Antioxidant profile of pepper (Capsicum annuum L.)
fruits containing diverse levels of capsaicinoids

José M. Palma	extsuperscript{1,*}, Fátima Terán	extsuperscript{1,2}, Alba Contreras	extsuperscript{1,3}, Marta Rodríguez-Ruiz	extsuperscript{4} and Francisco J. Corpas	extsuperscript{1}

	extsuperscript{1} Group Antioxidant, Free Radical and Nitric Oxide in Biotechnology, Food and Agriculture, Dpt. Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, Granada, Spain; tamara.molina@eez.csic.es (T.M.-M.); mariajesus.campos@eez.csic.es (M.J.C.); carmelo.ruiz@eez.csic.es (C.R.-T.); javier.corpas@eez.csic.es (F.J.C.); josemanuel.palma@eez.csic.es (J.M.P.)

	extsuperscript{2} Present address: Departament of Agricultural and Environmental Sciences, Universitat Jaume I, Castelló de la plana, Spain; fatimateca26@gmail.com (F.T.)

	extsuperscript{3} Present address: Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Valencia, Spain; aconrreras@iata.csic.es (A.C.)

	extsuperscript{4} Present address: Laboratório de Fisiologia do Desenvolvimento Vegetal; Instituto de Biociências-Universidade de São Paulo; Cidade Universitária-São Paulo-SP, Brazil; martarodriguezruiz@usp.br (M.R.-R.)

* Correspondence: josemanuel.palma@eez.csic.es; Tel +34-958-181600; Fax: +34-958-1816009

Abstract

Capsicum is the genus where a number of species and varieties have pungent features due to the exclusive content of capsaicinoids such as capsaicin and dihydrocapsaicin. In this work, the main enzymatic and non-enzymatic systems in pepper fruits from four varieties with different pungent capacity has been investigated at two ripening stages. Thus, a sweet pepper variety (Melchor) from California type fruits, and three autochthonous Spanish varieties were used, including Piquillo, Padrón and Alegría riojana. The capsaicinoids contents were determined in pericarp and placenta from fruits showing that these phenyl-propanoids were mainly localized in placenta. The activity profile of catalase, superoxide dismutase (SOD, total and isoenzymatic), the enzymes of the ascorbate-glutathione cycle (AGC) and four NADP-dehydrogenases indicate that some interaction with the capsaicinoid metabolism seems to occur. Among the results obtained on enzymatic antioxidant, the role of an Fe-SOD and the glutathione reductase from the AGC is highlighted. Additionally, it was found that ascorbate and glutathione content were higher in those pepper fruits which displayed the greater contents of capsacinoids. Taken together, all these data indicate that antioxidants may contribute to preserve capsaicinoids metabolism to maintain their functionality in a framework where NADPH is perhaps playing an essential role.

Keywords: ascorbate, ascorbate-glutathione cycle, capsaicin, catalase, dihydrocapsaicin, glutathione, NADP-dehydrogenases, superoxide dismutase

1. Introduction
Pepper (*Capsicum annuum* L.) fruits are one of the most consumed vegetables worldwide. Pepper fruits are mainly characterized by their high vitamin C and A, and mineral contents [1-8]. Thus, about 60-80 g intake of fruits per day can provide 100 and 25% of recommended daily amounts of vitamin C and A, respectively [5, 9]. Besides, this horticultural product contains important levels of other health-promoting substances with antioxidant capacity, and they include carotenoids, flavonoids and other polyphenols, among others [1, 10-12].

The diversity of pepper varieties is quite high and they are basically differentiated by shape, size, pulp (pericarp) thickness, and final color at the ripe stages. This diversity is also mirrored by the number of common names to designate pepper fruits which, in most cases, are used very locally. From culinary and gastronomic points of view pepper fruits are mainly classified as sweet and hot depending on the absence or presence of capsaicin, respectively [4, 5, 12, 13]. Within the sweet pepper (also amply known as bell pepper) varieties three main types are distinguished according to their shape and size: California, Lamuyo and Dulce italiano. Hot peppers include the highest number of varieties and names including chili, habanero, jalapeño, paprika, chipotle, and the Spanish Alegria riojana, Padrón, and Piquillo used in this work, among others.

Capsaicin is exclusive of genus *Capsicum* and is the responsible of the pungency trait. According to the pungent level, pepper fruits are ranked in the so-called Scoville scale which assigns a score to each fruit variety. In this scale, the highest value for the most pungent pepper fruit variety is around $3 \cdot 10^6$, being pure capsaicin $16 \cdot 10^6$ [14-19]. Capsaicin is an alkaloid with phenylpropanoid nature which has given rise to a family of capsaicinoids composed by, at least 22 primary compounds. Out of them, capsaicin and dihydrocapsaicin contribute to about 90-95% of total capsaicinoids present in most hot pepper varieties [20, 21]. These compounds are mainly localized in the epidermic vacuoles of the placenta and the septum from fruits, and can be separated and identified through the use of high performance liquid chromatography associated to electrospay ionization mass spectrometry (HPLC-ESI/MS) [20, 22, 23]. Capsaicin is useful for pepper plants to avoid biting by insects and other animals since this chemical has repellent/insecticide capacity [24-28]. From a pharmacological perspective, the research carried out so far has shown that capsaicinoids, particularly capsaicin, have a diversity of biological and physiological functions *in vitro*, so they play as antioxidants, stimulants of the energetic metabolism, fat accumulating suppressors, anti-inflammatory, neurostimulants and as apoptosis-alleviating agent in neurodegenerative disorders [21, 29-31]. Regarding to the mechanism of action, capsaicinoids act on a family of ion channels known as Transient Receptor Potential (TRP), which, in mammals are framed within the subtype TRP Vanilloid (TRPV1) [32, 33]. It has been also found that in many types of cancers the proapoptotic activity of capsaicin is also mediated by this TRPV, and the activation of the p53 tumor suppressing protein by a phosphorylation process is induced by capsaicin [34, 35].

Another relevant feature of pepper fruits is the ripening process, visibly characterized by a shift in the fruit color from green to red, yellow, orange or purple depending on the variety. This event implies chlorophyll breakdown and synthesis of new carotenoids and anthocyanins, emission of organic volatiles, new protein synthesis and cleavage of existing ones, and cell wall softening, among others [5, 7, 36-40]. Relevant differences between the transcriptomes from green immature and ripe pepper fruits have been also reported, involving thousands of genes [8, 41 and references therein]. From a redox view, it has been found that the reactive oxygen species (ROS) metabolism is also affected during fruit ripening, leading to major changes in total soluble reducing equivalents in fruits and the antioxidant capacity [42]. The profile of the major non-enzymatic antioxidants, including ascorbate, glutathione, carotenoids and polyphenols, have been followed during ripening in pepper fruits [4, 11, 12, 43-45], but less is known on how enzymatic antioxidants evolve with this physiological process. These enzyme systems basically include superoxide dismutase (SOD), catalase (CAT), the ascorbate-glutathione cycle as the primary defense barrier against ROS, and some NADP-dehydrogenases as secondary system to help the antioxidative enzymatic block. The profile of these enzymes throughout fruit ripening has been mostly carried out in sweet pepper [4, 11, 46, 47], but
scarce references have been reported on how those antioxidant enzymatic systems in the ripening of hot varieties [48-50 Ramirez-Serrano et al., 2008; Boonsiri et al., 2007; Tan et al., 2012]. Accordingly, using pepper varieties containing increasing capsaicin content, this work was aimed at characterizing the profile of the main antioxidants and their potential interaction with capsaicin during fruit ripening. This could provide a biochemical support and an added value of the particular features of each Spanish autochthonous cultivars which are included in the European Register of protected designations of origin for these horticultural products.

