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Non-destructive optical indices to estimate isoprenoids with nutritional value in packed rocket and spinach

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ABSTRACT

The quantification of phytochemicals with nutritional benefits relies on accuracy yet time-consuming, expensive and destructive methodologies. In contrast, optical indices widely employed in disciplines such as ecology can serve as fast, low-cost, and non-destructive tools for tracking pigment changes. However, their potential application in postharvest phases and commercialization (i.e., supermarkets) remains underexplored. In this context, this work aimed to investigate the feasibility of using the normalized difference vegetation index (NDVI), the chlorophyll content index (CCI) and the photochemical reflectance index (PRI) to estimate phytochemicals of nutraceutical interest (chlorophylls, carotenoids and tocopherols). A fully factorial and randomized experimental design (combining different illumination, packaging, and collecting time) was conducted in two green leafy packaged vegetables (rocket and spinach). With this design that induced differences in physiological parameters a database with variable content of isoprenoids (carotenoids, chlorophylls, and tocopherols) based on treatments was created. By establishing correlations and models between isoprenoids and optical indices, the NDVI was revealed as a valuable tool to estimate chlorophyll content in both species, while the PRI tracked both tocopherol and carotenoid (except for β-carotene) content. Taking this approach, estimation of the sum of total carotenoids and tocopherols was achieved using the PRI. This study confirms the possibility of extending the use of the NDVI and PRI to real-time nutritional monitoring in different stages of production and commercialization. A future application envisions integrating this technology into refrigerators for enhanced consumer access to nutritional information.

1. Introduction

Chlorophylls (Chls) and carotenoids (including neoxanthin, N; lutein, L; violaxanthin, V; antheraxanthin, A; zeaxanthin, Z and β-carotene, β-Car) play pivotal roles in plant functioning. They are involved in the light reactions of photosynthesis [1] and contribute to safeguarding the photosynthetic apparatus through the photoprotection mechanism mainly by the xanthophylls V, A and Z, which are involved in the so-called VAZ cycle [2,3]. Moreover, these compounds are indispensable nutraceuticals that significantly contribute to human health. Their intake is associated with notable benefits, including the prevention of ocular diseases [4–6], and reduced risk of cardiovascular diseases, diabetes, and certain types of cancer, among others [7,8] due to their antioxidant power [9]. The literature concerning the assimilation of Chls and their impact on human health is scarce. However, some authors have reported protective effects against cancer [10,11]. Additionally, α-tocopherol (α-Toc), known as vitamin E, is another antioxidant that plays multiple roles in plants, e.g., a stabilizer of the photosynthetic membranes [12]. This molecule, which can be absorbed by humans via the diet, also has a fundamental impact on human health, preventing cancer [13], and playing a significant role in bone health [14], among others. Humans cannot synthesize these three groups of nutraceuticals (carotenoids, chlorophylls and tocopherols), so they must be present in the diet in sufficient amounts, with fruits and leafy vegetables as their main dietary source.

Due to the importance that nutraceuticals play in human health, it is a desirable goal to achieve a transition from traditional agriculture to

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Abbreviations

α-Toc	α-tocopherol
β-Car	β-carotene
CCI	Chlorophyll Content Index
CD	Closed bags, darkness
Chl	Chlorophyll
CL:	Closed bags, light
DW	Dry weight
F_V/F_M	maximal photochemical efficiency of the photosystem II
FW	Fresh weight
L:	Lutein
LMEs	Linear mixed-effects models
NDVI	Normalized Difference Vegetation Index
OD	Open bags, darkness
OL:	Open bags, light
PRI	Photochemical Reflectance Index
RWC	Relative water content
t-Car	Total carotenoids
SATTC	Sum of α-tocopherol and total carotenoids
VAZ	Sum of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z)

nutrition-sensitive agriculture that promotes not only growth performance but also the nutritional quality of the products [15]. The content and type of nutraceuticals in plants, particularly carotenoids, are subject to variation due to multiple factors, as they respond differently to various environmental stimuli [16]. These factors may include genotype as well as pre- and post-harvest, such as cultivation methods and processing techniques [17]. Indeed, it has been described that storage conditions significantly affect the phytochemical concentrations of vegetables. Even the exposure to light that they receive while being on supermarket shelves can alter their nutritional value [18]. In this regard, it was found that the Z content of packed green leafy vegetables, stored on supermarket shelves, can be increased by modulating the light intensity and period [19]. Thus, to produce and offer highly nutritious food, it is desirable to quantify the nutritional value at every step of the process, i.e., from field storage conditions to our tables.

However, quantifying phytochemicals typically involves costly and destructive techniques, such as chromatography [20]. Thus, there is an increasing interest in developing non-destructive alternative methodologies to estimate plant physiological status and the phytochemical content of crops and food. In this regard, optical methods are useful tools for the estimation of coloured plant pigments. Specifically, by measuring leaf light reflectance at certain wavelengths, it is possible to calculate two main indices, the normalized difference vegetation index (NDVI) and the photochemical reflectance index (PRI), which can be used to assess the contents of chlorophylls and VAZ pigments, respectively [21,22]. Additionally, by measuring leaf light transmittance, it is possible to calculate the chlorophyll content index (CCI), which is an indicator of the Chl content in plant tissues [23]. These spectroradiometric determinations are proposed as fast, non-destructive and low-cost diagnostic tools to assess plant physiological status. Indeed, these indices can be used in both remote sensing of plant canopies and in physiological studies at the leaf level [24]. In particular, PRI is being increasingly employed to monitor metabolic changes and responses to stress in the field, usually for agriculture and ecology [22,25,26]. Nevertheless, the use of these indices to quantify the nutritional value in urban environments and/or at postharvest phases of commercialization of agricultural products is still to be developed. Reflectance data could even be used to elaborate models capable of estimating carotenoid composition contents and changes in plant tissues [27,28], a tool that could be implemented in the commercialization phase of nutrition-sensitive products at supermarkets. Despite the wide adoption of pigment assessments using reflectance measurements, its use for the assessment of tocopherol content is yet to be developed (being commonly determined by destructive analytical techniques). Some studies are using near-infrared spectroscopy [29], but generally, the use of optical techniques for the estimation of tocopherols is still a challenge.

