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Membrane Fatty Acids and Physiological Disorders in Cold-Stored 'Golden Delicious' Apples Treated with 1-MCP and Calcium Chloride

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Abstract: The present research intents to study skin fatty acids and physiological disorders developed during cold storage in 'Golden Delicious' apples treated with 1-MCP and calcium. Harvested fruits were treated with calcium chloride (Ca), 1-MCP (MCP), Ca + MCP or no treatment (control) and then subjected to cold storage at 0.5 °C for 6 months. Fatty acids' composition, malondialdehyde (MDA) and the physiological disorders bitter pit (BP), superficial scald and diffuse skin browning (DSB) were measured at harvest and after storage plus 7 days of shelf-life at room temperature ≈22 °C. Palmitic acid decreased and linoleic acid increased over time, while oleic and stearic acids had few changes. Generally, unsaturated/saturated fatty acids and MDA increased over the storage period. Treatment with Ca showed that, at the end of the experiment, the lowest MDA values and the highest unsaturated/saturated fatty acids ratio were mainly due to higher linoleic and lower palmitic acids concentrations, which are coincident with less severe BP. There was no clear correlation between the measured fatty acids (palmitic, linoleic, oleic and stearic), unsaturated-to-saturated fatty acids ratio or MDA with chilling skin physiological disorders. Further research is needed to clarify the changes in membrane properties and the effect of some treatments in response to chilling injury during storage.

Keywords: quality; MDA; bitter pit; superficial scald; chilling

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1. Introduction

To increase the postharvest life of fresh fruits, cold storage is the first aspect that needs to be considered, combined or not with other postharvest technologies. During storage, postharvest losses occur due to mechanical damage, diseases and physiological disorders [1,2]. Fresh apple marketing is an important subject worldwide. Apples (*Malus domestica* Borkh) can develop many physiological disorders during cold storage at 0 °C, one of the most prominent of which is bitter pit (BP) together with superficial scald, causing significant losses to apple growers worldwide [3,4]. BP occurs mainly during the period of cold storage and is characterized by black spots in the pulp, which dehydrate with time and form depressions in the skin of the fruit, reducing the marketability and quality of apple [5].

Calcium, in low concentrations in fruits, favors the formation of injuries that progress to the death of the tissues, leading to BP. This is because Ca has a role in the selective

permeability, the structure and functionality of the cell membranes by monogalactosyl diacylglycerols and phospholipids connections on the membrane surface [6]. Thus, the positive effects of Ca in preserving postharvest quality have been attributed to the fact that it is associated with the pectic substances of the middle lamella and cell membranes, stiffening tissues and preserving the characteristics of selective permeability in the cell membrane system [5,7].

Scald is also a physiological disorder appearing in the skin after long-term cold storage in apples and pears, which is related to the synthesis of α-farnesene and the accumulation of its conjugated trienols in the fruit epidermis and hypodermis. This event causes the rupture of the cell membranes, leading to polyphenoloxidase-mediated browning of the fruit peel [2,3,8,9].

Fatty acids and lipids are important structural and metabolic constituents of plant/fruit cells. Disturbances in membrane lipid composition often have severe consequences on the ability of the cell to adapt to extreme temperatures and other stress conditions, which, in fruit, may led to various storage physiological disorders [10,11].

Oxidative damage is the initial response of tissues to chilling. The production and accumulation of reactive oxygen species (ROS) are responsible for lipid oxidation. The end product of poly-unsaturated fatty acid oxidation is the toxic product malondialdehyde (MDA) [12]. Cheng et al. [13] reported a higher ratio of unsaturated/saturated fatty acids and lower levels of MDA in a pear treated with 1-MCP than in the control, explaining the lower CI in the first fruit. Additionally, cell membranes are considered to be the primary sites for the development of CI. A higher ratio of unsaturated/saturated fatty acids has been shown to improve tolerance to low-temperature storage in some fruits such as loquats [14], bananas [15] and mangoes [16].

Ethylene, known as the ripening hormone, triggers a series of biochemical changes that culminate in the ripening and senescence of fruits. The volatile compound 1-methylcyclopropene (1-MCP) binds permanently to ethylene receptors in fruit tissue [1,17] and prevents their action [18]. Thus, the postharvest application of 1-MCP can maintain the quality of apples, inhibit the growth of certain physiological disorders during storage such as superficial scald and demonstrate a positive effect on fruit quality preservation, leading to a delay in ripening and improved firmness retention [4,19].

