

Use of chlorophyll *a* fluorescence for evaluating the quality of leafy vegetables

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Abstract

The aim of this work is to evaluate leaf senescence rate via chlorophyll *a* fluorescence measurements and with the JIP test. Lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea* L.) vegetables were stored at 4 or 8 °C. The performance index (PI_{ABS}) was the only index that immediately changed, even after one day of storage. The cumulative respiration was higher at higher storage temperature (8 °C). At the end of storage, the cumulative respiration was 318 and 398 mg CO₂/100 g FW for lettuce and spinach, respectively. The data from the induction curves were correlated with cumulated respiration. A multivariate regression model showed a satisfactory predictive ability with a R² in validation of 0.67 and a standard error in prediction (RMSEP) of 170 mg CO₂/ 100 g FW corresponding to the CO₂ emission of 3.5 days at 4 °C and of 2.1 days at 8 °C.

Keywords: chlorophyll, carotenoids, respiration, lettuce, spinach, postharvest, senescence, quality.

Abbreviations: DI_o/RC = Dissipation of energy per reaction center; Fv/Fm = maximum quantum efficiency of photosystem II; PI_{ABS} = Performance Index on the absorption basis; TR_o/RC = Trapped energy flux per reaction center; ET_o/RC = Electron transport flux per reaction center; Fo/Fm = quantum yield of electron transport (at t=0); RC/CSm = Density of reaction centres (FP), φPo = 1-(Fo/Fm) = Fv/Fm maximum quantum yield of primary photochemistry; ψo = efficiency by which a trapped exciton, having triggered the reduction of Q_A to Q_A⁻, can move an electron further than Q_A⁻ into the electron transport chain.

Introduction

Changes in human life styles have led consumers to move towards ready-to-eat products. Particularly in recent years, there has been an increase in the consumption of fresh-cut vegetables or minimally processed fruit and vegetables due to their ease of use and nutritional properties (Artés et al., 2007). The preparation of fresh-cut products involves many steps such as cleaning, washing, trimming, coring, slicing, and shredding, which increase perishability (Artés and Allende, 2005). Therefore, these products have higher respiration rates because of wounds generated during processing, and they are often subjected to rapid loss of colour, organic acids, vitamins and other compounds that determine flavour and nutritional value. Usually the shelf life of these products is much shorter than that of unprocessed vegetables (Brecht et al., 2004). The selling period on the shelf of markets is limited to 5-7 days for most leafy vegetables (Rolle and Chism, 1987). This period of time is imposed for assuring the good quality of products to consumers. During storage and throughout the distribution chain, temperature is the most important parameter that must be continuously monitored and kept as low as possible for preserving quality. All fresh-cut items should be stored at 0-5 °C to maintain their quality safety and shelf life (Artés and Allende, 2005). The quality of vegetables during storage is difficult to determine. Considering the short period of marketability, the senescence symptoms are not visible, but the quality may be compromised. Consumer choices are oriented on products appearance (colour) and attractiveness (Ferrante et al., 2004). Fresh-cut vegetables must have a fresh appearance, be of consistent quality throughout the package and be free of defects. Research is currently focused on developing non-invasive systems that allow quality monitoring at any step of the distribution chain. The measurement of fluorescence of chlorophyll *a* in living plants

is a widely used tool in plant physiology to study the stress state of a plant; the fluorescence measurement evaluates the energy system of the photosynthetic complex (Strasser et al., 1995; Lazár, 1999). Chlorophyll molecules absorb light energy, which can be used for leaf photosynthetic activity, be dissipated as heat or be re-emitted as fluorescence. These three possible fates of the energy supplied by light compete with each other, and the increase of one is at the expense of others (Maxwell and Johnson, 2000). Thus, by measuring the fluorescence of chlorophyll *a*, it is possible to obtain information on the efficiency of photosynthesis and photochemical energy dissipation that occurs in a leaf (Strasser et al. 1995; Stirbet et al., 1998; Cha-um et al., 2010). The spectrum and kinetics of the fluorescence emitted by a leaf are biological parameters that are measured quickly and using non-invasive systems. The fluorescence of chlorophyll *a* was used to monitor senescence in different vegetables such as broccoli (Toivonen and DeEll, 2001) and peppers. In lettuce, potential shelf life has been calculated by considering the correlation between parameters of fluorescence (Fv/Fm) and aesthetic quality (Schofield et al., 2005). In apples, the fluorescence of chlorophyll was used as a selection tool to define different classes of quality (Moshou et al., 2005) and for managing oxygen in a controlled atmosphere in storage. In *Valerianella* leafy vegetables stored at 4 and 10 °C, some key parameters of chlorophyll *a* fluorescence and some derived indexes from the JIP test were able to describe the progression of senescence and loss of product quality (Ferrante and Maggiore, 2007). The aim of this work was to evaluate the quality changes and senescence process during storage using chlorophyll *a* fluorescence. In particular, analyses of the fluorescence transience and respiration were performed to evaluate the functionality of lettuce and baby spinach leaves during storage.