This study aimed to assess the feasibility of estimating isoprenoids of nutraceutical interest (i.e., chlorophylls, VAZ, β-carotene, α-tocopherol), in packed vegetables, using non-destructive optical indices (PRI, CCI, and NDVI), as commonly implemented in other disciplines such as agronomy or ecology. To do this, rocket (*Eruca sativa* Mill.) and spinach (*Spinacea oleracea* L.) were chosen due to their commercial significance and nutritional value [30,31]. It was hypothesized that the PRI, NDVI, and CCI can be used to estimate the concentration of isoprenoids of nutraceutical interest in leafy green vegetables, packed and stored under conditions comparable to those on supermarket shelves or in household fridges. This article will address the following questions: i) Which optical index (NDVI or CCI) will estimate the chlorophyll pool more accurately in packed spinach and rocket? ii) Will PRI estimate the total xanthophylls? iii) Can tocopherols be estimated using optical indices?

2. Materials and methods

2.1. Biological material and experimental design

Spinach and rocket, packaged in biaxially oriented polypropylene bags with modified atmosphere, were sourced from a local supermarket with at least 15 days until the date of expiry. To ensure comparable conditions in phytochemical profiles, both spinach and rocket were stored in darkness at 4 °C for 12 h before the experiment [32]. The design was a factorial experiment that manipulated three variables to achieve a pool of differential contents of pigments and tocopherols among the samples (Fig. 1): i) light conditions (exposing the plants to either light or darkness); ii) packaging (opening or keeping the bags closed); and iii) the time of collection post-experiment initiation (on days 1, 2, 3 and 4). With this design, the treatments over the four consecutive days were as follows: closed bags in darkness (CD), closed bags in light (CL), open bags in darkness (OD), and open bags in light (OL). For each combination of these factors, five replicates (leaves from independent bags) were selected. As the experimental design was randomized, we considered each bag an independent experimental unit.

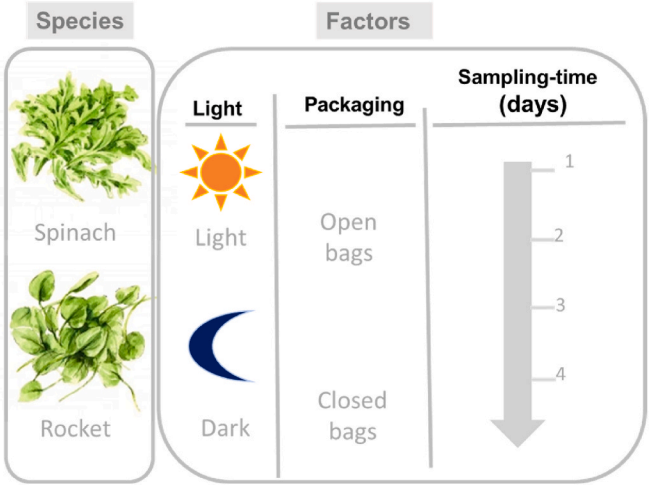

Fig. 1. The experimental randomized design implemented in this work combines three factors to generate differential levels of pigments and tocopherols in spinach and rocket leaves: light conditions (light or darkness), packaging (open or closed bags) and the collection time at the start of the experiment (days 1, 2, 3 and 4).

Accordingly, 100 independent bags (2 light condition x 2 packaging x 5 time collections x 5 replicates) were used for each species.

2.2. Sampling design

During the experiment, the bags were kept at room temperature (≈20 °C) to simulate the less favourable postharvest storage conditions. In dark-exposed treatments, bags were kept in darkness, and in light-exposed treatments, bags were illuminated using LED lamps at a 30 cm distance. This provided a white light that mimicked supermarket conditions (photosynthetic photon flux density 20 μmol photons $m^{-2}s^{-1}$). This light condition was maintained throughout the experiment to ensure consistent lighting conditions for the study.

The first collection and sampling was undertaken after 24 h of illumination (= day 1) and it was followed by three more samplings, each at 24 h intervals. Sampling was always performed at the same time each day, to avoid variations due to circadian rhythms. For each sampling, five spinach and five rocket leaves were selected randomly from independent bags. Non-destructive leaf reflectance measurements (i.e., PRI, CCI, and NDVI) were performed and compared with standard biochemical contents (i.e., chlorophyll, carotenoid and tocopherol contents). To assure the quality of the samples and to avoid pigment degradation (i.e., isomerization or pheophytinization), leaves presenting with yellowing or spoiled in appearance were discarded from the experiment. First, reflectance indices were measured on the adaxial surface of the same leaves intended for biochemical assessment, and secondly, five 3 mm diameter discs were collected from the same area of the same leaves where optical indices had been measured. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.3. Physiological status of plant material

