Different *Citrus* rootstocks present high dissimilarities in their antioxidant activity and vitamins content according to the ripening stage

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Abstract

"Lane Late" sweet orange grafted on six different citrus rootstocks and grown in the Guadalquivir valley (Seville, Spain) were picked at different ripening stages in two consecutive seasons to characterize their antioxidant activity (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and quantify their main antioxidant compounds (vitamin E and vitamin C). Linear discriminant analysis and 2-way ANOVA were applied to compare the effects induced by citrus rootstock and ripening stage. The results showed that differences in antioxidant activity and related compounds are mainly dependent on the citrus rootstock, despite ripening stage had also some particular effects. Changes observed in 2012 showed less marked differences among the citrus rootstock. Nevertheless, Cleopatra rootstock showed the highest antioxidant activity in both years, indicating that an increase in its cultivation might be a good solution to sweet orange farmers. Concerning the ripening stage, samples collected in January presented higher vitamin contents, while those collected in April showed higher antioxidant activity. This result allows deciding the harvesting period according to the desired effect.

Keywords: Antioxidant activity; Ascorbic acid; α-Tocopherol; *Citrus sinensis*; Ripening stage rootstock.

Introduction

Citrus cultivation is the second most important crop in Andalusia (Spain). Sweet orange is the most important product, representing more than 70% of the total Andalusian citrus production surface. Moreover, Seville is the Spanish region with the highest production of sweet orange, contributing with more than 40% of the total production (Consejeria de Agricultura, 2010).

However, the sweet orange production in this region has been directed on a single pattern, "Carrizo" citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) without taking into account important aspects such as the diversity in agroclimate conditions at Andalusia, the high concentration of supply in the market and the increasing concern about the fruit quality and a healthier lifestyle (Dauchet et al., 2008).

The introduction of new citrus rootstocks in sweet orange production is a good choice to adapt the crop to different requirements. But the kind of pattern used can influence the chemical composition and the antioxidant activity of the fruit (Sanchez-Rodriguez et al., 2012). On the other hand, the ripening stage might also exert important effects over the chemical composition and the antioxidant activity (Nuncio-Jauregui et al., 2014; Zhang et al., 2010).

With regard to chemical composition, orange is typically considered as one of the products with high contents of antioxidants and vitamins (Del Caro et al., 2004; Dhuique-Mayer et al., 2005). It is well known that the intake of antioxidant compounds is associated with the prevention of various human oxidative stress-related diseases such as neurodegenerative and cardiovascular diseases, stroke, cataractogenesis, diabetes and cancer (Halliwell, 2011; Mateos et al., 2005; Schreckinger et al., 2010). The principal reason is because the oxidative stress-related diseases are produced when the normal balance between the production of free radicals and their neutralization by antioxidant defences tends to the

overproduction of free radicals in the organism. In particular, the antioxidants present in orange such as tocopherols (vitamin E) and ascorbic acid (vitamin C) are believed to prevent us against degenerative malfunctions due to their role as scavengers of overproduced free radicals. Moreover, the intake of ascorbic acid (vitamin C) is critical because of the human inability to synthesize it.

In this context, the present work evaluates the quality of "Lane Late" sweet orange grafted on six different citrus rootstocks and grown in the Guadalquivir valley (Seville) according to the ripening stage (harvested at the beginning and end of the season) in two consecutive seasons (2011 and 2012). Besides characterizing the antioxidant activity in these different conditions, the main antioxidant compounds (which represent the most valued attributes in this fruit) were also quantified.

Materials and methods

Samples

Seven-years-old trees of Lane Late variety were grafted on three new citrus rootstocks, Forner-Alcaide nº5 (FA5), Forner-Alcaide nº13 (FA13) and Forner-Alcaide nº41 (FA41) (hybrids of *Cleopatra mandarin*×*Poncirus trifoliata*), and three traditional rootstocks, Carrizo citrange (CA) (*Citrus sinensis*×*P. trifoliata*), *Citrus macrophylla* (MP) and *Cleopatra mandarin* (Citrus reshni) (CL) to evaluate the effect of the citrus rootstocks on bioactive compounds and antioxidant activity. Moreover, samples were harvested in different ripening stages, at the beginning and end of the season (January and April) during two consecutive years (2011 and 2012). The production yield of each tree presented similar values (≈5 kg/m^3) for all rootstocks studied. After being collected and subsequently separation of the peel (eight by treatment and replication), the pulp was lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA), reduced to a fine dried

powder (20 mesh), mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

All samples reached the minimum requirements for quality standards imposed by regulation (colour index>6; maturity index>6.5; equatorial diameter>53mm and over 33% of juice) (*Regulation (CE) Nº1221/2008. European Commission, 5th December, Official Journal of the European Union, 1-80).*

Standards and Reagents

Ethyl acetate 99.8% and n-Hexane 95% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), α-tocopherol and ascorbic acid standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Methanol and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Antioxidant activity evaluation

Each lyophilized sample (0.5 g) was extracted by stirring with 20 mL of methanol/water (80:20, *v:v*) for 1h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 20 mL of methanol/water (80:20, *v:v*) for 1h. The combined hydromethanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness and re-dissolved in methanol/water (80:20, *v:v*) for antioxidant activity assays (40 mg/mL). Successive dilutions were made from the stock solution and submitted to the *in vitro* assays already described by Martins et al.

(2014), to evaluate the antioxidant activity of the samples. The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against sample concentrations (Pinela et al. 2012). Trolox was used as a positive control.

Reducing power by ferricyanide/Prussian blue assay. The extract solutions with different concentrations (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 °C 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 plate, the same with deionized water (0.8 mL) and ferric chloride (0.1% *w/v*, 0.16 mL), and the absorbance was measured at 690 nm in ELX800Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

DPPH radical-scavenging activity assay. This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 well plate consisted of a solution by the well of the extract solutions with different concentrations (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

Inhibition of β-carotene bleaching or β-carotene/linoleate assay. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and 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 test tubes containing extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (Absorbance after 2h of assay/initial absorbance)×100.

Thiobarbituric acid reactive substances (TBARS) assay. Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 *w/v* brain tissue homogenate which was centrifuged at 3000 *g* for10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 500 μL), followed by thiobarbituric acid (TBA, 2%, *w/v*, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color 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] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

Vitamins content

Tocopherols. Tocopherols were determined following a procedure previously optimized and described by the authors (Barros et al., 2010). Analysis was performed in a HPLC system consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), an auto-sampler (AS-2057 Jasco, Easton, MD, USA), and a fluorescence detector (FP-2020; Jasco) programmed for

excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry pulp.

Ascorbic acid. Ascorbic acid was analysed by ultra-fast liquid chromatography (UFLC, Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled with a photodiode array detector (PDA) as previously optimized and described by the authors (Pereira et al., 2013). The quantification was made by comparison of the area of the peaks recorded at 245 nm with the calibration curve obtained from commercial standard of L-ascorbic acid. The results were expressed in g per 100 g of dry pulp.

