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Changes in the content of chlorophylls and carotenoids in the rind of Fino 49 lemons during maturation and their relationship with parameters from the CIELAB color space

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ABSTRACT

In the present work, the coordinates L*, a* and b* from the CIELAB color space, as well as the chlorophyll, total carotenoids and the content of the carotenoids Lutein and β-cryptoxanthin were measured in the skin of fruits from the Fino 49 lemon during its development, with the aim of understanding the relationship that exists between the color changes of the fruit's skin (color coordinates) and the pigment content. Also, the understanding of the relative importance of the contents of lutein and β-cryptoxanthin with respect to the total content of carotenoids was sought. The period of study lasted three years; from September 2015 to January 2016, from September 2016 to January 2017, and from September 2017 to January 2018, the periods that comprised the color changes of the lemon fruit until its harvest. The fruits were measured every two weeks in the experimental plot of the IMIDA (Murcian Institute of Agricultural and Food Research and Development) located at La Alberca (Murcia, Spain) and in the experimental orchards from the CEBAS-CSIC, located in Santomera (Murcia). During the experiment, the color and chlorophyll, Lutein and β-cryptoxanthin concentrations were measured. The results showed that there was a good correlation between the color coordinates and the pigments responsible for the lemon's skin color: all the color pigments were correlated with the a*, b* color coordinates and the Hue angle index. Throughout the fruit's maturation, a degradation of the chlorophylls was observed, as well as an increase of β-cryptoxanthin, which is responsible for the green and yellow color of the fruits, respectively. Lutein, which was found in high concentrations, decreased with time, but did not contribute to the fruit's color.

1. Introduction

Citrus fruits are some of the most cultivated fruits in the world. In terms of production, more than 128 million tons per year have been harvested (FAOSTAT, 2018). Their fruits are very sought in the entire world for their consumption as fresh fruit, juice processing, jam, or as a food additive for dishes and drinks. The four most-important commercial groups of citrus are grapefruit, oranges, mandarins and lemons. In this last group, the color and caliber of the fruit are properties that indicate when the fruit should be harvested and its market price. There is a marked influence of the environmental and agronomical conditions such as light and temperature (Lado et al., 2018) on the color of the fruits. The color change begins with the lowering of the temperature in the autumn, which leads to the degradation of chlorophylls that are present in the fruit rind (green color). These results in the appearance of diverse pigments (carotenoids, flavonoids) that are responsible for the yellow color of the fruit, a color that was masked by the chlorophylls, or these pigments appear *de novo* due to the synthesis of new pigments (Casas and Mallent, 1988). Besides temperature, the change in color could be affected by other factors such as humidity, light, type of soil or rootstock (Manera et al., 2012; Brotons et al., 2013; Porras et al., 2014; Simón-Grao et al., 2016), and even due to the foliar applications of diverse products such as ascorbic acid (Rehman et al., 2018).

Carotenoids are the pigments responsible for most of the yellow, orange and red colors of fruits and vegetables, due to the presence of a chromophore in its molecule that has a complete or partial chain of

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double conjugated bonds. These compounds are present in every photosynthetic tissue along with the chlorophylls, as well as in non-photosynthetic plant tissues (Meléndez et al., 2004). The color of the citrus fruits has been widely studied, so that it is known that it is fundamentally due to the carotenoids, whose distribution changes qualitatively and quantitatively between different species and varieties and throughout the fruit's development and maturation (Gross, 1987).

There are many studies on the color and content of carotenoids in citrus (Lee and Castle, 2001; Shim and Kim, 2002; Cortés et al., 2006; Beltrán et al., 2008; Van-Wyk et al., 2009), but their relationship has not been studied in depth. The external color of the lemon fruits can be determined in many ways, from the purely visual to the use of color scales with color coordinates. At present, in order to study the color changes of citrus fruits, surface reflection equipment is being used, known as tristimulus colorimeters, with allow for the easier measurement of color, the making of fast measurements, *in vivo* and in a non-destructive manner. Starting with the tristimulus values of coordinates, X, Y and Z, the values L, a* and b* are calculated mathematically within the CIELAB color space, which closely corresponds to the color observed by the human eye (Gilabert, 1998). The L parameters refers to the luminosity of the fruit, the a* parameter represents the green to red color scale, and the b* parameter represents the range from blue to yellow.

The color change from green to yellow in the lemon fruits is due to alterations in the composition and concentration of the pigments, mainly chlorophylls and carotenoids. When the air temperature drops below 13 °C, the degradation of the chlorophylls begins at the same time as the synthesis of carotenoids, which are the compounds responsible for the yellow color (Manera et al., 2012). Besides temperature, other factors can influence the color change in fruits. Simón-Grao et al. (2016) observed that fruits from the Fino 49 lemon tree did not reach the characteristic yellow color when they were under netting or when they suffered drought conditions. However, irrigation with salinized water favored the degradation of chlorophylls and therefore natural de-greening. Small differences have also been observed in the color change models depending on the variety of the fruit. Thus, the 'Verna' lemon needs a lower starting temperature as compared to Fino, and once the natural de-greening process has begun, the speed of changing from green to yellow is slower as compared to 'Fino' (Conesa et al., 2015). Among the varieties of lemon Eureka Frost, Lisbon Frost, Fino 49 and Verna 51, color change studies have shown that Eureka, Lisbon and Fino 49 lose their green color two months before Verna, which occurs in the month of January for the latter variety (Porras et al., 2010).

There are many studies that have evaluated, on the one hand, the color changes using the a*, b* and L* coordinates, and on the other hand, how the composition of pigments change in the lemon fruit, as previously described. In the present experiment, however, a study was conducted on the changes of color of lemon fruits of the cultivar 'Fino 49', by combining physical parameters of color with chemical parameters (composition and concentration of pigments), in order to elucidate the relationship that exists between these parameters, and also to find out if it is possible to estimate the concentration of chlorophylls and carotenoids by using these color coordinates.

