Thermal Degradation Kinetics of Neoxanthin, Violaxanthin and Antheraxanthin in Virgin Olive Oils.

RAMÓN APARICIO-RUIZ AND BEATRIZ GANDUL-ROJAS*

Chemistry and Biochemistry Pigment Group. Department of Food Biotechnology.

Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain.

*Corresponding author

Telephone: 34-954691054

Fax: 34-954691262

E-mail: gandul@cica.es

ABSTRACT

A first-order kinetic mechanism was appropriate for describing the thermal degradation of epoxy xanthophylls in virgin olive oil (VOO). Consecutive reactions that involve reorganization of 5,6-epoxide groups to 5,8-furanoxide groups and subsequent rupture of the polyene chain occurred in the degradation pathways. Thermal stability was significantly affected by changes in the chemical structure (epoxy to furanoid structure), being the greatest stability for neoxanthin. A true kinetic compensation effect was found in a series of similar reactions, that is the degradation of 5,8-furanoxides into colorless products. An isokinetic study in different VOO matrices showed that the oily medium did not significantly affect the reaction mechanisms. Consequently, the kinetic parameters obtained as temperature functions according to the Arrhenius model can be used to develop a prediction mathematical model for 5,8-furanoxide xanthophylls in VOO over time. The potential usefulness of the parameter neoxanthin/neochrome ratio is discussed as a chemical marker of heat treatment in VOO.

Keywords: Virgin olive oil; carotenoids; isomerization, xanthophylls; thermal degradation; kinetics; Arrhenius parameters; isokinetic effect, thermal stability

INTRODUCTION

The main biological function of carotenoids in photosynthetic organisms is energy transfer in photosynthesis and photoprotection[1]. Among the carotenoids, in addition to β-carotene and lutein, 5,6-epoxy xanthophylls such as neoxanthin and violaxanthin are widely distributed in the photosynthetic organs of higher plants[2]. In mammals, which can incorporate carotenoids only through diet, the only so far known biological function of some carotenoids is their role as vitamin A precursors. The nutritional importance of this biological function has been studied for years and is still of interest today[3, 4]. Certain physiological responses following the ingestion of food or dietary supplements rich in carotenoids have been observed. These responses are known as biological activities, which have raised the interest of the scientific community in the context of improving health through diet and developing functional foods. These include antioxidant activity and its associated benefits in preventing degenerative diseases[4].

Carotenoids must be bioavailable to express these biological activities in tissues, i.e., they must be transferred from the food matrix to the bloodstream to be metabolised and/or stored by the body. In addition to the individual's physiological factors, many dietary factors will determine their bioavailability[5]. These include the characteristics of the food matrix[6] and the various technological alternatives for obtaining and/or preserving food, which may influence the type and proportion of carotenoid derivatives formed.

Virgin olive oil (VOO) is considered to be a healthy fat. Its beneficial properties are attributed mainly to its proper fatty acid composition. Recently, however, benefits from other minor compounds in VOO with vitamin E (tocopherols) and provitamin A (β-carotene and β-cryptoxanthin) functions have been reported, and other with potential biological activities as antioxidants (phenols, carotenoids, chlorophylls, squalene) or hypolipemiants (β-sitosterol) have been suggested[7]. Virgin olive oil is obtained from the olive fruit using only physical procedures under conditions, especially thermal, which do not involve alteration of the oil[8]. Thus, the composition of bioactive compounds that are transferred from the fruit remain potentially intact in

virgin olive oil. In terms of carotenoids, VOO mainly contains lutein and β-carotene, although there are also β-cryptoxanthin and 5,6-epoxy xanthophylls such as neoxanthin, violaxanthin, antheraxanthin and their furanoxides[9].

Carotenoids are susceptible to some reactions such as isomerization (*trans* to *cis*) and oxidation during food processing and storage due to the carbon-carbon double bonds of the polyene chain. Therefore, they react easily with acids, light, heat, and oxygen causing loss of colour and reduction of biological activity[10, 11]. Thus, these factors should be properly controlled to maximize carotenoids retention during storage. In the case of isomerisation, the *trans*-isomers are more common and stable in natural foods whereas *cis*-isomers are usually formed during food processing[12]. Organic acids liberated during the processing of fruit juices are strong enough to promote rearrangements of 5,6-epoxide groups to 5,8-furanoxide groups of carotenoids[13,14]. Therefore, the stability of carotenoids in foods varies greatly[15].