Statistical analysis

For each sample, all the extractions were performed in triplicate; each replicate was also measured in three times. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., USA).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors “citrus rootstocks” (CR) and “ripening stage” (RS). When a statistically significant interaction (CR×RS) was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means were compared using Tukey’s honestly significant difference (HSD) multiple comparison test.

Stepwise Linear Discriminant Analysis (LDA) was used to verify if differences found for antioxidant activity and bioactive compound contents were high enough to discriminate the evaluated CR (Carrizo, Cleopatra, Foner-Alcaide nº13, Forner-Alcaide nº41, Forner-Alcaide nº5 and Macrophylla) as also to conclude which of the RS (January or April) optimizes the indicated properties. A stepwise technique, using the Wilks's λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove) was applied to select variables. This procedure followed a combination of forward selection and backward elimination steps; *i.e.*, before a new variable is selected to be included, it is verified whether all previously selected variables remain significant. The combination of variables is defined in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on. To verify which canonical discriminant functions were significant, the Wilks's λ test was applied. To keep a more realistic data modulation, a leave-one-out cross-validation procedure was carried out to assess the model performance.

Results and discussion

The levels of ascorbic acid and α-tocopherol found, as well as the EC_{50} values obtained for each antioxidant activity assay are given in **Table 1**. The results are represented as the mean value of each CR (citrus rootstocks) for both RS (ripening stage), as well as the mean value of each RS, comprising values for all used CR. For all the performed analysis, the studied CR showed antioxidant activity, being particularly active in β-carotene bleaching inhibition (lowest EC_{50} values). The contents in ascorbic acid and α-tocopherol belong to the expected range of values for this kind of matrix, with ascorbic acid being quantified in much higher quantity (Miguel et al., 2009; Milella et al., 2011; Tsai et al., 2007).

In order to verify if differences in antioxidant profiles or vitamin levels are specific of a determined CR or RS, the interaction between these two factors was studied. The interaction among factors (CR×RS) was significant ($p < 0.05$) in all cases (**Table 1**), not allowing any multiple comparison tests. In these cases, the conclusions were drawn from the estimated marginal mean (EMM) plots obtained in each case. The results obtained for RS were compared using a simple *t*-test for equality of means (after checking the equality of variances through a Levene's test), since there were less than three groups.

Despite the significant interactions, some particular tendencies became evident from the analysis of the EMM plots: the α-tocopherol (in 2011, **Figure 1A** and 2012, **Figure 1B**) and ascorbic acid (in 2011, **Figure 1C** and 2012, **Figure 1D**) levels were higher in samples collected in January for almost all the tested CR, except for α-tocopherol in Carrizo and ascorbic acid in Macrophylla, both in 2011. These tendencies might also be depicted from the corresponding chromatograms (**Figure 2**), either for α-tocopherol (Cleopatra RS, **Figure 2A**), as for ascorbic acid (Carrizo RS, **Figure 2B**). Nevertheless, the higher levels in these antioxidant compounds were not reflected by the measured antioxidant activity. In fact, in all cases where significant differences were found, the EC_{50} values were lower in samples collected in April for all the tested CR: DPPH scavenging activity (in 2012, **Figure 3A**), reducing power (in 2011, **Figure 3B**), β-carotene bleaching inhibition (in 2011, **Figure 3C**) and TBARS formation inhibition (in 2012, **Figure 3D**). This apparent increase in the antioxidant activity might be related with the production of higher quantities of other bioactive compounds such as phenolic compounds and carotenoids, which usually augment with the physiological development of the fruit. In fact, similar results for antioxidant activity variation with the ripening stage have been already reported in other products like persimmon and apple (Ferreira Zielinski et al., 2014; Plaza et al., 2012).

Regarding the CR effect, some tendencies could also be identified (data not shown); namely, the higher levels of ascorbic acid in Cleopatra, which demonstrated also stronger DPPH scavenging activity and TBARS formation inhibition, in all cases for the results of the year 2011. The CR Forner-Alcaide 41, by its side, showed lower levels of ascorbic acid in 2012.

Until this point, the pointed out variations were analyzed considering each parameter at once. This might have the limitation of hindering having a global perspective of the true effects of CR or RS. Accordingly, two linear discriminant analyses (LDA) were applied to understand the concerted effects of both factors on the antioxidant activity and vitamin amounts, one for each year. The significant independent variables (results for antioxidant activity assays and antioxidant compound levels) were selected using the stepwise method of the LDA, according to the Wilks' λ test. Only those variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

In the case of CR effect, and starting with the year 2011, 5 significant functions were defined, from which the first three were plotted (**Figure 4A**), including 98.8% of the results variance (first, 91.7%; second, 4.9%; third, 2.2%). As it can be immediately depicted from the discriminant scores distribution, the tested groups (Carrizo, Cleopatra, Foner-Alcaide nº13, Forner-Alcaide nº41, Forner-Alcaide nº5 and Macrophylla) were almost completely separated. Function 1 was mostly correlated with TBARS formation inhibition and β-carotene bleaching inhibition, separating Cleopatra and Macrophylla, which presented the lowest and highest EC_{50} values, respectively, in those assays; function 2, more strongly correlated with DPPH scavenging activity and reducing power, somehow contributing to separate Forner-Alcaide nº5, which showed lower activity in these two assays; function 3 was more correlated β-carotene bleaching inhibition, but it did not separate any of the CR with satisfactory effectiveness. In terms of classification

performance, the corresponding contingency matrix (**Table 2**) gave values of sensitivity and overall specificity of 89 and 90% respectively within the leave-one-out cross-validation procedure, which may be considered as acceptable values. It is interesting to verify that only Carrizo, Forner-Alcaide nº41 and Forner-Alcaide nº5 showed misclassified cases (4 Carrizo were classified as Forner-Alcaide nº41, 4 Forner-Alcaide nº41 as Carrizo and 4 Forner-Alcaide nº5 were classified as Forner-Alcaide nº41).

Regarding the results for samples harvested during the year 2012, the discriminant model selected also 5 significant functions, which included 100.0% of the observed variance. The graph representation (**Figure 4B**) of the three first functions (function 1: 64.7%, function 2: 25.9%, function 3: 7%) did not show the assayed groups as separated as in the case of year 2011, but some differences among CR are still evident. Function 1 was mostly correlated with α-tocopherol and DPPH scavenging activity, but none of the CR became separated in a distinctive manner in result of this function; function 2 was more correlated with β-carotene bleaching inhibition, favoring the separation of Cleopatra rootstock (which presented higher EC_{50} values for that assay); function 3 correlated better with TBARS formation inhibition, contributing to separate Forner-Alcaide nº41 (which presented high EC_{50} values for this assay). The classification ability was not as high as the obtained for the year 2011 (**Table 2**), but the values for sensitivity (80%) and overall specificity (86%) within the leave-one-out cross-validation procedure, may still be considered as acceptable values. Despite some misclassified samples, it is relevant to say that all Forner-Alcaide nº5 samples were correctly classified.

