Progress in Nutrition 2018; Vol. 20, N. 3: 415-422 DOI: 10.23751/pn.v20i3.7097

Original article

Simple tools for monitoring chlorophyll in broccoli raab and radish microgreens on their growing medium during cold storage

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Summary. Microgreens have been recently introduced as a new category of vegetables, with unexploited potential as functional foods. Due to containerized production, they can be commercialized while growing on the medium, ready for being harvested before use. The chlorophyll content of vegetables is important for both health benefits and visual appearance of the produce. This paper aims to evaluate the feasibility of using simple tools to monitor chlorophyll content in microgreens of two different species, broccoli raab (*Brassica rapa* L., Broccoletto group) and radish (*Raphanus sativus* L.), in varying stages of cold storage in their growing vessel. Image acquisition with a CCD camera, followed by image analysis using preset algorithms of an open source software (ImageJ) was the approach used. Image color analysis (median values of L*, a*, and b* indices) and textural parameters obtained from the gray-level co-occurrence matrix (GLCM) allowed to obtain regression models for chlorophyll content with satisfactory fitting parameters (adjusted R^2 was 0.765 and 0.843 for broccoli raab and radish, respectively). These results point out the possibility to set up low-cost, real time, non-destructive monitoring systems for microgreens quality during their growing as well as during storage.

Key words: *Brassica rapa* L., image analysis, GLCM, nutritional quality, *Raphanus sativus* L., ImageJ

1. Introduction

Microgreens are young and tender edible seedlings produced using the seeds of different species of vegetables, herbaceous plants, aromatic herbs and wild edible plants. Depending on the species that has been used, they can be harvested 7-21 d after germination, when the cotyledon leaves have fully developed and the first true leaves have emerged (1). Microgreens represent a new category of vegetables with different traits as compared to the already known sprouts and the common fresh-cut leafy vegetables. They are characterized by a wide range of colors, flavors, textures (2, 3). Due to high content of functional components such as antioxidants, vitamins and minerals etc., microgreens are considered as potential "functional foods" (1). Moreover, microgreens can contribute to preserve and valorize biodiversity, and recover and use many local varieties that are at risk of genetic erosion (1). Microgreens can be produced in open air as well as in protected environment, both on soil and soilless. The latter growing system allows also containerized production, which can result in commercialization of the product while growing on the medium, ready for being harvested just before use. Harvest and many postharvest issues can be avoided with this approach (1, 4).

Chlorophylls are pigments that give green color to vegetables and several fruits, where they play key

roles in photosynthesis. The chlorophyll content of vegetables is important for both health benefits and visual appearance of the produce (5). Its decrease is associated with cellular degradation and/or senescence, and it is often used to estimate quality loss of green vegetables (6, 7). In fact, strong relation of chlorophyll content with overall visual quality of vegetables has been reported (8). Moreover, chlorophyll can be considered a bioactive compound, since its dietary naturally occurring derivatives showed antioxidant and antimutagenic activity (9-11).

The measurement of quality parameters (i.e. chlorophyll content) is generally carried out using traditional analytical techniques whose application in the food industry poses several problems: they require very long times, are expensive and destructive.

Nondestructive analytical approaches would be therefore required for quality control during both production and storage of microgreens. To the purpose, visible imaging coupled to image analysis using open source software (ImageJ) can be a cheap, effective and simple approach, allowing to provide both color-related and texture-related information useful for food inspection, grading, detection (12-15).

As far as we know, no attempt has been made to evaluate the potential of visible imaging coupled to image analysis for monitoring of microgreens directly on their growing medium. This approach could take advantage of the almost flat surface of the microgreen crops and overcome the flaws of another simple nondestructive instruments such as colorimeter. In fact, analysis by colorimeter requires multiple readings that can be hindered by the small leaf surface, the contact with the sample and an equipment with relatively high cost (16, 17). Moreover, image analysis requires low-cost equipments and can be carried out using open source software, such as ImageJ (18).

The aim of the present research was to evaluate the feasibility of using such simple tools to monitor chlorophyll content in microgreens of two different species, broccoli raab (*Brassica rapa* L., Broccoletto group) and radish (*Raphanus sativus* L.), in varying stages of cold storage in their growing vessel.