The physiological status of the leaves was monitored under each treatment (CD, CL, OD and OL) during the four consecutive days and considering two parameters: i) the maximal photochemical efficiency of photosystem II (F_V/F_M), which is an indicator of photosynthetic performance [33], and ii) the relative water content (RWC), which provides a measure of plant tissue hydration. F_V/F_M was measured on dark-adapted leaves (15 min) using a portable fluorimeter (FluorPen FP 100; Photon System Instruments, Brno, Czech Republic). This dark period permitted the determination of basal fluorescence (F_0). Subsequently, the maximal fluorescence (F_M) was determined considering a saturation pulse (3000 μmol photons m^{-2} s^{-1}). The maximal photochemical efficiency of photosystem II was then calculated based on Eq. (1). Values of Fv/Fm lower than 0.6 were excluded to create the linear mixed models because they typically indicate plant stress or photosystem II damage. RWC was determined by weighing the fresh weight (FW) of a 5 mm diameter leaf disc with a 0.1 mg precision balance (AB104, Mettler Toledo, USA). The turgid weight was obtained (TW) after hydrating the discs for 24 h in water at 4 °C. Finally, to obtain the dry weight (DW), the discs were dried at 80 °C in an oven for 48 h. To avoid rehydration of the desiccated tissues, the samples remained in an airtight jar with silica gel until weighing. The RWC (%) was then calculated based on Eq. (2).

$$F_V \Big/ F_M = \frac{F_M - F_0}{F_M} \tag{1}$$

$$RWC\ (\%) = \left(\frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}}\right) 100 \tag{2}$$

2.4. Optical indices

Three optical indices (CCI, NDVI and PRI) were determined on the adaxial surface of the spinach and rocket leaves before performing the phytochemical determinations (cf. 2.1.). In detail, the CCI was determined with a CCM-200 plus chlorophyll concentration meter (Opti-Sciences Inc., Hudson, NH) and calculated using the transmittance (T) at 931 and 653 nm, based on Eq. (3) [34]. Both the NDVI and PRI were determined using a handheld SpectraPen LM 500 spectrophotometer (Photon System Instruments, Brno, Czech Republic) [35]. The NDVI was calculated using the reflectance (R) difference in the visible and the near-infrared wavelengths (740 nm and 660 nm) based on Eq. (4). The PRI was calculated using a different set of reflectance wavelength bands (531 nm and 570 nm) based on Eq. (5).

$$CCI = \frac{\%\ T_{931}}{\%\ T_{653}} \tag{3}$$

$$NDVI = \frac{R_{740} - R_{660}}{R_{740} + R_{660}} \tag{4}$$

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R570} \tag{5}$$

2.5. Phytochemical determination

Samples were extracted following the method described by Esteban et al. [36]. Briefly, the plant material was extracted using a Tearor electric tissue homogenizer (BioSpec, Bartlesville, OK, USA) with 1 mL of acetone (100%) and 0.5 g/L of $CaCO_3$ to avoid acid traces that might change the composition of pigments and tocopherols. This extraction was done at ≤ 4 °C using cold racks (IsoPack, Eppendorf IsoTherm®, Madrid, Spain). Once homogenized, the samples were centrifuged at 16, 000×*g* for 20 min at 4 °C and syringe-filtered through a 0.22 μm PTFE filter (Teknokroma, Barcelona, Spain). The extracts were injected (15 μL) onto a reversed-phase C18 column HPLC system (Waters Spherisorb ODS1, 4.6 × 250 mm, Milford, MA, USA). The injector kept the samples at constant 4 °C to avoid compound degradation. Photosynthetic pigments were determined using a photodiode array detector (Waters model 996, Massachusetts, USA) in the range of 250–700 nm. Peaks were integrated at 445 nm for Chls (sum of Chla and Chlb) and carotenoids (V, A, Z, N, L, lutein epoxide, α-Car and β-Car). The retention times and conversion factors were as described by Lacalle et al. [37]. For the detection of α-tocopherol (α-Toc), a scanning fluorescence detector (Waters 474) was used with an excitation λ of 295 nm and an emission λ of 340 nm. Total carotenoids were calculated as being the sum of N, V, A, L, Z, and β-Car. The sum of α-tocopherol and total carotenoids (SATTC) was also calculated. The total chlorophyll pool (Chl a+b) was expressed on a dry weight basis (μmol g^{-1}), while carotenoids and α-Toc were expressed on both a dry weight basis (μg g^{-1} or mg g^{-1}) and total chlorophyll basis (mmol mol^{-1} Chl). Carotenoids and tocopherols used in the models are both expressed relative to chlorophyll content to mitigate variability and potential errors in these calculations, as expressing them on a weight basis may imply plant tissues.

2.6. Statistical analyses

The analysis proceeded in the following structured steps: *i*). Firstly, an initial comparison of the physiological values was performed using Student's *t*-tests. This statistical method tests whether the difference in physiological variables (F_V/F_M, RWC; Chl a+b, t-Car, α-Toc and Z/VAZ) between day 1 and day 4 was statistically significant. *ii*). Secondly, Spearman rank correlation tests were undertaken to see the overall relationships between isoprenoids with nutraceutical value (VAZ, L, β-Car, t-Car, Chl a, Chl b, Chl a+b, α-toc, SATTC) and optical indices (PRI, NDVI, CCI). To plot the correlation matrix, the 'rcorr' function of the 'corrplot' R package [38] from the 'Hmisc' R package [39] was used. *iii*). Thirdly, linear mixed-effects models (LMEs; LME; "nlme" Rpackage [40, 41]) were performed to analyse the effects of PRI separately on VAZ, L, β-Car, t-Car, α-Toc and SATTC. LMEs were also used to analyse the

