Moraga Ballesteros, G.; Igual Ramo, M.; García Martínez, EM.; Mosquera, LH.; Martínez Navarrete, N. (2012). Effect of relative humidity and storage time on the bioactive compounds and functional properties of grapefruit powder. Journal of Food Engineering. 112(3):191-199. doi:10.1016/j.jfoodeng.2012.04.002.

Effect of relative humidity and storage time on the bioactive compounds and functional properties of grapefruit powder

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Abstract

The modified state diagram of freeze-dried grapefruit powder was obtained in order to determine the critical water content and critical water activity that cause the glass transition of the amorphous matrix at storage temperature. At 20ºC these values were in the ranges of 0.031-0.057 g water/ g product and 0.089-0.210, respectively. Below those critical values, the glassy state of the amorphous matrix is guaranteed, thus avoiding an increase in the rate of the deteriorative reactions related to the loss of the bioactive compounds in the fruit (organic acids, vitamin C, main flavonoids, and total phenols) which contribute to the antioxidant capacity (AAO) of grapefruit. In the rubbery state, a certain time is needed for these degradative reactions to start. This time depends on the water content of the sample,

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the greater the water content the lower the time needed. In this study, the powder was stable for a relatively long storage time (3 months) regardless the relative humidity, due to the limited mobility of the molecular system. Between 3 and 6 months had to pass before a significant loss of bioactive compounds was observed; the higher the relative humidity, the greater the loss.

Keywords: freeze-drying, glass transition, water activity, antioxidant capacity, phenolic compounds, vitamin C, organic acids.

1. Introduction

Nowadays, there is a great deal of interest surrounding the healthy properties of fruit, as its intake appears to be associated with a reduced risk of chronic and degenerative diseases, such as cancer and cardiovascular diseases. This protective effect seems to be linked to its antioxidant activity, mainly related to the presence of vitamin C and a series of non-nutritive substances, called phytochemicals, which mainly include phenolic compounds (Igual et al., 2011). These compounds may inhibit the development of major oxidative reactions of the human body as they should neutralise free radicals (Xu et al., 2008). On the other hand, organic acids present in fruit, including citric, tartaric and malic acids, are important components which both contribute flavour attributes (Cen et al., 2007) and also help to stabilise ascorbic acid and phenolic compounds (Wang et al., 2007).

Grapefruit is a citrus fruit with especially high amounts of ascorbic acid and phenolic compounds which are beneficial for human health (Xu et al., 2008; Igual et al., 2010). As fresh fruit, however, its bitter taste limits its popularity among consumers and traditionally it has been used by the industry to obtain marmalades and beverages. Nowadays,

techniques like spray-drying or freeze-drying have opened up new alternatives for the fruit processing, as it is easier to handle, package and transport the obtained fruit powder at the same time that they provide a high-quality product with a prolonged shelf life. Freeze-drying is considered as a reference process. The sublimation of ice, coupled with a low process temperature, seems not only to preserve flavour and colour but also to minimize thermal damage to heat sensitive nutrients. Moreover, freeze-dried fruit powders can be reconstituted quickly to a good quality product (Mastrocola et al., 1997) or served as a functional ingredient for various food systems and new products as they provide numerous functional and nutritional benefits (Grabowski et al., 2008). Baby foods, sauces, soups, extruded cereal products, fruit purees for confectionary products and fillings for frozen toaster snacks are examples of these products.

Nevertheless, during freeze-drying the fruit is subjected to a rapid water removal that usually results in a very hygroscopic amorphous matrix. It is well known that the physical state of that phase, glassy or rubbery depending on the water content and temperature, is directly related to the powder's stability. The change from the glassy to the rubbery state occurs in a temperature range whose amplitude usually varies between 10 to 30 °C. Both the onset and midpoint temperatures of the glass transition temperature range are commonly referred to as glass transition temperature (T_g) (Roos, 1995). In powdered products, the T_g can be considered as a critical parameter, since collapse, stickiness, caking, agglomeration problems and re-crystallization phenomena may be avoided in the glassy state of the amorphous matrix (Roos, 1995; Ahmed & Ramaswany, 2006; Wang & Langrish, 2009; Fang & Bhandari, 2011). The structural changes caused by powder collapse affect some physical properties, such as color or mechanical behavior.

67 Nevertheless, in many cases not only is the rubbery state required but also a certain water
68 content, greater than the critical water content necessary for the glass transition to occur, is
69 needed for non-enzymatic and enzymatic browning development (Mosquera et al., 2011).
70 Also in the glassy state, some chemical and enzymatic reactions dependent on molecular
71 diffusion, which could cause a loss in the functional value of a product, may be prevented.
72 However, the work carried out in this field is much more limited.

73 The low molecular weight of the major solutes of fruits, sugars and organic acids,
74 implies low T_g values of the amorphous matrix (Telis & Martínez-Navarrete, 2009).
75 Moreover, in the range of non-freezable water content of these foods, the physical state of
76 this matrix is very sensitive to small changes in temperature and moisture content that can
77 occur at the usual storage conditions of these products. In this sense, a change from a glassy
78 to a rubbery state can occur in the powder product as a consequence of a slight increase in
79 the temperature or in the product water content during its storage. Depending on storage
80 temperature, the glass transition will occur at a critical value of water content (CWC) and
81 water activity (CWA) of the sample, which can be considered an important factor for the
82 stability of the product. From this point of view, it has been demonstrated that the modified
83 state diagram of the amorphous phase, which includes the relationships between the
84 product water content, water activity and its physical state as a function of temperature, is a
85 useful tool with which to improve product processing and stability (Roos, 1993; Fabra et
86 al., 2009). It allows us to predict the critical water content and water activity at which the
87 glass transition occurs at a determined storage temperature of the product.

88 The aim of this work was to study the effect of storage time at different relative
89 humidities on the main bioactive compounds (vitamin C, major flavonoids, total phenols,

major organic acids) and antioxidant capacity of freeze-dried grapefruit powder as related to the physical state of its amorphous matrix.

2. Materials and methods

2.1. Raw material

Grapefruits (*Citrus paradise* var. Star Ruby), from the city of Murcia, were purchased in a local supermarket (Valencia, Spain). They were selected on the basis of a similar soluble solid content (~10 ºBrix) and apparent fruit quality (firmness, size, visual colour and absence of physical damages). Fruit was processed in the laboratory immediately after purchase.

