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Chlorophyllase and Quality of 'Granny Smith' Apples during Storage

Chlorophyllase-Gehalt und Qualität von Äpfeln der Sorte 'Granny Smith' während der Lagerung

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Summary

'Granny Smith' apples are appreciated due to their bright green colour, which is usually associated to other expected sensory qualities. The main objective of the present work was to correlate the green colour and the chlorophyll levels with the enzyme chlorophyllase and quality parameters, such as weight loss, flesh firmness, acidity and pH during postharvest ripening of Granny Smith apples. Apples were stored during 147 days, with different treatments, to get a decrease in ethylene effect. Two groups of apples were stored at refrigerated temperature (4.6 ± 0.6 °C): one of them in 'Everfresh' bags (RE), which should adsorbe the produced ethylene, and a second group in polyethylene bags (RP); a third group was stored at ambient temperature (13.7 ± 1.0 °C) in polyethylene bags (AP). Skin colour and chlorophyll content were not significantly different ($p < 0.05$) under refrigeration (RE, RP) during the 147 days of storage. The fruits which are kept under ambient temperature (AP) showed however an increase in chroma and a decrease in the chlorophyll levels. Accordingly, chlorophyllase activity decreased faster in AP, not so fast in RP, and slower in RE stored apples, which correlates with the colour measurements.

Zusammenfassung

'Granny Smith' Äpfel sind nicht nur wegen ihrer geschmacklichen Qualität, sondern mehr noch wegen ihrer grünen Farbe bekannt. Das Ziel dieser Arbeit war es, die Korrelation zwischen der grünen Farbe und dem Chlorophyllgehalt mit dem Chlorophyllase Enzym und einigen Qualitätsindikatoren, wie Gewichtsverlust, Fruchtfleischfestigkeit, Säuregehalt und pH zu untersuchen. Um die Wirkung des Ethylens zu verringern, wurde während 147 Tagen Lagerung verschiedene Behandlungen durchgeführt. Zwei Äpfelgruppen wurden im Kühllager gelagert: eine in „Everfresh" Beuteln verpackt (RE), welche das Ethylen absorbieren sollten, die andere in Polyethylen Beuteln (RP). Die dritte Gruppe wurde bei Zimmertemperatur (AP) gelagert. In den 147 Tagen Kühllagerung wurden keine bedeutsame Unterschiede zwischen RE und RP ($p < 0.05$) weder in der Farbe noch im Chlorophyllgehalt gefunden. Die Äpfel in den AP Lagerversuche zeigten aber eine Zunahme im Chroma und eine Abnahme im Chlorophyllgehalt. Gleichzeitig damit nahm die Aktivität der Chlorophyllase schneller ab in AP, nicht so schnell in RP und noch langsamer in RE gelagerten Äpfeln, welches mit der Farbe übereinstimmte.

Introduction

The fruit skin of 'Granny Smith' apples has a bright green ground colour that becomes greenish yellow with the maturity. The flesh is greenish white, firm, rather coarse-textured, juicy and subacid (WARRINGTON, 1994; KAWANO, 1998). Recently, this cultivar has been grown experimentally in Temuco, southern Chile, being the apples smaller in size but of good eating quality.

Appearance, as an all-inclusive term involving size, shape, texture, mass, gloss, colour and others, is one of the major attributes of external quality (FRANCIS, 1995). The perception of quality of Granny Smith apples, as influenced by green colour, deals with chlorophylous pigments.

It has been long presumed that chlorophyllase may be involved in the breakdown of chlorophyll molecules, which is characteristic during ripening of fruits. The enzyme catalyses the removal of the phytol group of chlorophyll forming the corresponding chlorophyllide, an unstable and therefore easily degradable compound (RÜDIGER and SCHOCH, 1988). Ethylene accelerates the respiration, leading to the softening, ripening and senescence of many kinds of fruits. Furthermore, ethylene accumulation can cause yellowing of green vegetables and may be responsible for a number of specific postharvest disorders in fresh fruits and vegetables. This was well studied in citrus fruits treated with ethylene (AMIR-SHAPIRA *et al.*, 1987; SHIMOKAWA *et al.*, 1990a, 1990b). Ethylene appears to enhance the degreening of citrus fruit peel through *de novo* synthesis of the chlorophyllase protein (TREBISH *et al.*, 1993). HALDER-DOLL and BANGERTH (1987) showed that 'Golden Delicious' apples treated with aminoethoxyvinilglycine (AVG), an inhibitor of the autocatalytic ethylene production, didn't have an appreciable effect on the chlorophyll degradation of epidermal tissue during five months of storage. WARRINGTON (1994) pointed out that ethylene production cannot be used as a maturity marker for Granny Smith apples, because it remains very low throughout