However, 1-MCP treatment may also cause disorders such as bitter pit (BP) [2,20,21], which can be reduced by adding Ca to the 1-MCP treatment [4]. Larrigaudière et al. [22] and Gamrasni et al. [23] also reported a disorder known as diffuse skin browning (DSB) in 1-MCP-treated fruit, which can be misguidedly interpreted as superficial scald, with the appearance of diffuse browning of the skin; but in this case, the skin becomes very rough. Interestingly, this disorder appears only in countries that have very hot summers and little rainfall. Further, it was found that by progressive cooling and delaying the time after harvest to 1-MCP, application may prevent this disorder [22,23]. No induction [2] or a minor induction [4] of DSB by 1-MCP was found when the application was delayed 3 days after cooling the fruit.

The objective of this work was to investigate the effect of Smart fresh TM (625 nL/L^{-1} 1-MCP) and calcium chloride (1.5%) on the skin membrane fatty acids and their relation with the development of physiological disorders during cold storage (0.5 °C in normal atmosphere for 6 months) and subsequent shelf life (7 days) at room temperature ≈22 °C in 'Golden Delicious' apples.

2. Material and Methods

2.1. Plant Material and Treatments

Apples of the cultivar Golden Delicious were harvested from 10 orchards located in the west-center region of Portugal with ≈15% soluble solid content (°Brix), ≈15N firmness and 6–7 starch index. All the orchards were under commercial management conditions.

Half of the fruits from each producer (4 crates) were immersed for 2 min in a solution containing 1.5% $CaCl_2$ (calcium chloride anhydrous 95% PANREAC) and TECTO 500SC

(thiabendazole 42.9% p/p–200 mL/100 L water). The other half of the fruits were immersed for 2 min in a solution only with TECTO 500SC (thiabendazole 42.9% p/p –200 mL/100 L water). After, all fruits (not treated or treated with $CaCl_2$) were stored in a cold room at 0.5 °C, and after 3 days of storage, half of the fruits treated with $CaCl_2$ and half of the fruits without calcium treatment were treated with 625 nL/L 1-MCP using Smartfresh™ (AgroFresh, Spain) for 20 h at 0.5 °C. Treatments were as follows: control (no treatment), Ca (treated with $CaCl_2$), MCP (treated with 1-MCP) and Ca + MCP (treated with $CaCl_2$ and 1-MCP). All fruits were stored at 0.5 °C in normal atmosphere and 90–95% relative humidity for 6 months. Four replications were carried out for each treatment.

Sampling dates were at harvest (0 d) and 7 days of shelf-life at ≈22 °C and after 6 months storage at 0.5 °C (0 d poststorage) and 7 more days of shelf-life (7d poststorage) at room temperature (≈22 °C). On these dates, fatty acid identification and quantification as well as MDA content were carried out, and the incidence of superficial scald, DSB and BP on the apple exocarp were also observed and registered.

2.2. Fatty Acid Identification and Quantification

Fatty acids derived from the same exocarp tissue sample were extracted according to Meyer and Terry [24]. Briefly, 3 g of ground lyophilized exocarp tissue was homogenized with hexane and filtered under vacuum through a Fisherbrand QL 100 filter paper (Fisher Scientific, Leicester, UK). The solvent from the lipid-containing filtrate was evaporated under vacuum.

Fatty acid methyl esters (FAMEs) were produced according to the method prescribed by the International Olive Oil Council (IOOC) with modifications. Briefly, 0.2 mL of methanolic KOH (2 N) was added to 0.1 g of apple oil extract in 2 mL of hexane. Hexane was chosen as the preferred solvent due to improved peak resolution. The mixture was shaken vigorously for 30 s and left to stratify until the upper layer became clear. The hexane layer containing the methyl esters was decanted and kept for no more than 12 h at 5 °C until needed. This solution was diluted 1:100 (v/v) with fresh hexane immediately before injection into a Trace 1300 GC (Thermo Scientific, San Jose, CA, USA) equipped with a G1540N flame ionization detector (FID) and a 7683B autosampler. The identification and quantification of selected compounds were performed using two capillary columns: TG-17MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Thermo Scientific, San Jose, CA, USA) and TG-1MS (30 m × 0.25 mm i.d., 0.25 µm film thickness; Thermo Scientific, San Jose, CA, USA). Column temperature was programmed at 55 °C for 3 min and then raised to 175 °C at 13 °C min^{-1} intervals, followed by an isothermal period of 1 min, and increased again to a final temperature of 220 °C at 8 °C min^{-1}. The carrier gas was He at a constant flow rate of 1.6 mL min^{-1}. The injector and detector temperatures were set at 220 and 250 °C, respectively. The presence and abundance of fatty acids were calculated by comparison of peak area as a percentage.