2. Materials and Methods

2.1. Plant material

Fruits from four pepper (Capsicum annuum L.) varieties were used in this work: California-type (sweet), obtained from plants grown in plastic-covered greenhouses (Zeraim Iberica/Syngenta Seeds, Ltd., El Ejido, Almería, Spain); Padrón, provided by the Regulatory Council of Denomination of Origin “Pemento de Herbón” (Herbón, Coruña, Spain), and Piquillo and Alegria riojana, both provided by the Regulatory Council of Denomination of Origin “Pimiento del Piquillo-Lodosa” (Navarra, Spain). Padrón, Piquillo and Alegria riojana (onwards Alegria) fruits were obtained from plants grown in orchards under the local conditions. In all varieties, fruits at both green immature and red ripe stages were analyzed. Figure 1A shows representative pictures of the different varieties used in this work, and in Table 1 comparative data on the mean fresh weight (g) of each type of fruit are given. After harvesting, in all fruits set for analyses the pericarp and placenta were separated (Figure 1B), and each one was cut into small cubes (approximately 3-5 mm/side), frozen under liquid nitrogen and then stored at -80°C until use.

![Image of plant materials](image-url)

**Figure 1.** Representative pictures of plant materials used in this work. A, Fruits from the four varieties at two ripening stages: immature green and ripe red. Melchor is a variety of California type sweet pepper fruit. Piquillo Padrón and Alegria riojana contain different levels of capsaicin with the sequence Piquillo <<< Padrón < Alegria riojana. B, Different parts of the pepper fruit.
Table 1. Fresh weight (FW) of whole fruits from four pepper varieties at two ripening stages. Data are the means ± SEM of five fruits from three independent experiments.

| Variety  | FW Immature Green (g) | FW Ripe Red (g) |
|----------|------------------------|-----------------|
| Melchor  | 245.22 ± 13.41          | 212.05 ± 12.45  |
| Piquillo | 43.50 ± 2.62            | 40.91 ± 6.69    |
| Padrón   | 16.19 ± 1.91            | 24.37 ± 1.58    |
| Alegría  | 43.78 ± 1.94            | 36.13 ± 2.38    |

2.2. Determination of capsaicin and dihydrocapsaicin by high performance liquid chromatography-electrospray mass spectrometry (HPLC-ES/MS)

Samples were ground into a powder under liquid N₂ and using an IKA® A11 Basic mill. For each sample three extractions were made as follows. Plant materials (0.5 g powder) were suspended into 2.0 mL acetonitrile (AcN) containing 100 ppm N-[(3,4-dimethoxyphenyl)methyl]-4-methyl-octanamide (DMBMO), as internal standard. Mixtures were incubated in the following sequence: 1 h at room temperature and darkness with continuous shakings; 65°C and darkness for 1 h and short shakings every 15 min; and 1 h at room temperature in the dark. Then, samples were centrifuged at 16,000 g and room temperature for 15 min. Supernatants were passed through 0.22 µm pore size polyvinylidene fluoride filters and were used for analysis through HPLC-ESI/MS with mode Multiple Reaction Monitoring (MRM). A XBridge 2.1×10 mm pre-column and a XBridge 2.1×100 mm C18 3,5 µm (Waters) were used connected to an Allience 2695 HPLC system coupled to a Micromass Quattro micro API triple quadrupole mass spectrometer both obtained from the Waters Corporation. The chromatography was run at a flux of 0.3 mL/min with temperatures of 35ºC for the column and 5ºC for the auto-injector, with 5 µL being injected per sample. The gradient used was: 6 min with AcN:H₂O (60:40) containing 0.1% (v/v) formic acid; 10 + 5 min with AcN:H₂O (90:10); and 20 + 4 min with AcN:H₂O (60:40). Standard curves were prepared using pure capsaicin and dihydrocapsaicin (Cayman Chemical). Under these conditions, the retention time for capsaicin and dihydrocapsaicin was 1.88 min and 2.24 min, respectively. The concentration of capsaicin was expressed as µg g⁻¹ of fresh weight (FW).

2.3. Detection and quantification of ascorbate, GSH and GSSG by high performance liquid chromatography-electrospray mass spectrometry (LC-ES/MS)

Pericarps and placentas were ground under liquid N₂ with the use of a pestle and a mortar. Then, 0.4 g from each powdered tissue were suspended into 1 mL of 0.1 M HCl and further spinning for 20 min at 15,000 g and 4ºC. Supernatants were filtered through 0.22-µm polyvinylidene fluoride filters and rapidly analyzed. All procedures were performed at 4ºC and protected from light to avoid potential degradation of the analytes. Samples were analyzed by liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) using an HPLC system and mass spectrometer as indicated above. HPLC runs were carried out using an Atlantis® T3 3 µm 2.1×100 mm column obtained from the Waters Corporation. For the instrument control, data collection, analysis and management, the MassLynx 4.1 software package was used. This method allows simultaneous detection and quantification of ascorbate, reduced (GSH), and oxidized (GSSG) glutathione [7, 51]. The concentration of analytes was calculated using external standards and expressed as referred to fresh weight (FW).

2.4. Preparation of crude extracts for enzyme activity
Protein extracts from pericarps and placentas were powdered under liquid nitrogen and then prepared in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol in a final 1:1 (w/v) plant material:buffer ratio. Homogenates were centrifuged at 15,000 g for 30 min and the supernatants were used for enzymatic assays.

2.5. Enzyme activity assays

Catalase (EC 1.11.1.6) activity was determined mm by following the of H2O2 breakdown at 240 nm [52]. Ascorbate peroxidase (APX; EC 1.11.1.11) was monitoring at 290 nm by plotting the initial ascorbate oxidation by H2O2 [53]. Monodehydroascorbate reductase (MDAR; EC 1.6.5.4) was assayed by following the monodehydroascorbate-dependent NADH oxidation. In these assays, the monodehydroascorbate was generated through the ascorbate/ascorbate oxidase system as reported earlier [54], with the rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) being subtracted from the monodehydroascorbate-dependent reaction. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) was measured by monitoring at 265 nm the increase of ascorbate formation from NADP+ to NADPH. Glutathione reductase (GR; EC 1.6.4.2) was assayed by following the NADPH oxidation coupled to the reduction of GSSG to the GSH form [56]. The GR reaction rate was corrected for the very small, non-enzymatic oxidation of NADPH by GSSG.

Total SOD activity (EC 1.15.1.1) was determined by the ferricytochrome c reduction method using the system xanthine/xanthine oxidase as superoxide radicals (O2-) generator and considering one unit as the amount of protein necessary to inhibit 50% of the cytochrome c reduction [57]. For the analysis of the SOD isoenzymatic profile, proteins were separated by non-denaturing PAGEs on 10% acrylamide gels. Then, SOD isozymes were detected as acromatic bands in the gels by a specific staining method based in the photochemical nitroblue tetrazolium (NBT) reduction method [58]. For the identification of the different SOD isozymes, before staining pre-incubation of gels in the presence of specific inhibitors, either 5 mM KCN or 5 mM H2O2, was carried out. Copper, zinc-containing SODs (CuZn-SODs) are inhibited by both KCN and H2O2; iron-containing SODs (Fe-SODs) are inactivated by H2O2; and Mn-SODs are resistant to both inhibitors [59, 60].