2. Materials and methods

2.1. Plant material and experimental conditions

The plant material used in this work were fruits from the lemon variety Fino 49 (*Citrus limon* L. Osbeck), grafted onto the *Citrus macrophylla* Wester rootstock. The orchards where the trees were grown were located in two experimental orchards. One of them belonged to the Murcian Institute of Research and Agricultural Development and Food (IMIDA) from La Alberca (Murcia). The trees were 30 years old, and were planted at a spacing of 6 × 6 m. They were watered with a drip irrigation system with 5 drippers per tree and an output of 4 L/h. The average temperature was 18.7 °C, and the average rainfall was 321 mm/year; the soil was permeable and very calcareous (17.1% total calcium carbonate). The other experimental orchard belonged to the CEBAS-CSIC, located in the municipality of Santomera (Murcia, Spain), with climatic conditions characterized by the following values (averaged for 2007–2012): rainfall 285 mm, air temperature 17 °C. The soil was clay-loam with 11.10% active calcium carbonate. All the trees were in good health and in full production. The study was conducted for three years (2015/16, 2016/17, 2017/18) in the IMIDA experimental orchard, and for two years at the CEBAS experimental orchard (2016/17, 2017/18). In each year, fruit samples were harvested randomly every two weeks starting in October until harvest time. The samples consisted of 10 fruits per tree picked from the interior part of each tree chosen (5 fruits from the north-facing side and 5 from the south-facing side), from each of the four trees selected for the study. The color and the concentration of chlorophylls and carotenoids were measured in each fruit. The harvesting of the fruits was conducted in mid-September to mid-January in the three years of the study at the IMIDA experimental orchard, and in the two years of study at the CEBAS one.

2.2. Color measurements

During the autumn growing cycle in the years studied, data was recorded on the external color of the lemon fruit. The external color of the fruit was measured with a *Konica Minolta (Sensing Americas)* CM-700d portable spectrophotometer, using a view angle of 10°, standard illuminant D65, and the CIELab color space. For each of the measurements of color, three readings were performed on the equatorial zone of each of the fruits, thereby obtaining an average of the three readings (L*, a* and b*). The a* colorimetric coordinate corresponded to the green-red axis, where the negative values were related to the green color and the positive values with the orange and red (−60 green, +60 red). The b* corresponded to the blue-yellow axis, where the negative values were related to the color blue, and the positive values with the yellow color (−60 blue, +60 yellow). The L* coordinate (0 = black, 100 = white) measured the luminosity, and it was placed on the z-axis, with the a* and b* coordinates placed on the xy color planes. The chlorophyll content of the fruit was associated to the a* coordinate. The Hue angle index (arctan b*/a* was also utilized.

2.3. Extraction, identification and quantification of the Pigments

2.3.1. Extraction of pigments

The pigments were extracted from the external part of the fruit after previously separating the flavedo of the lemons by grating the skin with a thin blade around the entire equatorial perimeter of the fruits. Afterwards, it was ground with a grinder until obtaining a fine powder.

2.3.2. Extraction and quantification of chlorophylls

To measure the chlorophyll content, a spectrophotometric method was used as proposed by Hansmann (1973). The chlorophylls were extracted from 2 g of sample by adding 10 mL of acetone:water (9:1). The slurry was mixed in a mortar and was subjected to ultrasonics for 10 min. The sample was then centrifuged at 10,000 rpm for 5 min at 5 °C. The supernatant was measured at 665, 645 and 630 nm. For chlorophyll quantification, an equation proposed by Parsons and Strickland (1963) was used.

2.3.3. Extraction of carotenoids

The carotenoids are classified into carotenes (carbohydrates) and xanthophylls, with the latter containing oxygen atoms as well as carbon and hydrogen. In the case of the carotenoids that are typically found in products consumed as part of the human diet, oxygen is usually found in the xanthophylls in the shape of a hydroxyl (lutein, zeaxanthin), epoxy (violaxanthin, neoxanthin, anteraxanthin) or carbonyl

