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Effect of the Technological and Agronomical Factors on Pigment Transfer during Olive Oil Extraction

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The aim of this work was to determine the transfer of the chloroplast pigment fractions during the virgin olive oil extraction process, in relation to different factors: the ripening stage of the olive fruits, the irrigation water applied to the olive tree, and the addition of natural microtalc (NMT) during the oil extraction process. Results showed that the percentage of chloroplast pigments transferred from the olive paste to the oil increases with the ripening of the olive fruit (raw material). An excess of the water irrigation applied to the olive tree shows a reduction in the biosynthesis of chloroplast pigments in olive fruits, which is reflected in a low concentration in the virgin oils. Furthermore, the percentage of pigment transfer from the olive paste to the oil during the extraction process is reduced by irrigation, mainly of the chlorophyll fraction. The addition of NMT during the malaxation step produced an increase in the percentage of the total pigments transferred from the olive paste to the oil, in relation to nonaddition.

KEYWORDS: Virgin olive oil; pigment transfer; chlorophylls; carotenoids; ripening index; irrigation; natural microtalc

INTRODUCTION

Among vegetable oils, virgin olive oils are rich in pigment compounds, the total amount of which depends largely on the cultivar, the degree of maturation, and the industrial processes employed for oil extraction (*1*−*3*). Environmental factors and agronomic practices, such as irrigation, can also affect the pigment composition of virgin olive oil (*4*).

The olive fruit, *Olea europaea*, a well-known and widespread species of the Oleacea family, is a green, fleshy, edible drupe. During the ripening process, the photosynthetic activity decreases and the concentrations of both chlorophylls and carotenoids decrease progressively. At the end of the maturation process, the violet or purple color of the olive fruit is due to the formation of anthocyanins (*1*, *2*, *5*).

In their natural environments, the olive fruit pigments are fairly stable. However, during virgin olive oil extraction, the crushing and malaxation processes break cell walls and expose the olive matrix to enzymes, oxygen, and mild heat. As a consequence, many chemical and enzymatic processes take place. Chlorophylls are transformed into their respective magnesium-free derivatives. Moreover, there is a considerable loss of chloroplast pigments, this being greater from the chlorophyll fraction than for the carotenoids (*6*). In addition to pigments transformations, mass partitioning phenomena occur that determine the pigment distribution between the solid and the liquid phases. During the malaxation step, the lipoprotein membranes, which surround the oil droplets, are removed and reformed repeatedly, resulting in a mutual exchange of components between the oil and the solid phase. On the other hand, such membranes bind the minute oil droplets to the water droplets. The vegetable colloids thus form stable emulsions, which cannot be isolated or removed by mechanical means. The emulsion is carried away with the byproducts, pomace, and vegetation water (*7*).

Some olive varieties (such as Leccino and Cassanese Italian cvs. or Picual and Hojiblanca Spanish cvs.) (*8*, *9*) and, in general, olive fruits, which are only slightly ripe, give rise to the so-called "difficult olive paste" during the oil extraction process. In such cases, part of the oil is lost with the residues, lowering the oil yield. To break the emulsion, the mixing time or temperature can be increased, but a loss of oil quality has been observed as a result. To avoid this problem, the addition of micronized talc (hydrated magnesium silicate with a particle size lower than 40 μm) was introduced in Spain in the late 1970s. It is eliminated during centrifugation together with the solid residue. The addition of talc helps to yield oil with lower water and suspended solid contents, as well as residues with a lower fat content (*10*).

Previous studies by our group have found a negative linear relationship between the irrigation water applied to olive tree and the chlorophyll and carotenoid contents of virgin olive oil (*4*) when linear irrigation strategies are applied. This reduction could be related to an effect on pigment biosynthesis in the olive fruit or pigment losses during the oil extraction process as a consequence of the major incidence of difficult olive pastes.

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There is little scientific information about the partitioning behavior of the olive fruit pigment compounds and hence their distribution between the oil and the solid fractions during the oil extraction process. A previous study by Gallardo-Guerrero et al. (*3*) showed that the loss of the olive pigments during the olive oil extraction process is mainly due to retention in the pomace, rather than destruction during the process. Furthermore, a greater proportion of the chlorophyllic fraction than the carotenoid fraction is transferred to the oil.

Thus, the aim of this work was to determine the transfer, considering the individual components, of the chloroplast pigment fractions (chlorophylls and carotenoids) during the virgin olive oil extraction process, between the olive paste, the pomace, and the oil in relation to two different agronomical factors: the ripening stage of the olive fruits and the irrigation water applied to the olive tree. An additional objective was to evaluate the effect on the partitioning of pigment compounds of adding technological natural microtalc (NMT) during the oil extraction process.

MATERIALS AND METHODS

Method for Picking Olive Fruit. The experiments were carried out during the 2003 olive harvest period in two commercial orchards located in the Segrià region (Catalonia, Spain). For each experiment, homogeneous batches of 3 kg of olive fruits from the *Arbequina* cultivar were recollected by hand.

Ripening Index (RI). The fruits for each sample were picked from all around the perimeter of the tree, selecting fruits with a similar skin color. The RI of the olive fruit was determined according to the guidelines of the Spanish National Institute of Agronomic Research (*11*), based on an evaluation of the olive skin and pulp colors. The RIs used in this study were as follows: green with reddish spots (group $N = 2$), black with <50% purple flesh (group $N = 5$), and black with >50% purple flesh (group $N = 6$).