NADP-dependent dehydrogenase activities were determined by recording the formation of NADPH at 340 nm and 25°C. The assay was performed in a reaction medium (1 mL) containing 50 mM HEPES, pH 7.6, 2 mM MgCl2 and 0.8 mM NADP. The enzymatic reaction was initiated by the addition of the respective specific substrate [47]. Thus, glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was initiated by the addition of 5 mM glucose-6-phosphate. To monitor 6-phosphoglucononatedehydrogenase (6PGDH, EC 1.1.1.44) activity, the substrate used was 5 mM 6-phosphoglucuronate. NADP-isocitrate dehydrogenase (NADP-ICDH, EC 1.1.1.42) activity started by the addition of 10 mM 2R,3S-isocitrate [61, 62]. And for the NADP-malic enzyme (NADP-ME, EC 1.1.1.40) activity, the reaction was initiated by the addition of 1 mM L-malate [63]. Protein concentration was determined by the of Bradford method [64], with the Bio-Rad protein assay solution and using bovine serum albumin as standard.

2.6. Immunoblot analysis

Proteins separated by native- (10% acrylamide) and SDS-PAGE (12% acrylamide) were transferred onto PVDF membranes using a Trans-Blot SD equipment (Bio-Rad). The transfer buffer was composed by 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 11.0, containing 10% (v/v) methanol. The run was performed at 1.5 mA/cm² membrane for 2 h [65]. After the protein transfer, membranes were processed for a further blotting assay. An antibody against Fe-SOD from pepper fruits (dilution 1:5,000) was used, and the antibody-recognizing proteins were detected using the ClarityTM Western ECL Substrate kit according to the manufacturer’s instructions.
2.7. Statistical analysis

With the aid of the Statgraphics Centurion program, the t-student test was used to detect differences between the two ripening stages for each variety and each tissue. Values for p<0.05 were considered statistically significant.

Figures

3. Results

In this work, pepper fruits from four varieties with different pungency tasting were investigated. Thus, the concentration of the main capsaicinoids, capsaicin and dihydrocapsaicin, was analyzed. As shown in Table 2, Melchor, which is a sweet variety, did not contain any or the capsaicinoids, and Piquillo only displayed little values both in green and red fruits, with placenta being the tissue where both metabolites were present in higher amount. Regarding Padrón and Alegria, both varieties showed high capsaicinoids contents, with small amounts in the pericarp and the major levels being clearly observed in placenta. In the two varieties, the concentration of capsaicin and dihydrocapsaicin was remarkably increased in ripe red fruits.

Table 2. Content of capsaicin and dihydrocapsaicin in pericarp and placenta from fruits of four pepper varieties at two ripening stages. Data are the means ± SEM of at least three independent experiments. FW, fresh weight.

| Variety | Ripening stage | Tissue  | Capsaicin (g/g FW) | Dihydrocapsaicin (g/g FW) | Capsaicin ± Dihydrocapsaicin (g/g FW) |
|---------|----------------|---------|--------------------|---------------------------|---------------------------------------|
| Melchor | Green          | Pericarp | 0                  | 0                         | 0                                    |
|         |                | Placenta | 0                  | 0                         | 0                                    |
|         | Red            | Pericarp | 0                  | 0                         | 0                                    |
|         |                | Placenta | 0                  | 0                         | 0                                    |
| Piquillo| Green          | Pericarp | 0.40 ± 0.01        | 0.56 ± 0.01                | 0.96 ± 0.02                          |
|         |                | Placenta | 1.35 ± 0.63        | 0.24 ± 0.13                | 1.59 ± 0.76                          |
|         | Red            | Pericarp | 0.25 ± 0.02        | 0.54 ± 0.01                | 0.79 ± 0.03                          |
|         |                | Placenta | 0.59 ± 0.03        | 0.61 ± 0.01                | 1.20 ± 0.04                          |
| Padrón  | Green          | Pericarp | 2.11 ± 0.08        | 0.03 ± 0.02                | 2.14 ± 0.10                          |
|         |                | Placenta | 244.09 ± 34.85     | 33.10 ± 4.31               | 277.19 ± 39.16                       |
|         | Red            | Pericarp | 22.45 ± 2.26       | 3.02 ± 0.19                | 25.47 ± 2.45                         |
|         |                | Placenta | 553.47 ± 29.59     | 166.96 ± 5.00              | 720.43 ± 34.59                       |
| Alegria | Green          | Pericarp | 8.91 ± 1.69        | 1.55 ± 0.21                | 10.46 ± 1.90                         |
|         |                | Placenta | 205.23 ± 9.46      | 72.96 ± 3.42               | 278.19 ± 12.88                       |
|         | Red            | Pericarp | 51.06 ± 0.55       | 7.25 ± 0.35                | 58.31 ± 0.90                         |
|         |                | Placenta | 766.26 ± 37.00     | 269.44 ± 27.77             | 1035.70 ± 64.77                      |

As shown in Fig. 2, the higher ascorbate concentration was found in Melchor, and this parameter only changed due to ripening in the two varieties with higher capsaicinoid levels, Padrón and Alegria. In both, ascorbate was significantly enhanced after fruits ripened. Likewise, this tendency
also occurred with GSH, which only increased significantly in Padrón and Alegria after ripening, whereas it lowered in Melchor after this physiological process took place (Fig. 3A). The oxidized form of glutathione (GSSG) diminished in Melchor and Piquillo ripen fruits and no changes were observed in Padrón and Piquillo. As indicated in Table 3, total glutathione content (GSH + GSSG) increase in Padrón and Alegria and lowered in Melchor after ripening. The ratio GSH/GSSG was enhanced by ripening in the four varieties, thus indicating a shift to a higher reducing environment (Table 3).

**Figure 2.** Ascorbate content in pericarp from fruits of four pepper varieties at two ripening stages. Data are the means ± SEM of at least three independent experiments. Asterisks indicate significant differences of red fruits with respect to green fruits for each variety (t-student, p<0.05). FW, fresh weight.

**Figure 3.** Reduced (GSH) and oxidized (GSSG) glutathione contents in pericarp from fruits of four pepper varieties at two ripening stages. A, GSH. B, GSSG Data are the means ± SEM of at least three independent experiments. Asterisks indicate significant differences of red fruits with respect to green fruits for each variety (t-student, p<0.05). FW, fresh weight.
Table 3. Total glutathione (GSH + GSSG) and ratio GSH/GSSG from fruits of four pepper varieties at two ripening stages. GSG, reduced glutathione. GSSG, oxidized glutathione. FW, fresh weight.

| Variety | Ripening stage | GSH + GSSG (nmol · g⁻¹ FW) | GSH/GSSG |
|---------|----------------|----------------------------|----------|
| Melchor | Green          | 88.93 ± 12.84              | 18.55    |
| Melchor | Red            | 60.12 ± 1.38               | 32.02    |
| Piquillo| Green          | 78.83 ± 21.43              | 15.04    |
| Piquillo| Red            | 78.84 ± 13.10              | 33.71    |
| Padrón  | Green          | 65.24 ± 8.57               | 4.24     |
| Padrón  | Red            | 80.07 ± 5.75               | 5.08     |
| Alegría | Green          | 50.08 ± 2.08               | 7.62     |
| Alegría | Red            | 70.43 ± 4.16               | 13.89    |