the commercial harvest period. Otherwise, LELIÈVRE *et al.* (1995) found that ethylene production at 17 °C started to increase after 35 days, but if chilled for 5 days in the cold (4 °C), it increased sharply with accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase protein in fruits, indicating that ACC oxidase, in addition to ACC synthase, is induced during chilling of preclimacteric Granny Smith apples and accumulates before any transfer of the fruit to warmer temperatures.

To prolong shelf-life and maintain an acceptable visual and organoleptic quality, accumulation of ethylene in the packaging should be avoided (VERMEIREN *et al.*, 1999). One ethylene-adsorbing technology is based on inclusion of finely dispersed minerals such as zeolites and japanese oya into packaging polyethylene films, major components of which are silicic acid anhydride and aluminium oxide, as described by URUSHIZAKI (1987). These bags have the capacity to hold water and partially to adsorbe and to deodorize, because of their strong interactions with polar molecules like water, and with small molecules containing double bond, like ethylene.

A harvested fruit is left to survive on its own metabolites to maintain its physiological processes and are not replenished any more. The metabolic changes inside the fruit are reduced by lowering the temperature (MITCHELL, 1985). A study of critical storage temperatures on pigment degradation was conducted by Monreal et al. (1999) on green beans. They found that green beans kept better quality at 4 °C than either 8 or 12 °C, maintaining a bright green color and good texture, but temperature of 4 °C induced chilling injury after eight days of storage. Cold storage temperatures of 4, 8, and 12 °C, produced different changes on the green beans chlorophyll profile.

In the present work, an attempt has been made to determine the pattern of some biochemical changes and to correlate quality indexes during postharvest ripening, mainly the green colour with the enzyme chlorophyllase.

Materials and methods

Apple treatments

Apples (Malus domestica Borkh. cv. Granny Smith) were picked 187 days after full bloom in Temuco (39°15' LS with rainy climate and frequent frost in early spring and late autumn), and then randomised into 4 samples of 75 fruits each (polar diameter: 5.75 – 8.42 cm; equatorial diameter: 6.33 – 8.42 cm) and immediately stored. Two groups were placed in a thermostatically regulated chamber at 4.6 ± 0.6 °C, and a third directly at ambient temperature (13.7 ± 1.0 °C). During storage of apples, bags of polyethylene (size 1 x 0.5 m) were used, excepting one group of apples stored in special bags (URUSHIZAKI, 1987) with the commercial name Everfresh (size 0.5 x 0.4 m). Bags were renewed every 60 days.

The three treatment conditions were the following:
1) refrigerated, in Everfresh bags (RE)
2) refrigerated, in polyethylene bags (RP)
3) in ambient temperature in polyethylene bags (AP)

Non destructive analysis

Weight loss and skin colour were assessed weekly on 20 labeled apples, during the whole storage period. Colour was measured with a Minolta Chromameter CR-200b on two opposite sides of each apple, according to CIEL*a*b* system. The L* variable or lightness index ranges the scale from 0 for black to 100 for white. The a* scale measures the degree of red (+a*) or green (–a*) colour and the b* scale measures yellow (+b*) or blue (–b*) colour. The saturation index or chroma, given by the distance from the central chromatic axis along the corresponding radius, is given by the equation $C^* = (a^{*2} + b^{*2})^{1/2}$ (LITTLE, 1975; IHL *et al.* 1994b).

Destructive analysis

Each 14 days, In triplicate on the edible portion, were measured fruit flesh firmness, pH, titratable acidity, soluble solid and chlorophyll contents. Chlorophyllase activity was determined on the joined pericarp corresponding to the same apples.

Fruit flesh firmness was measured on both sides of the fruit, after removal of the peel, using an Effegi penetrometer fitted with a 7.9 mm diameter tip. Soluble solids content of juice squeezed from a slice of fruit was measured using a Reichert-Jung hand-held refractometer, temperature compensated. Titratable acidity was measured on 10 ml of juice after homogenisation and filtration and expressed in percent of malic acid equivalents (A.O.A.C., 1990).