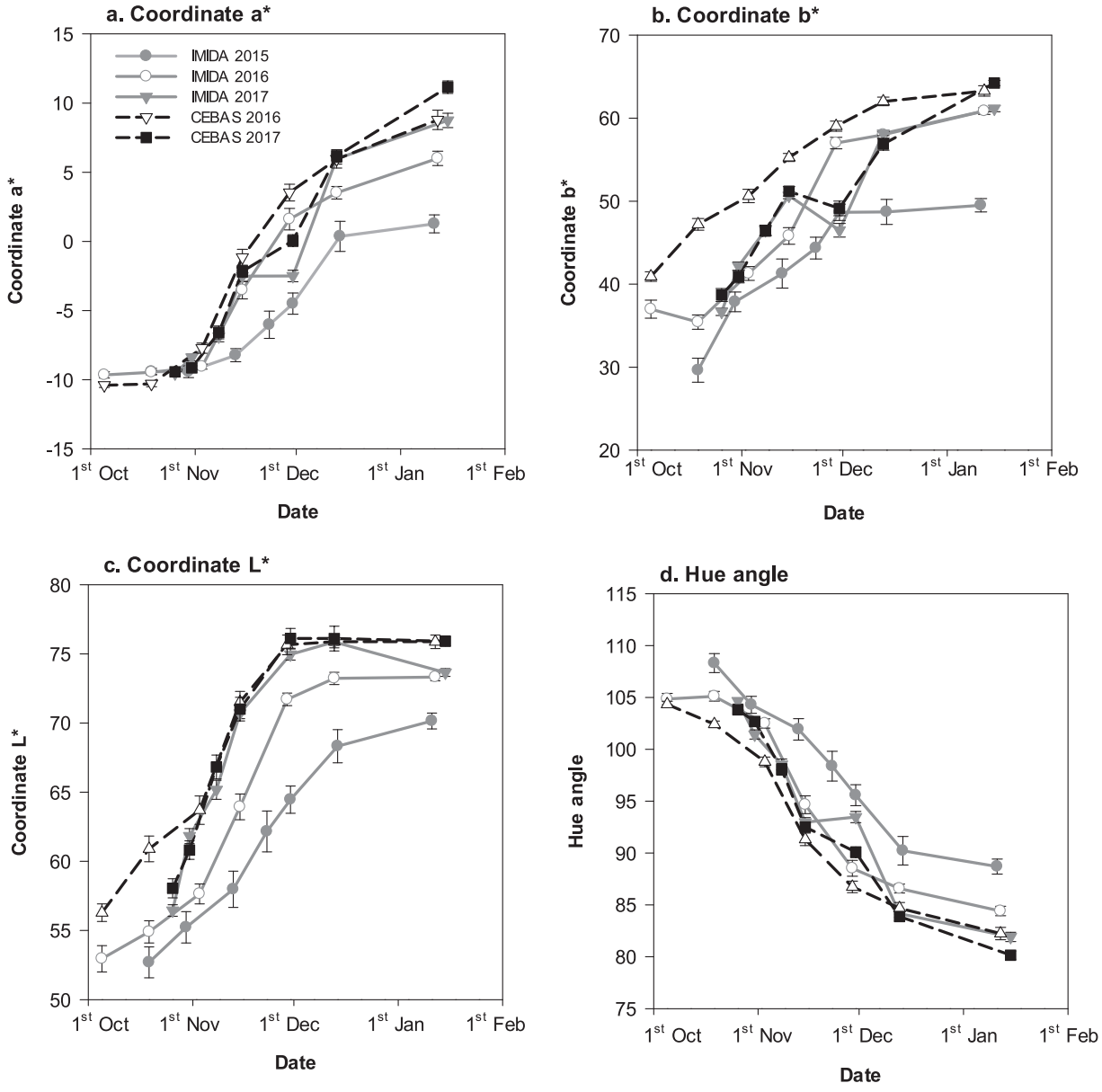

Fig. 1. Changes in the a* (1a), b* (1b), L* (1c) colorimetric coordinates and Hue angle (1d) in the three years of the study (2015/16, 2016/17 and 2017/18) for the IMIDA and CEBAS experimental orchards.

(canthaxanthin, astaxanthin) groups.

The carotenoids were extracted following the method described by Agócs et al., (2007); 10 mL of the extraction agent hexane:acetone:ethanol (2:1:1) +0.01% BHT were added to 5 g of sample. The sample was filtered and was then transferred to a decanting funnel, with this extraction conducted three times. Afterwards, the upper phase was placed together with anhydrous sodium sulfate and it was saponified with the addition of 20% KOH in methanol, in the dark and under agitation. The phases were separated with the addition of 50 mL of 10% NaCl in water. The upper phase was evaporated in a roto-evaporator and it was re-dissolved in hexane. Lastly, the sample was placed in a vial under Nitrogen gas (N_2) and stored at −30 °C until analysis.

2.3.4. Quantification and identification of carotenoids

The total content of the carotenoids was determined with a Shimadzu spectrometer (Shimadzu 1601 UV–vis), with a double beam at 450 nm. The results were expressed as mg of carotenoids equivalents to lutein per g of fresh weight of the skin.

The identification and quantification of individual carotenoids was performed with an HPLC (Hewlett Packard 1100) with the ChemStation software, coupled to a DAD photodiode array. The column used was a C18 Mediterranea (Teknokroma), with dimensions 5 µm 250 × 4.6 mm. A system of gradients was used, composed by A (12% (v/v) water in methanol), B (methanol), C (30% (v/v) dichloromethane in methanol). From 0 to 2 min 100% A, from 2 to 10 min until 80% A/20% B, from 10 to 18 min until 50% A/50% B, from 18 to 25 min until 100% B, from 25 to 27 min 100% B, from 27 to 34 min until 100% C, from 34 to 41 min 100% C. The flow rate was 1.25 mL/min and the column temperature was set at 30 °C. The carotenoids were identified by comparing their retention times and their absorption spectra (230–600 nm) with individual patterns of lutein and β-cryptoxanthin (Extrasynthese), as well as with the results obtained with the mass spectrometer. Apocarotenal was used as the internal standard to calculate the quality of the carotenoid extraction.

Agócs et al. (2007) identified β-cryptoxanthin and Lutein as the most common carotenoids found in lemon skin. In our study, the corroboration of the identification of the carotenoids was conducted with an HPLC (Agilent 1200 HPLC Infinity series), equipped with a