The activity of the main enzymatic antioxidants was studied. Catalase significantly was lower in ripe fruits from all varieties excepting in Padrón, where the activity increased after ripening (Fig. 4A). SOD activity increased as consequence of ripening but only significantly in Padrón and Alegría. No changes were observed in variety Piquillo at the two stages (Fig. 4B). This SOD activity pattern was partially confirmed by the analysis of the isoenzymatic profile. Thus, in the Padrón variety, no Fe-SOD activity was detected in green fruits, whereas this isozyme appeared in red fruits (Fig. 5A). Additionally, CuZn-SOD I and II were also higher in ripe fruits than in green ones. Regarding the Alegría variety, it was observed that Fe-SOD and CuZn-SOD II were more prominent in red fruits that in green fruits (Fig. 5A). To seek for the possible reason of the absent Fe-SOD activity in Padrón variety, immuno blot assays were performed under native and denaturing conditions. Thus, after native PAGE and western blotting analysis using an antibody against an Fe-SOD from pepper fruits, no cross-reacting bands were observed in green fruits from the Padrón variety. Additionally, the use of this approach confirmed that the activity pattern observed in Alegría was due to a higher Fe-SOD protein amount in red fruits (Fig. 5B). To further check that Padrón did not contain Fe-SOD protein, SDS-PAGE and western blotting was achieved. In all cases, a cross-reacting band, characteristic of the plant Fe-SOD monomeric size (23 kDa), was detected (Fig. 5C). Also, with a very little quantity in green fruits from the Padrón variety. This indicates that this isozyme is present in this variety, but in such a little amount that its contribution to the total SOD activity is possibly irrelevant.

The enzymatic side of the AGC was analized, following the activity of APX, MDAR, DHAR and GR. APX was little, but significantly, enhanced in ripe fruits with respect to green fruits in Melchor and Piquillo, and lower in red fruits from Padrón (Fig. 6A). Regarding MDAR, this enzyme did not show changes upon ripening in the four varieties (Fig. 6B). DHAR was only to be significantly lower in red fruits from those varieties with high capsaicinoids content, Padrón and Alegría (Fig. 6C). Finally, all varieties displayed significan enhanced GR activity after ripening (Fig. 6D).
Figure 4. Catalase and superoxide dismutase (SOD) activity in pericarp from fruits of four pepper varieties at two ripening stages. A, catalase. B, SOD. Data are the means ± SEM of at least three independent experiments. Asterisks indicate significant differences of red fruits with respect to green fruits for each variety (t-student, p<0.05).

Figure 5. Isoenzymatic superoxide dismutase (SOD) pattern in pericarp from fruits of four pepper varieties at two ripening stages. A, Native PAGE on 10% acrylamide gels and further in-gel SOD activity staining by the NBT reduction method; 34 mg protein per well were loaded. B, Immunoblotting after native PAGE on 10% acrylamide gels. C, Immunoblotting after SDS-PAGE on 12% acrylamide gels. In both immunoblotting assays an antibody against Fe-SOD from pepper fruits (dilution 1:5,000) was used. Data are representative of at least three independent experiments.
Figure 6. Activity of enzymes from the ascorbate-glutathione cycle in pericarp from fruits of four pepper varieties at two ripening stages. A, Ascorbate peroxidase (APX). B, Monodehydroascorbate reductase (MDAR). C) Dehydroascorbate reductase (DHAR). D) Glutathione reductase (GR). Data are the means ± SEM of at least three independent experiments. Asterisks indicate significant differences of red fruits with respect to green fruits for each variety (t-student, p<0.05).

Regarding the activity profile of NADP-dependent dehydrogenases (NADP-DHs), four enzymes were studied: G6PDH, 6PGDH, NADP-ICDH and NADP-ME. G6PDH and NADP-ICDH displayed parallel profiles with lower activities in red than in green fruits in the varieties Melchor and Alegría, but enhanced activity after fruits from Padrón variety ripened (Figs. 7A, 7C). No changes in those enzymatic systems were observed in fruits from the Piquillo variety. With respect to 6PGDH, this activity only changed in Padrón, with enhancement after ripening (Fig. 7B). NADP-ME showed opposite evolution depending on the varieties. Thus, it increased in Melchor and Piquillo upon ripening and lowered in Padrón, with no changes in Alegría (Fig. 7D).
Figure 7. Activity of NADP-dehydrogenases in pericarp from fruits of four pepper varieties at two ripening stages. A, Glucose-6-phosphate dehydrogenase (G6PDH). B, 6-Phosphogluconate dehydrogenase (6PGDH). C) NADP-dependent isocitrate dehydrogenase (ICDH). D) NADP-dependent malic enzyme (ME). Data are the means ± SEM of at least three independent experiments. Asterisks indicate significant differences of red fruits with respect to green fruits for each variety (t-student, p<0.05).

4. Discussion

4.1. The experimental design provided a gradual capsaicin concentration depending on the pepper variety and the ripening stage

Pepper varieties used in this work were selected because of their different pungency levels according to consumers taste, which is the basis where the Scoville scale resides. All four varieties are common in the Spanish food market and their culinary uses are diverse. Melchor is a type of sweet pepper characterized by its consistency and appropriateness for different purposes. This variety, along with other sweet pepper varieties provide the high production figures in Spain. Its tasting features in either green or red frame this variety in the non-pungent fruits at all. Piquillo is an autochthonous variety from Northern Spain and its main phenotypic feature is its triangle shape with sharp extreme. Upon intake, it is characterized by very slight pleasant pungency, but it is only consumed in its ripe red stage. Padrón is characteristic and original from Northwestern Spain, although lately it is also cultivated in many other lands in the Mediterranean area. This fruits are small and they are usually consumed as green after cooking. Commonly in the green stage they show a very slight spicy taste, but it the red stage it is not consumed due to its strong pungency. Finally, Alegria riojana is also autochthonous from Northwest Spain and it is usually used as spice in the red stage. Both green, but mainly red fruits are extremely pungent.

With this tasting background and considering the antioxidant quality attributed to capsaicinoids [21, 29, 30], we aimed at this work to investigate the potential influence of these compounds (capsaicin plus dihydrocapsaicin; Cap+DiCap) in the profile of the main enzymatic and non-enzymatic antioxidants of pepper fruits containing different levels of these alkaloids. Our experimental design established a gradual scale from null values of Cap+DiCap, both in green immature and red ripe stages (Melchor), to red Alegria which contained high levels of the two capsaicinoids. The content of the Cap+DiCap couple matched with tasting scale and the higher values, as expected, were found in
the placenta in the three pungent varieties. Based in these data, we found quite appropriate the selection of these varieties and ripening stages to target our objective.

Excepting Alegría which has been scarcely used for research purposes, reports on the other three varieties can be found in the literature. Thus, Melchor variety has been used to decipher the mechanisms involved in fruit ripening [18, 39, 66, 67] where some of their antioxidant system have been reported to be involved [68]. Variety Piquillo was used as model to address the effects triggered by infection with Verticillium [69-72] and how to protect pepper plants against it through diverse practices [73, 74], as well as to investigate the effect of sanitized sewage sludge on the growth, yield, fruit quality, soil microbial community and the physiology of pepper plants [75, 76]. On the other hand, variety Padrón was set, for example, to investigate how wounding induces local resistance but systemic susceptibility to Botrytis cinerea in pepper plants [77], as a reference to assess real-time PCR as a method for determining the presence of Verticillium dahliae in distinct solanaceae species [78], or to study virulence and pathogenesis issues of Phytophthora capsici [79], among others.