2.2. Sample preparation

Grapefruits were washed, peeled with careful removal of the albedo and the seeds removed to obtain the pulp. The pulp was cut and triturated in a bench top electrical food processor (Thermomix TM 21, Vorwerk, Spain). Part of the triturated pulp was taken to carry out the analytical determinations described in Section 2.3. ºBrix and pH were also measured at 20 ºC by using a refractometer (Abbe Atago 89553 by Zeiss, Japan) and a pH meter (model SevenEasy Conductivity, Mettler-Toledo, Switzerland), respectively. The rest of the pulp was placed in aluminum pans (approximately 200 g in 0.5 cm thickness by pan) and immediately frozen at -40 ºC for 24h before freeze-drying in a Telstar Lioalfa-6 Lyophyliser at 10^{-2} Pa and -40 ºC for 48h. The freeze-dried grapefruit (approximately 18 g

112 by pan) was ground in a mortar to obtain a free flowing powder and the water content and
113 water activity were measured as described in Sections 2.3.1 and 2.3.2, respectively.
114 For sorption experiments, the freeze-dried powder was placed at 20ºC (P. Selecta Hot
115 Cold B 0-60 ºC, Barcelona, Spain) in hermetic chambers containing saturated salt solutions
116 (LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$ and $NaNO_2$). Three replicates of about 18 g
117 were placed in each chamber with different relative humidity (RH) levels, ranging between
118 11- 68 % (Greenspan, 1977). The sample weight was controlled till a constant value was
119 reached ($\Delta m < \pm 0.0005$ g), when it was assumed that the equilibrium between the sample
120 and the atmosphere surrounding was reached (Spiess and Wolf, 1983). At this moment, the
121 a_w of each sample was considered to be equal to the corresponding RH/100. In each
122 equilibrated sample, the corresponding equilibrium water content was analyzed as
123 described in Section 2.3.1. These values were used in order to construct the sorption
124 isotherm. Calorimetric analyses were carried out in each equilibrated sample in order to
125 analyze the glass transition temperature (T_g) by differential scanning calorimetry (DSC).
126 About 10 mg of each sample were placed into DSC pans (P/N SSC000C008, Seiko
127 Instruments), sealed and analyzed using a DSC 220CU-SSC5200 (Seiko instruments Inc.).
128 Heating rate was 5 ºC/min and temperature range varied between -100 to 100ºC, depending
129 on sample water content.
130 Samples were maintained at 20ºC in the same hermetic chambers with different RH and
131 they were analyzed after 3 and 6 months of being freeze-dried, as described below.
132
133 *2.3. Analytical determinations*
134 All the analyses described below were carried out in triplicate on both fresh grapefruit
135 and in powdered samples. In order to compare the results, the powdered samples were

136 previously rehydrated to reach the same water content as the fresh fruit. From this point of
137 view, all the results are referred to 100 g of fresh fruit and expressed as mean value ±
138 standard deviation.
139
140 2.3.1. Water content
141 Mass fraction of water was obtained by vacuum drying the samples in a vacuum oven
142 (Vaciotem, J.P. Selecta) at 60 ºC ± 1 ºC under a pressure of < 100 mm Hg until constant
143 weight.
144
145 2.3.2. Water activity
146 Water activity of fresh grapefruit was measured by using a water activity meter
147 (Aqualab CX-2, Decagon Devices).
148
149 2.3.3. Organic acids
150 High performance liquid chromatography (HPLC, Jasco equipment, Italy) was applied
151 to the quantitative determination of citric (CA), malic (MA) and tartaric acid (TA),
152 according to Cen et al. (2007). Samples were centrifuged at 10,000 rpm for 15 min and
153 filtered through a 0.22 μm membrane. HPLC method and instrumentation was: Ultrabase-
154 C18, 5 μm (4.6x250 mm) column (Análisis Vínicos, Spain); mobile phase 0.01mol/L
155 potassium dihydrogen phosphate solution, volume injection 20 μL, flow rate 1mL/min,
156 detection at 215 nm and 25 ºC. Standard curves of each reference acid (Panreac, Spain)
157 were used to quantify the acids.
158

159 2.3.4. Ascorbic acid and vitamin C

160 Ascorbic acid (AA) and vitamin C (ascorbic acid + dehydroascorbic acid) were
161 determined by HPLC (Jasco equipment, Italy). The method proposed by Xu et al. (2008)
162 was used to determine the ascorbic acid. A 1 mL sample was extracted with 9 mL 0.1%
163 oxalic acid for 3 min and filtered through a 0.45 μm membrane filter before injection. The
164 procedure employed to determine vitamin C was the reduction of dehydroascorbic acid to
165 ascorbic acid, using DL-dithiothreitol as reductant reagent (Sánchez-Moreno et al., 2003).
166 A 0.5 mL aliquot sample was taken to react with 2 mL of a 20 g/L dithiothreitol solution
167 for 2 h at room temperature in darkness. Afterwards, the same procedure as that used for
168 the ascorbic acid method was performed. The HPLC method and instrumentation was:
169 Ultrabase-C18, 5 μm (4.6x250 mm) column (Análisis Vínicos, Spain); mobile phase 0.1 %
170 oxalic acid, volume injection 20 μL, flow rate 1mL/min, detection at 243 nm and at 25 ºC.
171 AA standard solution (Panreac, Spain) was prepared.
172
173 2.3.5. Total phenols

174 Total phenols (TP) were analysed by using the method based on the Folin-Ciocalteu
175 method, which involves the reduction of the reagent by phenolic compounds with the
176 concomitant formation of a blue complex. The extraction consisted of homogenizing 35 g
177 of the sample (T25 Janke and Kunkel turrax) for 5 min with 40 mL of methanol, 10 mL of
178 HCl (6 N) and NaF (2 mM) (Tomás-Barberán, Gil, Cremin, Waterhouse Hess-Pierce &
179 Kader, 2001). The homogenate was centrifuged (10,000 rpm, 10 min, 4 ºC). 15 mL of
180 distilled water and 1.25 mL of Folin Ciocalteu reagent (Sigma-Aldrich, Germany) were
181 added to 25 μL of the supernatant. The samples were mixed and allowed to stand for 8 min

182 in darkness before 3.75 mL of 7.5% sodium carbonate aqueous solution was added. Water
183 was added to adjust the final volume to 25 mL. Samples were allowed to stand for 2 h at
184 room temperature before absorbance was measured at 765 nm in a UV-visible
185 spectrophotometer (Thermo Electron Corporation, USA). The total phenolic content was
186 expressed as mg of gallic acid equivalents (GAE) per g of sample, using a standard curve
187 range of 0-800 mg of gallic acid (Sigma-Aldrich, Germany) /mL.
188
189 2.3.6. Flavonoids determination
190 The extraction of flavonoids was carried out in the same way as that of total phenols but
191 using bidistilled water instead of HCl. The obtained supernatant was filtered through a 0.45
192 μm membrane filter. The HPLC (Jasco, Italy) method and instrumentation was: Ultrabase-
193 C18, 5 μm (4.6x250 mm) column (Análisis Vínicos, Spain); mobile phase: linear gradient
194 elution made up of methanol and water (from 30:70 to 100:0 after 70 min) at 1mL/min ;
195 volume injection 25 μL. Chromatograms were recorded at 254, 284 and 286 nm and at 25
196 ºC. Standard curves of each reference flavonoid (narirutin (NAT), naringin (NAR),
197 hesperidin (HES), neohesperidin (NEOH), didymin (DID), poncirin (PON), naringenin
198 (NAG) and quercetin (QUER); Extrasyntesis, France) were used to quantify the flavonoids.
199 Naphthalene was used as internal standard.
200
201 2.3.7. Antioxidant capacity
202 Antioxidant capacity was assessed using the free radical scavenging activity of the
203 samples evaluated with the stable radical DPPH• (2,2-diphenyl-1-pierylhydrazyl)
204 (Sánchez-Moreno et al., 2003). Briefly, 0.1 ml of grapefruit juice sample was added to 3.9

ml of DPPH• (0.030 g/L, Sigma-Aldrich, Germany) in methanol. A Thermo Electron Corporation spectrophotometer (USA) was used to measure the absorbance at 515 nm at 0.25 min intervals until the reaction reached a plateau (time at the steady state). The changes in absorbance were measured at 25 ºC. Appropriately diluted juice samples were used on the day of preparation. The percentage of DPPH• was calculated by using equation (1).

$$\% \text{ DPPH} \bullet = \frac{(A_{control} - A_{sample})}{A_{control}} 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control (initial time) and A_{sample} the absorbance of the sample (time at the steady state).