Irrigation Experiment. The experiment was carried out during the olive harvest period (November, 2003). Homogeneous batches of olive fruit grown under nonirrigation (control) and irrigation treatments were handpicked at a similar RI. The experimental irrigation was based on a linear irrigation design in which the total applied irrigation water changed linearly with the effective crop efficient (K_c) used when the water budget method proposed by the FAO (*12*) was applied to determine the crop water requirements (ET_c). This used the reference crop evapotranspiration (ET_o) from an agricultural weather station and the effective crop coefficient (K_c) ($Etc = ET_o \times K_c$). The water budget method calculates the irrigation requirements by subtracting the effective precipitation (P_{ef}) from the ET_c. The irrigation treatment applied had a $K_c = 0.85$, corresponding to an approximate annual water application of 260 mm.

Olive Oil Extraction. The Abencor system (MC2 Ingeniería y Sistemas, Seville, Spain) was used to process the olives. The extraction process consisted of the following: The olives were crushed with a hammer mill, the olive paste obtained was malaxated at 28 ± 1 °C for 20 min, and then, a content of water of 300 g was added at 50 °C and homogenized for 10 min. Finally, centrifugation (1 min, 1445.5*g*) was performed by adding 100 g of water at 50 °C to obtain the byproducts: pomace, oil, and wastewater. The oil was separated from the wastewater by decantation, and all of the oil samples were filtered through an Ahlstrom paper filter with a porosity of 690 μm. Samples of olive paste and pomace were transferred into the glass recipients, and samples of olive oil were transferred into dark glass bottles; both were closed in nitrogen atmosphere and stored in the dark at 4 °C. To assess the mass balance of products and wastes from the Abencor in relation to olive paste, samples with the same RI or from the same irrigation strategy were processed in four lots.

To study the effect of the addition of a technological coadjuvant necessary in the process of oil extraction, the samples from the nonirrigation treatment (control) were malaxated with and without addition of NMT (10 g). All of the samples were processed in four batches.

Olive Paste and Pomace Analyses. The moisture and lipid contents of the solid phases were necessary to obtain a mass balance approach.

Moisture Content. Samples of 10 g of olive paste and pomace were weighed, then dried for 24 h at 105 °C, cooled for 30 min in a desiccator, and reweighed according to UNE Standard Spanish method (*13*).

Lipid Content. Dried samples of olive paste and pomace were measured in duplicate with a NMS 100 Minispec NMR Analyzer using ExpSpel Version 2.10 software (Bruker Analytik, Silberstreifen, Germany). The results were expressed as a percentage of oil obtained with respect to the raw material.

Pigment Extraction. Pigments were extracted from the olive fruit and the virgin olive oil following the procedure in Mínguez-Mosquera and Garrido-Fernández (*14*) with modifications (*15*). The method is based on a selective separation of components with *N*,*N*-dimethylformamide (DMF) and hexane. The *n*-hexane phase carried over lipids and the carotene fraction, while the DMF phase retained chlorophylls, chlorophyllic derivatives, and xanthophylls.

Solid Phases. Pigments were extracted from the olive fruit, olive paste, and pomace. Samples of 5−30 g were triturated for 1 min in a beater vessel containing 100 mL of DMF saturated with $MgCO_3$. This solution was filtered, and the operation was repeated until the filtrates were colorless. The extracts were combined and transferred to a funnel containing 70 mL of *n*-hexane where they were shaken and left until the phases had separated. The two phases, *n*-hexane and DMF, were treated separately according to the method described by Criado et al. (*15*). The dry residue was dissolved in 1 mL of acetone.

The final dried hexane residue was eluted in a known volume of hexane to measure the β-carotene concentration directly, using the coefficient of extinction $E_{454nm}^{1\%} = 2592$ (*16*). On the other hand, to identify the other isomers of carotene, the carotenoid pigments were saponified with the following extraction process. The *n*-hexane phases were mixed in a funnel and saponified with 100 mL of 15.6% KOH in methanol. After 1 h, distilled water was added and left until the phases had separated. The ether phase with the carotene fraction was washed three times with water and another three times with an aqueous solution of Na_2SO_4. It was concentrated by rotary evaporation at reduced pressure. The final residue was dissolved in 1 mL of acetone.

The chlorophyll and carotene extracts were stored in the dark in a freezer at −30 °C for high-performance liquid chromatography (HPLC) analyses. All extractions were performed in triplicate under a green light to prevent pigment alteration.

Olive Oil. The sample of virgin olive oil (15 g) was dissolved in 150 mL of DMF saturated with $MgCO_3$ and treated five times successively with 50 mL of *n*-hexane. Two phases, *n*-hexane and DMF, were also treated separately. The DMF phase was transferred to a 1000 mL funnel containing 400 mL of 2% sodium sulfate solution at approximately 0 °C. A 70 mL portion of *n*-hexane and another 70 mL portion of ethyl ether were added, and the mixture was shaken and kept until the phases had separated (about 20 min). The ether phase with the chloroplast pigments solution was washed three times with an aqueous solution of Na_2SO_4 (2%) at 0 °C. The ether was evaporated in a rotary evaporator at reduced pressure. The dry residue was dissolved in 1 mL of acetone.

The *n*-hexane phases were mixed in a 500 mL funnel containing 100 mL of ether and saponified with 100 mL of 15.6% KOH in methanol and strongly shaken to hydrolyze the lipids and purify the possible carotenoids. After 1 h, distilled water was added and kept until the phases had separated. The ether phase with the carotene fraction was washed three times with water to neutrality and another three times with an aqueous solution of Na_2SO_4. It was concentrated by rotary evaporation at reduced pressure. The final residue was dissolved in 1 mL of acetone. All extractions were performed in triplicate under a green light to prevent pigment alteration. The chlorophyll and carotene extracts were stored in the dark in a freezer at −30 °C awaiting the HPLC analysis according to Mínguez-Mosquera et al. (*17*) and Criado et al. (*15*).