effects of NDVI and CCI on chlorophylls (i.e., only Chl a+b was considered here because the correlation with the optical indices was very similar between chlorophylls, and there is no evidence of different impacts of Chl a and Chl b as nutrients). All these LMEs were performed for a deeper analysis of the responses of each species (spinach and rocket) by considering the different treatments to which they were exposed (light conditions, packaging, collecting time; cf. below). Prior to performing the LMEs, the normality (Shapiro-Wilk test) of the response variables was checked. When the assumption of normality was not met, the response variables were logarithmically (VAZ, L, t-Car, SATTC, Chl a+b) or square root (i.e., α-Toc) transformed. The fixed part of the LMEs included the PRI, NDVI or CCI, the species (spinach and rocket), and their interactions. The random part of the LMEs considered the effects of the replicates (i.e., leaves) nested within light conditions (light or darkness) nested within the packaging (open or closed bags). A first-order autoregressive covariance structure was used to account for temporal autocorrelation (collecting time starting from the initiation of the experiment, i.e., days 1, 2, 3 and 4). The 'emmeans' R package [42] was used for further determination of how the slope of the relationships between the PRI, NDVI or CCI and each of the response variables (cf. above) differed between species (spinach and rocket). The final coefficients of the LMEs were estimated using the Restricted Maximum Likelihood Method (REML). The conditional R^2 values (i.e., the proportion of variance explained by both the fixed and random parts of the LMEs), were calculated using the "rsquared" function from the "piecewiseSEM" R package [43]. Overall, the conditional R^2 values of the LMEs considered for this study were higher than 0.90. The residuals of the final models fulfilled the normality assumption. The final plots of the LMEs were made sing the 'sjPlot' [44], 'sjmisc' [45], 'ggplot2' [46] and 'gridExtra' [47] R packages. All statistical analyses were performed in R v 4.2.2 [48]. Statistical relationships were considered significant at $p < 0.05$.

3. Results

3.1. Impact of light and packaging factors on the physiological parameters and nutraceutical levels

Table 1 shows the analysed physiological parameters (F_V/F_M, RWC, Chl a+b, t-Car, α-Toc and Z/VAZ) of spinach and rocket leaves between days 1 and 4 in terms of the different treatments (i.e., light conditions and packaging).

For both species, F_V/F_M decreased in closed bags (under both the light and dark treatments; CD and CL), while in open bags, F_V/F_M tended to either increase (light exposed bags, OL) or to not vary significantly (dark exposed bags, OD). Specifically, for spinach, the percentage of variation observed was as follows: CD showed a decrease of 22.2%, and CL exhibited a significant decrease of 43.7%. Conversely, the CD rocket of showed a decrease of 79.5%. The RWC remained stable in closed bags (CD, CL), but it showed a tendency to decrease in open bags. Specifically, under light conditions, the spinach leaves showed a 23.7% decrease in RWC, while under dark conditions, the rocket leaves showed a 9.6% decrease in RWC. Regarding the content of pigments and tocopherols, no clear overall responses were observed between the different treatments. While under the CD treatment the Chl a+b of the spinach leaves decreased significantly (34.8%), this variable showed no significant changes under the other treatments. For t-Car (expressed on a dry weight basis; mg g^{-1}), a significant decrease in the spinach leaves under the CD treatment (42.9%) and in the rocket (32.8%) leaves under the OL treatment was found. For α-Toc (μg g^{-1}), a significant decrease in the spinach leaves under the CD treatment (44.9%) and a significant increase (81.5%) in the rocket leaves under the OD treatment was observed. Finally, the Z/VAZ index showed a significant increase in the spinach leaves of 25.8% under the CD treatment.

It should be noted that these percentage changes are the variations between days 1 and 4, whereas, the monitored parameters would have fluctuated throughout the whole experiment. Indeed, minimum and maximum values for each parameter were not necessarily recorded on days 1 and/or 4. The range of these data is indicated in Table 1. F_V/F_M values varied greatly among storage conditions and sampling times, particularly for rocket (0.09–0.78). In the case of RWC, spinach was the species with the highest water loss in open bags (Table 1). There was a wide variation in phytochemical contents observed as well, due to the different storage conditions and species. The chlorophyll content in the leaves of our experiment varied between 8.8 and 37.9 mg g^{-1}; t-Car values were between 1.84 and 7.59 mg g^{-1}; and α-Toc fluctuated between 20 and 768 μg g^{-1} (Table 1).

3.2. Pigments, carotenoids, and tocopherols as a function of optical indices

Fig. 2 shows the results (only the statistically significant results, $p <$

Table 1
The percentage (%) variation in physiological parameters of spinach and rocket leaves between day 1 and day 4: photochemical efficiency, F_V/F_M; relative water content, RWC; total chlorophylls, Chl a+b (mg g^{-1}); total carotenoids, t-Car (mg g^{-1} and mmol mol^{-1} Chl) and α-tocopherol, α-Toc (μg g^{-1} and mmol mol^{-1} Chl), Z/VAZ. The table is organized according to the treatments used in the experiment: CD: Closed bag, Darkness; CL: Closed bag, Light; OD: Open bag, Darkness; OL: Open bag, Light. Negative and positive variations are marked in red and blue, respectively. The degree of variation is represented by the saturation of each colour. The data range is indicated between the parentheses below the percentage variation. Significant differences between days 1 and 4 (Student's *t*-test) are indicated with asterisks (*).