Table 1. The chlorophyll and carotenoid contents in lettuce (*Lactuca sativa* L.).

| Time (d) | Chl a (mg/g FW) | | Chl b (mg/g FW) | |
|----------|-------------------|-------------|-----------------------|------------|
| | 4 °C | 8 °C | 4 °C | 8 °C |
| 0 | 0.32±0.010 | 0.32±0.010 | 0.08±0.002 | 0.08±0.002 |
| 3 | 0.31±0.013 | 0.30±0.012 | 0.09±0.015 | 0.08±0.005 |
| 6 | 0.31±0.012 | 0.32±0.015 | 0.08±0.006 | 0.08±0.010 |
| 9 | 0.32±0.018a | 0.26±0.010b | 0.07±0.011 | 0.05±0.008 |
| 13 | 0.32±0.017 | 0.27±0.020 | 0.02±0.006 | 0.03±0.008 |
| Time (d) | Chl a+b (mg/g FW) | | Carotenoids (mg/g FW) | |
| | 4 °C | 8 °C | 4 °C | 8 °C |
| 0 | 0.41±0.011 | 0.41±0.011 | 0.11±0.007 | 0.11±0.007 |
| 3 | 0.40±0.014 | 0.39±0.016 | 0.10±0.010 | 0.10±0.003 |
| 6 | 0.39±0.013 | 0.40±0.013 | 0.11±0.008 | 0.12±0.012 |
| 9 | 0.39±0.015a | 0.31±0.010b | 0.11±0.014 | 0.09±0.006 |
| 13 | 0.34±0.020 | 0.29±0.022 | 0.12±0.008 | 0.10±0.009 |

The values are the means with standard errors (n=4). Data were subjected to two-way ANOVA analysis. The differences were calculated with Bonferroni's test. Different letters indicate statistical differences at $p < 0.05$.