HPLC Analysis of Pigments. The HPLC Water (Waters Inc., Milford, MA) system was made up of an autosampler (717 plus), a pump (600 E), a column heater module, and a photodiode array detector (996). The column was a Spherisorb ODS-2 column and a Spherisorb S5

Table 1. Total Mass Balance during the Olive Oil Extraction Process in Relation to the Ripening Stage of the Olive Fruit ($n = 4$)

Mass Balance (g)								
	input				output			
RI	olive paste	malaxation water	centrifugation water[a]	total	oil	wastewater	pomace	total
2	801.9	301.3	106.0	1209.2	99.5	363.3	718.6	1181.5
5	700.0	305.0	100.0	1105.0	102.5	332.2	636.8	1071.5
6	713.0	302.66	103.6	1119.2	154.4	215.4	706.6	1076.4

Mass Balance (%)									
	input				output				
RI	olive paste	malaxation water	centrifugation water[a]	total	oil	wastewater	pomace	total	losses (%)
2	66.3	24.9	8.8	100.0	8.2	30.1	59.4	97.7	2.3
5	63.3	27.6	9.1	100.0	9.3	30.1	57.6	97.0	3.0
6	63.7	27.0	9.3	100.0	13.8	19.2	63.1	96.1	3.9

[a] Water added to the vertical centrifuge.

ODS-2 precolumn (Teknokroma, Barcelona, Spain). Separation was performed using an elution gradient (flow rate = 2 mL/min) with the mobile phases (A) water/ion pair reagent (0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water)/methanol (1:1:8 v/v/v) and (B) methanol/acetone (1:1 v/v). Detection was performed at 430 (to measure chlorophyll *a*), 435 (to measure chlorophyllide *a*), 440 (for neoxanthin, violaxanthin, and esterified xanthophylls), 445 (for antheraxanthin, lutein, and α-carotene), 452 (β-cryptoxanthin), 454 (β-carotene), 466 (chlorophyll *b*), and 468 nm (to measure chlorophyllide *b*). All peaks were identified by their chromatographic and spectroscopic characteristics.

External standard calibration was used for quantification. Chlorophyll *a* [no. C-6144 from *Synechococcus nidulans* (Pringsheim) Komárek], chlorophyll *b* (no. C-5878 from *Spinacia oleracea* L.), and β-carotene (no. C-4582) were supplied by Sigma (St. Louis, MO). Chlorophyllides *a* and *b* were obtained from the respective solutions of chlorophylls by enzymatic de-esterification (*18*). The enzymatic extract of chlorophyllase was obtained from *Ailanthus altissima* (Miller) Swingle leaves (*19*). Lutein, violaxanthin, and neoxanthin were obtained from a pigment extract of fresh spinach (*S. oleracea*) and separated by thin-layer chromatography (TLC) on silica gel GF_{254} (0.2 mm) on 20 cm × 20 cm plates using petroleum ether (65−95 °C)/acetone/diethylamine (10:4:1 v/v/v) (*17*). Standards of antheraxanthin and β-cryptoxanthin were obtained from a saponified extract of red pepper (*Capsicum annuum*) using HPLC for separation and quantification (*20*).

Pigment Identification. Spectrophotometric and chromatographic properties permitted the identification of the pigments. The spectral absorption maxima in different solvents, peak ratios, and TLC and HPLC chromatography with authentic samples were used to identify pigments (*14*, *21*). Individual pigments of olive fruit, olive paste, pomace, and olive oil are expressed as mg/kg.

Extraction and Assay of Chlorophyllase Activity. The chlorophyllase activitiy in the crude enzyme extracts obtained from fruit from the irrigation experiment was assayed following the procedure described by Mínguez-Mosquera et al. (*22*) with minor modifications (*5*). The chlorophyllase assay mixture consisted of 100 mM Tris buffer (0.5 mL; pH 8.5) containing Triton-X 100 (2.4 g/L) and crude chlorophyllase extract (0.5 mL), and the reaction was initiated by adding 0.1 mL of a 0.1 μmol solution of chlorophyll *a* (Sigma). Eppendorfs were incubated at 40 °C in the dark for 3 h. The chlase enzymatic activity was expressed as nanomoles of substrate hydrolyzed per second (nmol/s). The results are expressed as specific activity, nmol/s/kg olive drupe.

Percentage of the Pigment Transferred during Oil Extraction Process. The percentage of the pigments transferred from the olive paste to the oil and the percentage retained in the pomace were calculated considering the total mass balance (inputs and outputs) during the olive oil extraction process, according to the expressions:

$$\%\ T\ \text{pomace} = (A/B) \times (C/D) \times 100 \quad (1)$$

where % *T* pomace = percentage of pigments transferred from the olive paste to the pomace, *A* = mg pigment/kg of pomace (output), *B* = mg pigment /kg of olive paste (input), *C* = kg of pomace (output), and *D* = kg of olive paste (input).

$$\%\ T\ \text{oil} = (A'/B') \times (C'/D') \times 100 \quad (2)$$

where % *T* oil = percentage of pigments transferred from the olive paste to the oil, A' = mg pigment/kg of oil (output), B' = mg pigment/kg of olive paste (input), C' = kg of oil (output), and D' = kg of olive paste (input).