	Spinach				Rocket			
Physiological parameters	**CD**	**CL**	**OD**	**OL**	**CD**	**CL**	**OD**	**OL**
F_V/F_M	-22.2* (0.40-0.77)	-43.7* (0.36-0.76)	3.1 (0.65-0.75)	44.9* (0.40-0.77)	-79.5* (0.09-0.74)	-12.3* (0.57-0.74)	5.5 (0.62-0.75)	18.2* (0.48-0.78)
RWC	-0.3 (87-97)	2 (78-95)	-22.9 (42-91)	-23.7* (55-95)	3.7 (71-88)	10.8 (68-98)	-9.6* (78-96)	12.3 (64-92)
Chl a+b (mg g^{-1})	-34.8* (8.8-26.0)	-6.1 (8.8-26.7)	19.5 (18.7-29.4)	-6.7 (14.6-33.5)	28.8 (10.8-37.9)	-1.5 (11.6-20.1)	-19.1 (11.2-26.2)	-27.3 (9.3-20.5)
t-Car (mg g^{-1})	-42.9* (1.84-6.16)	4.6 (1.90-6.07)	21.6 (4.19-7.24)	-3.8 (3.16-7.55)	29.4 (2.27-7.59)	-7.5 (2.33-4.18)	-2.4 (2.70-5.05)	-32.8* (2.03-4.50)
t-Car (mmol mol^{-1} Chl)	-11.3* (309-390)	10.9* (307-382)	0.7 (317-389)	3.1 (339-399)	1.7 (316-345)	-5.5* (296-343)	20.9* (303-397)	-7 (289-367)
α-Toc (μg g^{-1})	-44.9* (75-388)	6.0 (145-296)	-7.9 (204-768)	10.4 (194-739)	-70.6 (20-286)	-19.0 (93-267)	81.5* (113-367)	-9.7 (134-261)
α-Toc (mmol mol^{-1} Chl)	-19.7 (4.3-12.9)	23.2 (5.4-13.7)	-27.7 (6.8-22.0)	19.9 (10.9-30.9)	-77.4* (1.5-12.0)	-13.2 (4.8-11.2)	129.2* (5.7-17.6)	30.9 (7.7-17.3)
Z/VAZ	25.8* (0.04-0.09)	18.2 (0.06-0.15)	-8.3 (0.04-0.08)	10.3 (0.03-0.07)	3.8 (0.03-0.08)	15.5 (0.03-0.18)	1.4 (0.04-0.07)	48.7 (0.02-0.07)

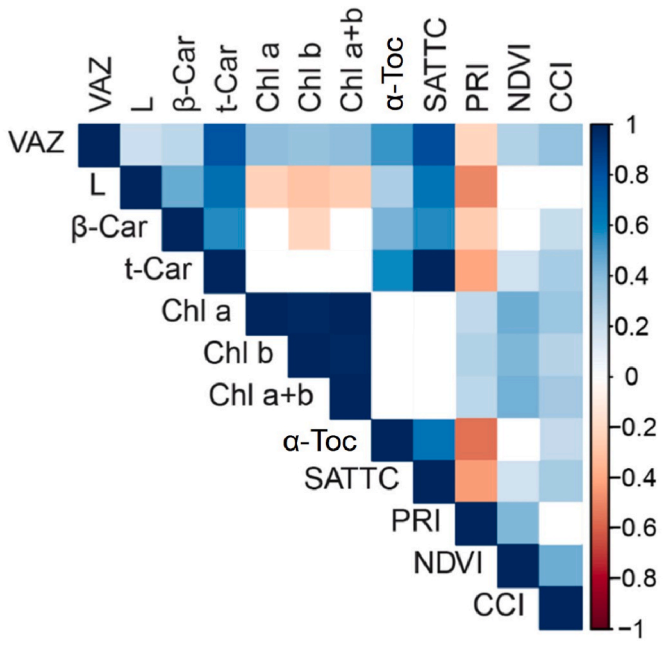

Fig. 2. Spearman's correlation matrix shows significant relationships between carotenoids (VAZ, total xanthophyll pool; L, lutein; β-Car, β-carotene; t-Car, total carotenoids; expressed in mmol mol^{-1} Chl), chlorophylls (Chl a, Chl b, Chl a+b; expressed in mg g^{-1}), tocopherols (α-Toc, expressed in mmol mol^{-1} Chl), the sum of pigments and tocopherols (SATTC, expressed in mmol mol^{-1} Chl) and the optical indices (PRI, photochemical reflectance index; NDVI, normalized difference vegetation index; CCI, chlorophyll content index). The statistically significant ($p < 0.05$) negative and positive correlations are indicated in red and blue, respectively. The strength of the correlation is indicated by the colour saturation. Note that the non-significant relationships ($p > 0.05$) are represented in white. On the right side of the correlogram, the colour legend shows the correlation coefficients and the corresponding colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.05, are shown) of the overall Spearman correlations between the isoprenoids with nutritional value and optical measurements. Since carotenoids, chlorophylls and tocopherols were significantly correlated, and because the main focus of this paper is the possible estimation of isoprenoids with optical indices, we will only focus on these results. To sum up, most of the relationships were positive (indicated by varying intensities of blue), with the NDVI showing correlations with VAZ, t-Car, Chl a, Chl b, Chl a+b and SATTC. Similarly, the CCI was positively correlated with all the isoprenoids with nutritional value except for L. In contrast, the PRI was positively correlated only with the chlorophylls (Chl a, Chl b and Chla + b) and negatively correlated with the rest of the isoprenoids with nutritional value (L, β-Car, t-Car, α-toc and SATTC).

The results of the LMEs detailed the responses of the chlorophylls (Chl a+b), carotenoids (VAZ, L, β-Car, t-Car), α-Toc and the sum of carotenoids and tocopherols (SATTC) to the optical indices (NDVI, CCI and PRI) by discriminating between the two species (spinach and rocket) with respect to the different treatments.