4.2. The ripening stage and the capsaicinoids content alter the metabolism of enzymatic antioxidants

The profile of antioxidant enzymes during the ripening process has been investigated in pepper fruits previously but, to our knowledge, no comparisons have been made between varieties with different capsaicinoid content. Thus, for example, in California-type pepper fruits it has been reported that the catalase activity decreases as the fruit ripens [80, 81] and this event was due to post-translational modification (PTMs) underwent by the enzyme and promoted by ROS and reactive nitrogen species (RNS) derived from nitric oxide (NO) [42, 82]. In fact, it has been proved that ripening of pepper fruits is controlled by NO [8, 41, 81]. This inhibitory effect of ripening in the catalase activity also occurred in the same California type fruits subjected to storage at 20°C [80], in other sweet pepper varieties from Lamuyo and Dulce italiano types [4] and during ripening of hot pepper Kulai [50]. Our data on the Melchor, Piquillo and Alegría varieties confirm this activity pattern of the catalase activity although, interestingly, this profile is opposite in the Padrón variety where catalase activity increases in ripe fruits. This same increasing catalase activity was reported in hot pepper varieties either under saline stress conditions [48], or preventing seed browning during low temperature storage [49].

Regarding SOD, the total activity was higher in ripe fruits from those varieties which contained higher capsaicinoids content, Padrón and Alegría. In Alegría this higher activity seems basically to be due to an enhancement of the isozyme Fe-SOD and CuZn-SOD II, whereas in Padrón the presence of an Fe-SOD (nearly absent in green fruits) and higher activity of both CuZn-SODs could be responsible for such changes. Because of this interesting behavior of the Fe-SOD isozyme in the Padrón variety, complementary immunoblot analyses were performed using an antibody against the isozyme from pepper fruits. Thus, by western blotting after both non-denaturing- and SDS-PAGEs, it was confirmed that the negligible Fe-SOD activity in ripe Padrón fruits was due to the little amounts of its corresponding protein, whose monomer (23 kDa) could only be detected after SDS-PAGE. This issue needs to be further investigated at molecular level (gene and protein expression) since it means that it might be an identity feature of this pepper variety. The SOD activity has been also studied earlier in pepper varieties including some of those included in the present work. So, recently, it has been reported that the SOD isoenzyme pattern and gene expression of California-type pepper fruits are regulated by ripening and NO [68], and this enzymatic system from sweet pepper is also involved in the response against low temperatures [4] and the storage of fruits at 20°C [80], as well as in the “accommodation” of fruits to nitrogen deprivation during plant growth [83]. The isoenzymatic SOD pattern was also investigated in the plastid population from sweet pepper fruits of different California-type varieties, and a protective role of these organelles by the different SOD internal isozymes during ripening was reported [46]. In the Piquillo variety, it was found that SOD is involved in the association of pepper plants with arbuscular mycorrhizal fungi (AMF) to avoid the negative effects promoted by Verticillium [74]. A number of studies have reported the involvement of SOD from
hot pepper in diverse processes including ripening and postharvest [50, 84], salt stress [48, 85], storage at low temperature [49], and iodine bio-fortification practices to improve fruit quality [86].

The activity of the four enzymes of the ascorbate-glutathione cycle (AGC), APX, MDAR, DHAR and GR were analyzed in this work. APX is responsible for the direct scavenging of hydrogen peroxide (H₂O₂) using ascorbate as electron donor, whereas MDAR and DHAR restore the reduced status of ascorbate using NAD(P)H and GSH, respectively. The last step of the AGC is carried out by GR, an enzyme which converts the oxidized form of glutathione (GSSG) to the reduced form (GSH) with the use of NADPH as reducing power. In our experimental design the most remarkable response of this cycle was found at the GR side which were significantly enhanced in ripe fruits from all four varieties. The profile of these AGC enzymes have been investigated in pepper fruits from diverse varieties, both sweet and hot, and different trends have been reported depending on the experimental conditions, including ripening, post-harvest, salt stress, defense mechanisms, or bioremediation practices [4, 11, 46, 50, 76, 83, 84, 87]. In our case, it is remarkable that APX behaved oppositely in sweet and hot varieties, with the activity increasing in ripe fruits from Melchor and Piquillo and decreases in hot ripe fruits from Padrón and Alegria. MDAR and DHAR shared similar trend with lower values in ripe fruits, but only significant in MDAR from Padrón and Alegria. According to the activity profile of APX, MDAR and DHAR from green to red stages, it could be hypothesized that the cycle seems to be operative in the first steps which involve the direct ascorbate metabolism, but more research at different levels is necessary to obtain a whole picture of this antioxidant metabolic pathway. According to our results, it seems that hot peppers have less capacity to recycle ascorbate but all varieties showed a great potentiality to provide GSH.

The activity pattern displayed by the NADP-dehydrogenases (NADP-DHs) can be framed in three main features: i) the behavior in the two varieties with less Cap+DiCap levels (Melchor and Piquillo) was quite similar with slight, although not strongly significant, decreases of G6PDH and little, although not significant enough, decreases of 6PGDH and NADP-ME in ripe fruits; ii) excepting for the NADP-ME, all other NADP-DHs rose after ripening in the variety Padrón (high capsaicinoids content), and this suggests a higher NADPH availability for different purposes in ripe fruits from this variety; iii) interestingly, the behavior of these enzymatic systems in the other variety with high capsaicinoids content was different to that showed by Padrón. Thus, green fruits from Alegria seemed to have higher capacity to generate NADPH. To our knowledge, no reports on NADP-DHs from hot pepper fruits have been published earlier, and the only data concerning these NADP-NADPH systems in pepper refer to sweet varieties. Our data mostly confirm those previously found for other California-type pepper varieties [47]. Recent data report that pepper fruit NADP-DHs are not only influenced by ripening in the Melchor variety [8], but also by NO through diverse PTMs [88, 89]. Moreover, it was also found that NADP-DHs are also involved in the response of sweet pepper plants to stress exerted by high Cd levels [90].

4.3. The higher capsaicinoids level the higher ascorbate and glutathione content

Capsaicinoids, specially capsaicin, have been reported to have, among others, antioxidant properties [21, 29-31]. In pepper fruits containing these alkaloids this feature is quite interesting since these horticultural products are one of those with the highest ascorbate levels [5], with ascorbate being perhaps the most paradigmatic molecular antioxidant for living beings. In fact, ascorbate is one of the parameters which is commonly determined in (sweet and hot) pepper fruit research either considering ripening and post-harvest, any type of stress (biotic, abiotic and environmental), or culture practices [4, 5, 7, 11, 14, 18, 46, 48, 50, 76, 85, 91]. As an appraisal of the potential roles attributed to ascorbate in pepper fruits, it was proposed that in the sweet varieties, ascorbate functions as a redox buffer to balance the great metabolic changes which undergo during ripening [5, 7]. Regarding the hot varieties (Padrón and Alegria), the pattern observed in this work where ascorbate levels increased in those fruits was also reported earlier for diverse hot pepper varieties [14, 18, 50, 91]. Perhaps, the redox stabilizing role of ascorbate indicated above for sweet pepper could be
also applicable to hot varieties to assure the capsaicinoids level. In fact, it was proved that during the capsaicinoids oxidation catalyzed by peroxidases, capsaicinoid radicals are formed, and ascorbate rapidly reduces capsaicinoid radicals, this being an important cue for capsaicinoid content and preservation in pepper fruits [92].