The percentage of pigment loses by degradation during oil extraction process was calculated with the expression:

$$\%\ \text{losses} = 100 - (\%\ T\ \text{pomace} + \%\ T\ \text{oil}) \quad (3)$$

Statistical Analysis. Statistical procedures were carried out with the 8.02 version SAS System package (SAS Institute Inc., Cary, NC). Separation of the means was obtained using the least significant differences test, and the significant difference was defined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of the Olive RI on the Pigment Partitioning during the Oil Extraction Process. During the olive oil extraction process, the temperature and malaxation time were considered constants to assess the real influence of the RI of the olive fruit on pigment transference between the different phases. The olive fruits were crushed, and a destruction of the tissue structure occurred; then, the olive paste (input solid phase) obtained was malaxed to join in a continuous oily phase. Finally, centrifugation allowed the oil contained in the paste to be separated. The system has two inputs, olive paste and water, and three outputs, two of which are byproducts: pomace and wastewater (byproducts) and oil (**Table 1**). In the experimental process, the olive paste (raw material) was processed with water to obtain a complex matrix system. The input of water in the process was around 35% of the total material input. From the results shown in **Table 1**, it is clear that pomace represented the majority of the byproducts obtained, whereas the oil was under 14% in all three samples. The higher the RI of the olive fruit is, the greater the oil yield in the process (*23*).

The individual chloroplast pigments identified and their concentrations in the different fractions (olive paste, pomace, and olive oil), in relation to the RI of the olive fruit, are shown in **Table 2**. The qualitative lipid-soluble pigment profile of the olive paste was made up of chlorophyll *a*, chlorophyll *b*, and

Table 2. Pigment Concentration (Chlorophylls and Carotenoids) in Olive Paste, Pomace, and Olive Oil (Expressed as mg/kg) in Relation to the RI of the Olive Fruit ($n = 4$)

	RI								
	olive paste[a]			pomace[a]			olive oil[a]		
pigment	2	5	6	2	5	6	2	5	6
chlorophyll *a*	8.68 a	2.3 b	0.596 c	7.20 a	1.07 b	0.422 c	5.83 a	2.09 b	0.445 c
chlorophyll *b*	4.56 a	1.3 b	0.425 c	3.83 a	0.876 b	0.342 c	0.711 a	0.308 b	0.073 c
chlorophyllide *a*	0.022	ND	ND	0.097	ND	ND	ND	ND	ND
chlorophyllide *b*	ND	ND	ND	0.018	ND	ND	ND	ND	ND
pheophytin *a*	3.51 a	0.852 b	0.249 c	2.52 a	0.804 b	0.357 c	1.16 a	0.230 b	0.101 b
pheophytin *b*	0.023 a	0.019 a	ND	0.033 a	0.016 b	0.006 b	0.020	ND	ND
pheophorbide *a*	0.675 a	0.160 b	0.060 c	1.01 a	0.227 b	0.083 c	0.015 a	0.007 a	ND
total chlorophylls	17.5 a	4.63 b	1.33 c	14.7 a	2.99 b	1.21 c	7.74 a	2.63 b	0.619 c
lutein	2.67 a	1.42 b	0.729 c	2.15 a	0.945 b	0.480 c	3.04 a	2.11 b	1.02 c
all-*trans*-β-carotene	0.844 a	0.450 b	0.300 b	0.671 a	0.303 b	0.151 c	1.65 a	0.661 b	0.401 b
neoxanthin	0.327 a	0.155 b	0.054 b	0.255 a	0.066 b	0.026 b	0.254 a	0.124 b	0.025 c
violaxanthin	0.264 a	0.107 b	0.054 b	0.149 a	0.012 b	0.012 b	1.07 a	0.508 b	0.118 c
antheraxanthin	0.264 a	0.113 b	0.055 b	0.165 a	0.025 b	0.013 b	0.767 a	0.310 b	0.102 c
luteoxanthin	0.111 a	0.034 b	0.015 b	0.069 a	0.004 b	0.004 b	ND	ND	ND
mutatoxanthin	0.047 a	0.021 b	ND	0.046 a	0.018 b	0.003 c	ND	ND	ND
β-cryptoxanthin	0.082 a	0.074 a	0.024 b	0.050 a	0.003 b	0.003 b	0.159 a	0.065 b	0.024 c
esterified xanthophylls	0.123 a	0.114 a	0.035 b	0.077 a	0.024 b	0.012 b	0.384 a	0.177 b	0.075 c
cis-α-carotene	0.007 a	0.004 a	tr	0.003 a	0.001 a	tr	0.014 a	0.003 b	tr
total carotenoids	4.74 a	2.49 b	1.27 c	3.63 a	1.40 b	0.704 c	7.34 a	3.96 b	2.03 c
total pigments	22.2 a	7.12 b	2.60 c	18.3 a	4.39 b	1.91 c	15.1 a	6.59 b	2.65 c
chlor *a*/chlor *b*[b]	1.90	1.77	1.40	1.88	1.22	1.23	8.20	6.79	6.10
chlor/carot[c]	3.69	1.86	1.05	4.05	2.14	1.72	1.05	0.664	0.305
lutein/β-carotene	3.16	3.16	2.43	3.20	3.12	3.18	1.84	3.19	2.54

[a] Different letters in the same row indicate significant differences between RIs at $p < 0.05$. [b] Chlorophyll *a*/chlorophyll *b* ratio. [c] Chlorophyll/carotenoid ratio; ND, not detected; tr, trace amounts.

the carotenoids that typically accompany the chlorophylls in the chloroplast, such as lutein, all-*trans*-β-carotene, neoxanthin, violaxanthin, and antheraxanthin. A remarkable fact is that although pheophytins *a* and *b* and pheophorbide *a* are not detected naturally in fruit from the *Arbequina* cultivar (*5*, *15*, *24*), these chlorophyll derivatives were detected in the olive paste (**Table 2**). They are probably formed from the olive fruit native chlorophylls during crushing as a consequence of the cell rupture and release of acids and tissue enzymes (mainly chlorophyllase). Similarly, luteoxanthin and mutatoxanthin are derivative pigments from native violaxanthin and antheraxanthin associated with the acidic medium of the oil extraction process. Besides these, additional characteristic pigments of the *Arbequina* cultivar were quantified. These included *cis*-α-carotene, β-cryptoxanthin, esterified xanthophylls, and chlorophyllides *a* and *b*. Finally, the presence of the pheophorbide *a* should be a consequence of the chlorophyllase activity observed in fruit from the *Arbequina* cultivar (*5*, *25*). From a qualitative viewpoint, all of the pigments that were found in the olive paste had been transferred to the oil, with the exception of chlorophyllides *a* and *b* (products of the enzymatic deesterification of the alcohol phytol in the molecules of chlorophyll) and luteoxanthin and mutatoxanthin (acidic derivatives).