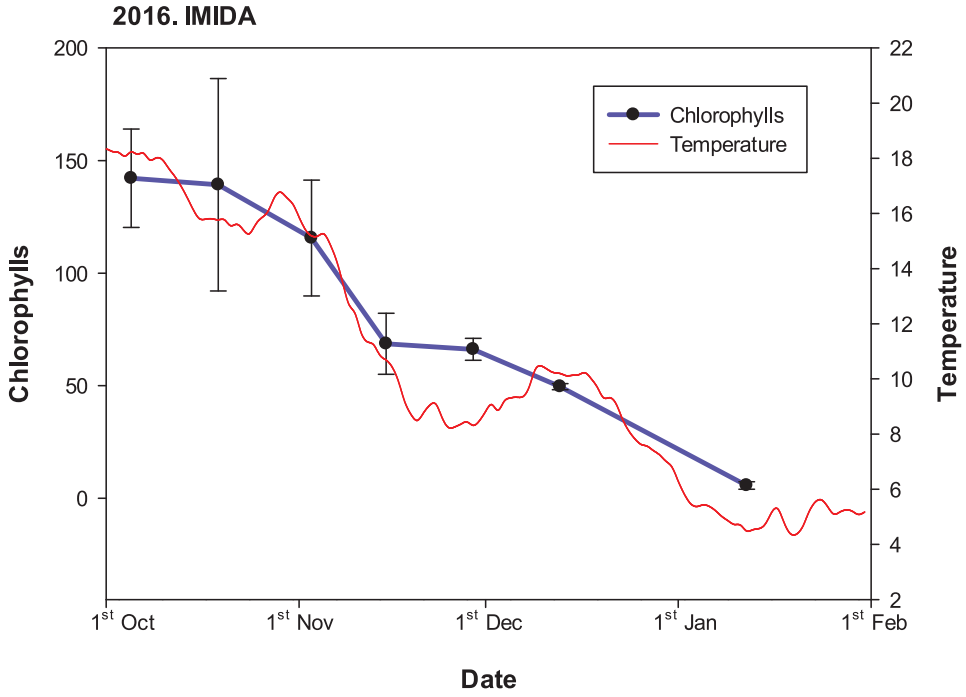

Fig. 2. Changes in the chlorophylls and average minimum temperatures for 14 days of the fruits harvested in the IMIDA (Murcia experimental orchard in 2016). The vertical bars indicate the standard error (± SE) for n = 3.

photodiode detector, connected to a mass spectrometer (Bruker Daltonics Ultra HCT-ESI Ion Trap, Bremen, Germany), controlled *via* the Esquire Control software. The chromatographic conditions were similar to the previously-described ones. The ionization conditions were 300 °C and 3 kV. The nebulizer pressure was 40 psig and the flow rate was 9.0 mL/min. The scanning of masses was set for the *m/z* range of 100 to 1200. The collision-induced fragmentation was achieved with an ion trap, using helium as the collision gas, with ramping cycles from 0.3 to 2 V. The spectrophotometry data was acquired in positive ionization mode. The quantification of the peaks was conducted using the Apocarotenal internal standard, and the sum of all the peaks was compared to the total content obtained by spectrophotometry.

2.4. Statistical analysis

A descriptive statistical analysis was conducted through charts, as well as second-degree polynomic regressions between the colorimetry coordinates and the concentration of pigments analyzed with the SPSS 19 computer software. More specifically, the change of the colorimetric coordinates a*, b*, L* and the Hue angle were correlated with the changes in the concentration of chlorophylls, Carotenoids, lutein and β-cryptoxanthin. The analysis was conducted for each of the experimental orchards and years. To adjust the data, quadratic regressions were used, and these were analyzed with the coefficient of determination r^2.

3. Results

3.1. Color changes

During the maturation of the lemons, the a* coordinate changed from a negative value (around −10; very green, every year) until values that oscillated between 1 and 11, depending on the year studied (absence of green color). The highest increase of a* was produced every year in the months of November and December, and the slowest occurred in January (Fig. 1a). Fig. 1b shows the changes of the b* coordinate with time. The value of b* began with values between 30 and 40 in October, depending on the year, and reached values close to 45 and 56 in mid-November, which progressively increased until reaching a maximum value of 65 (2016, CEBAS fruits in the second week of January). The L* coordinate (Fig. 1c) measured the fruit's luminosity, and its change throughout the de-greening process was characterized by its progressive increase as the fruit matured in every year studied, starting with values between 52 and 57 at the beginning of October, until reaching values between 68 and 75 in mid-December, varying little from this date onwards. In the first sampling dates, the Hue angle (Fig. 1d) was found in the fourth quadrant close to the 105° and 110° angle, as the a* coordinate was at first negative (green color) every year, and the b* had values around 45 and 56 (yellow colors), although these colors were not visible as they were masked by the green color of the chlorophylls, as indicated by the a* coordinate. Lemon maturation and its de-greening occurs in autumn, and as a result, the a* coordinate changes to positive values in the first quadrant, with the value of the b* coordinate (yellow color) increasing as well. Likewise, the angle, measured by the Hue angle, also decreased, so that the values were found in the first quadrant in every year studied.

3.2. Changes in chlorophyll, Carotenoids, Lutein and β-cryptoxanthin with time

Fig. 2 shows the changes of the chlorophyll and the minimum temperature in the autumn of 2016 and January 2017, to illustrate how the decrease of the minimum temperatures had an influence on the de-greening of the lemon rind, resulting in the degradation of the chlorophylls (Manera et al., 2012). This was found for both years and sites studied.

In the case of the chlorophylls (Fig. 3a), a decrease in the concentration was observed with time, moving from 120 and 142 mg kg^{-1} in October to values ranging between 20 and 52 mg kg^{-1} towards the end of December, and from this date on, the concentration decreased little, reaching somewhat lower values in the second week of January. The total carotenoids (Fig. 3b) progressively decreased throughout the process of maturation. In the beginning of October, the concentration was about 21 mg kg^{-1}, decreasing to values of 7 and 3 mg kg^{-1} in January depending on the year. As for Lutein, (Fig. 3c), its values