Glutathione is a ubiquitous and powerful antioxidant in eukaryotes [93]. In spite of its relevant role in many biological processes, this tripeptide has been less investigated in pepper fruits, mainly associated to ripening, or bioremediation purposes [50, 76, 83, 94]. But no much information is available on the glutathione metabolism in capsaicinoids-containing pepper varieties. This work provides the first comparison of the levels of both GSH and GSSG in different pepper varieties containing gradual amounts of capsaicinoids. It is noteworthy that, whereas the total glutathione content (GSH+GSSG) did not change or decreased after ripening in the varieties with no capsaicinoids (Merlchor and Piquillo), in the hot varieties this parameter augmented in mature fruits. And this was due to the evolution of the reduced form GSH during that physiological processes. This higher content of GSH in ripe fruits found in the hot pepper varieties could be due to an enhanced GR activity. In these cases, the enzyme GR is perhaps playing a role not linked to the AGC. GSH could be used, in cooperation with ascorbate, to preserve the capsaicinoids functionality in these hot varieties. However, more research is necessary to bring light to this emerging subject. Besides, GSH could be also driven to signaling processes by either glutathionylation events (another PTM), or as S-nitrosoglutathione, a chemical form which allows transporting NO among cells and tissues [51, 95-97]. GR uses NADPH to achieve the reduction of glutathione. But NADPH is also essential in intermediate steps of the capsaicin biosynthesis [98]. These eventualities points towards the necessity of investigating the interaction capsaicinoids-ascorbate-glutathione-NADPH in more detail, especially after the perspective of considering NADPH as a quality footprinting in horticultural crops, as it has been proposed recently [99].

5. Conclusions and future prospects.

The obtained data in this work points towards a close relationship among capsaicinoids and the antioxidant systems in pepper fruits. This interaction seems to maintain a redox and functional homeostasis to preserve the role of capsaicinoids with the cooperation of antioxidants, basically ascorbate and glutathione. But also, some antioxidant enzymatic systems are also involved. The exclusivity of the capsaicinoid metabolism in Capsicum species makes this research more attractive to look for an exclusive model that could provide interesting information both at plant physiological level, but also considering the pharmacological and nutraceutical uses of hot pepper fruits, based mainly in the content of capsaicinoids but also in vitamins C and A. On the other hand, another interesting cue is opened. The role of the Fe-SOD needs to be investigated in the pepper fruit physiology, due to the diverse behavior of this isozyme among varieties. Fe-SOD has been localized in peroxisomes from pepper fruits [5, 100] and lately its gene expression profile has been reported in sweet pepper during ripening and under NO treatment. Overall the interaction of NO in pungent pepper fruits is another issue that deserves to be investigated. Furthermore, this characterization contributes to providing a biochemical antioxidant pattern for each pepper cultivar which could be part of the particular features of these cultivar that are included in the European Register of protected designations of origin for these Spanish agricultural products. Besides, this provides an added value to these autochthonous products and may have some incidence at the marketing and economical levels in their respective producing sectors.

Author Contributions: FT, ACR and MRR carried out all biochemical experiments. FJC and JMP designed the work, drove and coordinated the tasks and wrote the manuscript.

Funding: This work was supported by the ERDF-cofinanced grants AGL2015-65104-P from MINECO, PID2019-103924GB-I00 from MICIT, and P18-FR-1359 from the Plan Andaluz de Investigación, Desarrollo e Innovación, Spain.
Acknowledgments: The technical support of the Scientific Instrumental Service from the Estación Experimental del Zaidín (CSIC, Granada, Spain) is acknowledged. The valuable assistance of Carmelo Ruiz-Torres, Tamara Molina-Márquez and María Jesús Campos is also appreciated. Authors are thankful to the different companies and Regulatory Councils of Denomination of Origin for their valuable and generous collaboration providing the different pepper cultivars: Melchor by the Zeraim Iberica/Syngenta Seeds, Ltd., El Ejido, Almería, Spain; Padrón by the Regulatory Council of Denomination of Origin “Pimento de Herbón” (Herbón, Coruña, Spain), and Piquillo and Alegría riojana, both provided by the Regulatory Council of Denomination of Origin “Pimiento del Piquillo-Lodosa” (Navarra, Spain).

Conflicts of Interest: The authors declare no conflict of interest

References

1. Howard LR, Talcott ST, Brenes CH, Villalon B (2000) Changes in phytochemical and antioxidant activity of selected pepper cultivars (Capsicum species) as influenced by maturity. Food Chem. 48, 1713–1720.
2. Proteggente AR, Pannala AS, Paganga G, Van Buren L, Wagner E, Wiseman S, Van De Put F, Dacombe C, Rice-Evans CA (2002) The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. Free Rad Res 36: 217-233.
3. Mariko N, Hassimoto A, Genovese MI, Lajolo FM (2009) Antioxidant capacity of Brazilian fruit, vegetables and commercially-frozen fruit pulps. J Food Comp Anal 22: 394–396.
4. Mateos RM, Jiménez A, Román P, Romojaro F, Bacarizo S, Leterrier M, Gómez M, Sevilla F, del Río LA, Corporas FJ, Palma JM. (2013) Antioxidant systems from pepper (Capsicum annuum L.): involvement in the response to temperature changes in ripe fruits. Int J Mol Sci 14: 9556-9580.
5. Palma JM, Sevilla F, Jiménez A, del Río LA, Corporas FJ, Álvarez de Morales P, Camejo DM (2015) Physiology of pepper fruit and the metabolism of antioxidants: chloroplasts, mitochondria and peroxisomes. Ann Bot 116: 627–636.
6. Palma JM, Corporas FJ, Ruiz C, Molina T, Campos-Ramos MJ, Juanena A, Torreira JR (2016) Los pimientos de las variedades Padrón, Piquillo y Alegría riojana: Una buena fuente de macró y microelementos para nuestra dieta. Horticultura 323: 60-64.
7. Rodríguez-Ruíz M, Mateos RM, Codesido V, Corporas FJ, Palma JM. (2017) Characterization of the galactono-1,4-lactone dehydrogenase from pepper fruits and its modulation in the ascorbate biosynthesis. Role of nitric oxide. Redox Biol. 12: 171-181.
8. Corporas FJ, Freschi L, Rodríguez-Ruíz M, Mioto PT, González-Gordo S, Palma JM (2018) Nitro-oxidative metabolism during fruit ripening. J Exp Bot 69: 3449–3463.
9. Song W, Derito CM, Liu MK, He HJ, Dong M, Hai Liu R (2010) Cellular antioxidant activity of common vegetables. J. Agric. Food Chem. 58, 6621-6629.
10. Palma JM, Corporas FJ, del Río LA (2009) Proteome of plant peroxisomes: new perspectives on the role of these organelles in cell biology. Proteomics 9: 2301-2312.
11. Martí MC, Camejo D, Vallejo F, Romojaro F, Bacarizo S, Palma JM, Sevilla F, Jiménez A (2011) Influence of fruit ripening stage and harvest period on the antioxidant content of sweet pepper cultivars. Plant Foods Human Nut 66: 416-423.
12. Fratianni F, d’Acierino A, Cozzolino A, Spigno P, Riccardi R, Raimo F, Pane C, Zaccardelli M, Lombardo VT, Tucci M, Grillo S, Coppola R, Nazzaro F. Biochemical characterization of traditional varieties of sweet pepper (Capsicum annuum L.) of the Campania Region, Southern Italy. Antioxidants 2020, 9, 556.
13. Basu SK, De AK (2003) Capsicum: Historical and Botanical Perspectives.In Capsicum. The Genus Capsicum (De AK, Ed.) Taylor & Francis. pp. 1-15.
14. Topuz A, Ozdemir F (2007) Assessment of carotenoids, capsaiacinoids and ascorbic acid composition of some selected pepper cultivars (Capsicum annuum L.) grown in Turkey. J Food Comp Anal 20: 596-602.
15. Roy A (2016) Bhut jolokia (Capsicum chinense jasc): a review. Internat J Pharmac Sci Res 7: 882-889.
16. Aguiar AC, Coutinho JP, Fernández-Barbero G, Godoy HT, Martínez, J (2016) Comparative study of capsaiacinoid composition in capsicum peppers grown in Brazil. Internat J Food Prop 19: 1292-1302.
17. Parvez GMM (2017) Current advances in pharmacological activity and toxic effects of various Capsicum species. Internat J Pharmac Sci Res 8: 1900-1912.
18. Kopta T, Sekara A, Pokluda R, Ferby V, Caruso G (2020) Screening of chilli pepper genotypes as a source of capsaiacinoids and antioxidants under conditions of simulated drought stress. Plants 9: 364.
19. Tomas K, Sekara A, Pokluda R, Ferby V, Caruso G (2020) Screening of Chilli pepper genotypes as a source of capsaicinoids and antioxidants under conditions of simulated drought stress. Plants-Basel 9, 364.