Chl a+b showed a positive response to the NDVI and CCI. These responses were both statistically significant ($p < 0.05$) (Table S1; Fig. 3). However, no significant differences between the two species (spinach and rocket) were obtained (Table S1). Nevertheless, the interaction between the NDVI and species was marginally significant ($p = 0.06$), while the interaction between the CCI and species was statistically significant ($p < 0.01$) (Table S1). Finally, when looking at how the slope of the relationships between the NDVI or CCI and Chl a+b differed between species (spinach and rocket), statistically significant results ($p < 0.01$) were obtained when the CCI was considered and marginally significant ones ($p = 0.06$) when the NDVI was considered (Table S1). For both the

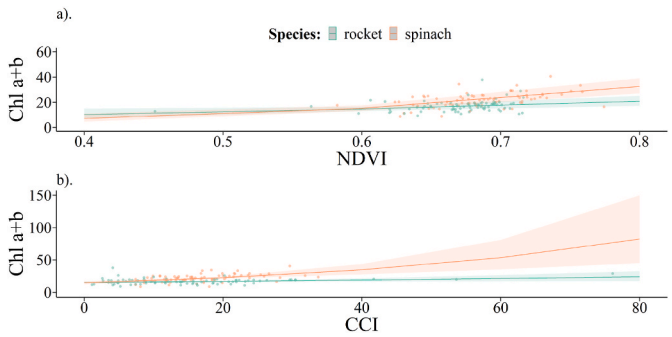

Fig. 3. Graphical representation of the linear mixed-effects model (LME) results showing the total chlorophyll pool (Chl a+b; expressed in mg g^{-1}) as a function of i) the normalized difference vegetation index (NDVI; **upper panel a**) and ii) the chlorophyll content index (CCI; **lower panel b**). The plots show the predicted values (marginal effects) for specific model terms (NDVI or CCI, species and their interactions). Back-transforming predictions to the original response scale have been applied to ease the interpretation of the plots. The shaded area represents the model's confidence interval at 95%.

NDVI and CCI LMEs, the value of the conditional R^2 was 0.99 (Table S1).

VAZ, L, β-Car, t-Car, α-Toc and SATTC showed a negative response to the PRI. This response was statistically significant for VAZ, L, t-Car, α-Toc and SATTC ($p < 0.001$) and marginally significant for β-Car ($p = 0.06$) (Table S1; Figs. 4–6). Statistically significant differences between spinach and rocket were found only when the VAZ response to the PRI was considered (Table S1). Nevertheless, the interaction between the PRI and species was never statistically significant (Table S1). Finally, when looking at how the slope of the relationships between the PRI and VAZ, L, β-Car, t-Car, α-Toc and SATTC differed between species (spinach and rocket), no statistically significant results were obtained (Table S1). The conditional R^2 values varied between 0.95 (the VAZ LME), 0.99 (the L LME), 0.99 (the β-Car LME), 0.94 (the t-Car LME), 0.95 (the α-Toc LME) and 0.93 (the SATTC LME) (Table S1).

4. Discussion

4.1. Storage conditions significantly influence the phytochemical content of the spinach and rocket leaves

Harvested vegetables keep their metabolism active during storage. Thus, their isoprenoids with nutritional values (including pigments and tocopherols) change over time, being affected by many factors such as packaging materials, protective atmosphere, temperature and/or illumination [18,49,50]. In our experiment, suboptimal storage conditions were simulated by maintaining spinach and rocket leaves at room temperature and then subjected to varying illumination (light or darkness) and packaging conditions (open and closed bags), which resulted in two outcomes: i) alterations in the environmental conditions within the bags, causing changes in photochemical efficiency, and ii) a varied composition of compounds (mainly xanthophylls and α-Toc) for subsequent modelling.

In the first outcome, examination of the treatment exerting the most significant impact on tissues, while considering the F_V/F_M trait as an indicator of vegetable quality [18], indicated a decrease in F_V/F_M in closed bags (both under the dark and light treatments, CD and CL) between day 1 and day 4 (Table 1). It is crucial to emphasize that the most significant decrease in F_V/F_M values (from 0.7 to 0.1) occurred on the final day of the treatment (data not shown). Indeed, the modified atmosphere packaging for salads and vegetables has been reported to decrease the maximal photochemical efficiency of green tissues, as compared to vegetables packed in natural air [51]. Nevertheless, this decrease was not attributed to water loss within bags, because closed bags were the most favourable conditions to maintain RWC and

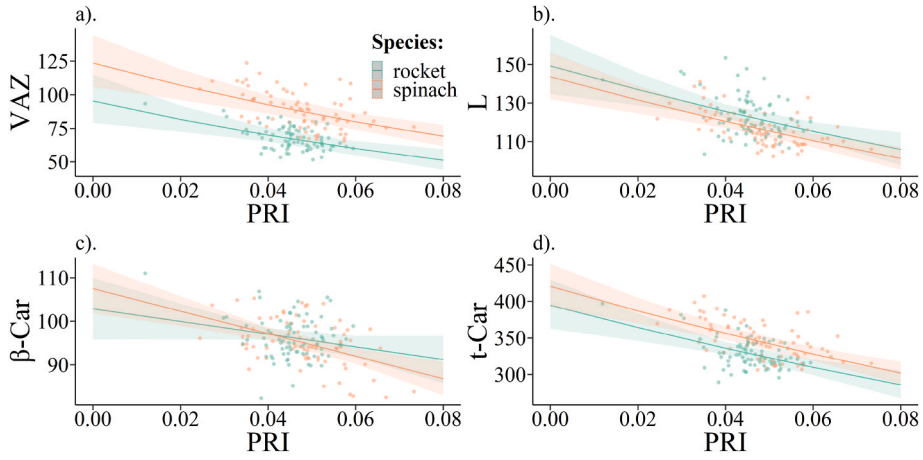

Fig. 4. Graphical representation of the linear mixed-effects model (LME) results showing the total xanthophyll pool (VAZ; **upper left panel a**), lutein (L; **upper right panel b**), β-carotene (β-Car; **lower left panel c**) and total carotenoids (t-Car; **lower right panel d**), with all four expressed in terms of mmol mol^{-1} Chl, as a function of the photochemical reflectance index (PRI). The plots show the predicted values (marginal effects) for specific model terms (PRI, species and their interactions). Back-transforming predictions to the original response scale have been applied to ease the interpretation of the plots. The shaded area represents the model's confidence interval at 95%.