In what concerns the combined effect of RS, samples harvested in each one of the months were clearly separated and classified with 100% of accuracy, with the same result being obtained for both years. A single function was defined in each case (the number of defined functions equals the number of levels for a determined factor less one), more correlated

with reducing power (lower EC_{50} values for samples collected in April) in 2011 and with TBARS formation inhibition (lower EC_{50} values for samples collected in January) in 2012. Overall, the results obtained in this work proved that the differences in antioxidant activity and related compounds are strongly dependent on the CR, but vary also due to the effects of the RS. In fact, some particular compounds, especially α-tocopherol, as well as specific antioxidant activity indicators such as the TBARS formation inhibition are strongly altered by the ripening stage of the studied sweet orange CR. When comparing the results for the consecutive years, changes observed in 2012 showed less marked differences among the CR. Nevertheless, Cleopatra rootstock was a very active CR in both years (as confirmed in the LDA), indicating that an increase in its production might be a good solution in sweet orange production. Concerning the ripening stage, samples collected in January presented higher vitamin contents, while those collected in April showed higher antioxidant activity. This result allows deciding the harvesting period according to the desired effect.

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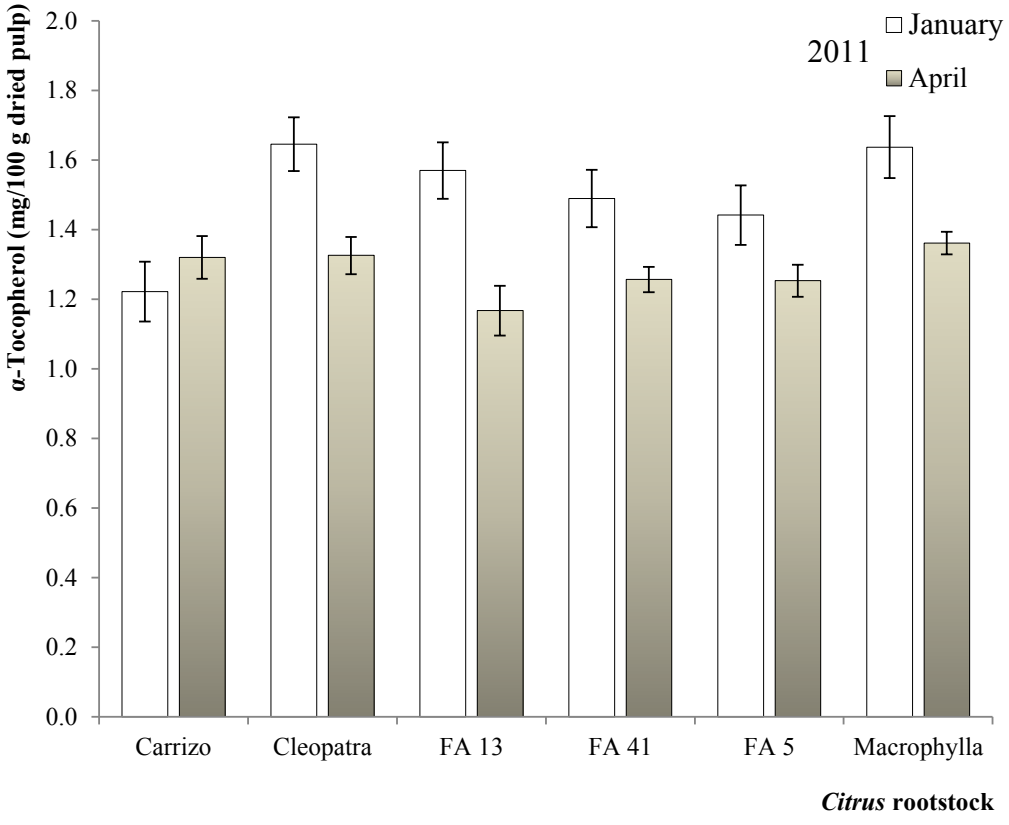
Table 1. Antioxidant properties (EC_{50} values in mg/mL) and major antioxidant compounds divided by citrus rootstock (CR) and ripening stage (RS). The results are presented as mean±SD*.

		DPPH scavenging activity	Reducing power	Lipid peroxidation inhibition		α-Tocopherol (mg/100 g of dried pulp)	Ascorbic acid (g/100 g of dried pulp)
				TBARS formation inhibition	β-Carotene bleaching inhibition		
				2011			
Citrus Rootstock (CR)	Carrizo	8.0±0.4	2.4±0.1	3.2±0.3	1±1	1.3±0.1	0.3±0.1
	Cleopatra	6±1	1.9±0.5	2.1±0.5	0.8±0.4	1.5±0.2	0.35±0.03
	Forner-Alcaide 13	7±1	2±1	2.3±0.5	0.7±0.2	1.4±0.2	0.31±0.03
	Forner-Alcaide 41	7±1	2.1±0.5	3.6±0.1	2±1	1.4±0.1	0.32±0.05
	Forner-Alcaide 5	11±2	2.6±0.1	3.3±0.3	1±1	1.3±0.1	0.36±0.05
	Macrophylla	9.1±0.2	3.0±0.5	3.5±0.2	2±1	1.5±0.2	0.34±0.03
p-value (n = 18)	Tukey's test	<0.001	<0.001	<0.001	0.001	<0.001	0.088
Ripening stage (RS)	January	8±1	2.7±0.4	3±1	2±1	1.5±0.2	0.36±0.05
	April	8±3	1.9±0.5	3.1±0.4	0.4±0.1	1.3±0.1	0.30±0.05
p-value (n = 54)	*t*-student's test	0.128	<0.001	0.123	0.696	<0.001	<0.001
p-value (n = 108)	CR×RS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
				2012			
Citrus Rootstock (CR)	Carrizo	10±2	2.9±0.1	2.9±0.2	1.02±0.04	1.1±0.2	0.32±0.03
	Cleopatra	9±1	3.1±0.5	3.3±0.1	3±2	1.2±0.2	0.36±0.03
	Forner-Alcaide 13	8±1	3±1	3±1	0.6±0.3	1.3±0.1	0.34±0.03
	Forner-Alcaide 41	11±2	2.6±0.1	3.6±0.3	0.20±0.03	1.3±0.4	0.29±0.03
	Forner-Alcaide 5	8±1	3.8±0.2	3±1	0.3±0.1	1.1±0.2	0.31±0.03
	Macrophylla	9.2±0.5	3.0±0.3	3±1	1.0±0.1	1.1±0.2	0.31±0.03
p-value (n = 18)	Tukey's test	<0.001	<0.001	0.600	<0.001	0.030	<0.001
Ripening stage (RS)	January	10±2	3±1	2.5±0.5	1±1	1.4±0.1	0.33±0.03
	April	8±1	2.9±0.4	3.5±0.3	0.6±0.3	1.0±0.1	0.31±0.04
p-value (n = 54)	*t*-student's test	<0.001	<0.001	<0.001	0.002	<0.001	0.001
p-value (n = 108)	CR×RS	<0.001	0.002	<0.001	<0.001	<0.001	<0.001