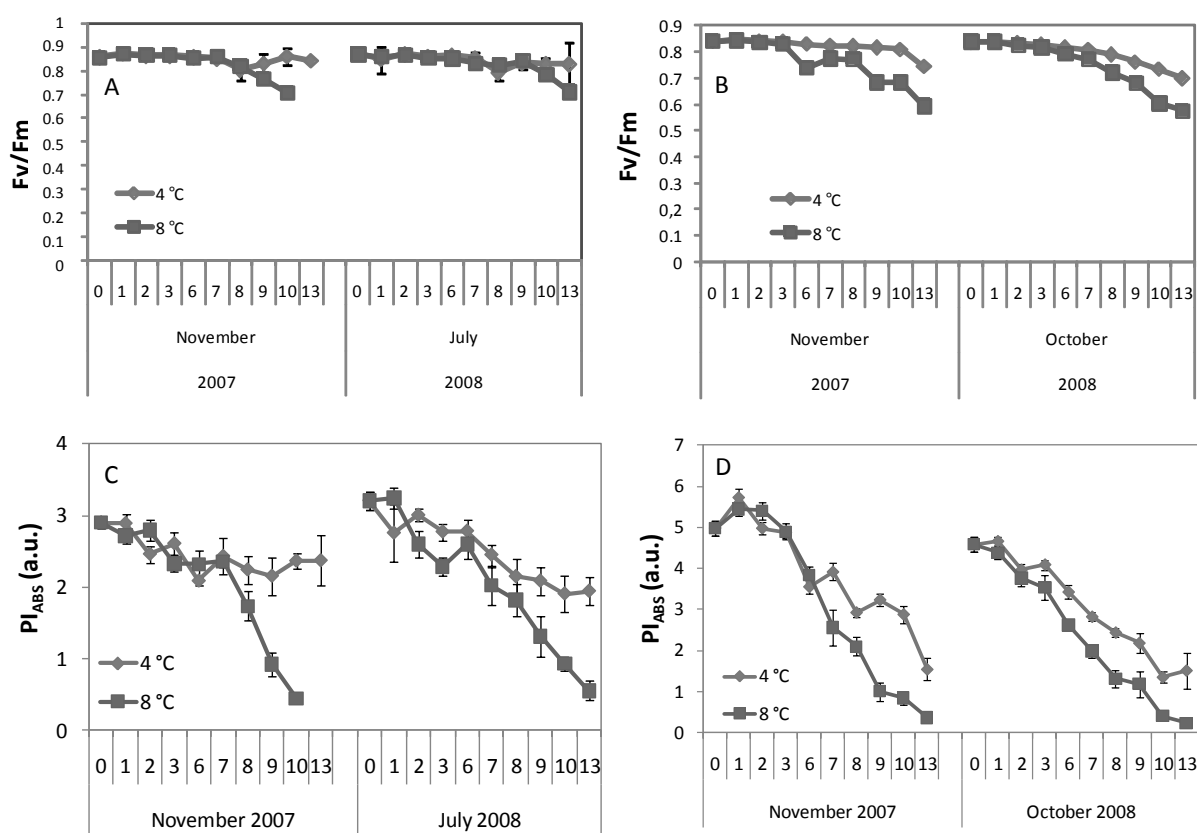


Fig 1. Fv/Fm ratio in lettuce (A), or spinach (B), PI in lettuce (C), or spinach (D) stored at 4 or 8 °C. Values are means ± standard errors (n=10).

Materials and methods

Plant material and storage treatments

Lettuce (*Lactuca sativa* L. [var. *acephala* type Batavia]) cv. Rubia flava and spinach (*Spinacia oleracea* L.) cv. Bella leaves were harvested at the commercial stage when the plants reached the 13 cm height and 4-6 leaves were expanded, minimally processed (as required by the industry procedures), packed in plastic bags and transported at 4 °C to the laboratory (provided by La Linea Verde spa). The packages (plastic bags) were stored at 4 or 8 °C in dark conditions for 13 days.

Determination of chlorophyll, carotenoid content and chlorophyll a fluorescence

Leaf pigments were extracted using 99.9% methanol as solvent. The samples were kept in a dark cold room at 4 °C for 24 h. The absorbance of the extracts was measured at 665.2 and 652.4 nm for the chlorophyll pigments and 470 nm for total carotenoids. Chlorophyll *a* and *b* and carotenoids were calculated by Lichtenthaler's formula (1987). Chlorophyll *a* fluorescence was determined on leaves randomly taken from the stored boxes. The leaves were dark adapted with leaf clips and incubated for 30 min at room

Table 2. The chlorophyll and carotenoid contents in spinach (*Spinacia oleracea* L.).

| Time (d) | Chl a (mg/g FW) | | Chl b (mg/g FW) | |
|----------|-----------------|------------|-----------------|-------------|
| | 4 °C | 8 °C | 4 °C | 8 °C |
| 0 | 0.90±0.015 | 0.90±0.015 | 0.22±0.014 | 0.22±0.014 |
| 3 | 0.84±0.041 | 0.82±0.018 | 0.20±0.020 | 0.22±0.009 |
| 6 | 0.86±0.031 | 0.83±0.022 | 0.23±0.008 | 0.20±0.010 |
| 9 | 0.85±0.019 | 0.78±0.019 | 0.22±0.031a | 0.15±0.016b |
| 13 | 0.81±0.023 | 0.81±0.028 | 0.16±0.005 | 0.19±0.014 |

| Time (d) | Chl a+b (mg/g FW) | | Carotenoids (mg/g FW) | |
|----------|-------------------|-------------|-----------------------|------------|
| | 4 °C | 8 °C | 4 °C | 8 °C |
| 0 | 1.12±0.026 | 1.12±0.026 | 0.28±0.006 | 0.28±0.006 |
| 3 | 1.04±0.035 | 1.04±0.019 | 0.25±0.005 | 0.25±0.008 |
| 6 | 1.09±0.034 | 1.04±0.030 | 0.26±0.010 | 0.26±0.005 |
| 9 | 1.07±0.047a | 0.93±0.021b | 0.25±0.008 | 0.26±0.007 |
| 13 | 0.97±0.024 | 1.00±0.037 | 0.27±0.009 | 0.27±0.010 |

Values are means with standard errors (n=4). Data were subjected to two-way ANOVA analysis. The differences were calculated with Bonferroni's test. Different letters indicate statistical differences at $p < 0.05$