2.4. Statistical analysis

Analyses of variance (ANOVA) were carried out to evaluate the effect of water content and storage time. When p value was lower than 0.05, significant differences between samples were assumed. Furthermore, an analysis of the correlation between the antioxidant activity and all the studied compounds, with a 95 % significance level, was carried out. All statistical analyses were performed using Statgraphics Plus 5.1.

3. Results and discussion

3.1. Grapefruit characterization

227 The fresh grapefruit batch used in this study showed the following characteristics: pH
228 3.08 ± 0.01, 10.0 ± 0.1 °Brix, 89.3 ± 0.1 g water/100g and a_w of 0.993 ± 0.003. Table 1
229 shows the concentration of the analyzed bioactive compounds present in the fresh
230 grapefruit. In general, the values obtained were similar to those shown in the bibliography
231 for grapefruit juice (Igual et al., 2010). The difference between analyzed vitamin C and AA
232 is the content of dehydroascorbic acid (DHAA) (13 ± 2 mg/100g). This compound is an
233 oxidized form of AA but also presents biological activity as vitamin C (Rusell, 2004). As
234 deduced from the different analyzed flavonoids (Table 1), 96% of total phenolic
235 compounds are related to flavonoids. As has been described by other authors, NAR was
236 the most abundant flavonoid in grapefruit, followed by NAT, QUER and NAG (Vanamala
237 et al., 2006; Gorinstein et al., 2006; Peterson et al., 2006; Igual et al., 2011). These four
238 components represent about 94% of the analysed flavonoids. The values found for all
239 compounds were in the same range as those reported in other publications (Xu et al., 2008;
240 Igual et al., 2011). The percentage of DPPH in fresh grapefruit was similar to the values
241 found for citrus fruits by other authors (Klimczak et al., 2007; Igual et al., 2010).
242 The freeze-dried grapefruit had an equal or smaller content of the analysed compounds,
243 except for MA (Table 1). The freeze-drying process caused a significant ($p < 0.05$) drop in
244 the content of TA, CA and AA of 31 %, 9 % and 17 %, respectively, while a 28 % increase
245 of MA was detected. Neither Vitamin C, nor TP, nor flavonoids nor % DPPH were
246 significantly affected by the freeze-drying process. A high retention rate of total phenols
247 has been also observed in spray dried bayberry powder, at least under controlled operating
248 conditions (Fang & Bhandari, 2011).
249

3.2. Sorption behavior and glass transition temperature of freeze-dried grapefruit powder

After the freeze-drying process the grapefruit powder's a_w was 0.205 ± 0.003, the mass fraction of water being 0.0477 ± 0.0002 g water/g sample. This powder took between 1 and 4 weeks to reach the equilibrium water content, depending on storage RH. The amount of water adsorbed in the equilibrated samples, expressed in dry basis (w_e), as a function of a_w allowed us to obtain the water sorption isotherm at 20ºC. Figure 1 shows these values, the water content being expressed in wet basis (x_w). This important tool can be used to optimise the drying or rehydration conditions and to determine the stability of the powder product during storage as a function of its water content. Several empiric and theoretical equations have been described to predict the sorption properties of foods, but the GAB (Guggenheim, Anderson and de Boer) model (Eq. 2) is the most extensively used one for fruit (Chirife & Iglesias, 1978; Van den Berg & Bruin, 1981).

$$w_e = \frac{w_o \cdot C \cdot K \cdot a_w}{(1 - K \cdot a_w) \cdot (1 + (C - 1) \cdot K \cdot a_w)} \quad (2)$$

where: w_e: equilibrium water content (g water/ g solids); a_w: water activity; w_o: monolayer moisture content (g water/ g solids); C: constant related to monolayer sorption heat and K: constant related to multilayer sorption heat.

The parameters of the fitted GAB model were w_o=0.077 g water/g dry solids, C=6.193 and K=0.995. The value of the monolayer moisture content is of particular interest since, as it indicates the amount of water that is strongly adsorbed to specific sites on the food surface, it has been related by some authors to certain product stability (Roos, 1995).

Changes in the relative humidity of the atmosphere in contact with the fruit powder imply changes in the water content and the evolution of its a_w value, according to sorption

273 isotherm, which, in turn, can induce the glass transition of the amorphous phase.
274 Thermograms obtained in all the equilibrated samples showed glass transition. No first
275 order transitions were observed in any case. The increase in the water content leads to the
276 depression of the T_g, which can be accurately modelled by the Gordon and Taylor equation
277 (1952) (Eq. 3). Table 2 shows the Gordon and Taylor parameters, *k* and $T_{g(as)}$, obtained by
278 considering the onset, midpoint and endpoint temperatures of the transition.

279 $$T_g = \frac{(1 - x_w) \cdot T_{g(as)} + k \cdot x_w \cdot T_{g(w)}}{(1 - x_w) + k \cdot x_w} \quad (3)$$

280 where: x_w: mass fraction of water (g water/ g product); T_g: glass transition temperature
281 (ºC); $T_{g(w)}$: glass transition temperature of amorphous water (-135 ºC); $T_{g(as)}$: glass
282 transition temperature of anhydrous solids (ºC) and k: constant model.
283
284 In order to obtain the critical water content and water activity values related to glass
285 transition, the combined T_g-x_w-a_w data and the corresponding GAB and Gordon and Taylor
286 fitted models were used (Figure 1). At room temperature (20ºC), the CWA for the glass
287 transition of the freeze-dried grapefruit was 0.089-0.210 and, therefore, the maximum
288 relative humidity of the atmosphere that would ensure the glassy state of the product
289 throughout the entire storage period is 8.9-21%. The corresponding CWC was 0.031-0.057
290 g water/ g product, lower than the monolayer moisture content. As in powdered products
291 the glassy state prevents significant quality changes (Mosquera et al., 2011), this indicates
292 that w_o cannot be considered a water content that assures quality preservation during the
293 storage of freeze-dried fruits, in agreement with that found by other authors (Roos, 1993;
294 Fabra et al., 2009; Moraga et al., 2011). Moreover, these critical values indicate that the
295 freeze-drying process applied in the study was not sufficient to ensure the completely