20. Ishikawa K (2003) Biosynthesis of capsaicinoids in Capsicum. In Capsicum. The Genus Capsicum (De AK, Ed.) Taylor & Francis, pp. 87-95.

21. Reyes-Escogido ML, González-Mondragón EG, Vázquez-Tzompantzi E (2011) Chemical and pharmacological aspects of capsaicin. Molecules 16: 1253-1270.

22. García-Claver A, Arnedo-Andrés MS, Abadía J, Gil-Ortega R, Álvarez-Fernández A (2007) Determination of capsaicin and dihydrocapsaicin in Capsicum fruits by Liquid Chromatography-Electrospray/Time-of-Flight Mass Spectrometry. J Agric Food Chem 54: 9303-9311.

23. Al Othman ZA, Ahmed YB, Habila MA, Ghafar AA (2011) Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography. Molecules 16: 8919-8929.

24. Liu X, Lin Y. The bioactivity of capsaicin on peach aphid and its combination with several insecticides (2003) J Pest Sci 52: 94-96.

25. Jin MS, Liu SW, Gu ZM, Wei SH, Wang YZ (2008). Repellent activity of capsaicin and its effects on glutathione-S-transferase and Na+, K+- ATPase activity in Plutella xylostella (Lepidoptera: Plutellidae). Acta Entomol Sin 51: 1039-1043.

26. Aley JP, Adams NJ, Ladyman RJ, Fraser DL (2015) The efficacy of capsaicin as an equine repellent for chewing wood. J Vet Behav 10: 243-247.

27. Dickens JC, Bobbot JD (2015) Neuromolecular basis of repellent action. In: Insect Repellents Handbook, 2nd Edition (Debboun M, Frances SP, Strickman DA, Eds) CRC Press, pp. 31-42.

28. Li B, Yang M, Shi R, Ye M (2019) Insecticidal activity of natural capsaicinoids against several agricultural insects. Nat Prod Comm 10: 2909.

29. Sancho R, Lucena C, Macho A, Calzado MA, Blanco-Molina M, Minassi A, Appendino G, Muñoz E (2002). Immunosuppressive activity of capsaicinoids: capsaicin derived from sweet peppers inhibits NF-kappaB activation and is a potent antiinflammatory compound in vivo. Eur. J. Immunol. 32, 1753-1763.

30. Matserska M, Perucka I (2005) Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (Capsicum annuum L). J Agric Food Chem 53: 1750-1756.

31. Liu J, Liu H, Zhao Z, Wang J, Guo D, Liu Y (2020) Regulation of Actg1 and Gsta2 is possible mechanism by which capsaicin alleviates apoptosis in cell model of 6-OHDA-induced Parkinson’s disease. Biosci Rep 40: BSR20191796, doi: 10.1042/BSR20191796.

32. Tabrizi MA, Baraldi PG, Baraldi S, Gessi S, Merighi S, Borea PA (2017) Medicinal chemistry, pharmacology, and clinical implications of TRPV1 receptor antagonists. Medic Res Rev 37: 936-983.

33. Yang F, Zheng J (2017) Understand spiciness: mechanism of TRPV1 channel activation by capsaicin. Protein Cell 8, 169-177.

34. Chapa-Oliver AM, Mejía-Teniente L (2016) Capsaicin: From plants to a cancer-suppressing agent. Molecules 21: 931.

35. Clark R, Ho Lee S (2016) Anticancer properties of capsaicin against human cancer. Antican Res 36: 837-844.

36. Klie S, Osorio S, Tohge T, Drincovich MF, Fait A, Giovannoni JJ, Fernie AR, Nikoloski Z (2014) Conserved changes in the dynamics of metabolic processes during fruit development and ripening across species. Plant Physiol 164: 55-68.

37. Palma JM, Corpas FJ, del Rio LA (2011) Proteomics as an approach to the understanding of the molecular physiology of fruit development and ripening. J Proteomics 74: 1230–1243.

38. Barsan C, Zouine M, Mazza E, Bian W, Egea I, Rossignol M, Pichereaux C, Purgatto E, Bouzayen M, Latché A, J-C Pech (2012) Proteomic analysis of chloroplast to chromoplast transition in tomato reveals metabolic shifts coupled with disrupted thylakoid biogenesis machinery and elevated energy-production components. Plant Physiol 160: 708–725.

39. Corpas FJ, Palma JM (2018) Nitric oxide on/off in fruit ripening. Plant Biol 20: 805-807.

40. Zhang L, Zhu M, Ren L, Li A, Chen G, Hu Z (2018) The SIFSR gene controls fruit shelf-life in tomato. J Exp Bot 69: 2897–2909.

41. González-Gordo S, Bautista R, Claros M G, Cañas A, Palma JM, Corpas FJ (2019). Nitric oxide-dependent regulation of sweet pepper fruit ripening. J Exp Bot 70: 4557–4570.
42. Rodriguez-Ruiz M, González-Gordo S, Cañas A, Campos MJ, Paradela A, Corpas FJ, Palma JM (2019) Sweet pepper (Capsicum annuum L.) fruits contain an atypical peroxisomal catalase that is modulated by reactive oxygen and nitrogen species. Antioxidants 8, 374.

43. Hamed M, Kalita D, Bartolo ME, Jayanty SS (2019). Capsaicinoids, polyphenols and antioxidant activities of Capsicum annuum: comparative study of the effect of ripening stage and cooking methods. Antioxidants 8; 364.

44. Cisternas-Jamet J, Salvatierra-Martínez R, Vega-Gálvez A, Stoll A, Uribe E, Goñi MG (2020) Biochemical composition as a function of fruit maturity stage of bell pepper (Capsicum annuum) inoculated with Bacillus amyloliquefaciens. Sci Horticult 263: 109107.

