	Samples	DPPH scavenging Activity	Reducing power	β-carotene bleaching inhibition	TBARS inhibition
Grapefruit	Peels volatile fraction	338.31 ± 26.50 a	2.56 ± 0.02 e	30.39 ± 1.27 a	67.37 ± 1.30 a
	Peels polar fraction	5.15 ± 0.32 de	0.77 ± 0.03 gh	0.57 ± 0.04 hi	0.51 ± 0.01 hi
	Juice polar fraction	12.78 ± 0.53 de	2.57 ± 0.21 e	2.12 ± 0.04 g	2.49 ± 0.35 f
	Crude juice	9.38 ± 0.36 de	4.96 ± 0.05 b	3.28 ± 0.19 f	2.42 ± 0.07 fg
Lemon	Peels volatile fraction	116.25 ± 10.56 b	1.55 ± 0.01 f	23.90 ± 0.58 c	21.04 ± 1.89 c
	Peels polar fraction	3.77 ± 0.16 e	0.43 ± 0.02 hi	0.21 ± 0.01 i	0.19 ± 0.00 i
	Juice polar fraction	11.15 ± 3.08 de	2.67 ± 0.39 e	2.27 ± 0.01 g	2.52 ± 0.18 f
	Crude juice	6.41 ± 1.00 de	3.95 ± 0.75 c	2.36 ± 0.11 g	2.18 ± 0.48 fg
Lime	Peels volatile fraction	124.52 ± 1.28 b	2.41 ± 0.01 e	27.85 ±0.89 b	24.61 ± 1.61 b
	Peels polar fraction	1.72 ± 0.40 e	0.36 ± 0.01 i	0.13 ± 0.01 i	0.10 ± 0.00 i
	Juice polar fraction	15.92 ± 1.20 d	3.85 ± 0.41 c	2.79 ± 0.04 fg	6.23 ± 0.21 e
	Crude juice	12.47 ± 0.45 de	6.07 ± 0.15 a	4.91 ± 0.07 e	2.89 ± 0.22 f
Orange	Peels volatile fraction	95.67 ± 2.21 c	1.09 ± 0.02 g	18.44 ± 0.87 d	17.94 ± 0.43 d
	Peels polar fraction	4.99 ± 0.31 de	0.53 ± 0.01 hi	0.26 ± 0.02 i	0.23 ± 0.00 i
	Juice polar fraction	5.55 ± 0.16 de	1.69 ± 0.37 f	1.23 ± 0.04 h	1.99 ± 0.51 fg
	Crude juice	5.30 ± 0.13 de	3.19 ± 0.04 d	1.12 ± 0.23 h	1.36 ± 0.04 gh

Table 4. Antioxidant compounds present in the polar factions obtained from citrus fruits. The results are expressed as mean ± SD (n=9). In each column different letters mean significant differences (p<0.05).

	Samples	Phenolics (mg GAE/g extract)	Flavonoids (mg CE/g extract)	Ascorbic acid (μg/g extract)	Carotenoids (μg/g extract)	Reducing sugars (mg/g extract)
Grapefruit	Peels polar fraction	55.88 ± 2.45 d	2.29 ± 0.19 de	822.77 ± 5.65 c	2.18 ± 0.03 b	273.68 ± 20.23 b
	Juice polar fraction	8.93 ± 0.16 gh	1.96 ± 0.18 e	519.34 ± 8.65 f	0.22 ± 0.03 f	6.56 ± 0.05 e
	Crude juice	9.46 ± 0.17 g	0.32 ± 0.08 gh	97.31 ± 5.49 k	0.20 ± 0.02 f	10.44 ± 0.67 de
Lemon	Peels polar fraction	87.77 ± 1.42 b	15.96 ± 0.24 a	938.00 ± 1.35 b	1.59 ± 0.04 c	291.26 ± 17.03 b
	Juice polar fraction	8.43 ± 0.02 gh	1.43 ± 0.07 f	348.76 ± 3.30 h	0.07 ± 0.00 h	6.88 ± 0.09 e
	Crude juice	11.17 ± 0.05 f	0.22 ± 0.02 h	417.44 ± 10.87 g	0.06 ± 0.02 h	22.43 ± 0.03 de
Lime	Peels polar fraction	124.63 ± 0.52 a	13.61 ± 0.64 b	1779.55 ± 77.95 a	1.27 ± 0.18 d	38.34 ± 5.47 cd
	Juice polar fraction	7.51 ± 0.06 h	2.36 ± 0.04 d	280.40 ± 1.70 i	0.17 ± 0.01 fg	6.83 ± 0.03 e
	Crude juice	9.01 ± 0.09 g	0.43 ± 0.03 gh	190.52 ± 3.82 j	0.08 ± 0.01 gh	23.81 ± 2.79 de
Orange	Peels polar fraction	79.75 ± 1.25 c	3.97 ± 0.21 c	766.80 ± 20.20 d	31.57 ± 0.06 a	358.93 ± 61.61 a
	Juice polar fraction	13.43 ± 0.10 e	0.56 ± 0.06 gh	693.01 ± 1.08 e	0.26 ± 0.01 f	33.62 ± 2.94 cde
	Crude juice	12.41 ± 0.07 ef	0.62 ± 0.09 g	523.89 ± 5.10 f	0.80 ± 0.02 e	63.07 ± 1.86 c

Table 5. The most important parameters defined for discrimination between different fruit components considering antioxidant activity assays (A), bioactive compounds with all samples (B) and bioactive compounds with juices (C).

	Wilks' Lambda	*F*-remove	*p*-Level	Tolerance	1-Tolerance (R^2)
A					
TBARS inhibition	0.0000	43.113	< 0.001	0.9861	0.0139
β-carotene bleaching	0.0000	1107.9	< 0.001	0.8897	0.1103
Reducing power	0.0000	436.06	< 0.001	0.9983	0.0017
DPPH scavenging	0.0000	6.1685	< 0.001	0.8824	0.1176
B					
Carotenoids	0.0000	61064	< 0.001	0.9963	0.0037
Phenolics	0.0000	816.82	< 0.001	0.8877	0.1123
Flavonoids	0.0000	1410.0	< 0.001	0.4815	0.5185
Ascorbic acid	0.0000	701.37	< 0.001	0.4848	0.5152
Reducing sugars	0.0000	139.49	< 0.001	0.9327	0.0673
C					
Ascorbic acid	0.0000	547.02	< 0.001	0.0685	0.9315
Reducing sugars	0.0000	114.70	< 0.001	0.2554	0.7446
Phenolics	0.0000	38.229	< 0.001	0.0878	0.9122
β-carotene bleaching	0.0000	77.323	< 0.001	0.8111	0.1889
TBARS inhibition	0.0000	33.591	< 0.001	0.1693	0.8307
Carotenoids	0.0000	27.288	< 0.001	0.7918	0.2082
Reducing power	0.0000	7.6667	< 0.001	0.9203	0.0797
Flavonoids	0.0000	4.3288	< 0.001	0.1334	0.8666