296 glassy state of the obtained grapefruit powder and, therefore, should be optimized with this
297 purpose in mind. Different materials, such as isolated protein, polysaccharides like
298 maltodextrin with different dextrose equivalent and anti-caking agents can be used to
299 increase the glass transition temperature and consequently the CWC, thus decreasing the
300 stickiness and producing free flowing powders with improved handling and quality
301 properties. The film-forming capacity of maltodextrins has been used during the production
302 of food powders not only to extend the shelf-life of the product, but also to protect the
303 product against oxidation (Ersus & Yurdagel, 2007; Telis & Martínez-Navarrete, 2009).
304
305 **3.3. Changes in phytochemical compounds as a function of relative humidity and**
306 **storage time.**
307 The powder grapefruit samples were analyzed after being maintained for 3 and 6
308 months at each ambient. Figure 2 shows TA, MA and CA content of the stored grapefruit
309 powder samples. In general, no significant changes in the amount of organic acids were
310 observed after 3 months of storage at any RH. After 6 months, however, all the studied
311 organic acids decreased sharply when stored in the range 23 %<HR<52 % . The MA
312 content dropped the most sharply in this relative humidity range. A certain recovery of TA
313 and CA was observed above RH of 52 %. Citric and malic organic acids are intermediate
314 components in tricarboxylic acid cycle of cellular metabolism. These series of chemical
315 reactions are catalyzed by a multienzyme system which produces the oxidative
316 decarboxylation of these acids (Cox & Nelson, 2005).
317 The AA, DHAA and vitamin C content of the freeze-dried grapefruit powder stored for
318 3 and 6 months at the seven different relative humidities considered are presented in Figure
319 3. After 3 months of storage, the AA content of freeze-dried grapefruit powder was

practically constant till RH = 52 %, but decreased significantly over the range of RH from this value up to 68 %, which coincided with a rise in DHAA. The water content of the grapefruit powder with this level of water activity could be considered high enough to provoke an increase in the degradation reactions of AA at this storage time. When stored above RH = 68 %, there was no more significant change in AA content, while further hydrolysis reactions of DHAA to form other nutritionally inactive compounds seem to occur (Gregory, 1996). So, as reflected by the experimental data, vitamin C decreased. Although no longer of nutritional importance, the various reactions involved in the final phases of ascorbate degradation are important in the formation of flavor compounds and as precursors of non-enzymatic browning (Belitz, Grosch & Schieberle, 2009). However, at a lower storage RH (23 %), there was an observed decrease not only in AA but also in vitamin C after 6 months of storage; the higher the RH, the greater the decrease. At RH = 68 % there was no AA at all and the value of vitamin C in this sample corresponded to the DHAA content (18.1±0.2 mg/100g). From the results commented on, a certain amount of water is needed for a decrease in both AA and vitamin C to occur; the greater the water content, the less time is needed (3 months if $x_w \geq 0.13$ g water/g sample ($a_w \geq 0.520$) and 6 months if $x_w \geq 0.058$ ($a_w \geq 0.230$)).

No significant changes in the amount of total phenols and total flavonoids, considered as the sum of individual analyzed flavonoids, were observed after 3 months of storage (Figure 4), maintaining ≈156 mg/100g and ≈144 mg/100g, respectively. After 6 months of storage, an increase in water activity affected the TP and total flavonoid content of the grapefruit powder. Although only a small decrease was observed in the product stored at RH 23 %, a sharp decrease was detected in the range 43.0 – 52.0 %, especially in the sum

343 of the different analyzed flavonoids. In regard to polyphenol degradation, the most
344 important biochemical mechanism to occur is enzymatic oxidation (De la Rosa et al. 2010).
345 Phenolic compounds are substrates for polyphenol oxidases. These enzymes hydroxylate
346 monophenols to o-diphenols and also oxidize o-diphenols to o-quinones. o-Quinones can
347 enter into a number of other reactions, thus giving the undesired brown discoloration of
348 fruits and fruit products (Belitz, Grosch & Schieberle, 2009).

349 Figure 5 shows the individual flavonoid content of the freeze-dried grapefruit powder at
350 different times and relative humidities of storage considered. In general, the flavonoid
351 content of all the samples was maintained after 3 months and sample water activity led to
352 no significant differences ($p > 0.05$). Nevertheless, after 6 months of storage, NAT and
353 NAR decreased significantly ($p < 0.05$) when RH was equal to or higher than 43 and, since
354 these flavonoids are the most abundant, this behavior is reflected in the trend of total
355 flavonoids (Figure 4). NAG content was practically constant after 6 storage months till
356 RH=52 % but presented a significant ($p < 0.05$) decrease above this value. QUER stored for
357 6 months behaved similarly to that stored for 3 months, with lower values at every relative
358 humidity. As far as minority flavonoids are concerned, after 6 months of storage NEOH
359 decreased significantly ($p < 0.05$) as the sample a_w increased. Although minor fluctuations
360 in HES content were observed after both 3 and 6 months, it was not significantly ($p > 0.05$)
361 affected either by storage time or relative humidity. The DID content did not change
362 significantly ($p > 0.05$) after 3 storage months, but increased after 6 months when stored in
363 the range 11.3 - 52 % RH and decreased significantly ($p < 0.05$) at RH=68 %. The PON
364 content decreased significantly ($p < 0.05$) at an RH of under 43.0 % and increased
365 significantly ($p < 0.05$) at a greater RH.

366 The observed evolution of bioactive compounds seems to be related with the
367 availability of water to participate in degradative reactions or to act as a vehicle that allows
368 the mobility of the different substrates involved. As has been commented on, most of the
369 studied compounds remained stable during the first 3 months and started to decrease after 6
370 months from the lowest storage RH. This decrease is small until a sample a_w of about 0.23
371 is reached and greatly increases as the water content of the samples rises. As it has been
372 stated that the CWA for the onset of glass transition at 20 ºC is 0.080 and for the endpoint
373 is 0.21, it can be deduced that the completely rubbery state of the amorphous matrix of the
374 powder is needed for degradative reactions to occur and, moreover, in this state, a certain
375 time is needed for the reactants to contact one to another. This is due to the high viscosity
376 of the liquid phase in the samples considered, none of them having freezable water content.
377 In the rubbery state, the greater the relative humidity and the longer the storage time, the
378 lower the bioactive compound content. Similar results have been found by Fang &
379 Bhandari (2011) and Syamaladevi et al. (2011) when studying the stability of spay dried
380 bayberry polyphenols or freeze-dried raspberry anthocyanins, respectively. These results
381 point to a diffusion limited behavior. Vitamin C was the only studied component that
382 started its degradation in the first 3 months of storage if the RH was over 68 %. In fact,
383 only Vitamin C and AA showed a negative, significant correlation with a_w, when a Pearson
384 correlation statistical analysis was performed using all the data obtained both after 3 and 6
385 storage months (Table 3).

386 Figure 6 shows the % DPPH evolution as a function of a_w of grapefruit powder after 3
387 and 6 months of storage. Samples stored for 3 months showed significantly higher
388 antioxidant capacity values than samples stored for 6 months at every a_w. After 3 months,
389 the storage of samples at relative humidity greater than 11.3 % caused a significant ($p <$

390 0.05) decrease of its antioxidant capacity. In the case of samples stored for 6 months,
391 despite the fact that % DPPH was lower than after 3 months, it dropped sharply ($p < 0.05$)
392 over the RH range of 11.3-23 % and it remained stable ($p > 0.05$) at a higher RH. In this
393 case, as expected from the previous results, the decrease in the antioxidant capacity also
394 starts at the same time as the glass transition and is more marked at greater water contents
395 and longer storage times.