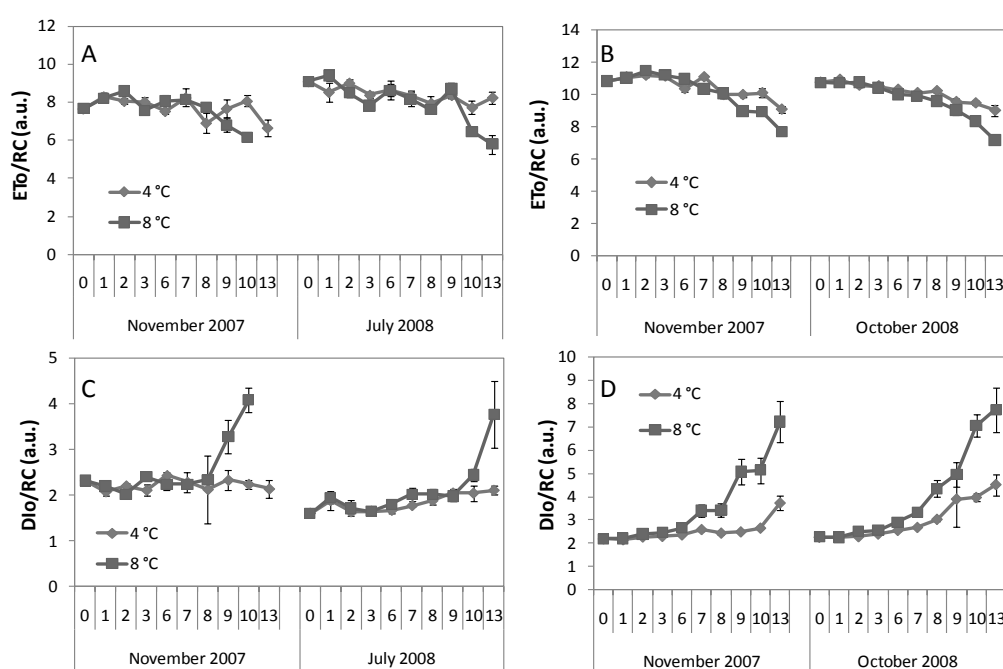


Fig 2. ETo/RC in lettuce (A) or spinach (B), Dto/RC in lettuce (C) or spinach (D) stored at 4 or 8 °C. Values are means ± standard errors (n=10).

temperature. Chlorophyll *a* fluorescence transients were measured using a portable Handy Plant Efficiency Analyser (PEA, Hansatech, UK). The measurements were taken on the leaf surface after illumination with a light intensity (LED with maximum emission peak at 650 nm) of $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Respiration measurements

The respiration rate was measured daily using the alkaline trap method (Brewer and Sullivan 2003); at each sampling time during the shelf life, 20 g of leaves for each treatment were stored in triplicate in individual sealed glass jars containing alkali traps (10 mL 1 N NaOH). The trapped CO_2 released by biological respiration during incubation intervals was quantified by back titration.

Statistical analysis

The data are reported as the mean values with standard error (SE). Each treatment was comprised of 100 packages. Data

analysis on fluorescence spectra was performed using Matlab R2007 (The Mathworks, USA) and PLS Toolbox 5.2 (Eigenvector Research, Inc. Wenatchee, WA, USA). The chlorophylls and total carotenoids data were subjected to a two-way ANOVA, and differences among the means were calculated with Bonferroni's post test (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Chlorophyll and carotenoid content

In lettuce stored at 4 °C, the chlorophyll *a* (chl *a*) content did not change, but a reduction of 25% between the initial content and that found at the end of experiment was observed at 8 °C (Table 1). Statistical differences between the two storage temperatures were observed after 9 days of storage. In comparison, the chlorophyll *b* (chl *b*) content drastically declined at both storage temperatures without showing