Chlorophylls *a* and *b* were the major pigments in olive pastes from green reddish spotted fruit (RI, 2) (**Table 2**), and the chlorophyll content was four times higher than carotenoid, as can been observed in the chlorophyll/carotenoid ratio. However, in olive pastes from fruits with the more advanced RI (RI 6), the relation between both fractions decreased to near one, and lutein was the more important pigment. The ratio between lutein and β-carotene also decreased with the ripening of the olive fruit. These tendencies related to the RI of the olive were similar in the pomace and oil. All of the pigments that were found in the olive paste had been occluded in pomace and transferred to the oil.

In general, pigment retention in pomace was high, and this means important losses of the olive fruit pigment during virgin olive oil extraction process. Additionally, the losses of pigment by degradation are important, and as a consequence, the percentage of pigments from olive fruit in virgin olive oils is relatively low.

The percentage of the pigments transferred from the olive paste (100% as reference) to the oily phase (oil) and the percentage of pigments retained in the pomace (byproduct) are shown in **Table 3**, considering the three RIs of the original olive fruit (raw material). Additionally, the pigment losses associated with the degradation during the oil extraction process are shown. Water and oil balances were performed to observe their distribution of each component in all of the out products/byproducts (**Table 1**). The percentage of transference between phases was calculated according to the expressions (*1*−*3*) described in the Material and Methods.

The results showed higher pigment transference from olive paste to the oil with the more advance ripening stage of the olive fruit, independently of the pigment content. It is observable that pigment retention in the pomace phase was high, mainly in the process corresponding to fruit with a RI of 2 (green with reddish spots). On the other hand, the transfer of the carotenoid fraction to the oil was higher than that of the chlorophyll fraction for all of the ripening stages (**Table 3**). At the same time, important losses from degradation were observed, mainly in the process that corresponded to RI 5. In relation to the chlorophyll fraction, the percentage of chlorophyll *a* transferred to the oil was higher than that of chlorophyll *b*, which was probably related to the more important degradation during the oil extraction process. A previous study with olive fruit from the *Arbequina* cultivar (*5*) showed a slight increase in the chlorophyllase activity at the latest stages of ripening. That could explain the higher pigment degradation in olive pastes from RI of 5 (<50% purple flesh). Different studies have focused on

Table 3. Percentage of the Olive Paste Pigments (100%) Retained in the Pomace, Transferred to the Olive Oil, and Degraded (Losses) during the Virgin Olive Oil Extraction Process in Each Ripening Stage of the Olive Fruit (Raw Material)

	RI								
	2			5			6		
pigment (%)	pomace	oil	losses	pomace	oil	losses	pomace	oil	losses
chlorophyll *a*	75.1	8.4	16.5	42.3	13.3	44.4	68.2	15.9	15.9
chlorophyll *b*	75.9	2.0	22.1	61.3	3.5	35.2	77.7	3.6	18.7
total chlorophylls[a]	75.4	6.2	18.4	49.2	9.8	41.0	72.1	10.8	17.1
lutein	72.9	14.2	12.9	60.6	21.7	17.7	63.3	29.9	6.8
all-*trans*-β-carotene	71.9	24.5	3.6	61.3	21.6	17.1	47.2	28.0	24.8
minor carotenoids[b]	59.4	31.2	9.4	21.1	30.7	48.2	32.5	33.1	34.4
total carotenoids	69.7	20.1	10.2	51.6	23.8	24.6	54.4	30.2	15.4
total pigments	74.1	9.8	16.1	50.3	15.4	34.3	62.5	21.5	16.0

[a] Sum of chlorophylls *a* and *b*. [b] Sum of neoxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, and esterified xanthophylls.

Table 4. Pigment Concentration (Chlorophylls and Carotenoids) in Olive Fruit, Olive Paste, Pomace, and Olive Oil (Expressed as mg/kg) Comparing Olives from Nonirrigated (N-IR) and Irrigated (IR) Olive Trees ($n = 4$)[a]