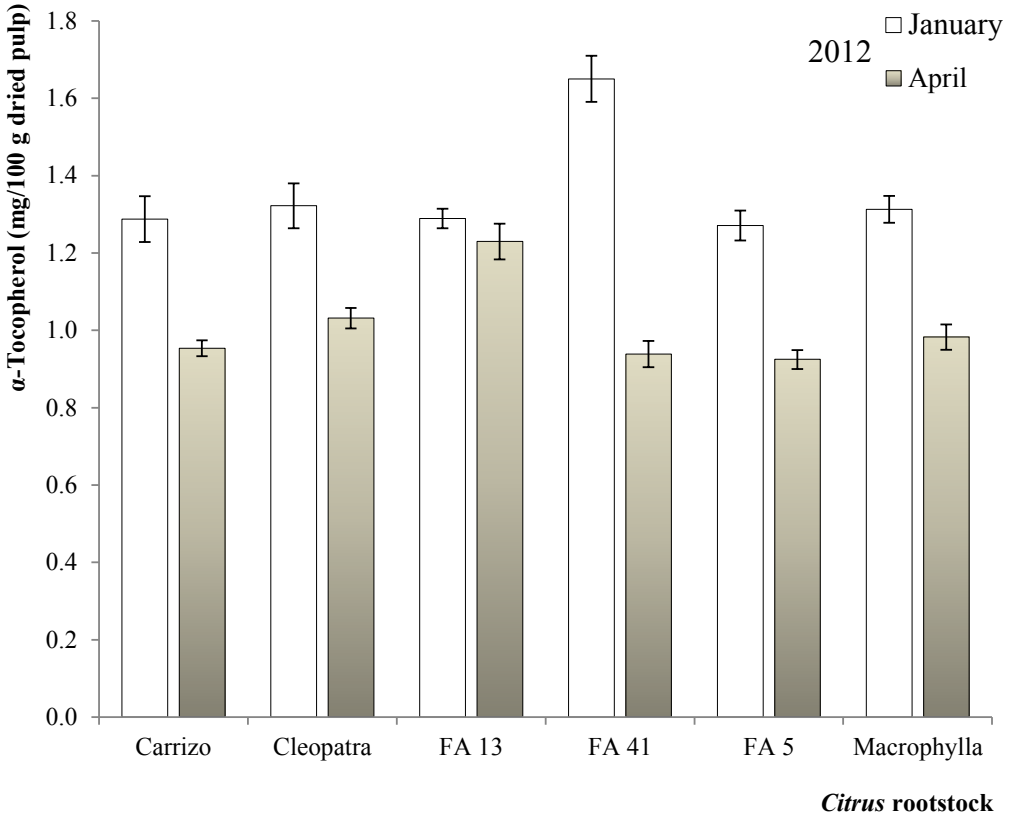
*Mean and standard deviation (SD) values reflect the results obtained for samples in different conditions (corresponding to different levels of the non-fixed factor, CR or RS). Accordingly, the SD values should not be considered as a measure of accuracy of the applied methodologies.

Table 2. Contingency matrix obtained using LDA based on antioxidant activity and antioxidant contents of sweet orange Lane Late grafted in different Citrus Rootstocks (CR).

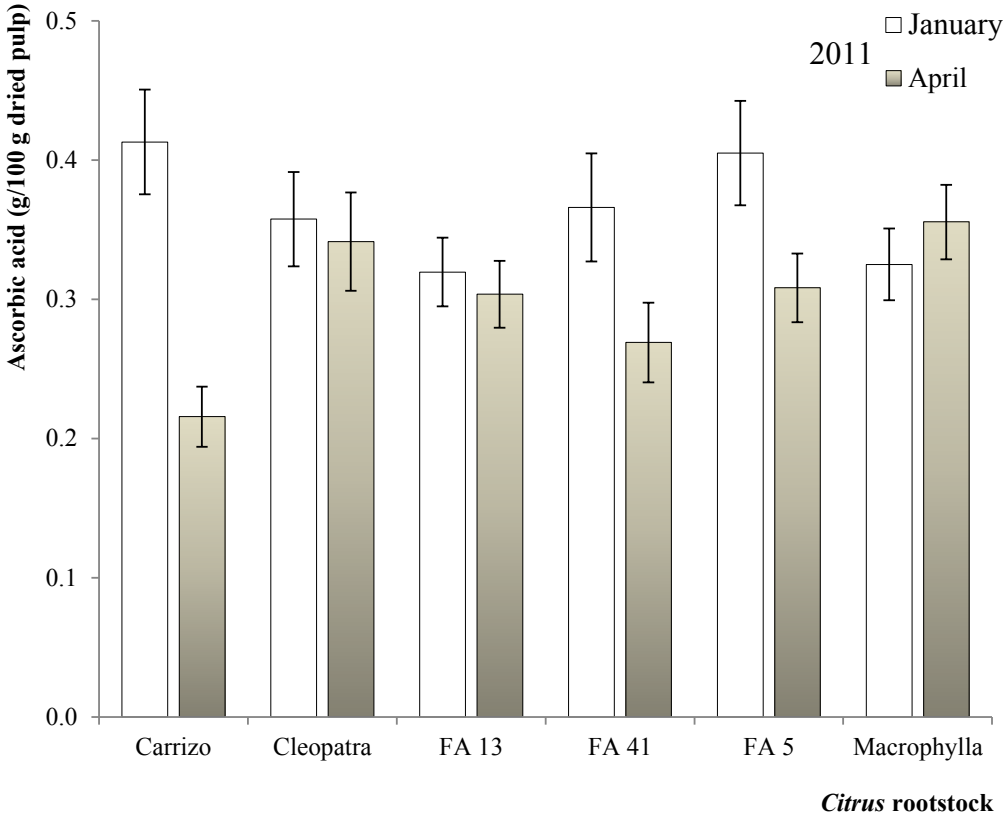
CR	Predicted Group Membership						Total	Sensitivity (%)
	Carrizo	Cleopatra	Forner-Alcaide 13	Forner-Alcaide 41	Forner-Alcaide 5	Macrophylla		
				2011				
Carrizo	14	0	0	4	0	0	18	78
Cleopatra	0	18	0	0	0	0	18	100
Forner-Alcaide 13	0	0	18	0	0	0	18	100
Forner-Alcaide 41	4	0	0	14	0	0	18	78
Forner-Alcaide 5	0	0	0	4	14	0	18	78
Macrophylla	0	0	0	0	0	18	18	100
Total	18	18	18	22	14	18	108	89
Specificity (%)	78	100	100	64	100	100	90	
				2012				
Carrizo	13	0	0	0	0	5	18	72
Cleopatra	0	15	0	0	0	3	18	83
Forner-Alcaide 13	0	0	9	0	9	0	18	50
Forner-Alcaide 41	1	0	0	17	0	0	18	94
Forner-Alcaide 5	0	0	0	0	18	0	18	100
Macrophylla	1	0	0	1	1	15	18	83
Total	15	15	9	18	28	23	108	80
Specificity (%)	87	100	100	100	64	65	86	