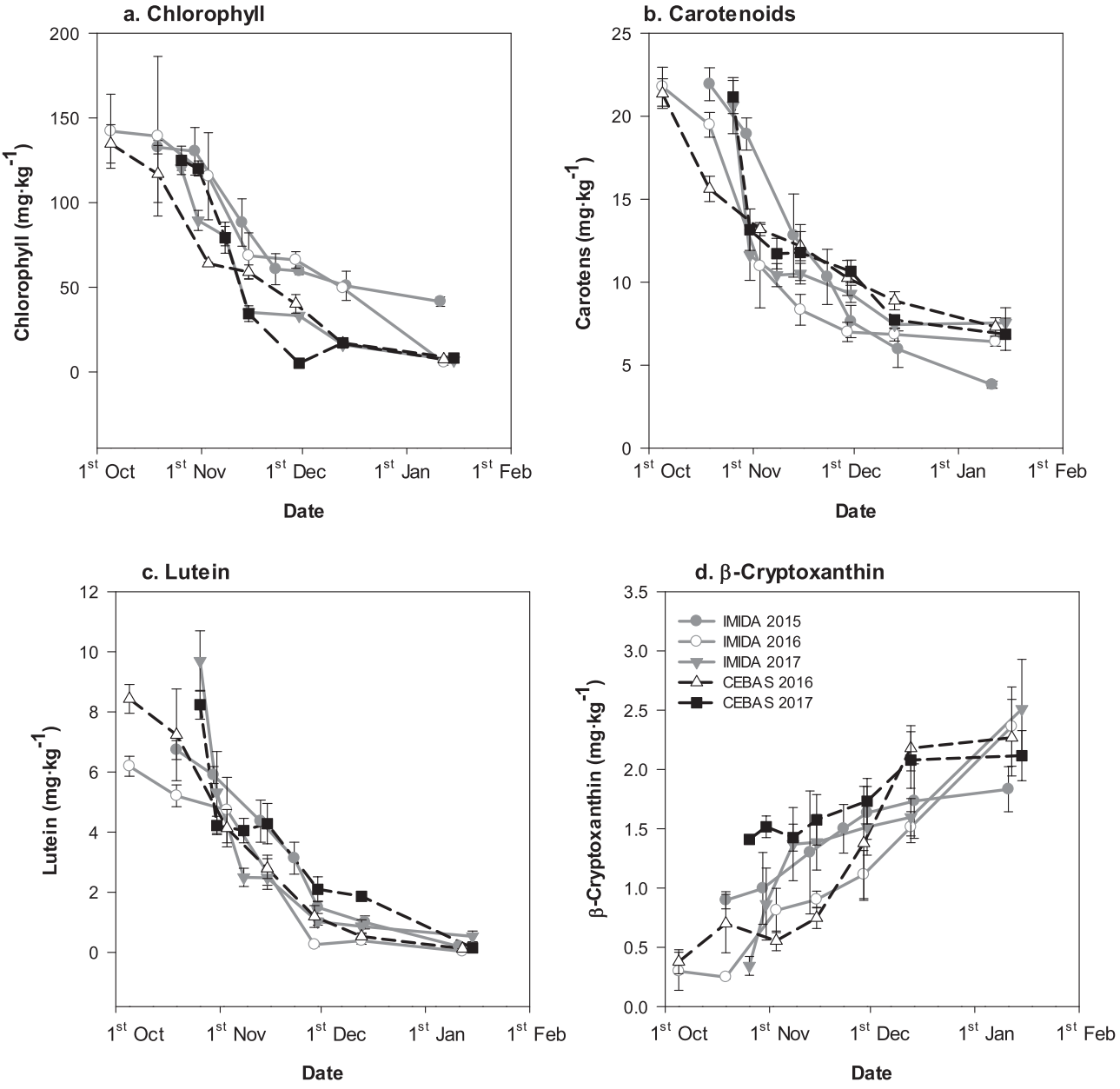

Fig. 3. Changes in the chlorophylls (2a), carotenoids (2b), Lutein (2c) and β-cryptoxanthin (2d) in the three years of study (2015/16, 2016/17 and 2017/18) for the IMIDA and CEBAS experimental orchards. The vertical bars indicate the standard error (± SE) for n = 3.

Table 1
Changes in the content of carotenoids (mg/kg) and percentages of Lutein and β-cryptoxanthin, as compared to total carotenes during the 2016/17 season in fruits from the IMIDA experimental orchard.

Date	Carotenoids mg kg^{-1}	Lutein (%)	β-cryptoxanthin (%)
10/05/2016	21.78 ± 1.17	28.42 ± 1.52	1.38 ± 0.73
10/19/2016	19.49 ± 0.75	26.73 ± 1.85	1.28 ± 0.10
11/03/2016	10.97 ± 2.51	43.21 ± 9.94	7.38 ± 1.73
11/16/2016	8.34 ± 0.93	32.01 ± 6.83	10.79 ± 0.84
11/30/2016	7.00 ± 0.58	3.71 ± 1.00	15.86 ± 2.86
11/13/2016	6.85 ± 0.40	5.69 ± 1.75	22.04 ± 1.90
01/11/2017	6.41 ± 0.27	0.62 ± 0.16	36.82 ± 5.15

oscillated between 9.69 and 6.2 mg kg^{-1} in the month of October, decreasing throughout the year until reaching values close to zero (0.13–0.04 mg kg^{-1}) in January. β-cryptoxanthin (Fig. 3d) had an opposite behavior, with its concentration increasing, ranging from 0.34–1.41 mg kg^{-1} (beginning of October) to 2.10–1.81 mg kg^{-1} (end of December), lightly decreasing at the end of maturation. Table 1 shows the percentage of Lutein and β-cryptoxanthin as related to the total content of Carotenoids. It can be observed that the concentration of carotenoids decreased with time, but the percentage of β-cryptoxanthin increased at the same time that Lutein decreased, so that at the end of the cycle, the percentage of β-cryptoxanthin was 36% of the total carotenoids.