396 The results of antioxidant capacity were analyzed for both data series, 3 and 6 months,
397 by the least square fit of general linear model, including relative humidity as variable.
398 Figure 6 shows the obtained models. Like the models themselves, the parameters obtained
399 for them were also statistically significant at 95%. The predicted antioxidant capacity
400 obtained using these models agreed closely with the experimental data.

401 In order to explain the relationship of the different compounds quantified in this study
402 with the antioxidant capacity of the samples and the water activity, correlation statistical
403 analyses were performed using all the data obtained both after 3 and 6 storage months
404 (Table 3). The greatest contribution to the antioxidant activity was provided by the content
405 of AA ($r=0.7603$, $p<0.05$), followed by MA ($r=0.6160$, $p<0.05$) and vitamin C ($r=0.5559$,
406 $p<0.05$). Other studies into citrus fruit confirm the existence of a greater positive
407 relationship between the AA content of the fruit and its antioxidant capacity (Xu et al.,
408 2008). Moreover, table 3 shows a close relationship between the MA content and the AA or
409 vitamin C content. Although organic acids are not widely considered as potential free
410 radical scavengers of DPPH•, previous studies attribute a direct chelating action
411 (Kayashima & Katayama, 2002). Specifically, organic acids have a complexing action on
412 inorganic metal ions which, in turn, can catalyse the degradation of AA under different

conditions, preventing or slowing its degradation and increasing its stability (Biolatto et al., 2005; Lo Scalzo, 2008).

4. Conclusions

In order to ensure the functional quality preservation of freeze-dried grapefruit powder during long term storage, the glassy state of the amorphous matrix must be guaranteed. This will avoid an increase in the rate of deteriorative reactions related to the loss in the bioactive compounds of the fruit with antioxidant capacity. In the rubbery state, this loss was not observed during the first 3 months of storage, but began to be significant from 6 months of storage at 23 % relative humidity and was significantly much greater at RH of over 52%.

Acknowledgment

The authors wish to thank the Ministerio de Educación y Ciencia for the financial support given throughout Project AGL 2010-22176 and the Generalitat Valenciana for the financial support given throughout Project GVPRE/2008/253.

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FIGURE CAPTIONS

Figure 1. Water activity - water content (——) and temperature - water activity (------) relationships of grapefruit powder considering onset (-), midpoint (■) and endpoint temperatures of the glass transition. Experimental points and the GAB and Gordon & Taylor fitted models.

Figure 2. Evolution of tartaric acid (TA), malic acid (MA), citric acid (CA) and total organic acids as a function of relative humiditie (RH) for grapefruit powder after 3 (▲) and 6 (□) months of storage.

Figure 3. Evolution of ascorbic acid, dehydroascorbic acid and vitamin C as a function of relative humiditie (RH) for grapefruit powder after 3 (A) and 6 (B) months of storage.

Figure 4. Evolution of total phenols and total flavonoids as a function of relative humiditie (RH) for grapefruit powder after 3 (▲) and 6 (□) months of storage.

Figure 5. Evolution of individual flavonoids (narirutin (NAT), naringin (NAR), naringenin (NAG), quercetin (QUER) hesperidin (HES), neohesperidin (NEOH), didymin (DID) and poncirin (PON)) as a function of relative humiditie (RH) for grapefruit powder after 3 (▲) and 6 (□) months of storage.

Figure 6. Evolution of % DPPH as a function of relative humiditie (RH) for grapefruit powder after 3 (▲) and 6 (□) months of storage. Predictive general linear models of evolution of % DPPH (equations and lines).

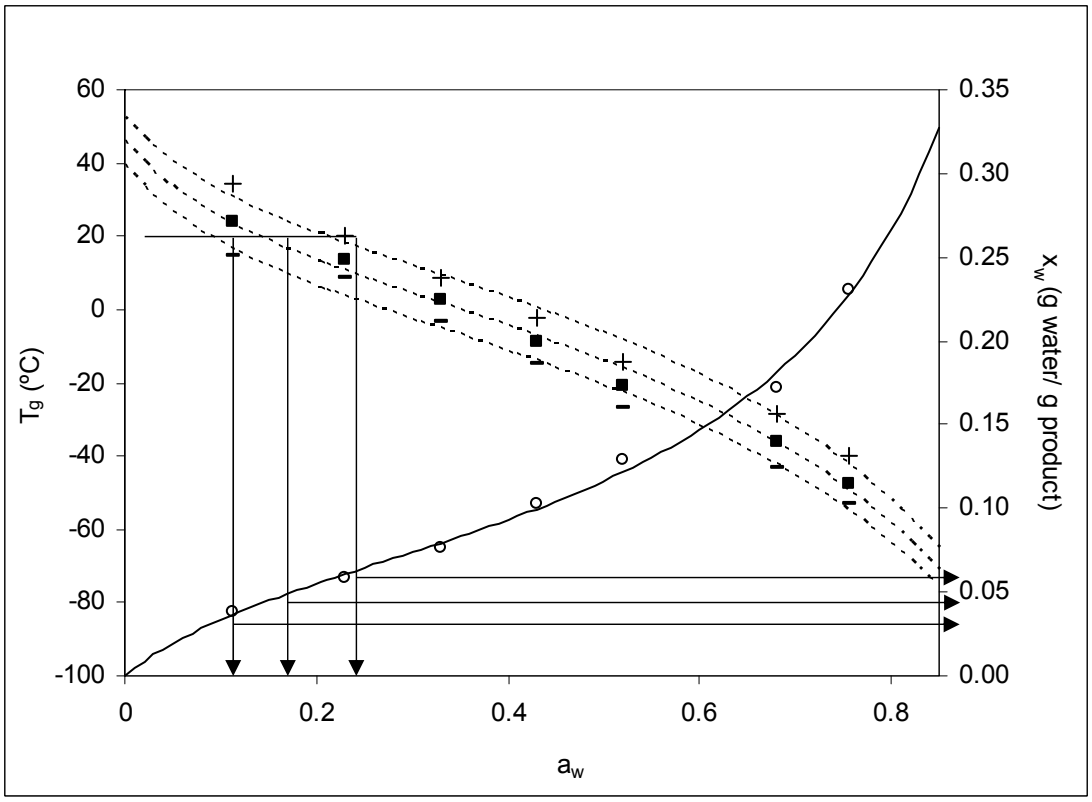
60
40
20
0
-20
-40
-60
-80
-100
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
0.2
0.4
0.6
0.8
T_g (°C)
x_w (g water/ g product)
a_w

Figure 1.