α-Tocopherol (mg/100 g dried pulp)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2011
January
April
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

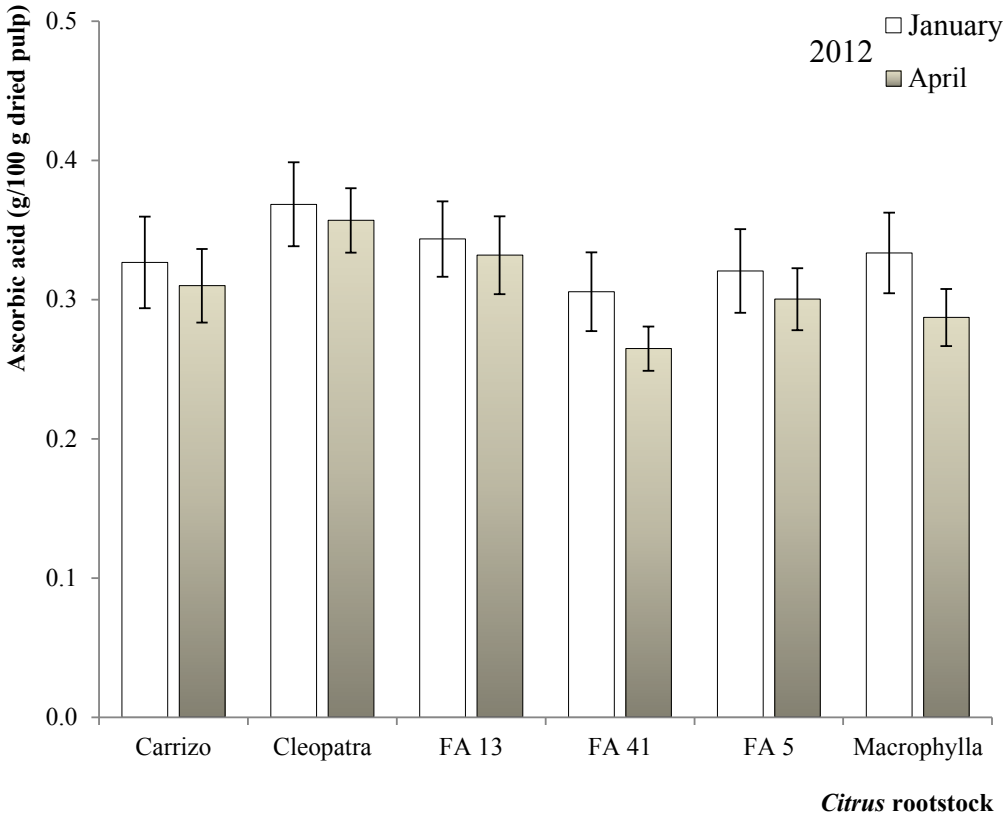
(A)


α-Tocopherol (mg/100 g dried pulp)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2012
January
April
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(B)

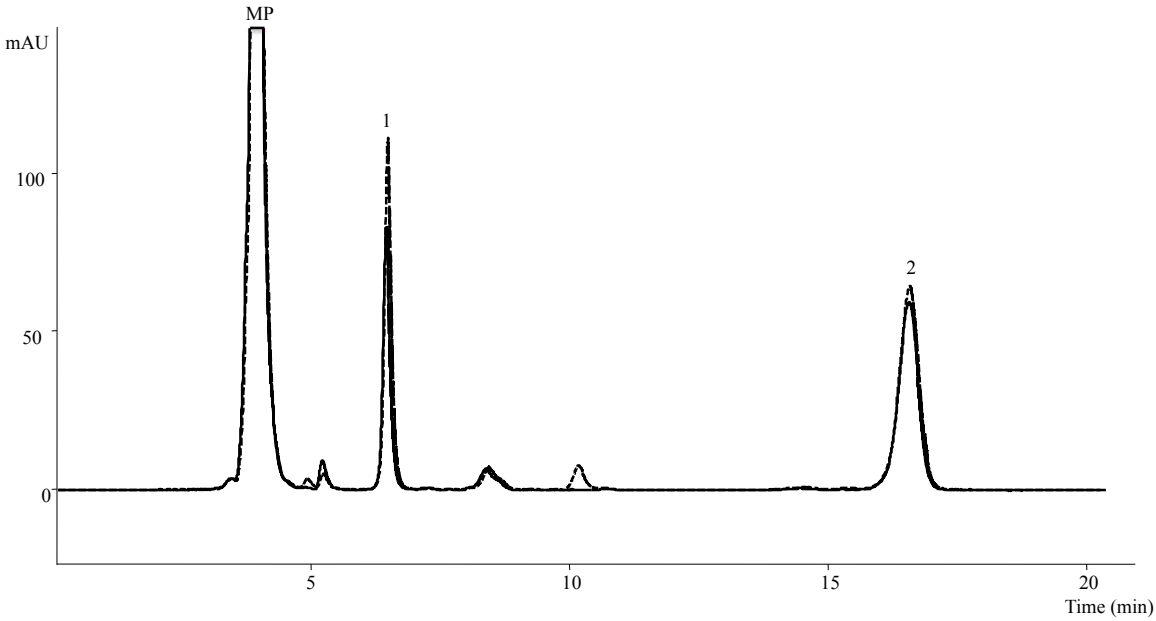

Ascorbic acid (g/100 g dried pulp)
0.5
0.4
0.3
0.2
0.1
0.0
2011
January
April
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(C)