3.3. Correlations between the physical and chemical parameters

As can be observed in Figs. 4–6, the correlations of the color coordinates with the concentration of the pigments analyzed were high during the period of color change of the lemon fruits, showing a second degree polynomic model. The concentration of β-cryptoxanthin had the weakest correlation with the color parameters, with an r^2 that did not

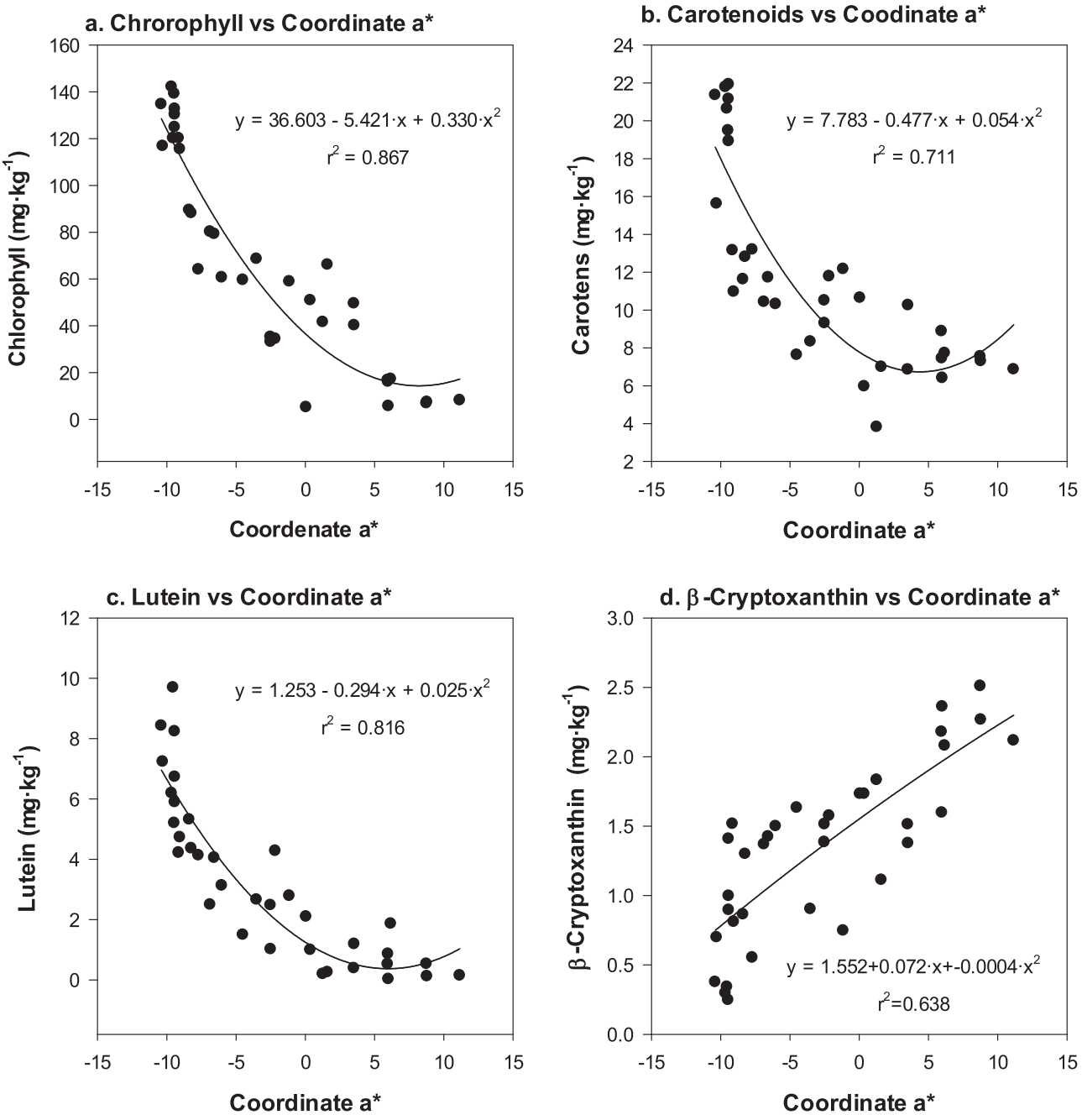

Fig. 4. Correlations between the a* coordinate and chlorophylls (4a), carotenoids (4b), Lutein (4c) and β-cryptoxanthin (4d) in the three years of study (2015/16, 2016/17 and 2017/18) for the IMIDA and CEBAS experimental orchards.

exceed 0.64. The correlations were slightly higher for the concentrations of chlorophylls and carotenoids with the Hue angle than with the a* and b* coordinates. The chlorophylls had a coefficient of determination of 0.896 with the Hue angle, while for the a* and b* coordinates, these were 0.867 and 0.773, respectively. The coefficient of determination of the carotenoids with the Hue angle was 0.804, while for the a* and b* coordinates, these were 0.711 and 0.695, respectively. However, for lutein this coefficient was slightly higher with the a* coordinate (0.816) than with the Hue angle (0.807) and the b* coordinate (0.663). With respect to β-cryptoxanthin, the coefficient of determination was lower than the previous results shown, so that with the a* coordinate it was 0.638, with b* it was 0.509, and with the Hue angle it was 0.631.

4. Discussion

Chlorophylls are the predominant pigments found in the skin of lemons when it is green (immature fruit), while the yellow color of the mature fruit is mainly due to carotenoids (Rodrigo et al., 2013). In this study, we have observed that the change from the green color to yellow (increase of the a* values) experienced by the Fino 49 lemon fruit during their maturation stage, was due to the degradation of the chlorophylls, in parallel to the low concentration of the carotenoid β-cryptoxanthin, which is made visible when the chlorophyll degrades. Thus, in the maturation stage of the fruit, a decrease in the concentration of the chlorophylls, from the highest values of 142 mg kg^{-1}–20 mg kg^{-1}, and a change in the a* coordinate during this decrease, from −9 to 5, was observed. Nevertheless, from this point on, the a* coordinate continued to increase once the concentration of