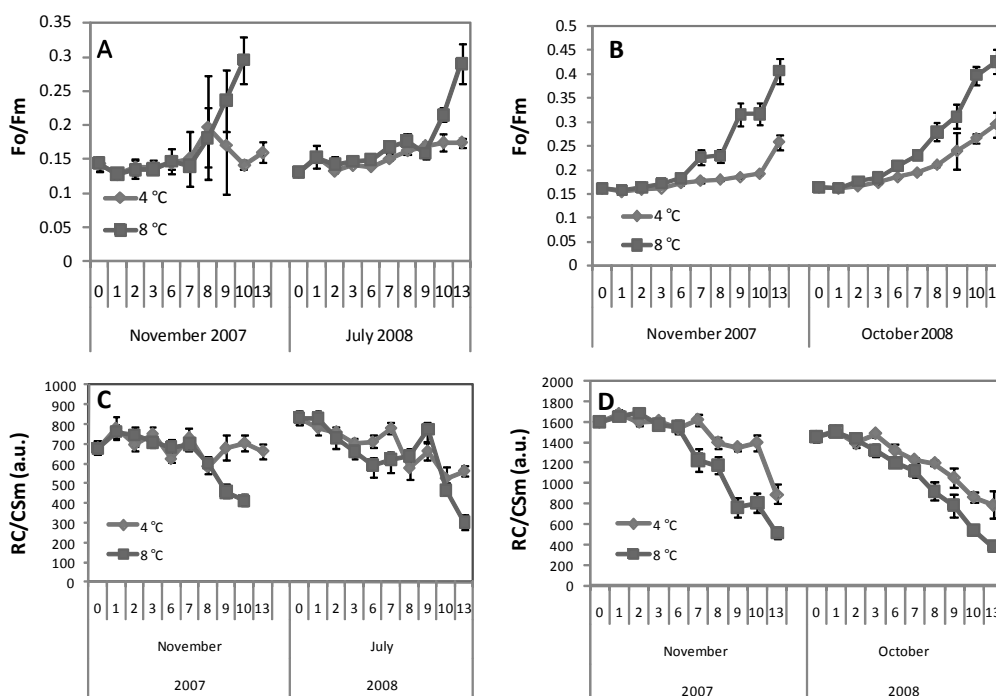


Fig 3. Fo/Fm in lettuce (A), B) Fo/Fm in spinach, C) RC/CSm in lettuce, D) RC/CSm in spinach baby leaf stored at 4 or 8 °C. Values are means \pm standard errors ($n=10$).

significant differences between the two temperatures. At the end of experiment, the content of chl *b* was 25% of the initial value. The reduction was observed after 6 days of storage. Total carotenoids, however, did not change during the whole experimental period in both storage conditions. In spinach leaves, the chlorophyll content showed the same trend at both storage temperatures, even if greater variability was observed in leaves stored at 8 °C (Table 2). In spinach, the chl *b* content only declined after 13 days of storage at 4 °C; statistical differences between 4 or 8 °C were observed after 9 days of storage. At the end of the storage period, the chlorophyll content was 1.0 mg g⁻¹ FW in leaves stored at 8 °C and 1.1 mg g⁻¹ FW in leaves stored at 4 °C (Table 2). In spinach, the carotenoid content did not change, and ranged from 0.25 to 0.28 mg g⁻¹ FW.

Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence was investigated for evaluating its application in monitoring quality during storage and the shelf life of baby leaf vegetables destined for the ready-to-eat market.

The Fv/Fm ratio did not change in lettuce stored at 4 °C during the whole experimental period, but a decline was observed at 8 °C after 8-9 days (Fig. 1). In spinach leaves, the Fv/Fm ratio declined after 6 days in samples stored at 8 °C, whereas at 4 °C, the ratio started to decrease after 8-10 days of storage. The Fv/Fm ratio was lower than 0.83 after 10 days of storage for lettuce and after 3 days for spinach.

The PI_{ABS} was 3-3.5 in lettuce at the beginning of the experiments and declined daily at both storage temperatures. However, after 8-9 days of storage, the PI_{ABS} drastically declined at 8 °C, reaching values close to zero (Fig. 1C). In spinach leaves, however, the PI_{ABS} initial values were higher than in lettuce, averaging 4.5-5.5. Differences between the two storage temperatures were observed after 6-7 days. The ETo/RC did not change in lettuce stored at 4 °C, but it started

to decline after 9 days when stored at 8 °C (Fig. 2). In spinach, the electron flux declined at both storage temperatures after 6 days. Differences between 4 and 8 °C were observed after 9 days of storage (Fig. 2B). The energy that is not transferred to carbon dioxide fixation is also dissipated as heat. The DIO/RC index measures this energy fraction and was dramatically increased at 8 °C in both species. In lettuce, the DIO/RC increased after 9-10 days of storage at 8 °C, whereas no differences were observed for samples stored at 4 °C. At the end of storage, the DIO/RC was almost double the initial values. In spinach, the DIO/RC increased after 6 days in both experiments (November 2007 and October 2008), and the values at the end of the experimental period were about 4-fold higher than the initial values (Fig. 2D). In contrast to spinach, the DIO/RC also increased in lettuce stored at 4 °C, but only after 8 days of storage. The Fo/Fm ratio strongly increased after 7-8 days of storage in lettuce kept at 8 °C, and the values were double the initial values (Fig. 3); in contrast, a slight increase was observed in leaves of lettuce stored at 4 °C. In spinach, however, the Fo/Fm increased after 3-6 days, reaching values 2.5-fold higher than the initial values (Fig. 3). Spinach leaves kept at 4 °C slowly, the Fo/Fm increased with increasing storage time, changing from 0.16 to 0.25-0.32 after 13 days. The density of active reaction centres at FP declined with time, especially in spinach. In lettuce, the RC/CSm was almost halved at the end of storage in samples stored at 8 °C, whereas at a temperature of 4 °C, this index did not change in the experiment performed in November 2007 but declined in July 2008 (Fig. 3). In spinach, the RC/CSm started to decrease after 3-6 days at both storage temperatures. At 8 °C, the RC/CSm decreased more rapidly compared to samples stored at 4 °C. At beginning of the experiment, the RC/CSm in lettuce was 700-800, i.e. half the number compared to that of spinach leaves (1500-1600). However, at the end of the experiment, both species reached almost the same value (300-400).