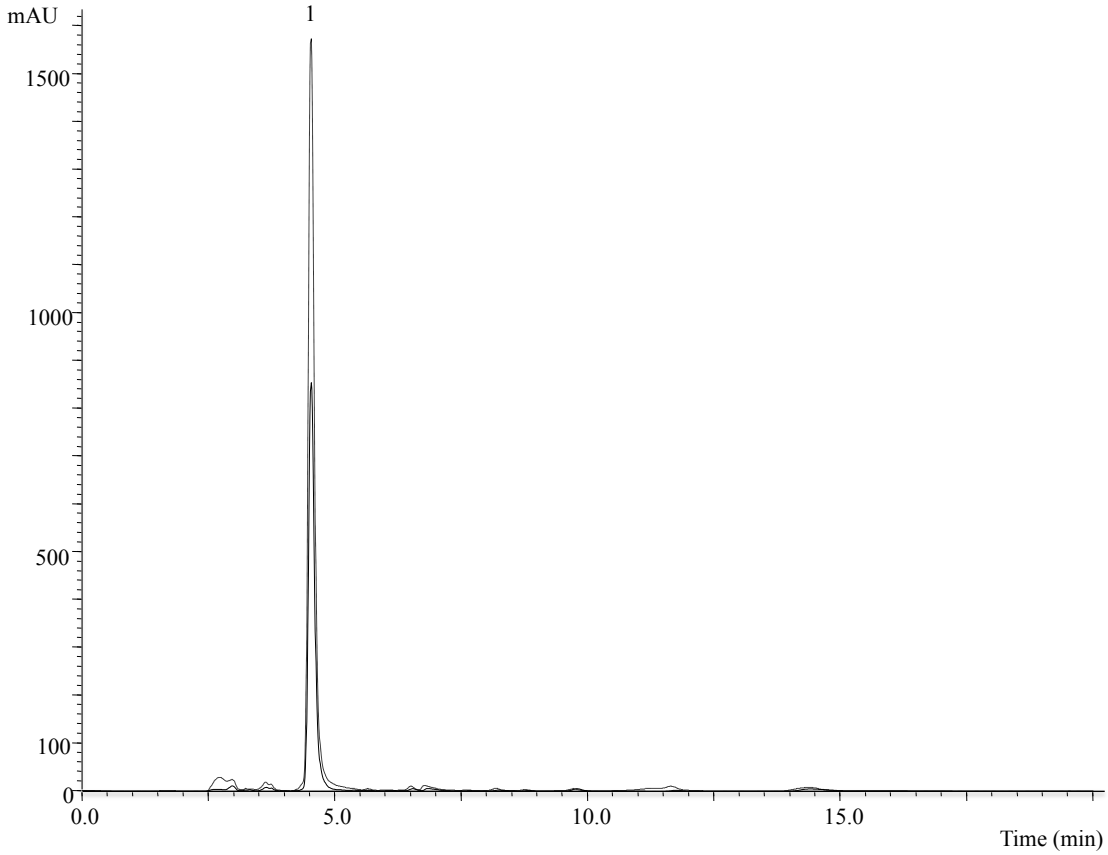

Ascorbic acid (g/100 g dried pulp)
0.5
0.4
0.3
0.2
0.1
0.0
2012
January
April
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(D)

Figure 1. Estimated marginal mean plots representing the effect of RS on the antioxidant vitamins of *C. sinensis* fruits. **A**- α-tocopherol (2011); **B**- α-tocopherol (2012); **C**- ascorbic acid (2011); **D**- ascorbic acid (2012).

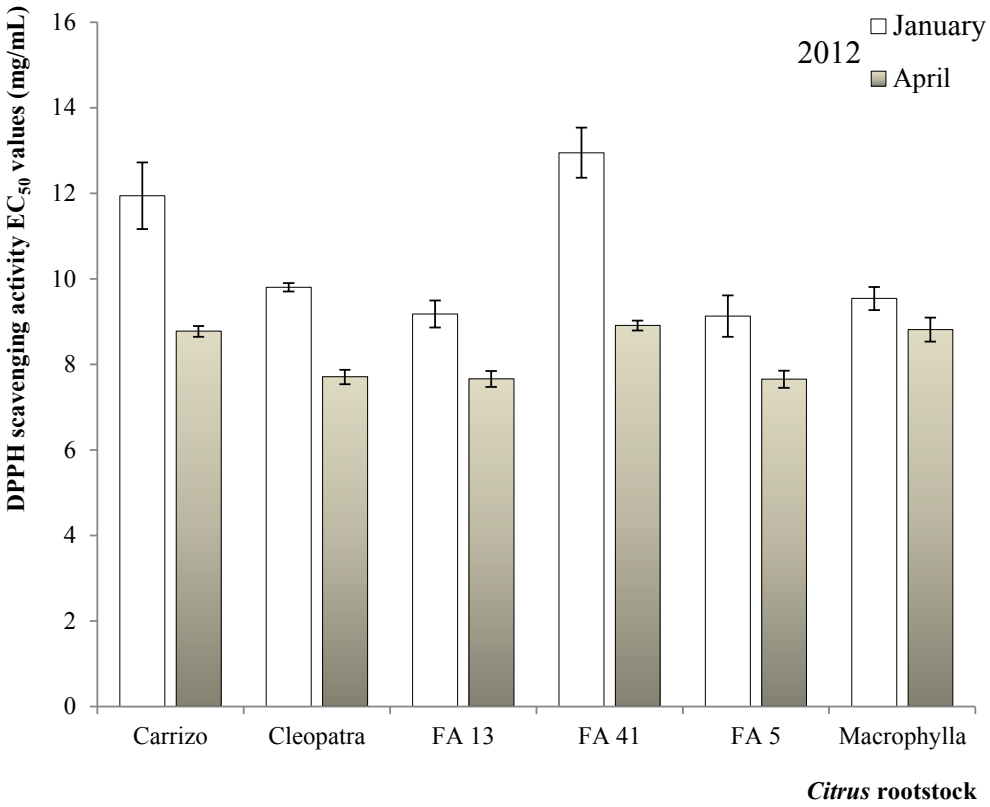

MP
mAU
1
2
100
50
0
5
10
15
20
Time (min)

(A)

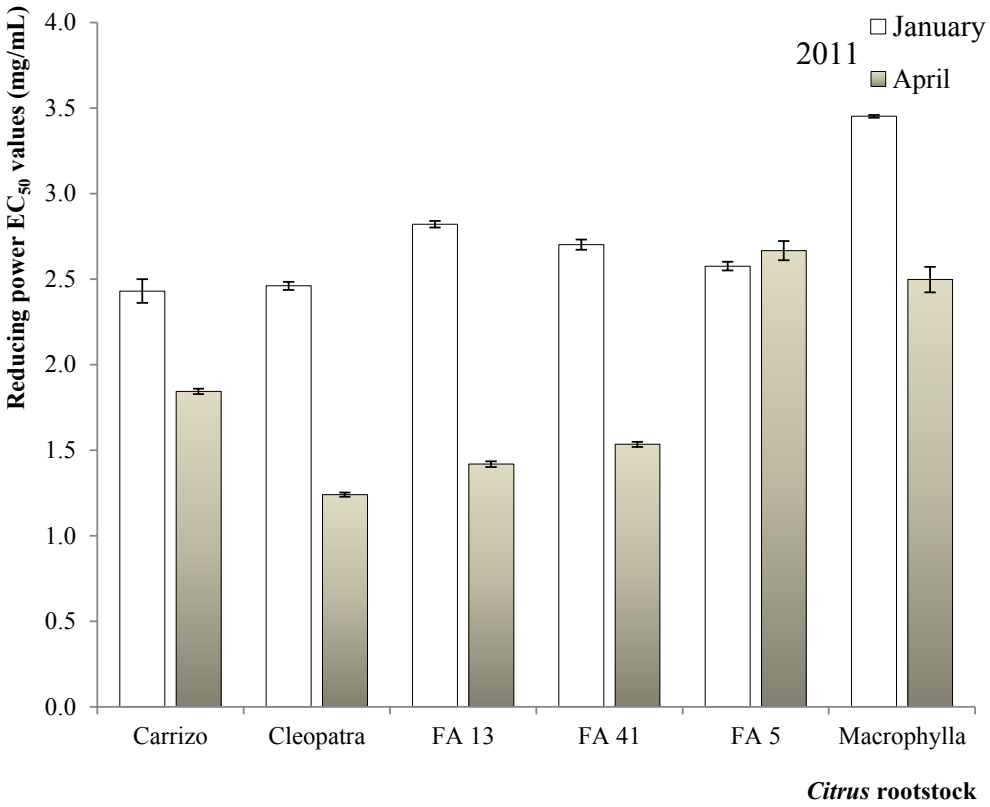

mAU
1
1500
1000
500
100
0
0.0
5.0
10.0
15.0
Time (min)