2. Materials and methods

2.1. Microgreens production and storage

Two different species were produced: broccoli raab (*Brassica rapa* L., Broccoletto group) also known as 'rappini' or 'rapini' and radish (*Raphanus sativus* L.). Seeds of a local variety ('*Sessantina*') produced by Puglia's hold-farmers were used for broccoli raab, while radish seeds cv Saxa were purchased (Riccardo Larosa company, Andria, Italy). The two genotypes were sown in four plastic trays (with holes at the bottom) filled with a mixture of peat (50% white-50% black peat mixture, Brill 3 Special, Brill Substrates, Georgsdorf, Germany), using a density of 3 seed cm^{-2}. Microgreens were grown in a growth chamber at controlled temperature (22°C) and relative humidity (85%). After germination, the seedlings were exposed for a 12 h photoperiod to a light irradiance of 200 $\mu mol\ m^{-2}\ s^{-1}$, determined by LICOR LI-190 (Li-Cor Inc., USA) quantum sensors. Seedlings were fertigated daily using a nutrient solution containing all the essential macro- and micro-nutrients at the following concentrations (mg L^{-1}): N 105, P 15, K 117, Ca 100, Mg 24, B 0.25, Cu 0.01, Fe 2.5, Mn 0.25, Zn 0.025, Mo 0.005.

Microgreen vessels were sampled ten days after germination, between fully development of cotyledons and first true leave. Four growing vessels per each species were sampled, put in low density polyethylene bags and stored in dark at 5°C. At day 0 and after 1, 2, 5 and 13 d of storage chlorophylls analysis, spectrocolorimetric determination and image acquisition were performed (n =4).

2.2. Chlorophyll analysis

Total chlorophyll content was determined spectrophotometrically using the method of Lichtenthaler and Buschmann (19) with minor modifications. Excised leaves (0.5 g, corresponding to about twenty leaves, sampled throughout the vessel) were homogenized and added with 15 mL acetone (HPLC-UV grade, Pharmco-Aaper, Brookfield, CT, USA) and stirred for 20 min. The mixture was filtered (Grade 413 Filter Paper, Qualitative, VWR International, West Chester, PA, USA) and transferred into spectrophotometric cuvettes. Absorbance was read at 661.6 nm

and 644.8 nm with a Cary 60 UV-VIS (Agilent Technologies, Santa Clara, PA, USA) and total chlorophyll ($chl_{a,b}$, mg L^{-1}) was calculated as the sum of chlorophyll *a* (chl_a, mg L^{-1}) and chlorophyll *b* (chl_b, mg L^{-1}) calculated by the following formulas:

$$chl_a = 11.24\, A_{661.6} - 2.04\, A_{644.8}$$
$$chl_b = 20.13\, A_{644.8} - 4.19\, A_{661.6}$$

where A_n was the absorbance of the extract at *n* nm of wavelength.

2.3. Colorimetric analysis

Colorimetric evaluations of lightness (L*), red index (a*), and yellow index (b*) were carried out under D65 illuminant by using a spectro-colorimeter CM-700d (Konica Minolta Sensing, Osaka, Japan) equipped with a pulsed xenon lamp. At least five readings were performed on different areas of each sample and the mean values were considered.

2.4. Visible imaging

Image acquisition was carried out using a low-cost equipment composed of the following elements: a black shooting box (50 x 42 x 28 cm) with two fluorescent lamps (40 W, 480 lm, 6400 K) and a small window on the top for the camera; a DMC-FS10 digital camera (Panasonic Corporation, Osaka, Japan) with a 12 Mpixel CCD, held above the light box by a moving mount at 28 cm from the bottom of the light box. Settings of the camera were in automatic mode (17).

2.5. Image analysis: RGB measurement

The acquired images were processed using the free ImageJ software (NIH, USA). Color thresholding was applied, in RGB color space, adjusting the parameters in order to select the microgreens and separate them from the background. The *RGB measure* plugin was run to obtain the mean RGB values of the image.

2.6. Image analysis: L a* b* measurement*

The image type was subsequently converted in a L* a* b* stack. Using the wand tool and changing tolerance parameters, the whole leaf area was selected and separated from the background. Then, the *measure* function allowed to measure mean, median, modal values and standard deviations for the selected pixels in all the three stacks.