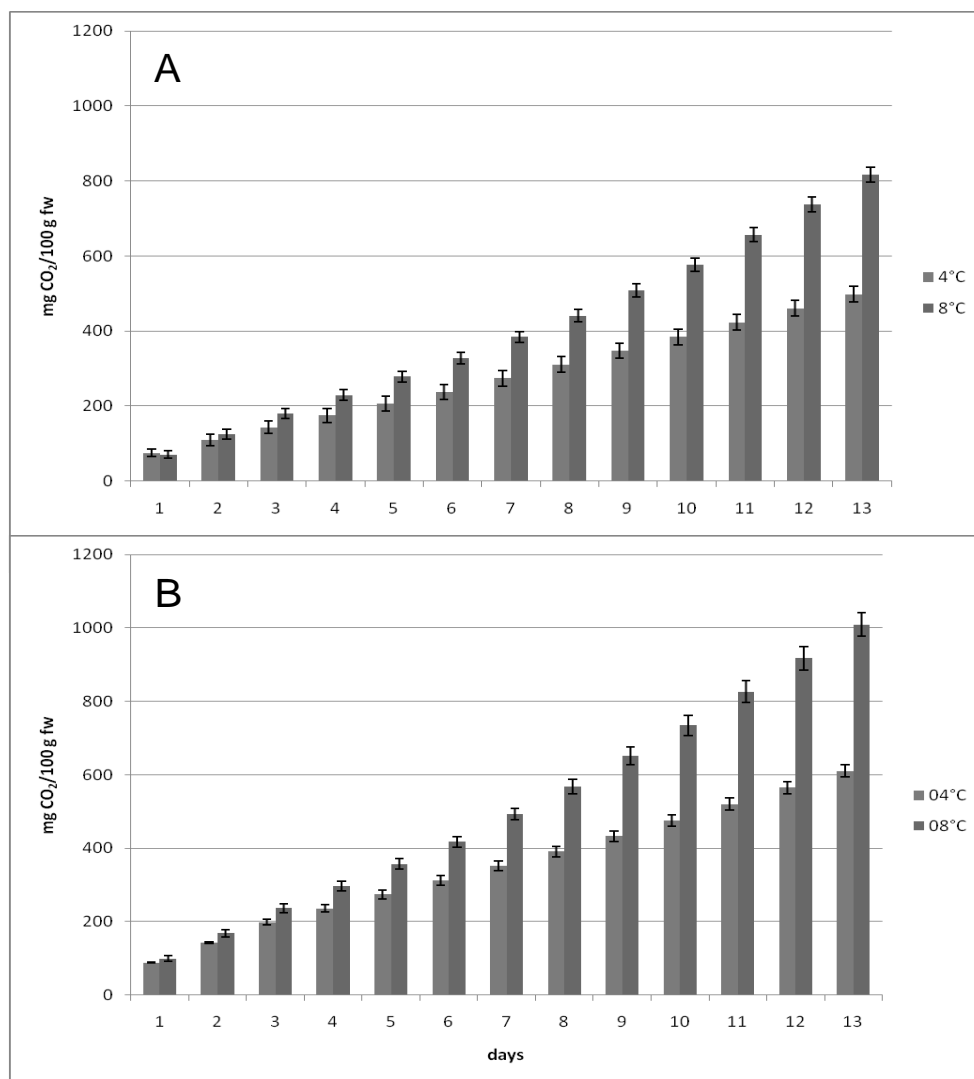


Fig 4. Cumulative respiration curves of lettuce (A) and spinach (B) baby leaves stored at 4 and 8°C. Values are means ± standard errors ($n=3$).