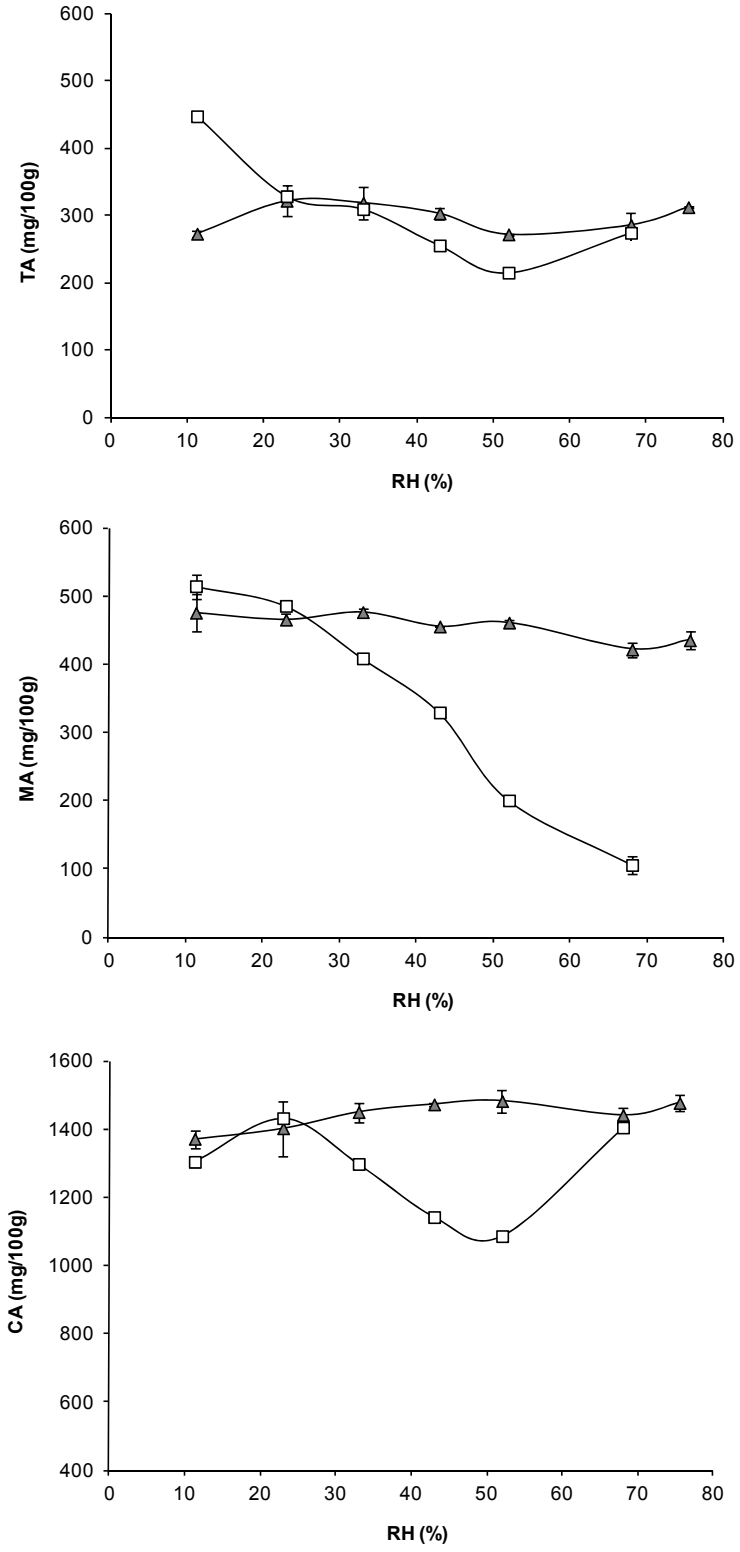

Figure 2.

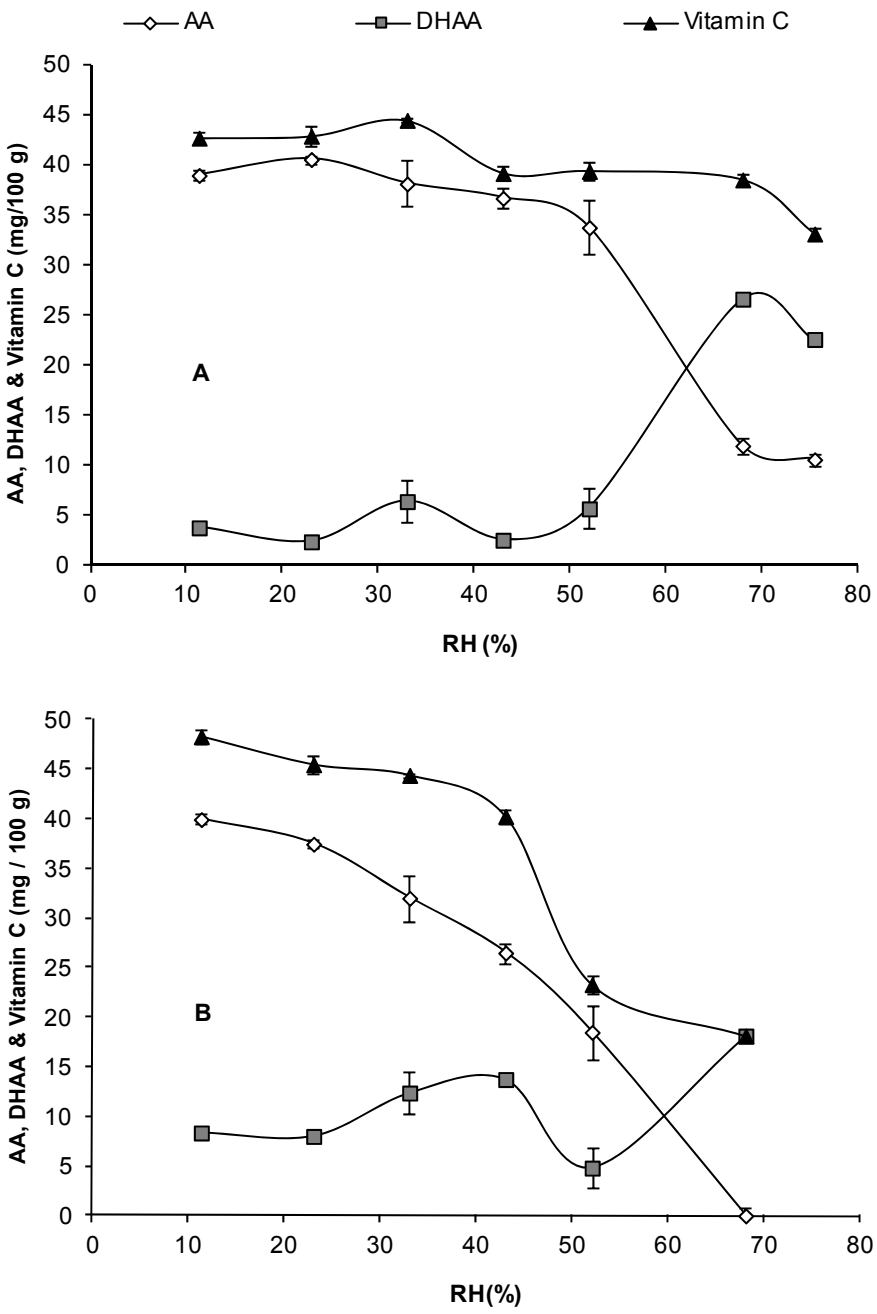
AA
DHAA
Vitamin C
AA, DHAA & Vitamin C (mg/100 g)
A
RH (%)
AA, DHAA & Vitamin C (mg / 100 g)
B
RH(%)

Figure 3.