(B)

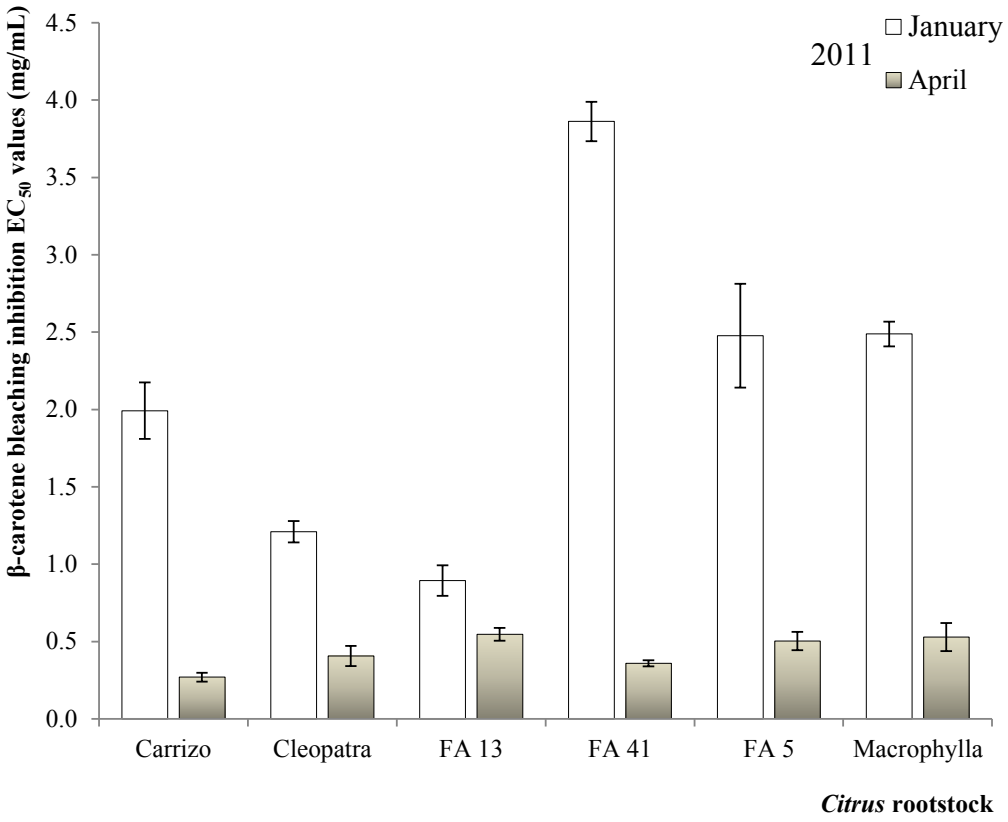
Figure 2. HPLC chromatograms of α-tocopherol (**A**, MP- mobile phase; 1- α-tocopherol; 2- tocol) in the dried pulp of Cleopatra rootstock and ascorbic acid (**B**, 1-ascorbic acid) in the dried pulp of Carrizo rootstock both for the year 2011(------January and ——April).


2012
January
April
DPPH scavenging activity EC50 values (mg/mL)
16
14
12
10
8
6
4
2
0
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

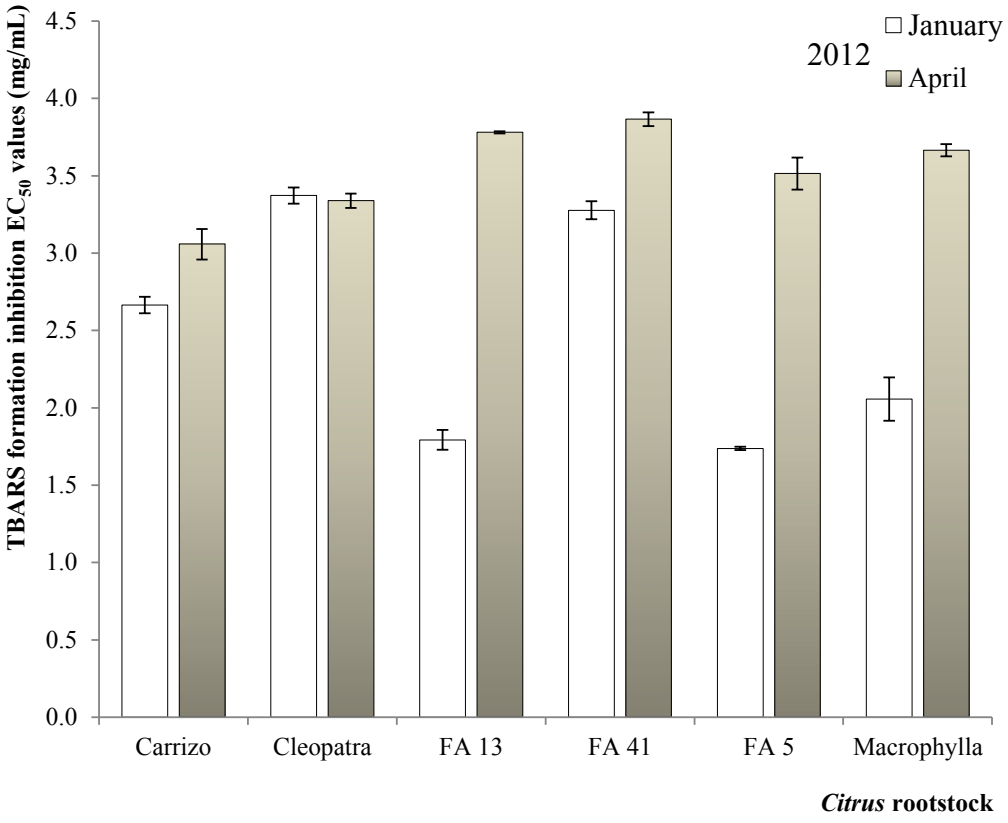
(A)