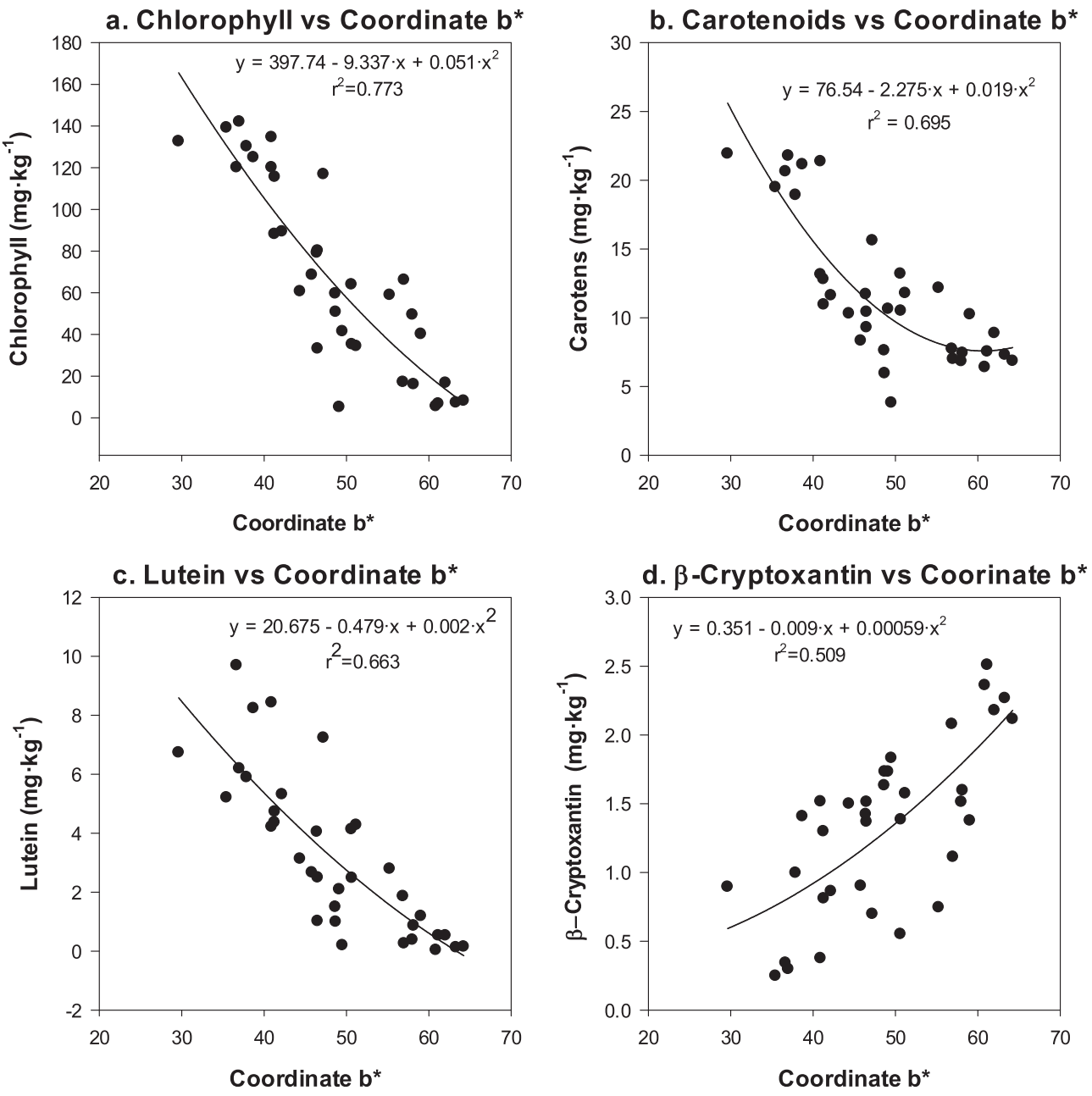

Fig. 5. Correlations between the b* coordinate and chlorophylls (5a), carotenoids (5b), Lutein (5c) and β-cryptoxanthin (5d) in the three years of study (2015/16, 2016/17 and 2017/18) for the IMIDA and CEBAS experimental orchards.

chlorophylls reached a stable value of 7 mg kg^{-1}, as this coordinate shifted towards the red color spectrum.

The yellow coloration observed in the lemon skin is due to the concentration of β-cryptoxanthin, although this compound, in greater concentrations than the one found in lemon fruits, usually results in an orange color. In other fruits such as oranges, where the concentration is higher, the orange color can be clearly observed. Initially, β-cryptoxanthin was found in the fruit in a low concentration of 0.30 mg kg^{-1} when the concentration of chlorophylls was still high, so that the yellow color was masked by the green color. Chlorophylls degradation, and the slight increase in the concentration of β-cryptoxanthin, which reached the low value of 2.51 mg kg^{-1}, resulted in that at the end of the maturation stage, the fruit acquired its typical yellow coloring. The changes of this latter pigment were well correlated with the changes of the a* coordinate throughout the period of maturation of the fruit (December). For the a* coordinate, ranging from −9.53 to 8.44, the values of β-cryptoxanthin were in the 0.4–2.51 mg kg^{-1} range.

It is also interesting to note that lutein, another of the carotenoids found to be present in the rind of lemon fruit, had little influence on the yellow color of the fruit. Although this pigment was found in greater quantities than β-cryptoxanthin, the degradation pattern was similar to chlorophylls. When the fruits were green, the concentration of lutein reached values between 7 and 9 mg kg^{-1}, but its color was masked by the chlorophylls. However, when these degraded, a decrease in lutein was also found, so that this pigment barely contributed to the color of the lemons, as shown in Table 1. In an experiment conducted in Hungary on different species of citrus (Agócs et al., 2007), it was observed that the main carotenoid in the lemon fruits was β-cryptoxanthin (19.9% with respect to the total), followed by lutein, with 8.3% (with respect to the total carotenoids). However, in our assay, lutein was barely found when the fruits had reached the characteristic yellow color.