	olive fruit		olive paste		pomace		olive oil	
pigment	N-IR	IR	N-IR	IR	N-IR	IR	N-IR	IR
chlorophyll *a*	2.17 a	0.195 b	2.3 a	0.484 b	1.47 a	0.246 b	2.64 a	0.242 b
chlorophyll *b*	0.771 a	0.098 b	1.3 a	0.356 b	1.05 a	0.287 b	0.409 a	0.014 b
chlorophyllide *a*	ND	ND	ND	ND	ND	ND	ND	ND
chlorophyllide *b*	ND	ND	ND	ND	ND	ND	ND	ND
pheophytin *a*	ND	ND	0.852 a	0.497 b	0.681 a	0.633 a	0.299	ND
pheophytin *b*	ND	ND	0.023 a	0.013 a	0.011 a	0.013 a	ND	ND
pheophorbide *a*	ND	ND	0.160 a	0.020 b	0.241 a	0.015 b	0.0089	ND
total chlorophylls	2.94 a	0.293 b	4.64 a	1.37 b	3.45 a	1.19 b	3.36 a	0.256 b
lutein	0.853 a	0.516 b	1.42 a	0.354 b	0.934 a	0.272 b	2.44 a	0.870 b
all-*trans*-β-carotene	0.391 a	0.136 b	0.450 a	0.222 b	0.225 a	0.175 b	1.07 a	0.175 b
neoxanthin	0.053 a	0.007 b	0.155 a	0.021 b	0.067 a	0.014 b	0.172 a	0.008 b
violaxanthin	0.134 a	0.007 b	0.107 a	0.013 b	0.015 a	0.011 a	0.610 a	0.031 b
antheraxanthin	0.077 a	0.010 b	0.113 a	0.012 b	0.025 a	0.011 a	0.374 a	0.027 b
luteoxanthin	ND	ND	0.034	ND	0.0039	ND	ND	ND
mutatoxanthin	ND	ND	0.021	ND	0.015	ND	ND	ND
β-cryptoxanthin	0.0046	ND	0.074	ND	0.015	ND	0.077	ND
esterified xanthophylls	ND	ND	0.114	ND	0.014	ND	0.193 a	0.007 b
cis-α-carotene	tr	ND	0.0038	ND	0.0011	ND	0.0068	ND
total carotenoids	1.51 a	0.676 b	2.49 a	0.622 b	1.32 a	0.483 b	4.94 a	1.12 b
total pigments	4.45 a	0.973 b	7.13 a	1.99 b	4.77 a	1.67 b	8.30 a	1.38 b
chlor a/chlor b[b]	2.82	1.99	1.77	1.36	1.40	0.857	6.45	17.29
chlor/carot[c]	1.95	0.433	1.86	2.20	2.61	2.46	0.680	0.229
lutein/β-carotene	2.18	3.79	3.16	1.59	4.15	1.55	2.28	4.97

[a] Different letters in the same row indicate significant differences between nonirrigated and irrigated at $p < 0.05$. [b] Chlorophyll *a*/chlorophyll *b* ratio. [c] Chlorophyll/carotenoid ratio; ND, not detected; tr, trace amounts.

the possible role of this enzyme in the degradation of chlorophylls (*26*). With regard to the carotenoid fraction, while the percentage of transference of all-*trans*-β-carotene is higher than lutein in oils from RI 2 (green with reddish spots) in oils from the more advanced RI (6, >50% purple flesh), the percentage of transference of lutein was higher. These results do not fully agree with the results in Gallardo-Guerrero et al. (*3*) in oils from *Hojiblanca* cultivar, in which a greater destruction of lutein is observed. On the other hand, in this study, no effect of the RI on the transference of minor carotenoids was observed.

As a conclusion to the present study, it is possible to affirm that an important percentage of the pigments (chlorophylls and carotenoids) present in olive paste is retained in the pomace, independently of the ripening stage of the olive fruit (raw material). Similarly, the percentage of the total pigment transferred to the oil ranging between 9.8 and 21.5%, showing an increase in the percentage of transference with the ripening stage of the olive fruit independently of the minor pigment content in fruit from the more advanced ripening stages. The most important losses through degradation during oil extraction corresponded to the ripening stage of 5, which is probably related to the more important chlorophyllase activity in the original olive fruit (raw material) (*5*).

Effect of Irrigation Treatment on the Partition of Pigment Compounds during the Oil Extraction Process. Table 4 shows the pigment content of olive fruit, olive paste, pomace, and oil from nonirrigated and irrigated olive trees. The results showed minor qualitative differences in the olive fruit pigment profiles between the samples from irrigated trees and the samples from nonirrigated trees. These differences are related to the presence of *cis*-α-carotene (traces) and a low quantity of β-cryptoxanthin in fruits from the nonirrigated treatment. The pigment characteristics of the *Arbequina* cultivar (chlorophyllides *a* and *b*, esterified xanthophylls, and *cis*-α-carotene) were not detected in this experiment. However, significant quantitative differences were observed that showed an effect of the water application to the olive tree on the biosynthesis of chloroplast pigments in the olive fruit. Thus, the fruits from nonirrigated treatments showed significantly higher total pigment contents than fruits from irrigated treatments (4.45 and 0.973 mg/kg of oil, respectively) (**Table 4**). Chlorophylls *a* and *b* and lutein were the most abundant pigments, and their concentration was

Table 5. Total Mass Balance during the Olive Oil Extraction Process, Comparing Olives from Nonirrigated (N-IR) and Irrigated (IR) Olive Trees ($n = 4$)

	Mass Balance (g)							
	input				output			
	olive paste	malaxation water	centrifugation water[a]	total	oil	wastewater	pomace	total
N-IR	692.5	305.0	107.5	1105.0	35.4	368.5	585.0	1088.9
IR	681.0	298.4	100.0	1079.4	58.6	497.1	510.8	1066.4

	Mass Balance (%)								
	input				output				
	olive paste	malaxation water	centrifugation water[a]	total	oil	wastewater	pomace	total	losses (%)
N-IR	62.7	27.6	9.7	100.0	12.3	33.4	52.9	98.6	1.4
IR	63.1	27.6	9.3	100.0	5.4	46.1	47.3	98.8	1.20

[a] Water added to the vertical centrifuge.

significantly higher in olive fruits from nonirrigated trees. Another remarkable difference between irrigation and nonirrigation was the chlorophyll *a*/chlorophyll *b* relationship. In higher plants, this relation is usually 3 to 1 and constitutes a parameter of the physiological status. Chlorophyll *b* is a constituent of the light-harvesting system, and the reaction centers are rich in chlorophyll *a* (*26*, *27*). It is thus possible to deduce that the higher water availability in the olive tree could be one of the factors responsible for a greater development of the light-harvesting complex, relatively rich in chlorophyll *b*.