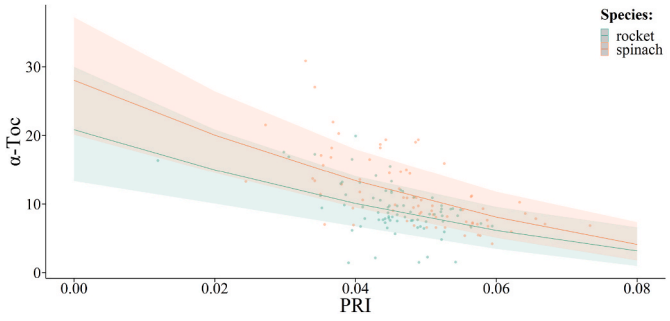

Fig. 5. Graphical representation of the linear mixed-effects model (LME) results showing the α-tocopherol (α-Toc; expressed in terms of mmol mol^{-1} Chl), as a function of the photochemical reflectance index (PRI) The plot shows the predicted values (marginal effects) for specific model terms (PRI, species and their interactions). Back-transforming predictions to the original response scale have been applied to ease the interpretation of the plot. The shaded area represents the model's confidence interval at 95%.

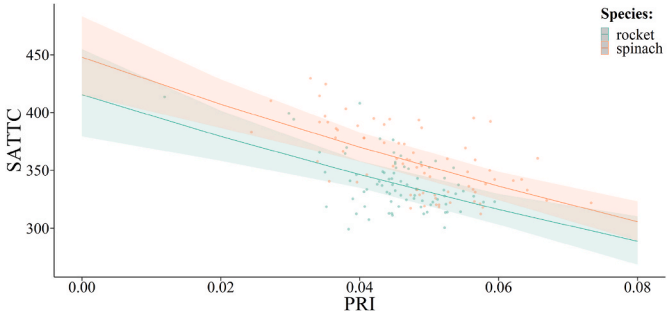

Fig. 6. Graphical representation of the linear mixed-effects model (LME) results showing the sum of all carotenoids and tocopherol (SATTC; expressed in terms of mmol mol^{-1} Chl), as a function of the photochemical reflectance index (PRI). The plot shows the predicted values (marginal effects) for specific model terms (PRI, species and their interactions). Back-transforming predictions to the original response scale have been applied to ease the interpretation of the plot. The shaded area represents the model's confidence interval at 95%.

turgidity, regardless of light or dark exposure (Table 1). One possible explanation for this phenomenon is linked to either high relative humidity or high CO_2 concentration, both of which cause stomatal closure [52]. As explained in the Material and Methods, values of Fv/Fm lower than 0.6 were not used to create the mixed linear models.

Regarding the changes in the pigment and tocopherol content (the second outcome of the treatments), our results indicated that light conditions and the packaging affected the composition of the spinach and rocket leaves. The total chlorophyll content ranged between 8.8 and 37.9 mg g^{-1}. Similarly, the t-Car pool ranged between 1.8 and 8.0 mg g^{-1}, and α-Toc ranged from 20 to 768 μg g^{-1}. These results agree with previous works reporting that temperature, lighting cycles, and atmosphere can significantly modify the phytochemical concentrations of the vegetables, and therefore their nutritional value, and that these effects can differ between species [18,19]. Focusing on α-Toc (vitamin E), an overall increase in this factor was observed in most of the illuminated bags and, to a lesser degree, in open bags as well (Table 1). This compound has an important antioxidant action [53], but it is unlikely that reactive oxygen species associated with oxidative stress were produced under this low illumination intensity (20 μmol m^{-2}s^{-1}). A more plausible explanation might be that dehydration (open bags decreased the RWC of the tissues; Table 1) may have caused oxidative stress (and therefore led to an increase in the antioxidant α-Toc). In fact, the findings indicated a negative correlation between CHR and α-Toc content, expressed on both a dry weight basis and a chlorophyll basis (correlation coefficients of −0.430 and −0.315, respectively; data not shown).

An increase in Z/VAZ levels within closed (dark) spinach bags (Table 1) was observed. However, in the case of rocket, the light was the primary factor responsible for the increase in Z/VAZ. This can be explained by CO_2-enriched protective atmospheres that may contribute to cytoplasm acidification, activating violaxanthin de-epoxidase, a process mediated by pH [54]. Consequently, this leads to an increase in the Z content, as observed in three leafy vegetables (corn salad, spinach and lettuce) and due to the high CO_2 content in bags [18].

4.2. *Optical indices correlate significantly with pigments and tocopherols*

Given that storage conditions can significantly impact the nutritional quality of packed vegetables, it is a matter of interest to develop cost-effective tools that can easily assess, in real-time, their nutritional value in warehouses, supermarkets, etc. In this context, optical and hyperspectral-based technology represent novel approaches. Specifically, for carotenoids and chlorophylls, many attempts have been

developed to predict the carotenoid pool using non-invasive techniques [55–59]. However, for the development of accurate indices to assess plant isoprenoid content via spectroscopic techniques, the dataset quality and wavelength selection are essential [60]. The optical indices employed in this study, such as the NDVI, PRI, and CCI, are introduced as promising tools that are fast, simple, and cost-effective for monitoring the postharvest nutritional value of vegetables. Initially designed to monitor and determine the health status and pigment composition of plants in natural ecosystems [22,61], their application in evaluating harvested vegetables has had less attention. The relationships between pigments (i.e., chlorophylls, carotenoids) and optical indices (NDVI, CCI, PRI) found in our work (Fig. 2) align with findings from previous research. However, the limitations of these indices may be considered, as they do not account for the leaf surface properties associated with reflective substances in the leaves, such as hairs, anthocyanins, or epicuticular waxes, which could lead to inaccurate predictions of the chlorophyll [62] and carotenoid contents [63]. This study was conducted with green leaves, so further research is needed for other consumed leaves that are not green (e.g., red-leaf lettuce, purple kale, borage).