Chlorophyll analysis

Chlorophyll and chlorophyllide pigments, extracted from 1 g of homogenised pericarp (with 1 mm mesocarp) of 3 fruits from different sizes in 80% acetone final concentration, were determined by spectral analysis measuring the absorbance at 664, 647 and 750 nm. Chlorophyll was separated from chlorophyllide by adding n-hexane to the acetone phase, in the proportion 2:1, together with an ionic exchanger. The mixture was shaken, centrifuged and the hexane phase determined at 644, 662 and 750 nm (SCHOCH and BROWN, 1987; HELFRICH *et al.*, 1994). The analysis was carried out on a Hewlett Packard 8452 spectrophotometer, using the following equations, previously described (PORRA *et al.* 1989; PORRA, 1991; IHL *et al.* 1998a):

$$\text{Chlorophyll } a \text{ (nmol/mL)} = 11{,}30\,(A_{662} - A_{750}) - 1{,}11\,(A_{644} - A_{750})$$

$$\text{Chlorophyll } b \text{ (nmol/mL)} = 18{,}07\,(A_{644} - A_{750}) - 2{,}87\,(A_{662} - A_{750})$$

Experimental procedure for crude chlorophyllase extraction and assay

Apple pericarp with 1 mm mesocarp was obtained from 3 fruits of different size and chopped together. Chlorophyllase (from 10 g tissue) was extracted as described previously (IHL *et al.* 1998, IHL *et al.* 1999). The enzyme was incubated with its substrate for 2 h at 27 °C at darkness on a shaker. The reaction was stopped in 80 %

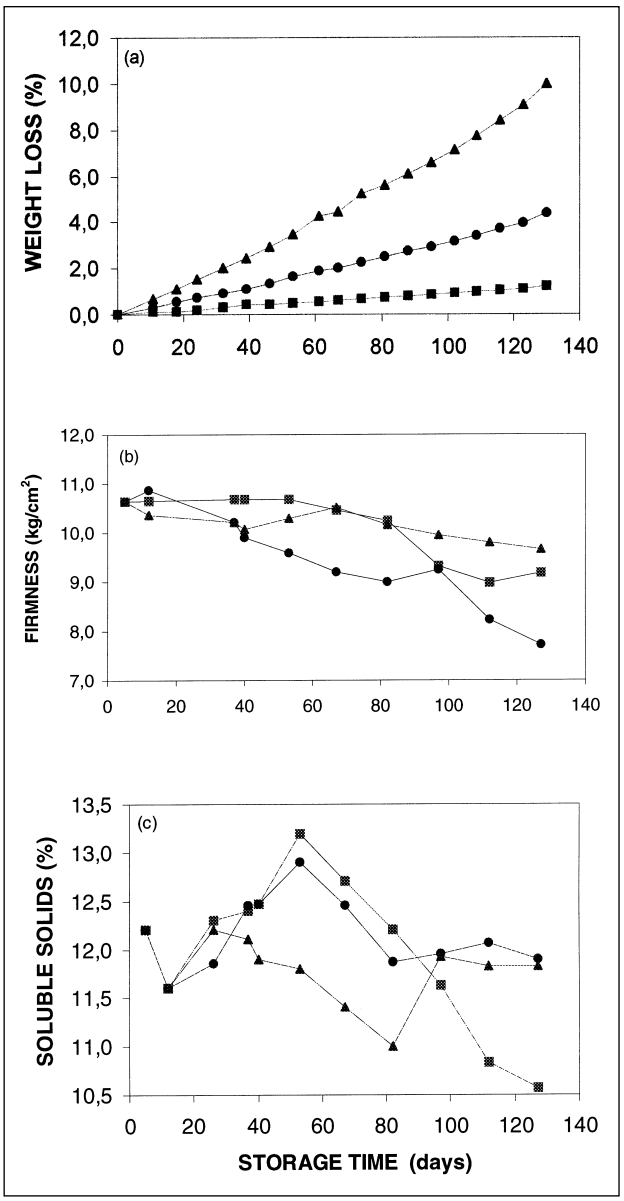


Fig. 1. Weight loss (a), flesh firmness (b) and total soluble solids (c) of 'Granny Smith' apples stored refrigerated in 'Everfresh' bags RE (–■–); refrigerated in polyethylene bags RP (–●–); at ambient temperature in polyethylene bags AP (–▲–).