45. Ribes-Moya AM, Adalid AM, Raigón MD, Hellín P, Fita A, Rodríguez-Burruezo A (2020). Variation in flavonoids in a collection of peppers (Capsicum sp.) under organic and conventional cultivation: effect of the genotype, ripening stage, and growing system. J Sci Food Agric 100: 2208–2223.

46. Martí MC, Camejo D, Olmos E, Sandalio LM, Fernández-García N, Jiménez A, Sevilla F (2009) Characterisation and changes in the antioxidant system of chloroplasts and chromoplasts isolated from green and mature pepper fruits. Plant Biology 11: 613–624.

47. Mateos RM, Bonilla-Valverde D, del Río LA, Palma JM, Corpas FJ (2009) NADP-dehydrogenases from pepper fruits: Effect of maturation. Physiol Plant 135: 130–139.

48. Ramirez-Serrano R, Larrinaga-Mayoral JA, Murillo-Amador B, Hernández-Saavedra NY, Fujiyama H (2008) Nitroxi dant enzymatic response of hot pepper (Capsicum annuum L.) under saline stress conditions. Interciencia 33: 377-383.

49. Boonsiri K, Ketsa S, van Doorn WG (2007) Seed browning of hot peppers under low temperature storage. Postharv Biol Technol 45: 358-365.

50. Tan CK, Ali,ZM, Zainal Z (2012) Changes in ethylene production, carboxyhydrate activity and antioxidant status in pepper fruits during ripening. Sci Horticult 142: 23-31.

51. Airaki M, Sánchez-Moreno L, Leterrier M, Barroso JB, Palma JM, Corpas FJ (2011) Detection and quantification of S-nitrosoglutathione (GSNO) in pepper (Capsicum annuum L.) plant organs by LC-ES/MS. Plant Cell Physiol 52: 2006-2015.

52. Aebi H (1984) Catalase in vitro. Methods Enzymol 105: 121–126.

53. Hossain MA, Asada K (1984) Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide: its protection by ascorbate. Plant Cell Physiol 25: 1285–1295.

54. Hossain MA, Nakano Y, Asada K (1984) Monodehydroascorbate reductase in spinach chloroplast and its participation in regeneration of ascorbate for scavenging of hydrogen peroxide. Plant Cell Physiol 25: 385–395.

55. Dalton DA, Baird LM, Langeberg L, Taugher CY, Anyan WR, Vance CP, Sarath G (1993) Subcellular localization of oxygen defense enzymes in soybean (Glycine max [L.] Merr.) root nodules. Plant Physiol 102: 481–489.

56. Edwards EA, Rawsthorne S, Mullineaux PM (1990) Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (Pisum sativum L.). Planta 180: 278–284.

57. McCord JM, Fridovich I (1969) Superoxide dismutase: An enzymic function for erythrocuprein. J Biol Chem 244: 6049–6055.

58. Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287.

59. Houmani H Rodriguez-Ruiz M, Palma JM, Abdelly C, Corpas FJ (2016) Modulation of superoxide dismutase (SOD) isozymes by organ development and high long-term salinity in the halophyte Cakile maritima. Protoplasma 253: 885–894.

60. Pinilla M, Iglesias-Moya J, Campos MJ, Corpas FJ, Palma JM (2019) Pomegranate (Punica granatum L.) Fruits: Characterization of the main enzymatic antioxidants (peroxisomal catalase and SOD isozymes) and the NADPH-regenerating system. Agronomy 9: 338.

61. Leterrier M, Airaki M, Palma JM, Chaki M, Barroso, JB, Corpas FJ (2012a) Arsenic triggers the nitric oxide (NO) and S-nitrosoglutathione (GSNO) metabolism in Arabidopsis. Environ Pollut 166: 136–143.

62. Leterrier M, Barroso, JB, Palma JM, Corpas FJ (2012b) Cytosolic NADP-isocitrate dehydrogenase in Arabidopsis leaves and roots Biol Plant 56: 705–710.
Not available
86. Li R, Li DW, Liu HP, Hong CL, Song, MY, Dai ZX, Liu JW, Zhou J, Weng HX (2017) Enhancing iodine content and fruit quality of pepper (Capsicum annuum L.) through biofortification. Sci Horticult 214: 165-173.
87. Padilha HKM, Madruga ND, Aranha BC, Hoffmann JF, Lopes Crizel RL, Barbieri RL, Chaves FC (2019) Defense responses of Capsicum spp. genotypes to post-harvest Colletotrichum sp. inoculation. Phytoparasitica 47: 557-573.
88. Muñoz-Vargas MA, González-Gordo S, Cañas A, López-Jaramillo J, Palma JM, Corporas FJ (2018) Endogenous hydrogen sulfide (H2S) is up-regulated during sweet pepper (Capsicum annuum L.) fruit ripening. In vitro analysis shows that NADP-dependent isocitrate dehydrogenase (ICDH) activity is inhibited by H2S and NO. Nitric Oxide 81: 36-45.
89. Muñoz-Vargas MA, González-Gordo S, Palma JM, Corporas FJ (2020) Inhibition of NADP-malic enzyme activity by H2S and NO in sweet pepper (Capsicum annuum L.) fruits. Physiol Plant 168: 278-288.
90. León AM, Palma JM, Corporas FJ, Gómez M, Romero-Puertas, MC, Chatterjee D, Mateos RM, del Río LA, Sandalio LM (2002) Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. Plant Physiol Biochem 40: 813–820.
91. Kopta T, Slosar M, Andrejiova A, Jurica M, Pokluda R (2019) The influence of genotype and season on the biological potential of chilli pepper cultivars. Folia Hortic 31: 365-374.
92. Goodwin DC, Hertwig, KM (2003) Peroxidase-catalyzed oxidation of capsaicinoids: steady-state and transient-state kinetic studies. Arch Biochem Biophys 417: 18-26.
93. Díaz-Vivancos P, de Simone A, Kiddle G, Foyer CH (2015) Glutathione--linking cell proliferation to oxidative stress. Free Radic Biol Med 89: 1154–1164.
94. Mateos RM, León AM, Sandalio LM, Gómez M, del Río LA, Palma JM (2003) Peroxisomes from pepper fruits (Capsicum annuum L): Purification, characterization and antioxidant activity. J Plant Physiol 160: 1507–1516.
95. Broniovska KA, Diers AR, Hogg N (2013) S-nitrosoglutathione. Biochim Biophys Acta 1830: 3173-3181.
96. Corporas FJ, Alché JD, Barroso JB (2013) Current overview of S-nitrosoglutathione (GSNO) in higher plants. Front Plant Sci 4, 126.
97. Belcastro E, Gaucher C, Corti A, Leroy P, Lartaud I, Pompella A (2017) Regulation of protein function by S-nitrosation and S-glutathionylation: processes and targets in cardiovascular pathophysiology. Biol Chem 398: 1267-1293.
98. Mazourek M, Pujar A, Borovský Y, Paran L, Mueller L, Jahn MM (2009) A dynamic interface for capsaicinoid systems biology. Plant Physiol 150: pp. 1806–1821.
99. Aghdam MS, Palma JM, Corporas FJ (2020) NADPH as quality footprinting in horticultural crops marketability. Trends Food Sci Technol 168: 111244.
100. Palma JM, Álvarez de Morales P, del Río LA, Corporas FJ (2018) The proteome of fruit peroxisomes: Sweet pepper (Capsicum annuum L.) as a model. Subcell Biochem 89: 323-341.