As far as the olive fruit carotenoid content was concerned, the ratio of lutein/β-carotene (2.18 and 3.79 for fruits from nonirrigated and irrigated treatments, respectively) could indicate that the biosynthesis of β-carotene may be affected by irrigation more than lutein biosynthesis; this ratio is maintained in the olive oil fraction. Qualitatively, all of the pigments that were found in the fruit had been quantified in the solid phases (olive paste and pomace) and transferred to the oils. However, the acids released during the crushing of the fruit to olive paste meant that a proportion of the native chlorophylls were transformed into pheophytins. Besides these acidic conditions, the chlorophyllase activity favored the presence of pheophorbide *a*. In spite of the higher chlorophyllase activity quantified in fruits from irrigated trees (17.54 nkat/kg olive fruit) in comparison with fruits from nonirrigated trees (10.65 nkat/kg olive fruit), the phephorbide *a* concentration in the olive paste and pomace from nonirrigated treatments was higher. This fact could be related to the higher subtract concentration (pheophytin *a*) in the olive paste from nonirrigation treatment that favors the enzyme activity.

The acidic conditions during the milling of the fruit also favor the presence of acid derivatives of carotenoids, such as luteoxanthin and mutatoxanthin, as well as esterified xanthophylls and β-cryptoxanthin, which were only quantified in the olive paste and pomace from nonirrigation treatment.

The olive oil pigment profile and content reflect the effect of tree irrigation on olive fruits (**Table 4**). From a qualitative viewpoint, all of the pigments that were quantified in the olive pastes had been transferred to the oils. However, the oils from irrigated treatment showed a very low pigment concentration. These results agree with those from a previous study by Tovar et al. (*4*) that found a negative linear relationship between the pigment content of the oil and the water applied to the olive tree, which was attributed to the higher water content of the olive fruit from more heavily irrigated treatments. Consequently, the olives pass quickly through the hammercrush sieves during the crushing, suffering less tissue damage and a reduction in the extraction of the pigments. Besides this, the present study shows a relationship between the water application to the olive tree and a reduction in the biosynthesis of chloroplast pigments in olive fruits, which is reflected in a low concentration in the virgin oil.

Table 6. Percentage of the Olive Paste Pigments (100%) Retained in the Pomace, Transferred to the Olive Oil, and Degraded (Losses) during the Virgin Olive Oil Extraction Process, Comparing Olives from Nonirrigated and Irrigated Olive Trees

	nonirrigated			irrigated		
pigment (%)	pomace	oil	losses	pomace	oil	losses
chlorophyll *a*	54.1	22.5	23.4	38.2	4.2	57.6
chlorophyll *b*	68.2	6.1	25.7	60.8	0.3	38.9
total chlorophylls[a]	59.0	16.6	24.4	47.7	2.6	49.7
lutein	55.5	35.6	8.9	57.7	21.2	21.1
all-*trans*-β-carotene	42.3	46.5	11.2	58.9	6.6	34.5
minor carotenoids[b]	20.5	49.7	29.8	58.1	12.9	29.0
total carotenoids	45.1	39.8	15.1	58.1	15.4	26.5
total pigments	53.4	25.8	20.8	52.2	8.0	39.8

[a] Sum of chlorophylls *a* and *b*. [b] Sum of neoxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, and esterified xanthophyl.

Total mass balance during the olive oil extraction process was performed to observe the distribution of pigments (chlorophylls and carotenoids) in all of the out products/byproducts (**Table 5**). The results showed an important influence of tree irrigation on the oil yield. Olives from nonirrigation had an oil yield of 12.25%, whereas the olives from irrigation had a yield of 5.43%. Similarly, the wastewater percentage was higher in the irrigation experiment, probably related to the higher water content in the olives that supposes a more fluid olive paste that makes the separation of the solid/liquid phases during vertical centrifugation difficult.

Considering the mass balance, the percentage of transference of pigments between phases (**Table 6**) was calculated according to the expressions (eqs 1−3) described in the Materials and Methods. The percentage of the pigment transfer, mainly the chlorophyll fraction, from the olive paste to the oil was affected by irrigation. During the oil extraction process, only 8.0% of the total pigments in the olive paste from irrigated trees was transferred to the oil and 52.2% was retained in the pomace, while 25.8% of the total pigments in pastes from nonirrigated olives was transferred to the oil. The most remarkable was the

Table 7. Pigment Concentration (Chlorophylls and Carotenoids) in Olive Paste, Pomace, and Olive Oil (Expressed as mg/kg) Comparing the Oil Extraction Process without (NMT–) and with (NMT+) Addition of NMT ($n = 4$)[a]

pigment	olive paste	pomace		olive oil	
		NMT–	NMT+	NMT–	NMT+
chlorophyll *a*	2.30	1.07 a	1.47 b	2.09 a	2.64 b
chlorophyll *b*	1.30	0.876 a	1.05 b	0.308 a	0.409 b
chlorophyllide *a*	ND	ND	ND	ND	ND
chlorophyllide *b*	ND	ND	ND	ND	ND
pheophytin *a*	0.852	0.804 a	0.681 b	0.230 a	0.299 b
pheophytin *b*	0.019	0.016 a	0.011 a	ND	ND
pheophorbide *a*	0.160	0.227 a	0.241 a	0.0065 a	0.0089 a
total chlorophylls	4.63	2.99 a	3.45 b	2.63 a	3.36 b
lutein	1.42	0.945 a	0.934 a	2.11 a	2.44 b
all-*trans*-β-carotene	0.450	0.303 a	0.225 a	0.661 a	1.07 b
neoxanthin	0.155	0.066 a	0.067 a	0.124 a	0.172 b
violaxanthin	0.107	0.012 a	0.015 a	0.508 a	0.610 b
antheraxanthin	0.113	0.025 a	0.025 a	0.310 a	0.374 b
luteoxanthin	0.034	0.004 a	0.004 a	ND	ND
mutatoxanthin	0.021	0.018 a	0.015 a	ND	ND
β-cryptoxanthin	0.074	0.003 a	0.015 b	0.065 a	0.077 a
esterified xanthophylls	0.114	0.024 a	0.014 a	0.177 a	0.193 a
cis-α-carotene	0.004	0.0008 a	0.0011 a	0.0032 a	0.0068 b
total carotenoids	2.49	1.40 a	1.32 a	3.96 a	4.94 b
total pigments	7.12	4.39 a	4.77 b	6.59 a	8.30 a
chlor a/chlor b[b]	1.77	1.22	1.40	6.79	6.45
chlor/carot[c]	1.86	2.14	2.61	0.664	0.680
lutein/β-carotene	3.16	3.12	4.15	3.19	2.28