During the mechanical process of extracting virgin olive oil, a total transfer of carotenoids from the fruit to the oil does not occur despite their lipophilic character. A high percentage remains in the *alperujo* (a subproduct from the olive oil extraction process), whereas some of it undergoes oxidation to colorless products[16]. The other structural changes of carotenoids associated with the processing are, however, of special importance, because they generate colored products and these compounds leave a "footprint" in the oil, which is used as a tracking parameter. These reactions are mostly mediated by the release of acid into the medium, the greater accessibility of enzymes and substrates, and the oxygenation that occurs during the milling of the fruit and the beating of the paste. In the fraction of xanthophylls, of note is the partial transformation of 5,6-epoxy xanthophylls to their corresponding 5,8-furanoxides[17].

Kinetic models are becoming more popular for studying the changes in the chemical composition of food. These models are capable of predicting shelf life in keeping with the different variables that can affect the degradation of the food item. Studies describing the kinetics of carotenoids in fruit- and vegetable-based products are rather limited, although this information

would be very useful and industrially relevant for predicting changes in functional compounds during fruit and vegetable processing[18]. In those studies, analysis of kinetic data suggested a first-order model to describe the thermal degradation of carotenoids as in paprika oleoresins[19], citrus juice[13] or carrot puree[18]. The thermal and oxidative degradation of lycopene, lutein, and 9-*cis* and all-*trans* β-carotene has been studied in an oil model system[20] to determine their relatives stabilities. The degradation kinetics also followed a first-order model, and the thermodynamic parameters indicated a kinetic compensation effect between all the carotenoids, with lutein being the most stable to degradation. A higher thermal resistance of lutein than β-carotene has been suggested by Achir et al.[21] in model systems with two different frying oils reporting the influence of the oil initial composition in all degradation rates.

There are numerous experimental works in the literature describing VOO degradation, but recently the kinetic performance in oxidation parameters as peroxide value (PV), absorbance at 232nm (K_{232}) and 270nm (K_{270}) has been described[22]. The first kinetic and thermodynamic study of pigment thermodegradation products in VOO is referred to chlorophylls and was reported in 2010[23].

Our most recent research in this field has been aimed at the kinetic study and characterization of the thermodynamic parameters governing the thermal degradation reactions of carotenoids in VOO, to advance our understanding of the thermal stability of these compounds in an oily matrix, and to establish for the first time mathematical models enabling the prediction of the degradation of this pigment during VOO storage and/or thermal processing. This study necessarily had to be separated into two parts due to the large amount of data. Recently the results for lutein, β-carotene and β-cryptoxanthin has been reported [24] and in this work the results concerning to 5,6-epoxide xanthophylls are presented.

MATERIALS AND METHOD.

Chemicals and Standards. Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands). HPLC reagent grade solvents were purchased from Teknokroma (Barcelona, Spain), and analytical grade solvents were supplied by Panreac

(Barcelona, Spain). For the preparation, isolation, and purification of carotenoid pigments, analytical grade reagents were used (Panreac). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Bedford, MA, USA). Reference samples of neoxanthin, violaxanthin, and antheraxanthin were obtained from a pigment extract of fresh spinach saponified with 3.5 M KOH in methanol and isolated by TLC on silica gel GF254 (0.7 mm thickness) on 20 x 20 cm plates using petroleum ether (65-95 °C)/acetone/diethylamine (10:4:1)[25]. Luteoxanthin, auroxanthin, neochrome, and mutatoxanthin were obtained by acidification with 1 M HCl in ethanol[26]. All standards were purified by TLC using different eluents[25].

Samples. The study of thermal degradation of pigments was carried out with virgin olive oils obtained from a single industrial mill (Cooperativa Sor Ángela de la Cruz, Estepa, Seville, Spain) to avoid any effect of pedoclimatic and agricultural parameters and the industrial variables of the extraction systems in the comparative studies. To have three lots of oil with differing pigment content, the starting material used was a mixture of two oil variety olives – Hojiblanca and Manzanilla – picked in three different months: November (sample N), December (sample D), and January (sample J). The proportion of fruits between varieties was 20:80, 80:20 and 100:0 respectively. The dates of picking correspond to high, medium, and low pigment levels (referring to the green color) and correlated inversely with the degree of fruit ripening according to the method of Walalí-Loudiyi et al.[27].