2.7. Image analysis: gray level co-occurrence matrix (GLCM)

Tournier et al. (20) defined GLCM as the description of the second-order statistics in the images, permitting the calculation of textural features which are expected to represent the texture characteristics of the image studied. This approach allows to calculate how often pairs of pixels with specific values and in a specified spatial relationship occur in an image (21). Before calculating GLCM parameters, the original image was finally converted in a 8-bit gray scale image. The GLCM-texture plugin was then run to perform texture analysis. The displacement vector (D) was set with a distance of 1 pixel, while the angle was 0°. The following parameters were measured (22):

- angular second moment (ASM), describing the regularity of the image;
- inverse difference moment (IDM), describing the local homogeneity of the image;
- entropy (e), measuring the statistical randomness;
- contrast (c), also evaluating the local homogeneity.

For regression analysis, a preliminary screening was performed (data not shown) to select the variable subset giving the best results.

2.8. Statistical analysis

Regression models were built using Minitab 17 (Minitab Inc., State College, PA, USA). Full quadratic models including second order terms and first order interactions. Mean subtraction was applied as coding option, in order to reduce collinearity. Backward removal was applied for model selection, with p = 0.01 as removal threshold.

3. Results and discussion

3.1. Chlorophyll content

Figure 1 reports the variability of chlorophyll contents in the microgreens considered. Both species were characterized by quite high chlorophyll content (11,23–25), particularly radish which showed a medi-

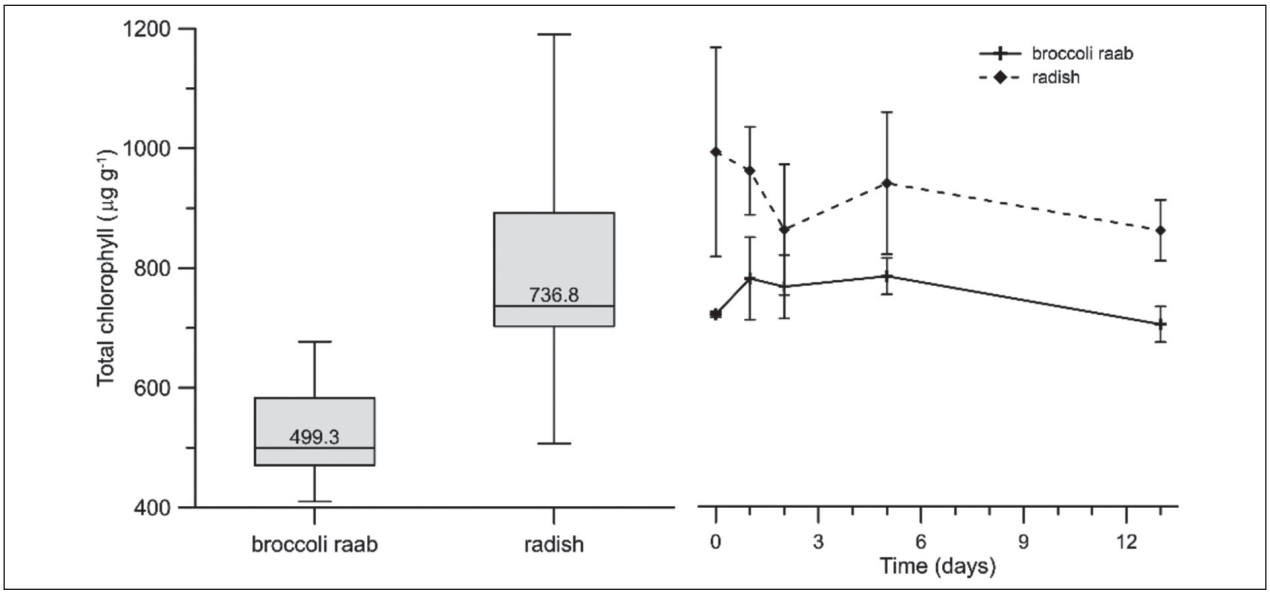

Figure 1. Box-Whisker and line plots of total chlorophyll ($chl_{a,b}$) content of microgreens during storage. Minimum, maximum, median, lower quartile, and upper quartile are reported in the Box-Whisker plot.

an content of 736 µg g^{-1}, while the median content in broccoli raab was 499 µg g^{-1}. The variability of data was higher for radish than for broccoli raab. Total chlorophyll did not show significant decreases during storage, contrarily to fresh cut produce stored at similar temperatures (3).

3.2. Image analysis. Comparison of different algorithms for chlorophyll monitoring

Figure 2 reports sample images of fresh and 13-days stored microgreens trays. Both broccoli raab and radish showed appreciable variations of visual aspect, such as incipient etiolation and chlorotic cotyledons. Therefore, sample images corresponded to a wide range of visual quality conditions.