Abb. 1. Gewichtsverlust (a), Fruchtfleischfestigkeit (b) und lösliche Trockensubstanz (c) von 'Granny Smith' Äpfel, während kühl gelagert in 'Everfresh Beutel RE (–■–), Polyethylen Beutel RP (–●–); oder gelagert bei Raumtemperatur in Polyethylen Beutel AP (–▲–).

acetone final concentration, followed by a centrifugation (2200 rpm, 3 min). Chlorophylls, before and after the incubation of the enzyme, are quantified in hexane, as described before.

Statistical analysis

The results were submitted to a statistically analysis by the use of a design of repeated measures completely randomiced, as to determine significally differences between the three storage treatments, by the Analysis of Variance (ANOVA) method, using a 95% confidence level with the statistical computer program Harvey (HARVEY, 1988); statistical differences between treatments were analysed by the test of the average standard deviation with a 95% confidence level, using the M-Stat program.

Results and discussion

Weight loss, firmness and soluble solids

Fig. 1a shows weight loss during storage under the studied conditions; AP treated apples achieved a significantly greater weight loss ($p<0.05$) than refrigerated apples, although weight loss in RE apples were significantly lower ($p<0.05$) than for the RP refrigerated treatment. Flesh firmness (Fig. 1b) was significantly different ($p<0.05$) for storage time within each treatment and not between treatments; the higher values of flesh firmness for AP apples can be explained by the larger dehydration, as can also be seen in Fig. 1a, but not by turgidity. The total soluble solids of fruit juice (Fig. 1c) increased for RE and RP apples as the storage period advanced, it reaches a peak value at 53 days of storage and declined thereafter whereas AP-stored apples did not show this behaviour change during storage. Likewise MAHAJAN (1994), for 'Red delicious' apple fruits, found similar results and explain that the initial increase may be due to hydrolysis of starch into sugars; on completion of hydrolysis of starch, no further increase in total soluble solids and sugars did occur.

Acidity and pH

The pH increase during storage (Fig. 2b) was not significantly different ($p<0.05$) between the two refrigerated treatments, being both significantly lower than ambient temperature stored apples. The pH of an extract is one way to express the acidity of the fruit. Free weak acids associated in the cell with their potassium salts constitute buffer systems, which play an important role in the cell, particularly in relation to the enzymes (ULRICH, 1970). For titratable acidity measurements, with end point of the assay at pH 8.5, in the final result are involved organic acids, and only a part of phosphoric acid and phenols (ULRICH, 1970). Fig. 2a shows how the titratable acidity changed in relation to storage time: the decrease in acidity was significantly ($p<0.05$) larger in AP treated apples compared to RP treated apples, but was not significantly different within the refrigerated treatments, neither between AP and RE treatments.

Colour, chlorophyll contents and chlorophyllase activity

Initially, apples showed epidermal values of $L^* = 51.0 \pm 3.3$, $a^* = -20.8 \pm 1.0$ and $b^* = 38.2 \pm 3.3$; darker and greener than Granny Smith apples reported by AUBERT *et al.* (1992). In Fig. 3a, colour expressed as chroma or saturation index did not change during the storage time for the refrigerated treatments, but it showed an increase in AP treated apples. Accordingly, when the chlorophyll content in AP treated apples de-