[a] Different letters in the same row indicate significant differences between NMT– and NMT+ at $p < 0.05$. [b] Chlorophyll *a*/chlorophyll *b* ratio. [c] Chlorophyll/carotenoid ratio; ND, not detected.

percentage of pigment losses by degradation, mainly in the irrigation experiment. These results may be due to the higher chlorophyllase activity detected in olive fruits from irrigated trees, which could be responsible for the higher chlorophyll degradation during the oil extraction process. It is important to consider that the irrigation experiment in this study corresponded with an effective crop coefficient (K_c) of 0.85. Preliminary studies by Tovar et al. (*4*) have shown that the excess of irrigation water applied to the olive tree can determine important reductions in the chlorophyll and carotenoid contents of the oil. As a consequence, it is important to find a balance between the oil yield and the concentration of minor components in the oil before selecting the irrigation regime.

Effect of Adding NMT on the Partition of Pigment Compounds. During the oil extraction process, stable emulsions between oil droplets, water droplets, and vegetable colloids (made up of hemicellulose, protein, pectin, etc.) are formed. The malaxation operation, inducing coalescence phenomena, causes the minutely bound oil droplets to merge into large drops, thus separating them from both colloids and water droplets. In addition, this step disrupts a proportion of the oily cells remaining uncrushed from the first step (crushing), allowing the recovery of another oil fraction (*7*). The formation of emulsions that are too stable to be removed by mechanical means may be involved in the loss of pigments during processing, mainly with olive pastes from trees with noncontrolled irrigation strategies. The addition of NMT may reduce the stability of the emulsion by absorbing part of the water and thus reducing the complexation of pigments trapped in the emulsion and improving their release in the oil during the extraction process.

The pigment concentration of pomace and olive oil from extraction process without and with addition of NMT is shown in **Table 7**. Not all of the pigments were affected in the same way by the addition of NMT during the malaxation step. Among the carotenoids, there were no significant changes in the retention in pomace; however, the transference to the oil was significantly higher with the addition of NMT. On the other hand, a significant increase in the transference of the more important components of the chlorophyll fraction (mainly chlorophylls *a* and *b*) and carotenoids was observed by addition of the NMT. Considering the pigment transfer as percentage, the addition of NMT during the malaxation step showed an increase in the percentage of the total pigments transferred from the olive paste to the oil and retained in the pomace (byproduct), in relation to nonaddition (**Table 8**). Similarly, the losses by degradation during the oil extraction process were reduced by NMT, mainly chlorophyll *a* degradation. Among the carotenoid fraction, transference of all components was significantly increased by the NMT, especially all-*trans*-β-carotene; there was also a significant reduction in the losses through degradation. After the comparison of the results obtained from the experiments with and without NMT addition, it was concluded that the use of this coadjuvant increases the affinity of chloroplast pigments (chlorophylls and carotenoids) for the oily phase and reduces the pigment losses by degradation during the different phases of the oil extraction process.

Table 8. Percentage of the Olive Paste Pigments (100%) Retained in the Pomace, Transferred to the Olive Oil, and Degraded (Losses) Comparing the Oil Extraction Process without (NMT–) and with (NMT+) Addition of NMT

pigment (%)	NMT–			NMT+		
	pomace	oil	losses	pomace	oil	losses
chlorophyll a	42.3	13.3	44.4	54.1	25.5	23.4
chlorophyll b	61.3	3.5	35.2	68.2	6.1	25.7
total chlorophylls[a]	49.2	9.8	41.0	59.0	16.6	24.4
lutein	60.6	21.7	17.7	55.5	35.6	8.9
all-*trans*-β-carotene	61.3	21.6	17.1	42.3	46.5	11.2
minor carotenoids[b]	21.1	30.7	48.2	20.5	49.7	29.8
total carotenoids	51.6	23.8	24.6	45.1	39.8	15.1
total pigments	50.2	15.5	34.3	53.4	25.8	20.8

[a] Sum of chlorophylls *a* and *b*. [b] Sum of neoxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, and esterified xanthophyl.

Different conclusions can be drawn from the factors evaluated and the results observed in this study. The percentage of chloroplast pigments transferred from the olive paste to the oil increases with the ripening of the olive fruit (raw material). The degradation of the chlorophyllic fraction during the oil extraction process is higher than that of the carotenoid fraction, resulting in a higher carotenoid transference to oil.

On the other hand, an excess of water irrigation applied to the olive tree shows a reduction in the biosynthesis of chloroplast pigments in olive fruits that are reflected in a low concentration in the virgin olive oils. Furthermore, the percentage of pigment transfer from the olive paste to the oil during the extraction process is reduced by mainly the chlorophyll fraction, and the pigment degradation is increased probably as a consequence of the higher chlorophyllase activity quantified in olives from the irrigated treatment. Finally, the addition of NMT to "difficult pastes" during the malaxation step produced an increase in the percentage of the total pigments transferred from the olive paste to the oil and retained in the pomace (byproduct), in relation to nonaddition.

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