Table 1 reports the comparison of the results of the regression models obtained for the chlorophyll content of microgreens as a function of image analysis parameters obtained from different algorithms. Bold characters in table highlight the model with the best performances. In fact, the models obtained showed quite different fitting performances. As regards broccoli raab, while colorimetric analysis did not allow to obtain a satisfactorily significant model, two out of the three image analysis algorithms provided significant

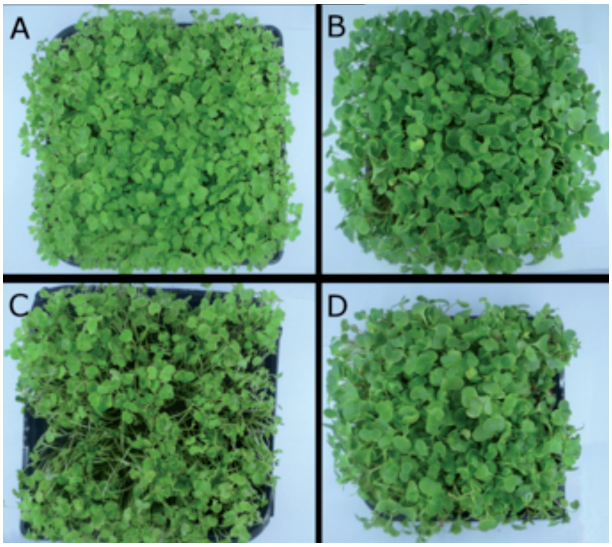

Figure 2. Representative samples of broccoli raab and radish microgreens after 1 d (A and B, respectively) and 13 d of storage (C and D, respectively).

models, though the best performances were obtained using L*, a*, b* median values: both adjusted R^2 and R^2 for prediction were by far higher than those of the other models.

Table 1. Comparison of the regression models for chlorophyll content[a]

broccoli raab									
method	algorithm	model parameters	model terms	*p*-value	adj. R^2	R^2 pred.	MSE	SSE	PRESS
colorimeter		L*, a*, b*	L,b	0.081	0.233	0.108	4298	51577	69995
image analysis	RGB	R, G, B	R,G,B,R*B,G*B	0.039	0.502	0.149	2789	25105	66752
	Lab	L*, a*, b* (mean value)	L,a,b,a*a,b*b,L*b,a*b	0.018	0.700	0.623	1683	11780	73553
		L*, a*, b* (standard deviation)	L,L*L	0.064	0.263	0.338	4130	49561	75786
		L*, a*, b* (modal value)	a,a*a	0.032	0.344	0.000	3674	44083	80249
		L*, a*, b* (median value)	**L,a,b,a*a,b*b,L*b**	**0.004**	**0.765**	**0.488**	**1315**	**10523**	**40138**
	GLCM	ASM, contrast, IDM	ASM,c,IDM,ASM*IDM,c*IDM	0.114	0.343	0.222	3682	33135	76702
radish									
method	algorithm	model parameters	model terms	*p*-value	adj. R^2	R^2 pred.	MSE	SSE	PRESS
colorimeter		L*, a*, b*	L,a,b,L*L,a*a,b*b,L*b,a*b	0.175	0.408	0.000	16240	97440	1215378
image analysis	RGB	R, G, B	G,G*G	0.011	0.449	0.367	15126	50505	243306
	Lab	L*, a*, b* (mean value)	L	0.098	0.135	0.000	23745	306688	464585
		L*, a*, b* (standard deviation)	-	-	-	-	-	-	-
		L*, a*, b* (modal value)	-	-	-	-	-	-	-
		L*, a*, b* (median value)	L	0.093	0.140	0.000	23597	306767	462036
	GLCM	**ASM, contrast, IDM**	**ASM,c,IDM,ASM*ASM,c*c,ASM*c**	**<0.001**	**0.843**	**0.668**	**4314**	**34513**	**127447**

[a] Second order terms and first order interactions were included in the models. Mean subtraction was applied as coding option. Backward removal was applied for model selection, with $p = 0.01$ as removal threshold. Selected models are in bold.