4.3. The NDVI for estimating the total chlorophyll pool in packed green vegetables

The LMEs that were run confirmed all the simple negative and positive relationships obtained with the Spearman correlations (Fig. 2) but went a step further to explore these relationships more deeply by discriminating between the two species with respect to the different treatments that they have been exposed to. For Chl a+b, even though both optical indices (NDVI and CCI) were found adequate to estimate chlorophylls (Fig. 3), the model utilizing the NDVI is considered a better option for estimating chlorophyll content. This is because, unlike the model where the CCI was used, only a marginally significant relationship was found between the NDVI and species (Table S1), meaning that the NDVI does not have a specificity for one species in particular, and it thus has a broader application in other species. Additionally, with the treatments applied here, we achieved chlorophyll levels ranging from ~9 mg g^{-1}DW to 38 mg g^{-1}DW, which is inside the theoretical ranges of variation for chlorophylls [64]. Indeed, the highest values were obtained for spinach, which has highly chlorophyll-concentrated leaves. Given this, it is not anticipated that green leaves would be encountered with significantly higher or lower chlorophyll contents, owing to the constrained configuration of plant photosystems [64]. To date, the NDVI as a chlorophyll content proxy has been a tool commonly used in the field to assess seasonal variations, stress responses and even to predict yield [65,66], but its use in urban and/or commercial environments has been little explored. The results obtained in our study represent a step forward in this regard, indicating a promising use of the NDVI (being aware of the limitations) when it comes to estimating nutritional value based on chlorophylls in stored vegetables that can be bought in supermarkets.

4.4. The PRI for estimating xanthophylls in packed green vegetables

The PRI is a good proxy for monitoring changes in carotenoids, such as during seasonal variations [25,28,67]. Our results lend further support to this finding and open the possibility of using it not only as a nutritional quality tool in field measurements, but also in greenhouses, warehouses, or even in supermarkets, i.e., the last stage of nutrition-sensitive production. In this regard, the results of the LMEs indicated that the PRI can be used for to estimate VAZ, L and t-Car but also that the PRI is less suitable to estimate β-Car (Table S1; Figs. 4–6). However, when implementing such tools it is essential to consider the methodological limitations (i.e., inaccuracy due to leaf structure that changes the reflectance of the leaf) and that these predictive models perform optimally within a specific range of values (not extreme values). This lack of sensitivity to low and high carotenoid content within these indices has been proven in several studies [60].

4.5. Tocopherols may be included in the models to estimate the nutritional content using the PRI

Recent studies have employed non-destructive chemical analysis techniques (i.e., Raman and infrared spectroscopy) to determine tocopherols in seeds and oil [29,68]. However, determining tocopherols using the PRI is challenging due to their narrow absorbance maximum band in the ultraviolet region, which is between 280 and 305 nm. The relationship found in the present study between the PRI and α-Toc was stronger than the one obtained between the PRI and VAZ (Fig. 2). It should be stressed that there is an interplay between α-Toc and Z [69]. Indeed, the covariation between these compounds might enable the detection of changes in tocopherols through optical indices, following the model employed to estimate isoprene using the PRI in temperate forests [70]. Therefore, the relationship found between the PRI and α-Toc (Fig. 2) could also serve as an indicator of the suitability of the PRI as a proxy of α-Toc in urban and/or commercial environments. The LME provided further support in this regard because the PRI showed a significant relationship with SATTC but no significant relationship with species (Table S1). As a significant relationship between the PRI and species was found only for the VAZ model, our results indicate that the use of the PRI can be considered broad when it comes to evaluating the content of carotenoids, as well as tocopherols, in different green-leaf vegetables. Thus, the simultaneous estimation of carotenoids and tocopherol from a single index (PRI) is a significant advance.

5. Conclusion

In this work it was found feasible to accurately estimate the content of chlorophylls, carotenoids, tocopherols, and the combined content of the latter two (SATTC) in rocket and spinach leaves using the NDVI and PRI under various storage conditions. These conditions encompassed different treatments used in our experiment, such as exposure to light or darkness and storage in open or closed bags for four consecutive days. Considering the nutritional significance of these phytochemicals and their variations in green-leaf vegetables during the postharvest storage and commercialization phases, our findings represent a significant step forward in the development of real-time nutritional value monitoring tools. Furthermore, while our statistical models are effective for both spinach and rocket, extending them to other species with different pigmentary compositions could be an interesting avenue for further research. In the context of nutrition-sensitive agriculture and consumption, our findings hold the potential for application at multiple stages of the production and commercialization chain, as well as in research projects. One potential future application includes the use of refrigerators equipped with such technology, enabling consumers to access real-time data on the nutritional quality of the fresh vegetables they purchase. Additionally, this technology could assist distributors in optimizing their product storage conditions. Nevertheless, future research is needed in this regard, as the white backgrounds of the fridges might have an additional reflectance effect and thus lead to erroneous nutritional value predictions.

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CRediT authorship contribution statement

Rafael G. Lacalle: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Idoia Iratzoki:** Methodology, Investigation. **Ana-Maria Hereş:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **José María Becerril:** Writing – review & editing, Funding acquisition, Conceptualization. **José Ignacio García-Plazaola:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Raquel Esteban:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Declare that the authors of this manuscript do not have any competing interests no conflicts. Authors have nothing to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2024.101151.

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