Fatty acid identification was carried out by comparison of the retention times of each peak with retention times of standards of oleic acid, linoleic acid, stearic acid and palmitic acid from Sigma-Aldrich, which were injected using the same conditions. The unsaturated/saturated fatty acid ratio was calculated by the formula: (18:1 + 18:2)/(16:0 + 18:0) where: 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid; and 18:2 = linoleic acid.

2.3. Malondialdehyde (MDA) Content

Lipid peroxidation was determined by measuring the MDA content of frozen ground tissue according to the method of Hodges et al. [25] and Lee et al. [26]. Frozen ground apple skin tissues (1.0 g) were homogenized in 8 mL of 80% (v/v) ice-cold ethanol and 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) with an Ultra-Turax then centrifuged at 3000× g at 4 °C for 10 min in Microfuge® 18 Centrifuge (Beckman Coulter, Brea, CA, USA). One aliquot (0.6 mL) was mixed with 0.6 mL of a solution without thiobarbituric acid (−TBA), which consisted of 20% trichloroacetic acid (TCA) and 0.01% butylated hydroxyltoluene (BHT), while the other aliquot was mixed with 0.6 mL of TBA solution

(+TBA), which was composed of the above with 0.65% TBA. After vigorous mixing, the sample was incubated at 95 °C for 25 min, cooled down quickly on ice and then centrifuged at 3000× *g* at 4 °C for 10 min.

The absorbance of the sample was recorded at 440, 532 and 600 nm using a spectrophotometer. MDA equivalents were calculated in the following manner:

(1) $[(\text{Abs } 532_{+\text{TBA}}) - (\text{Abs } 600_{+\text{TBA}}) - (\text{Abs } 532_{-\text{TBA}} - \text{Abs}600_{-\text{TBA}})] = \text{A}$;

(2) $[(\text{Abs } 440_{+\text{TBA}} - \text{Abs } 600_{+\text{TBA}}) \times 0.0571] = \text{B}$;

(3) MDA equivalent (nmol g^{-1} FW) = $[(\text{A} - \text{B}/157{,}000) \times 10^6 \times$ (adjusted sample FW) $\times$ (buffer volume)].

2.4. Physiological Disorders and Rots

The presence of diffuse skin browning (DSB), superficial scald, bitter pit (BP) and rot was visually evaluated for 100 fruits per orchard per treatment after 6 months of cold storage and 7 d of shelf-life. The incidence of each disorder or rot was calculated as the percentage of affected fruits compared to the total number of fruits per replicate [2].

2.5. Statistical Analysis

The experimental was conducted with a complete randomized block design. Statistical analysis was carried out with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA) was performed using treatments and storage time as factors. Duncan's multiple-range tests ($p < 0.05$) for means comparison were conducted.

3. Results and Discussion

3.1. Changes in Fatty Acids Composition

Four main fatty acids were identified in the apple peel: palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids (Figures 1 and 2). Palmitic acid, a saturated fatty acid (SFA), was found in the highest percentage in the fruits at the harvest stage; nevertheless, after 7 days of shelf-life, there was a significant reduction in its percentage (Figure 1A). After 6 months of cold storage, palmitic acid was reduced and was similar for all treatments, maintaining these values during posterior shelf-life, except for Ca-treated fruits, which had significantly lower values. Stearic acid, another SFA, was significantly reduced during shelf-life after harvest but did not change throughout the cold storage period except for showing a significant decrease in Ca + MCP-treated fruits, nevertheless increasing again after 7 days of shelf-life poststorage (Figure 1B).

The percentage of oleic acid, an important mono-unsaturated fatty acid, increased significantly during the shelf-life after harvest, but it is important to notice that after cold storage, there was a reduction in Ca + MCP-treated fruits, which recovered during the posterior shelf-life as stearic acid did (Figure 2A). The linoleic acid content, an essential poly-unsaturated fatty acid (PUFA), increased after 7 days of shelf-life after harvest (Figure 2B). After cold storage, the content of linoleic acid continued to increase; the Ca + MCP-treated fruits had higher values than the other treatments. After the shelf-life poststorage, the percentage of linoleic acid continued to increase except for Ca + MCP.