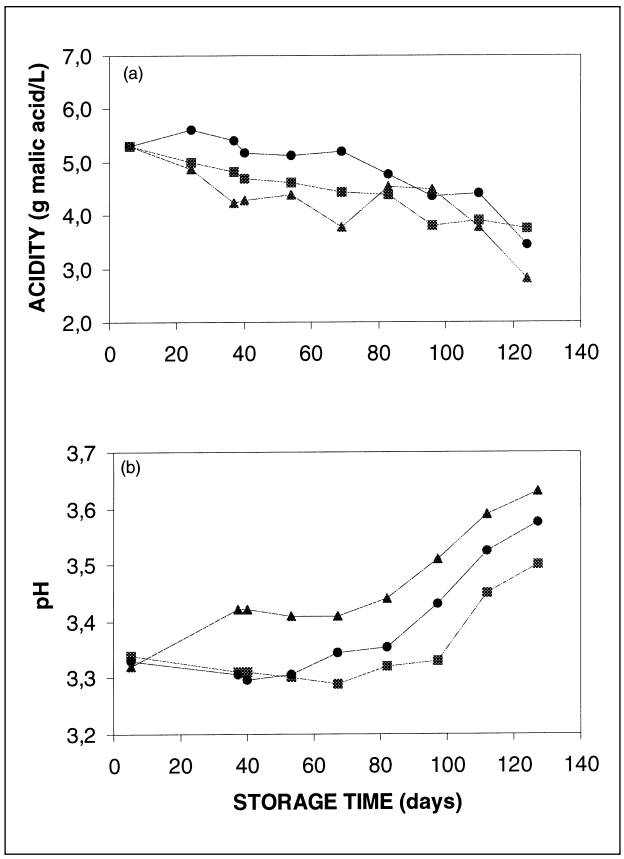


Fig. 2. Titratable acidity content (a) and pH (b) of 'Granny Smith' apples stored refrigerated in 'Everfresh' bags RE (–■–); refrigerated in polyethylene bags RP (–●–); at ambient temperature in polyethylene bags AP (–▲–).

Abb. 2. Abnahme der titrierbare Säure (a) und Zunahme des pH-Wertes (b) von 'Granny Smith' Äpfel, während kühl gelagert in 'Everfresh Beutel RE (–■–), Polyethylen Beutel RP (–●–); oder gelagert bei Raumtemperatur in Polyethylen Beutel AP (–▲–).

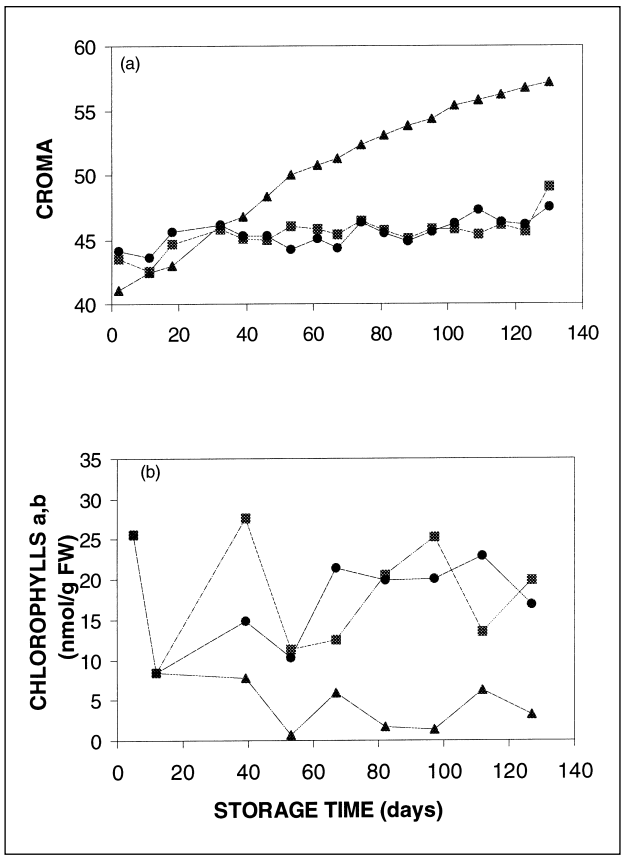


Fig. 3. Saturation index Chroma (a) and total chlorophylls (b) of 'Granny Smith' apples stored refrigerated in 'Everfresh' bags RE (–■–); refrigerated in polyethylene bags RP (–●–); at ambient temperature in polyethylene bags AP (–▲–).