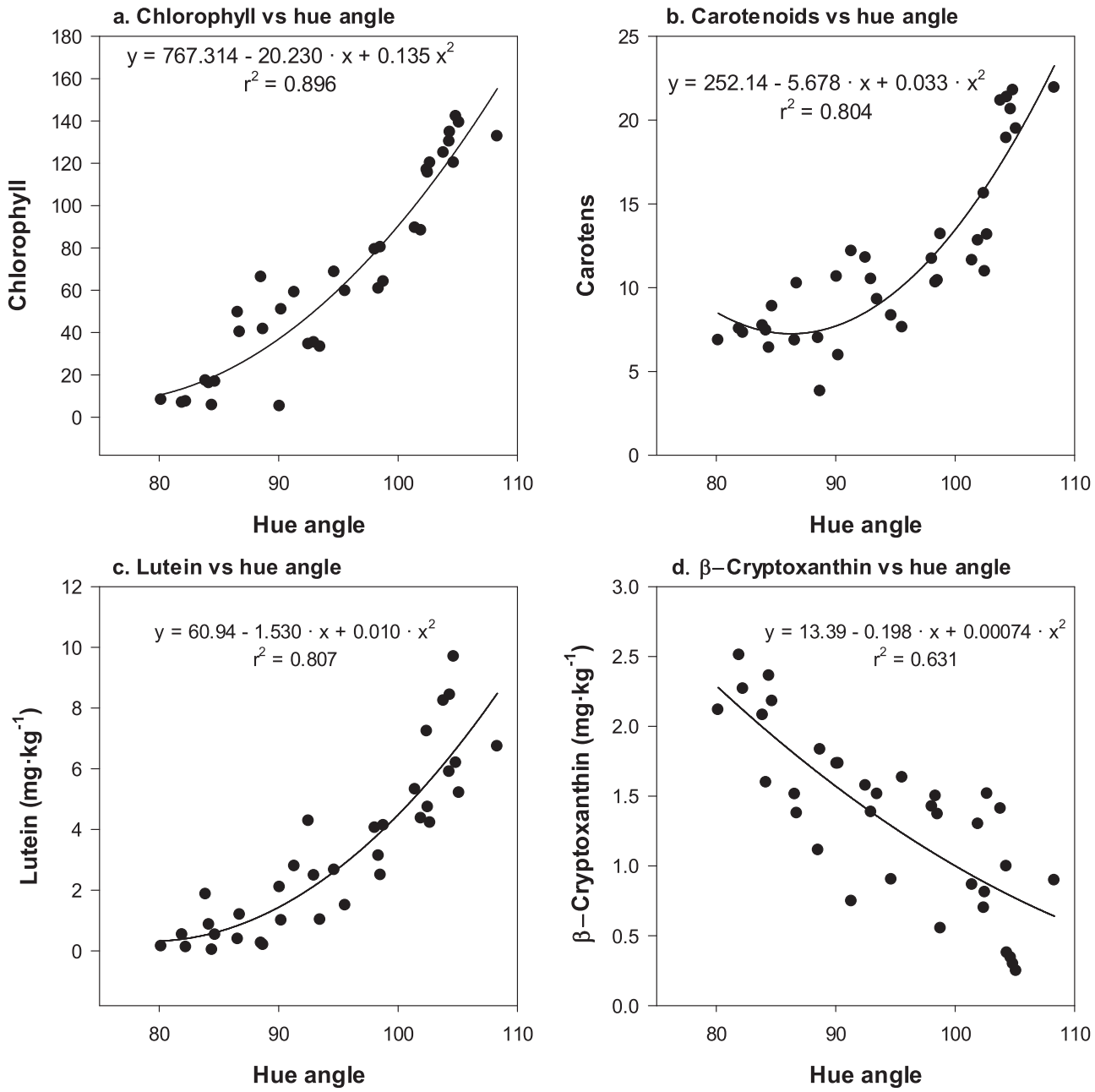

Fig. 6. Correlations between the Hue angle and the content of chlorophylls (6a), carotenoids (6b), lutein (6c) and β-cryptoxanthin (6d) during the period of color change of lemon fruits in the three years of study (2015/16, 2016/17 and 2017/18) for the IMIDA and CEBAS experimental orchards.

The regression study conducted between the different pigments and the color coordinates resulted in good second degree polynomic correlations for the concentration of chlorophylls, carotenoids and lutein with respect to all the color parameters (L, a*, b*, and Hue angle); however, for the concentration of β-cryptoxanthin, these were not well-adjusted as the rest of the parameters. Nevertheless, from all the parameters, Hue angle could be used to better estimate the concentration of these pigments, as indicated by its high r^2 values and its sensitivity, which encompassed the entire range where the concentrations were found. It seems logical that this parameter, which was calculated as arctan b*/a*, was the best, as it simultaneously utilized both the a* and b* color coordinates, and was thus able to correct possible deviations that could occur in these coordinates when they were used separately. For example, for the concentration of chlorophylls, the a* parameter stopped being ideal when it shifted towards values greater than 5, because from this point on, it moved to the red color spectrum.

5. Conclusions

In this experiment, we have shown, for three years of study and with two sites located in different geographical areas, that there is a good correlation between the CIELAB color coordinates and the composition of pigments in lemon fruits. The chlorophylls, carotenoids and lutein were strongly correlated with the a*, b* and Hue angle parameters, while β-cryptoxanthin had low correlations with these same parameters. Thus, in the lemon variety Fino 49, the color parameters could be used, preferably Hue angle, to estimate the concentration of pigments in the fruits, except for β-cryptoxanthin.

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