Studies by Wu et al. [27] found that palmitic acid and linoleic acid were the dominant fatty acids in eight commercially harvested apple cultivars, constituting 70–80% of the total fatty acids in the fruits. Similar results were obtained in the present work for 'Golden Delicious' apples, which had at harvest nearly 50% palmitic acid and 27% linoleic acid; nevertheless, after shelf-life and after storage, there was an increase in the percentage of linoleic acid and a decrease in the percentage of palmitic acid. As far as essential fatty acids are concerned, this is of nutritional interest, since diets rich in unsaturated fatty acids are healthier.

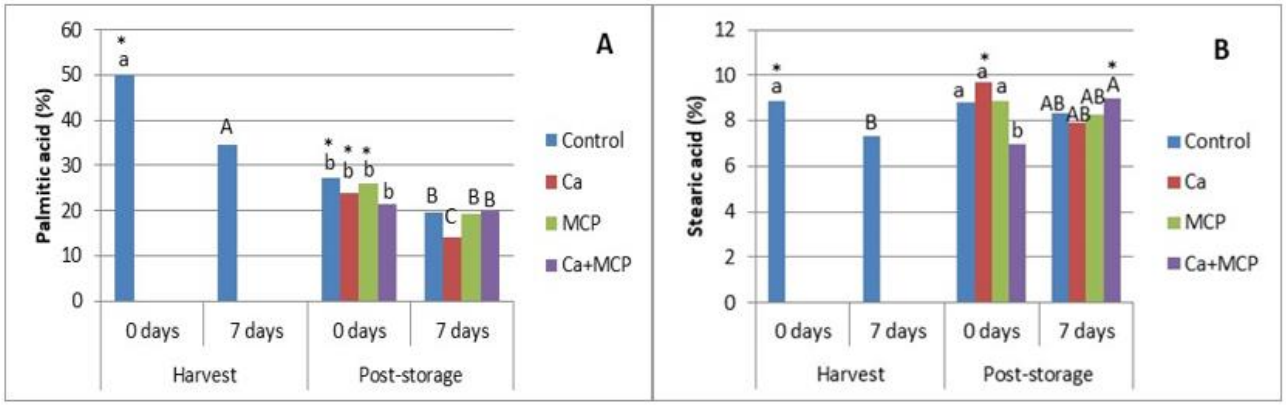

Figure 1. Changes in the saturated fatty acids, palmitic (**A**) and stearic (**B**), at harvest and after 6 months of storage at 0.5 °C and their respective shelf-life (7 days at ≈22 °C), in 'Golden Delicious' apples subjected to postharvest treatments with calcium chloride (Ca), 1-MCP (MCP), calcium chloride plus 1-MCP (Ca + MCP) and control. Lower-case letters compare treatments at harvest and after 6 months, and upper-case letters compare treatments after shelf-life. Columns with the same lower- or upper-case letter are not significantly different as determined by Duncan's multiple range test at $p < 0.05$. * represents significant differences between harvest and shelf-life or between poststorage and shelf-life for each treatment, at $p < 0.05$.