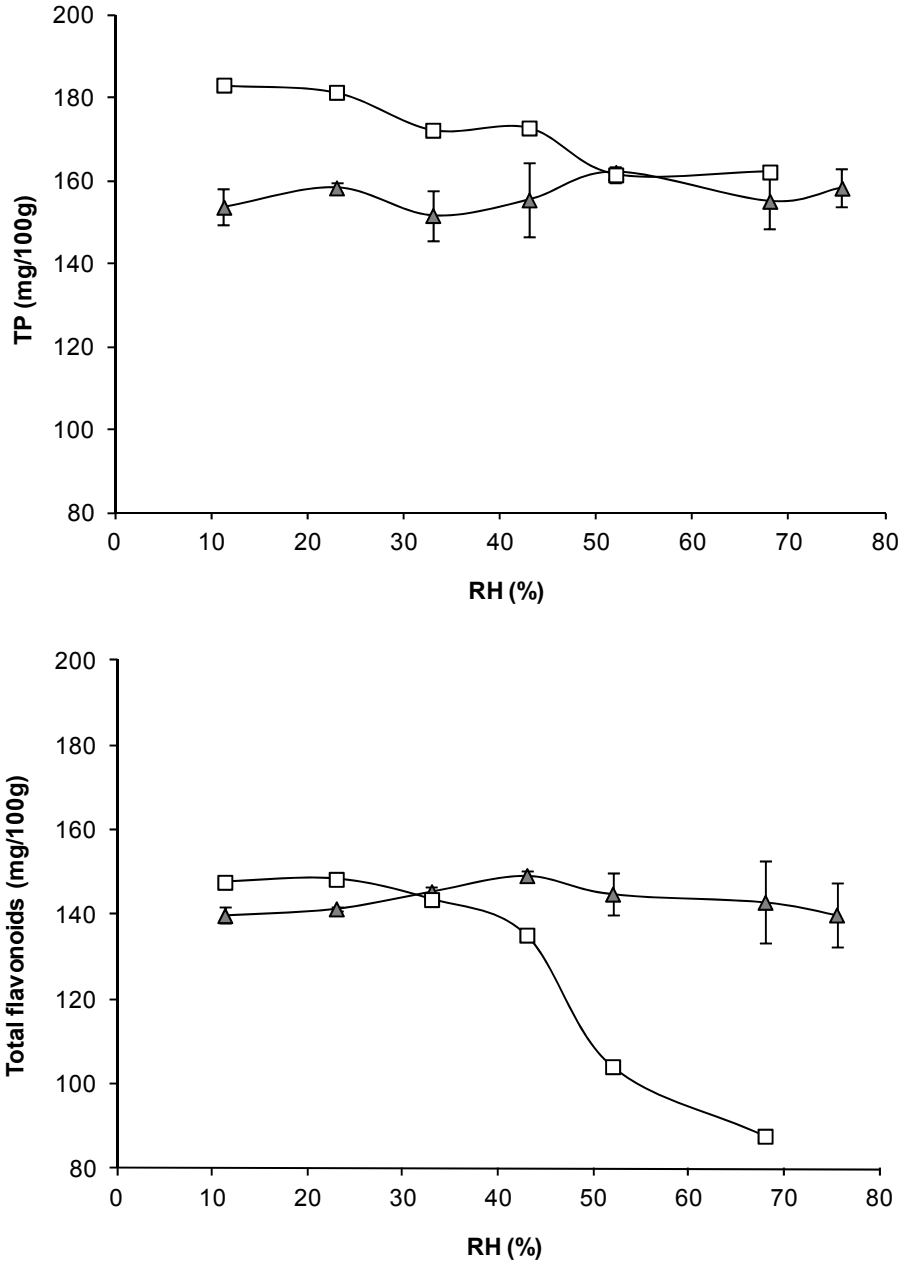
200
180
160
140
120
100
80
TP (mg/100g)
0
10
20
30
40
50
60
70
80
RH (%)
200
180
160
140
120
100
80
Total flavonoids (mg/100g)
0
10
20
30
40
50
60
70
80
RH (%)

Figure 4.

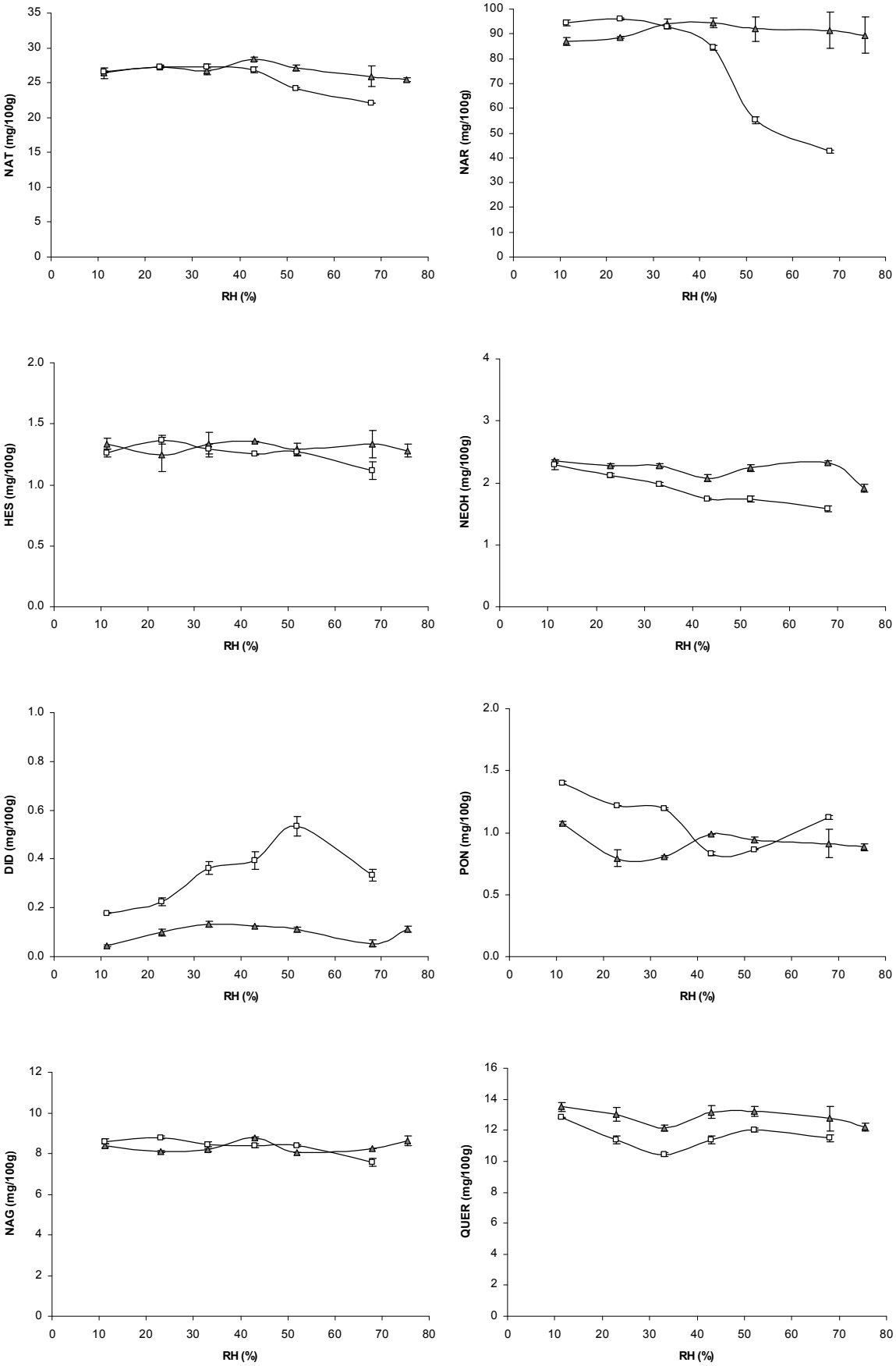
NAT (mg/100g)
RH (%)
NAR (mg/100g)
RH (%)
HES (mg/100g)
RH (%)
NEOH (mg/100g)
RH (%)
DID (mg/100g)
RH (%)
PON (mg/100g)
RH (%)
NAG (mg/100g)
RH (%)
QUER (mg/100g)
RH (%)