2011
January
April
Reducing power EC50 values (mg/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(B)

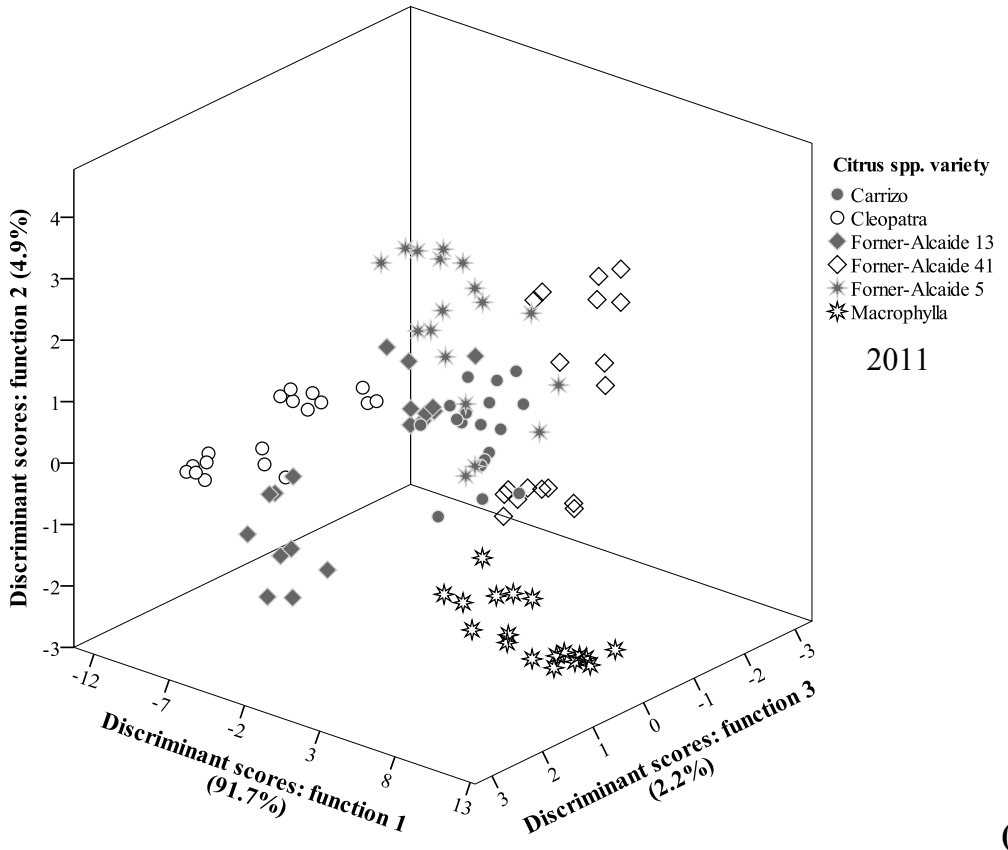

2011
January
April
β-carotene bleaching inhibition EC50 values (mg/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(C)

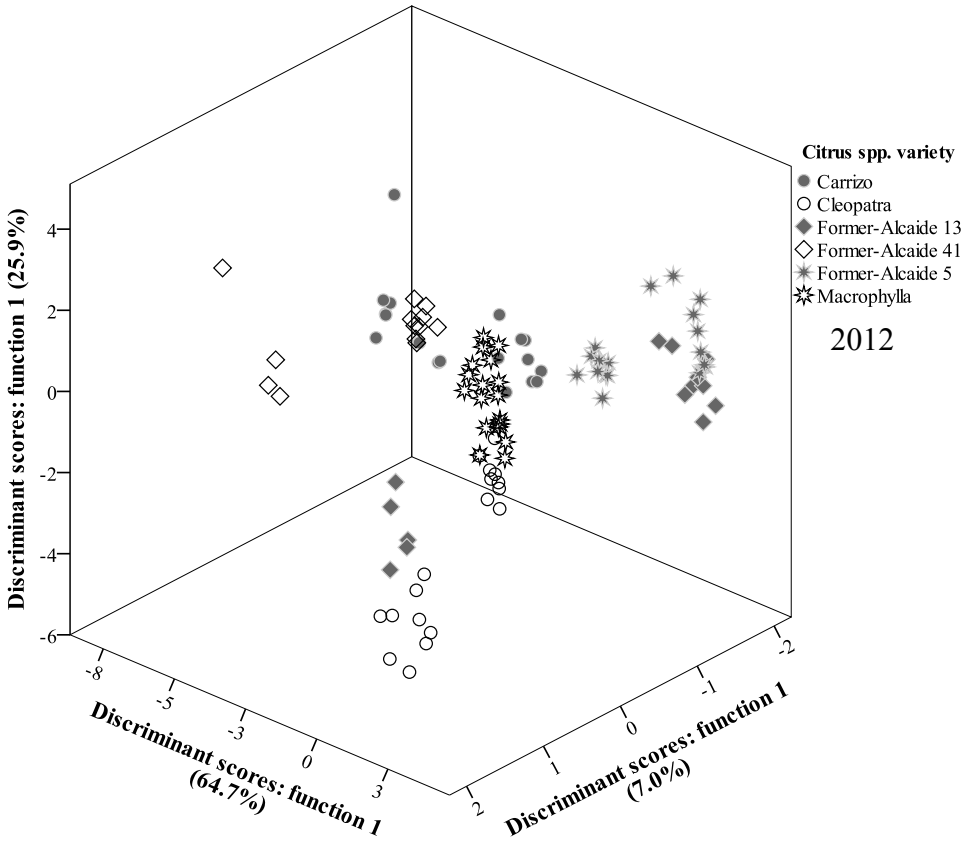

2012
January
April
TBARS formation inhibition EC50 values (mg/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Carrizo
Cleopatra
FA 13
FA 41
FA 5
Macrophylla
Citrus rootstock

(D)

Figure 3. Estimated marginal mean plots representing the effect of RS on the antioxidant properties of *C. sinensis* fruits. **A**- DPPH scavenging activity (2012); **B**- reducing power (2011); **C**- β-carotene bleaching inhibition (2011); **D**- TBARS formation inhibition (2012).


Citrus spp. variety
Carrizo
Cleopatra
Forner-Alcaide 13
Forner-Alcaide 41
Forner-Alcaide 5
Macrophylla
2011
Discriminant scores: function 2 (4.9%)
Discriminant scores: function 1 (91.7%)
Discriminant scores: function 3 (2.2%)

(A)


Citrus spp. variety
Carrizo
Cleopatra
Former-Alcaide 13
Former-Alcaide 41
Former-Alcaide 5
Macrophylla
2012
Discriminant scores: function 1 (25.9%)
Discriminant scores: function 1 (64.7%)
Discriminant scores: function 1 (7.0%)

(B)

Figure 4. Discriminant scores scatter plot of the canonical functions defined for antioxidant parameters results according with CR for the years 2011 (**A**) and 2012 (**B**).