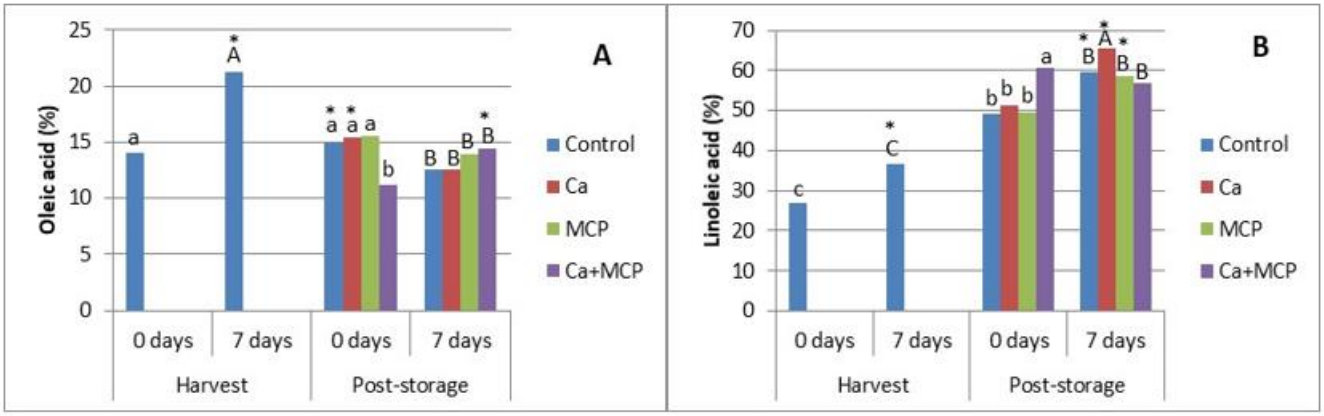

Figure 2. Changes in the unsaturated fatty acids, oleic (**A**) and linoleic (**B**), at harvest and after 6 months of storage at 0.5 °C and their respective shelf-life (7 days at ≈22 °C), in 'Golden Delicious' apples subjected to postharvest treatments with calcium chloride (Ca), 1-MCP (MCP), calcium chloride plus 1-MCP (Ca + MCP) and control. Lower-case letters compare treatments at harvest and after 6 months, and upper-cases letters compare treatments after shelf-life. Columns with the same lower- or upper-case letters are not significantly different as determined by Duncan's multiple range test at $p < 0.05$. * represents significant differences between harvest and shelf-life or between poststorage and shelf-life for each treatment, at $p < 0.05$.

3.2. Changes in the Ratio of Unsaturated/Saturated Fatty Acids

The ratio of unsaturated/saturated fatty acids in our experiment increased over time in both storage and shelf-life conditions (Figure 3A). This is mainly due to the decrease in the saturated palmitic acid and the increase in the PUFA linoleic acid (Figures 1A and 2B). According to our experiments, it appears that Ca treatment had a positive effect on the ratio of unsaturated/saturated fatty acids, since after 6 months of cold storage Ca + MCP-treated apples showed significantly higher values, and after 7 more days, the same happened in Ca-treated apples (Figure 3A).

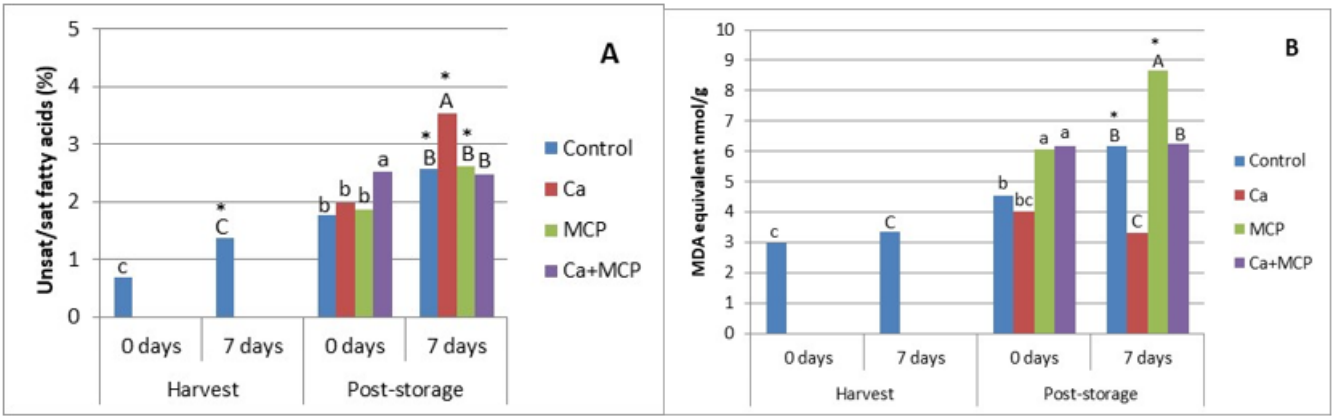

Figure 3. Changes in the unsaturated/saturated fatty acids ratio (**A**) and MDA equivalent (**B**) at harvest and after 6 months of storage at 0.5 °C and their respective shelf-life (7 days at ≈22 °C), in 'Golden Delicious' apples subjected to postharvest treatments with calcium chloride (Ca), 1-MCP (MCP), calcium chloride plus 1-MCP (Ca + MCP) and control. Lower-case letters compare treatments at harvest and after 6 months, and upper-cases letters compare treatments after shelf-life. Columns with the same lower- or upper-case letters are not significantly different as determined by Duncan's multiple range test at $p < 0.05$. * represents significant differences between harvest and shelf-life or between poststorage and shelf-life for each treatment, at $p < 0.05$.