Figure 5.

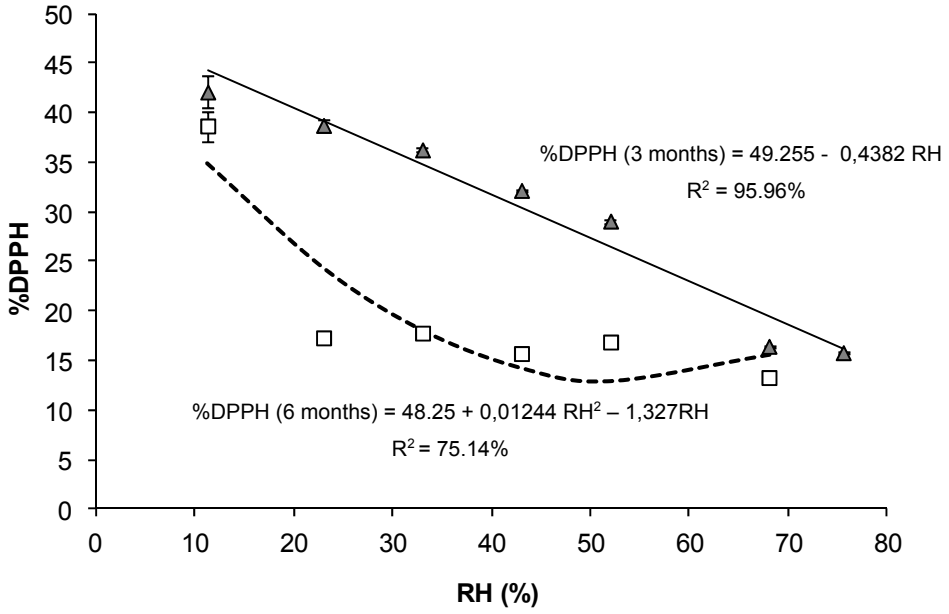
%DPPH
RH (%)
%DPPH (3 months) = 49.255 - 0,4382 RH
R2 = 95.96%
%DPPH (6 months) = 48.25 + 0,01244 RH2 – 1,327RH
R2 = 75.14%

Figure 6

Table 1. Characterization of fresh grapefruit (FG) and freeze-dried grapefruit powder (PG). Values of each compound expresed in mg/100g FG.

		FG	PG
Organic acids	**TA**[1]	456 (18)[a]	316 (26)[b]
	MA[1]	614 (17)[b]	785 (6)[a]
	CA[1]	1,707 (17)[a]	1,556 (32)[b]
	AA[1]	46 (2)[a]	38.4 (0.6)[b]
Flavonoids	**NAT**[1]	28 (4)[a]	26 (5)[a]
	NAR[1]	106 (22)[a]	81 (18)[a]
	HES[1]	3.0 (0.3)[a]	1.38 (0.05)[a]
	NEOH[1]	3.1 (0.3)[a]	2.3 (0.2)[a]
	DID[1]	1.48 (0.96)[a]	0.96 (0.03)[a]
	PON[1]	2.0 (0.2)[a]	1.4 (0.2)[a]
	NAG[1]	9.85 (0.97)[a]	8.40 (0.57)[a]
	QUER[1]	14 (2)[a]	10.7 (0.5)[a]
Total phenols[1]		174 (3)[a]	156 (12)[a]
Vitamin C[1]		59 (4)[a]	46.4 (0.4)[a]
Antioxidant capacity	**(%DPPH)**	46 (2)[a]	35 (3)[a]

The same letter in superscript within rows indicates homogeneous groups established by the ANOVA ($p<0.05$). [1]Units: mg/100g FG. TA: tartaric acid; MA: malic acid; CA:citric acid; AA: ascorbic acid; NAT: narirutin; NAR: naringin; HES: hesperidin; NEOH: neohesperidin; DID: didymin; PON:poncirin; NAG: naringenin; QUER: quercetin; TP: total phenols.

Table 2. Parameters of the Gordon & Taylor model (Eq. 3) fitted to experimental data (R^2: determination coefficient). Critical water content (CWC, g water/g product) and critical water activity (CWA) of grapefruit powder related to glass transition considering onset, midpoint and endpoint T_g values.

	Gordon & Taylor			Critical values (20ºC)	
	$T_{g(as)}$	k	R^2	CWC	CWA
$T_{g\ onset}$	41.1 ± 3.6	4.1 ± 0.3	0.991	0.031	0.089
$T_{g\ midpoint}$	48.2 ± 2.6	3.9 ± 0.2	0.995	0.043	0.140
$T_{g\ endpoint}$	55.8 ± 3.8	3.7 ± 0.3	0.990	0.057	0.210

$T_{g(as)}$: glass transition of anhydrous solids (ºC)

k: Gordon and Taylor constant model

Table 3. Pearson correlation coeficients among all studied components

	RH	Vitamin C	AA	CA	MA	TA	TP
%DPPH	-0.7088*	0.5559*	0.7603*	0.2499	0.6160*	0.4374	-0.2235
RH		-0.7134*	-0.8752*	0.1407	-0.5413	-0.4837	-0.3662
Vitamin C			0.8325*	0.2048	0.9075*	0.6023*	-0.3065
AA				0.064	0.7528*	0.4246	0.2018
CA					0.4651	0.2959	-0.3565
MA						0.5896*	0.0726
TA							0.4840

* Correlation is significant at the 0.05 level. AA: ascorbic acid; CA:citric acid; MA: malic acid; TA: tartaric acid; TP: total phenols.