Respiration

The trend of cumulative respirations was strictly linear for both storage temperatures; Fig. 4 reports the cumulative respiration data at 4 or 8 °C for spinach (A) and lettuce (B). Spinach showed a respiration rate increase of 20% compared to that of lettuce at both storage temperatures. At the end of storage, the CO₂ production at 4 °C was 498 and 610 mg CO₂/100 g FW for lettuce and and 817 and 1009 mg CO₂/100 g FW at 8 °C for spinach. We correlated the fluorescence data and the biological age of stored leaves using CO₂ production (Fig. 5), which depends on storage temperature, as the dependent variable (y-axis) of a predictive model based on fluorescence curves (x-axis, Fig. 6). Only spinach was investigated because lettuce showed lower variation in respiration and chlorophyll *a* fluorescence parameters. A chemometric model using PLS (partial least squares) regression was constructed using the data obtained from the leaves stored at 8 °C as the calibration set and those at 4 °C as the validation set. The model showed a satisfactory predictive ability (Fig. 6) using only five latent variables, with a R² in validation of 0.67 and a standard error in prediction (RMSEP) of 170 mg CO₂/ 100 g FW,

corresponding to a CO₂ emission of 3.5 days at 4 °C and of 2.1 days at 8 °C.

Discussion

The quality losses of leafy vegetables occur at a very late stage of storage and are not usually visible. The physiological and non-destructive parameters that change at the earliest stage of storage might be very useful for monitoring the quality of vegetables. In our experiments, the total chlorophyll and carotenoid content changed at the end of the storage period after the common selling period of 7 days. These results match the findings of previous works performed in rocket and in minimally processed Swiss chard baby leaves; in fact, the loss of colour occurred over 8 days of storage (Ferrante et al., 2004). In our work, differences in these parameters were marked between the two species. The lowest chlorophyll content was observed in lettuce and ranged from 0.3 to 0.4 mg g⁻¹ FW versus 1-1.2 mg g⁻¹ FW for spinach. Chlorophyll reduction was observed late during storage. Lower chlorophyll contents were measured in leafy vegetables stored at the higher temperature; a greater reduction was observed for chl *b* compared to chl *a*. During leaf senescence, chlorophyllase is considered to be the first