Phospholipids and fatty acids are metabolic constituents of plant/fruit cells, and disturbances in membrane lipid composition frequently have severe consequences on the ability of the cell to adapt to extreme temperatures and other stress conditions, leading to several storage-induced physiological disorders [10,11]. Ge at al. [28] and Antunes and Sfakiotakis [29] found an increase in unsaturated/saturated fatty acids in kiwifruits and bell peppers, respectively, after cold storage. However, in peppers, the increase only lasted until 15 days storage at 4 °C, while chilling injury started to develop after 5 days; nevertheless, unsaturated/saturated fatty acids decreased thereafter, although chilling injury continued. Gao et al. [30] reported decreased unsaturated/saturated fatty acids as peaches developed chilling injury, while there were no reported unsaturated/saturated fatty acid ratio changes in pineapples developing chilling injury [31]. It is noteworthy that the fatty acids used in the formulas to calculate the unsaturated/saturated ratio were not always the same in all mentioned studies.

3.3. Changes in Malondialdehyde (MDA)

The content of MDA is often used as an indicator of lipid peroxidation stress and cell damage [32,33]. At 7 days of shelf-life after harvest, apples had a similar MDA content to that found at harvest, but it increased in storage, except for the Ca-treated apples (Figure 3B).

After 6 months of cold storage, MCP- and MCP+Ca-treated apples showed a higher MDA content than the other treatments. Interestingly, after 7 more days of shelf-life, the control and MCP treatments continued to increase, resulting in MCP-treated apples having the highest values and the Ca-treated apples having the lowest. After shelf-life post storage, it seems that in fruits treated with 1-MCP (MCP) or not (control), when calcium was added (Ca) or (Ca + MCP) there was an effect of reducing MDA. Additionally, as observed after the 6 months of cold storage, it seems that when MCP treatment was included (MCP and Ca + MCP), MDA increased, suggesting a temperature-dependent effect. Further research is needed to understand this point. Moreover, lower temperatures promote lipid oxidation, affecting the structural integrity of the plant membrane cells [12]. Interestingly, the higher value of unsaturated/saturated fatty acids obtained after storage plus shelf-life in Ca-treated apples was coincident with the lowest MDA value (Figure 3B), which may reveal a positive effect of Ca on reducing lipid peroxidation.

As in our case, 'Golden Delicious' and 'Fuji' apples in cold storage had increased concentrations of peroxides and MDA [34,35]. However, the last study reported a reduced MDA content in apples treated with MCP. Nevertheless, those authors reported MDA content on flesh. Our results are based on apple peels, and the higher MDA values found at the end of storage plus shelf-life could be related to the higher BP damage present in fruits treated with MCP (Figure 3B and Table 1).

Table 1. Physiological disorders and rot in 'Golden Delicious' apples stored for 6 months at 0.5 °C plus 7 days at ≈22 °C, as a percentage of total fruit subjected to postharvest treatments with calcium chloride (Ca), 1-MCP (MCP), calcium chloride plus 1-MCP (Ca + MCP) and control.

Disorder	Control	Ca	MCP	Ca + MCP
BP (%)	18.61 ^{b}	13.95 ^{b}	27.21 ^{a}	17.74 ^{b}
Scald (%)	3.53 ^{a}	1.99 ^{a}	0.10 ^{a}	3.73 ^{a}
DSB (%)	2.50 ^{b}	5.02 ^{a}	0.62 ^{b}	1.82 ^{b}
Rot (%)	5.56 ^{a}	1.15 ^{b}	2.56 ^{b}	0.80 ^{b}

Rows with the same case are not significantly different as determined by Duncan's Multiple Range Test at $p < 0.05$.

3.4. Physiological Disorders

Table 1 depicts the effect of the postharvest treatments on the peels' physiological disorders after 6 months of storage at 0.5 °C plus 7 days of shelf-life in 'Golden Delicious' apples.

As previously observed, 1-MCP treatment significantly increased the percentage of fruit with BP as compared with the other treatments or control [2,4]. Nevertheless, 1-MCP has been applied to apples to increase their storage life and reduce superficial scald, as has been previously reported [2,19]. In fact, MCP showed lower superficial scald in our apples as compared to the other treatments, although in this experiment superficial scald was not a big problem (Table 1).