The relative error of calibration (REC) was 3.7%, while the regression equation was the following:

$$\text{Total chlorophyll } (\mu g\ g^{-1}) = -4.165 \times 10^4 + 427.3\ L^* - 362\ a^* + 1.419 \times 10^3\ b^* - 6.59\ a^{*2} - 5.40\ b^{*2} - 14.44\ L^* \times b^* \quad \text{(eq. 3.1)}$$

Predicted data are plotted versus observed data in Figure 3, which also reports the regression residuals for the selected model for broccoli raab.

The reason why median values provided much better results than mean values can be explained considering that the differences between mean and median values changed during storage of broccoli raab. As regards lightness, median values were higher than mean values and the difference tended to a slight, linear increase ($p < 0.01$) during storage; as regards red index, the difference *mean value – median value* tended to increase ($p < 0.05$); finally, as regards yellow index, the difference *mean value – median value* was positive and tended to decrease ($p < 0.001$). These changes could be due to the increasing effect of outlying pixels deriving either from background or from individual leaves. As a consequence, being median values less affected by outliers than mean values, the regression resulted more powerful in modeling the overall content of total cholophyll in spite of possible imperfections of image segmentation.

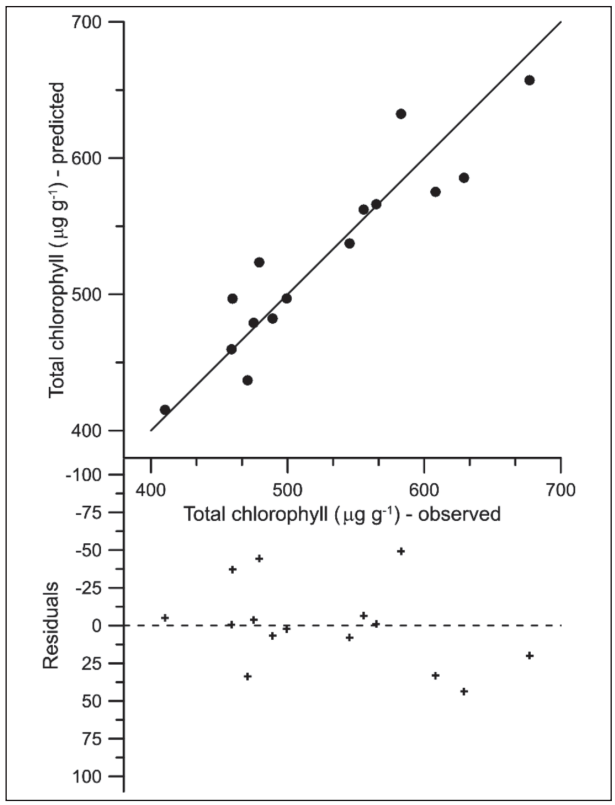

Figure 3. Regression analysis of total chlorophyll ($chl_{a,b}$) content in broccoli raab microgreens as a function of image analysis parameters. Plots of predicted values versus observed values (top) and residuals versus observed values (bottom). See Table 1 for regression indices (bold line) and equation 3.1 for regression equation.

A difference was observed between calibration performances of the model and its prediction capabilities, as can be observed comparing adjusted and prediction R^2, as well as SSE and PRESS. Model robustness could be increased by using larger sample sizes. Nevertheless, at this preliminary stage, algorithm comparison showed that image analysis can provide sufficient information to relate to the cholorphyll content. Further work is required for the improvement of the predictive capability of the selected model.

As regards radish, neither colorimeter nor L*, a*, b* image analysis algorithm provided significant models. On the other hand, the RGB algorithm allowed to obtain a significan regression ($p < 0.05$). Nevertheless, the best results were obtaind applying GLCM to the acquired images. Preliminary evaluation allowed to select, as starting variables, ASM, IDM and c. The best model showed values for both adjusted R^2 and R^2 for prediction equal to 0.843 and 0.668, respectively. The REC was 5.5% while the regression equation was the following:

$$\text{Total chlorophyll } (\mu g\ g^{-1}) = -6.750 \times 10^4 + 9.366 \times 10^7\ ASM + 858\ c + 1.861 \times 10^4\ IDM - 3.723 \times 10^{10}\ ASM^2 - 3.112\ c^2 - 6.048 \times 10^5\ \text{ASM} \times c \quad \text{(eq. 3.2)}$$

Predicted data are plotted versus observed data in Figure 4, which also reports the regression residuals for the selected model for radish (in bold in Table 1).