Abb. 3. Chromatischer Index (a) und Chlorophyllgehalt (b) von 'Granny Smith' Äpfel, während kühl gelagert in 'Everfresh Beutel RE (–■–), Polyethylen Beutel RP (–●–); oder gelagert bei Raumtemperatur in Polyethylen Beutel AP (–▲–).

creased (Fig. 3b), the chlorophyllase activity (Fig. 4) decreased. The RE treatment showed the highest chlorophyllase activity (Fig. 4) between 40 and 67 days of storage and declined after that. The RP treatment presented an intermediate activity of the enzyme. The chlorophyll content (Fig. 3b) and colour (Fig. 3a) for RP apples fluctuated during the whole storage period, likely because of irregular degreening in refrigerated apples; fluctuations in firmness, pH and acidity could be of the same origin. Indeed, in Fig. 4, chlorophyllase enzyme activity in RE and RP apples was high during 97 days of storage and fall to a minimum between 127 and 147 days, but in AP apples, the activity fall already to a minimum in 67 to 82 days. Previously, in 1993 and 1994, IHL *et al.* (1994a) observed in Granny Smith apples after 133 storage days, also a very low chlorophyllase activity in ambient temperature and high enzyme activity in 5 °C stored apples; besides, there was a low chlorophyllase activity in immature apples which increased with ripening. Although after 133 days storage the enzyme was found still with maximum activity, this difference with the present work could correspond to the proper seasonal variability of an horticultural produce. Othersides, chlorophyllase activity of 'Cox's Orange Pippin' apple tissue (RHODES and WOOLTORTON, 1967) was found to increase somewhat before the climacteric started, increased three-fold during the climacteric rise, while the chlorophyll content was declining, and continued to increase beyond the peak. LELIÈVRE *et al.* (1995) in preclimacteric 'Granny Smith' apples, found an increase in ethylene production after 5 days of storage in air (4 °C) while at 17 °C the ethylene production begun to increase only at 35 days. In agreement with RHODES and WOOLTORTON (1967) and LELIÈVRE *et al.* (1995), in Fig. 4, between 12 and 53 days of storage, AP treated apples could be at the postclimacteric stage. In a further research, it would be of interest to measure the ethylene produced in the fruit, in order to compare it to the chlorophyllase activity.

A sensory test panel, not presented here (IHL *et al.*, 1998b), showed that panelists prefered the apples stored in common polyethylene bags, being in disagreement to RE treatment probably to the adsorption of the aroma volatiles together with ethylene in the components of the 'Everfresh' bags.

In the present results, RE and RP apples, with a slower ripening process than AP apples, correspond to apples

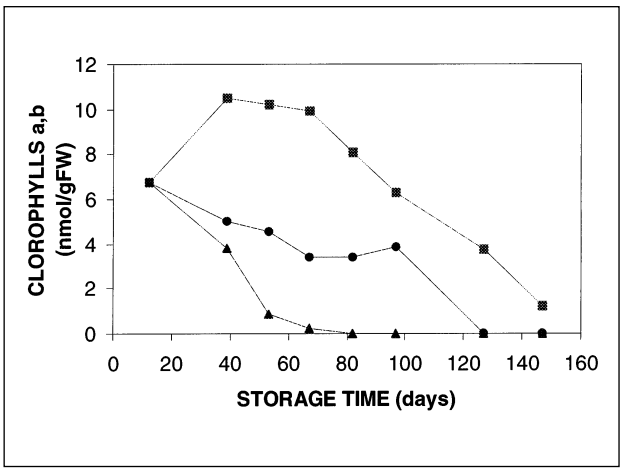


Fig. 4. Chlorophyllase activity, expressed as amount of chlorophyll degraded (difference in chlorophylls a and b content before and after 1 hour incubation) in the test conditions by the crude enzyme extract, of 'Granny Smith' apples stored refrigerated in 'Everfresh' bags RE (–■–); refrigerated in polyethylene bags RP (–●–); at ambient temperature in polyethylene bags AP (–▲–).

Abb. 4. Chlorophyllase Aktivität, dargestellt als abgebautes Chlorophyll Gehalt (Differenz zwischen dem Chlorophyll a und b Gehalt, bevor und nach einer Stunde Inkubation) von 'Granny Smith' Äpfel während der Lagerung: kühl gelagert in 'Everfresh Beutel RE (–■–), Polyethylen Beutel RP (–●–); oder gelagert bei Zimmertemperatur in Polyethylen Beutel AP (–▲–).

of final better quality, due mainly to the typical green colour, more acidity lower pH and lower weight loss, all important quality attributes of 'Granny Smith' apples; although firmness was not significantly different between treatments. The chlorophyllase analysis showed a longer lasting activity for both RE and RP apples, but higher for RE than for RP apples. Therefore, in this preliminary assay, chlorophyllase activity could reflect apple quality.

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