The degree of inhibition of scald produced by 1-MCP is cultivar-dependent. Inhibition is almost complete for 'Granny Smith' [36,37]. However, scald inhibition is less consistent for many other cultivars, being affected by factors such as the type of storage (air versus controlled atmosphere) and storage length [19,38,39].

Studies by Miqueloto et al. [40] in apples 'Catherine' and 'Fuji' showed that the fruits with BP had lower Ca in the tissues of the skin. Similar results were found in 'Golden Delicious' apples [2,4]. When Ca was added to MCP, BP was reduced to a value similar to that of the control (Table 1). The treatments where calcium was added showed a positive effect on reducing BP induced by 1-MCP [2,4]. Calcium chloride was already considered as having a positive effect on ROS homeostasis in loquat fruit (*Eribotrya japonica*). The authors suggested that $CaCl_2$ treatment alleviated chilling injury through rising antioxidant enzyme activities and the ascorbate–glutathione (AsA-GSH) cycle system to scavenge ROS [41], which could, therefore, prevent the lipid peroxidation.

Interestingly, DSB, which has been reported in countries with warm summers and low rainfall, in fruits treated with 1-MCP [22,23], had higher development in fruit treated only with Ca (Table 1). The same authors reported that by gradually decreasing the storage temperature and delaying 1-MCP application, this disorder can be avoided. In our experiment, fruits were put in the cold rooms, and 1-MCP treatment occurred only after 3 days, which was proved to be effective to reduce DSB development.

3.5. Correlations among Fatty Acids, MDA and Physiological Disorders

There was no found correlation between palmitic, linoleic, oleic, stearic, unsaturated/saturated fatty acids ratio or MDA and any of the peel chilling physiological disorders studied in this experiment (Table 2). Previous authors also found no correlation between that ratio and chilling in apples [31]. There was a negative correlation

between palmitic acid and MDA and a positive correlation between linoleic or unsaturated/saturated fatty acids ratio and MDA, as was expected and explained above (Table 2).

Table 2. Pearson's correlations among the different fatty acids, unsat/sat ratio, MDA and chilling physiological disorders.

Parameters	Palmitic	Linoleic	Oleic	Stearic	Unsat/sat	MDA
Palmitic acid	1	−0.930 **	0.106	−0.026	−0.957 **	−0.554 *
Linoleic acid	−0.930 **	1	−0.440	−0.108	0.944 **	0.577 *
Oleic acid	0.106	−0.440	1	0.239	−0.189	−0.183
Stearic acid	−0.026	−0.108	0.239	1	0.014	0.135
Unsaturated/saturated	−0.957 **	0.944 **	−0.189	0.014	1	0.639 **
MDA	−0.554 *	0.577 *	−0.183	0.135	0.639 **	1
BP	0.151	−0.074	−0.166	0.032	−0.199	0.139
Scald	−0.151	0.076	0.168	−0.028	0.203	−0.135
DSB	−0.056	0.057	0.091	0.079	0.148	0.035
Rot	−0.083	0.068	0.118	0.066	0.178	0.004

*. Significance level $p < 0.05$ (2-tailed). **. Significance level $p < 0.01$ (2-tailed).

With the results of this work, it can be concluded that there is no clear correlation between the measured fatty acids (palmitic, linoleic, oleic or stearic fatty acids), unsaturated/saturated fatty acids ratio and MDA with chilling skin physiological disorders BP, scald and DSB in 'Golden Delicious' apples. However, after 6 months of storage at 0.5 °C plus 7 days of shelf-life, the treatment with Ca showed the lowest MDA values and the highest unsaturated/saturated fatty acids ratio, mainly due to higher linoleic acid and lower palmitic acid concentrations, which was coincident with lower BP occurrence. More research is needed to clarify the properties of the membranes' effect on physiological disorders, namely the identification and quantification of other membrane fatty acids' evolution during storage.

Author Contributions: M.D.A., A.C.G. and C.G. conceptualized the study and designed the experiments; A.C.G. and C.G. performed the experiments; A.G. and M.G.M. assisted in laboratory analysis and results interpretation; J.P. assisted in the statistics and laboratory analyses; E.V.B. assisted in the revision of the research; M.D.A. wrote the article, with the contribution of all authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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