It clearly appears that GLCM texture analysis was particularly suited to assess total chlorophyll content in this species of microgreens. Previous applications of GLCM texture parameters of visible images mainly regarded classification or structural characterization of food samples (26). Most of the reported models reached an accuracy higher than 0.7, that is satisfactory for industrial applications (25). Values higher than those reported in the present study were obtained only for classification models. Few applications are reported of the use of GLCM texture for the assessment of chemical indices. Kondo et al. (27) coupled applied artificial neural networks to GLCM texture for determining sugar content in lyokan orange, gaining an accuracy of 0.84. Quevedo et al. (21) obtained highly significant ($R^2 > 0.976$) power law models for non enzymatic browning kinetics in avocado. The present application of GLCM texture on visible images of radish microgreens therefore expands the possibility of use of this algorithm for an effective chemical characterization of food samples. Also in this case, the differences between calibration and prediction pointed out the need to build more robust models with larger datasets. Nevertheless, the possibility to choose among several image analysis approaches and algorithms expands the potential of this technique and its adaptation to different crops.

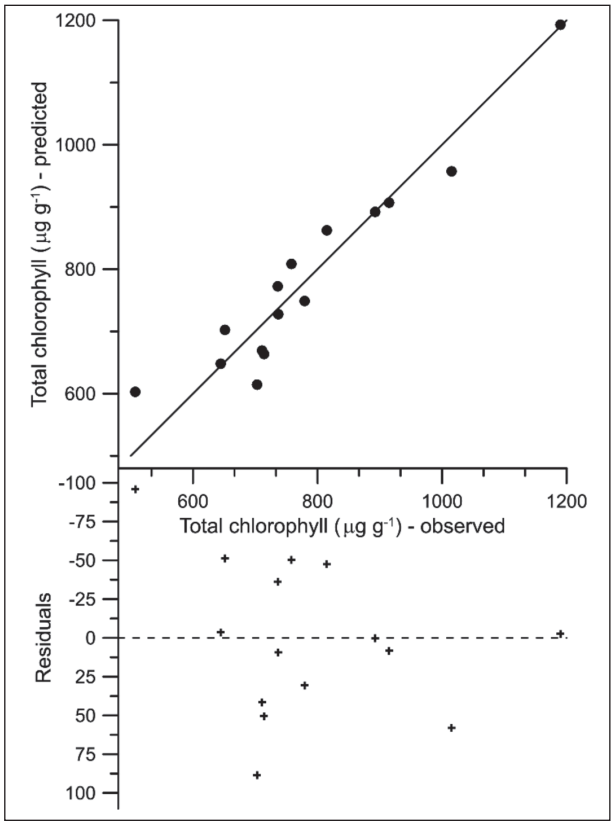

Figure 4. Regression analysis of total chlorophyll ($chl_{a,b}$) content in radish microgreens as a function of image analysis parameters. Plots of predicted values versus observed values (top) and residuals versus observed values (bottom). See Table 1 for regression indices (bold line) and equation 3.2 for regression equation.bold line) and equation 3.1 for regression equation.

4. Conclusion

Analysis of visible images of microgreens on their growing vessel resulted effective in monitoring a

chemical index (total chlorophyll content). The models described in this paper could be used for the automatic prediction of an important nutritional quality trait. This could be a significant achievement, since the strenght of commercialization of microgreens on their own growing vessel is strictly related to the possibility of keeping sensory and nutritional properties of the fresh product. The performances of statistical models relating image features with the monitored parameter can be optimized for each microgreens species. Different image analysis algorithms offer, to this scope, the opportunity of adopting the more appropriate model on the basis of the considered species, to allow the building up real time, non-destructive monitoring systems, using low-cost and simple pre-calibrated tools.

Acknowledgements

This work was funded by Fondazione Puglia [Bando Ricercatori 2015 – project "Caratterizzazione nutrizionale e shelf-life di micro-ortaggi confezionati – Nutritional characterization and shelf-life of packaged microgreens"].

This research was also supported by Regione Puglia Administration [Intervento cofinanziato dal Fondo di Sviluppo e Coesione 2007–2013–APQ Ricerca Regione Puglia – Programma regionale a sostegno della specializzazione intelligente e della sostenibilità sociale ed ambientale FutureInResearch – project "Innovazioni di prodotto e di processo per la valorizzazione della Biodiversità Orticola pugliese (InnoBiOrt) – Product and Process Innovation for the Exploitation of the Puglia's Vegetable Biodiversity"]

Chemical compounds studied in this article
Chlorophyll (PubChem CID: 6449992)

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