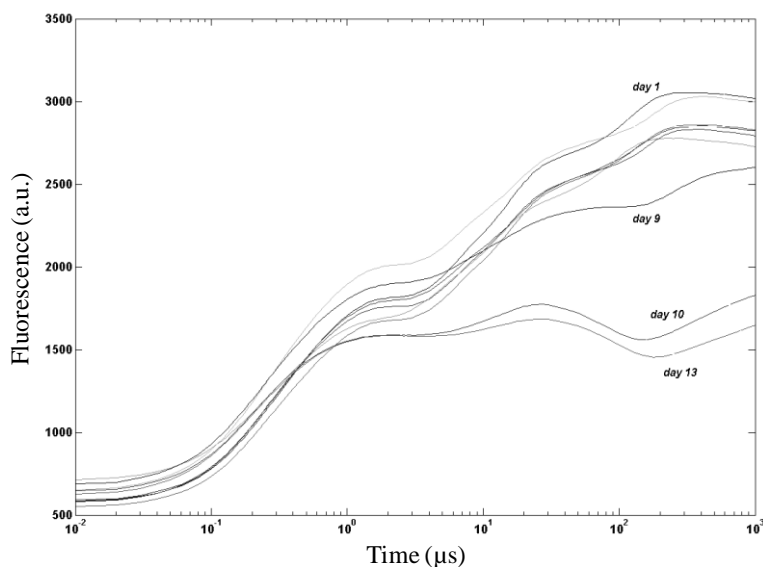


Fig 5. Fluorescence curves of spinach leaves stored at 8 °C for 12 days

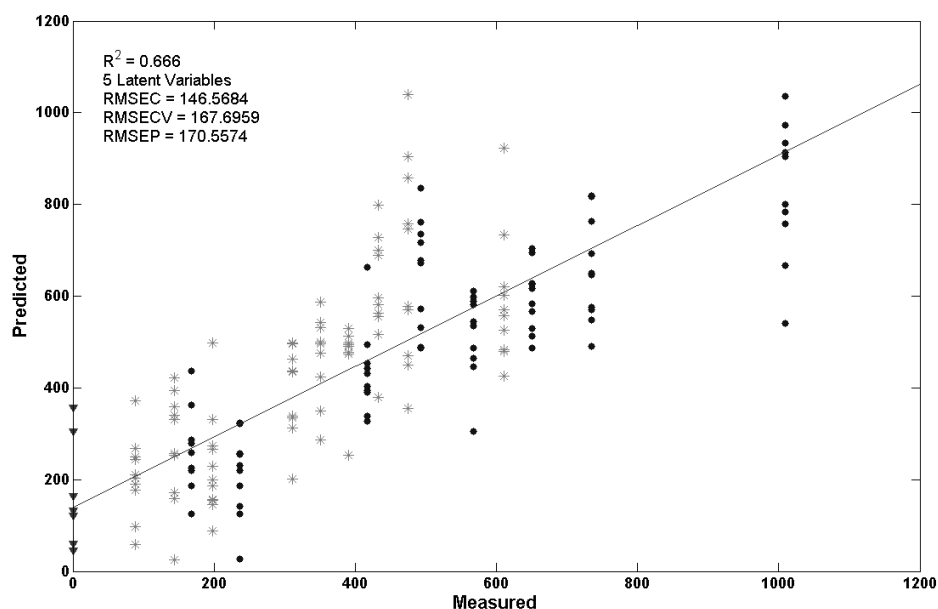


Fig 6. Scatterplot of measured vs. predicted cumulative respiration. The data are expressed in mg CO₂/100 g FW.

enzyme involved in the chlorophyll breakdown pathway, and it degrades both chl *a* and chl *b*. When Chl *b* is degraded and converted into chl *a* (Ito et al., 1993), the *a/b* ratio increases. Chlorophyll *a* fluorescence measurements and the JIP test provide good markers for evaluating stress conditions in leafy vegetables. The first application of the tools we used on minimally processed vegetables was performed on lamb's lettuce stored at 4 or 10 °C (Ferrante and Maggiore, 2007), whereas for adult vegetables, they were applied on iceberg lettuce. In iceberg lettuce, chlorophyll fluorescence was used to determine the storage potential (Schofield et al., 2005). The specific energy flux JIP indexes are primarily reported in the literature (Strasser et al., 1995). The Fv/Fm ratio, which represents the maximum quantum yield of PSII, declined during late storage and occurred mainly in leaves stored at 8 °C. Generally, a Fv/Fm ratio of 0.83 is considered the stress threshold for most herbaceous plants. In our experiments,

values above 0.83 were measured only in spinach during the common commercialisation period and only for those samples stored at 8 °C. PI_{ABS} was the only index that decreased with storage time and was also affected by temperature. PI_{ABS} (PI_{ABS} = (RC/ABS)·[φPo/(1-φPo)]·[Ψo/(1-Ψo)]) is the performance index at t = 0 expressed on the absorption basis; it is a multifactorial parameter and includes trapped energy, electron flux and active reaction centre density (Strasser et al., 2000). PI_{ABS} has been successfully used for screening dark chill-tolerant genotypes in soybean (Strauss et al., 2006). In *Jatropha curcas* seedlings exposed to cold stress, PI_{ABS} was particularly sensitive to low temperature, especially at early stages of the treatments (Liang et al., 2007). Interestingly, PI_{ABS} has been considered an optimal indicator of phosphorous deficiency in tea plants (Lin et al., 2009). The reduction in PI_{ABS} was due to electron flux, dissipation energy and density of active

reaction; in spinach, the adsorbed energy per RC also contributed later in storage. We analysed the variations in the PI_{ABS} components for spinach stored at 8 °C as described in the soybean genotypes study (Strauss et al., 2006), and the ratio $\psi_o/(1-\psi_o)$ that represents the efficiency of the conversion of excitation energy to electron transport was the index that showed higher changes during the storage period. Intermediate value oscillations were measured for the efficiency of primary photochemistry (trapping) $\phi Po/(1-\phi Po)$, and lower changes were observed for RC/ABS. Therefore, the $\psi_o/(1-\psi_o)$ ratio is the most important in determining PI_{ABS} changes, as reported in the soybean dark chill-tolerance study (Strauss et al., 2006). To evaluate the senescence process, the respiration data of spinach were recorded and linked to a predictive model based on fluorescence data. Respiration is known to be temperature dependent, and higher values accelerate all deterioration processes (Platenius, 1942). Our model gave good results for spinach because it had higher respiration rates and a higher chlorophyll content. A similar model has been applied in apples for evaluating the optimal harvesting time using respiration data correlated with FT-NIR spectra data. The model gave RMSEPs ranging from 4.2 to 6.9 days, which are good values for apples (Peirs, 2001). Respiration is the metabolic parameter used most often in models developed for postharvest applications, and a wide range of works have been published in that field (Fonseca, 2002). In conclusion, the aim of this study was to identify non-destructive indexes to describe the quality of the product and to build a model for describing the quality status of the product. The identification of these indexes has the dual goal of i) deciding whether the product can be stored longer or needs to be removed from the supermarket on the basis of objective parameters and ii) ensuring that the product on sale is still of good quality. In the first case, the information provided will be useful for managing the product during production and along the distribution chain. The latter indexes can be used for ensuring the quality of the product to the consumer.

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