Heat treatment. Preliminary assays, with a commercial sample of virgin olive oil, enabled an approximate determination of the degree of conversion for the main reactions to be studied and established a range of times for an appropriate sampling at each temperature. The total time of each experiment changed depending on the assay temperature: 42 h (120 ºC), 64 h (100 ºC), 370 h (80 ºC), and 744 h (60 ºC). At least 128 aliquots (32 for each of the four assay temperatures) were separated from each oil lot (samples N, D, and J). These aliquots were put into glass tubes that were sealed in the absence of air (using nitrogen as neutral gas) and placed in thermostated ovens at the temperatures fixed for each experiment. These four temperatures were used to determine the kinetic

and thermodynamic parameters (reaction order, reaction rate, and activation energies).

For each oil lot, two samples were analyzed for each time/temperature pair. The samples were removed from the thermostated ovens at fixed time intervals, depending on each experiment, to obtain a total of at least 16 duplicate samples. The samples were cooled rapidly in an ice bath and then kept at -20 ºC until analysis of the pigments.

Extraction and Analysis of Xanthophyll Pigments. All procedures were performed under green lighting to avoid any photooxidation of xanthophyll compounds. Pigment extraction was performed by liquid-phase distribution. This method was developed for VOO by Mínguez-Mosquera et al.[17]. The technique is based on the selective separation of components between N,N-dimethylformamide (DMF) and hexane. The oil sample (10-15g) was dissolved directly in 150 mL of DMF and treated with five 50 mL successive portions of hexane in a decanting funnel. The hexane phase carried over lipids and carotene fraction whereas the DMF phase retained chlorophyll pigments and xanthophylls. This system yielded a concentrated pigment solution that was oil free and could be adequately analyzed by chromatographic techniques.

HPLC analysis of carotenoid pigments was performed according to the method described by Mínguez-Mosquera et al.[25] using a reverse phased column (20 cm x 0.46 cm) packed with 3 μm C18 Spherisorb ODS2 (Teknokroma) and an elution gradient with the solvents (A) water/ion-pair reagent/methanol (1:1:8, v/v/v) and (B) acetone/methanol (1:1 v/v), at a flow rate of 1.25 mL/min. The ion-pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The pigments were identified by co-chromatography with the corresponding standard and from their spectral characteristics described in detail in previous papers[25, 28]. The online UV-vis spectra were recorded from 350 to 800 nm with the photodiode array detector. Pigments were detected at the wavelength of maximum absorption (430 nm for neoxanthin, neochrome, violaxanthin, mutatoxanthin, and auroxanthin, and 450 nm for antheraxanthin) and were quantified from the corresponding calibrated curves (amount versus integrated peak area). The calibration equations were obtained by least-squares linear regression analysis over a concentration range according to

the levels of these pigments in VOO. Injections in duplicate were made for five different volumes at each standard solution.

Kinetic Parameters. Changes in experimental data of pigment concentration, expressed in micromoles per kilogram, were used to calculate kinetic parameters by least-squares non linear regression analysis. The reaction order (*n*) and rate constant (*k*) were determined by trial and error using the integral method: a reaction order is initially assumed in the rate equation and then is integrated to obtain a mathematical expression that relates pigment concentration (*C*) with time (*t*). The mathematical expression that best fits the changes in the experimental data with the reaction time was selected to verify the order (assumed ad initio) and used to obtain the rate constant (*k*).

Thermodynamic Parameters. The effect of temperature on the rate constant was evaluated by means of the Arrhenius equation with a simple reparametrization[29] by using a reference temperature T_{ref}:

$$k = k_{ref} \times \exp\left[\frac{-E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right]$$

where R is the molar gas constant (1.98 cal mol^{-1} K^{-1}), *T* is the absolute temperature (K), E_a is the activation energy (cal mol^{-1}), *k* is the specific reaction rate constant at the temperature T, and k_{ref} is the specific reaction rate constant at the reference temperature T_{ref}. The reference temperature should preferably be chosen in the middle of the studied temperature regimen.

Therefore, E_a was estimated on the basis of non-linear regression analysis of k_i *versus* $1/T_{ij}$ (being i = N, D, J; j = 60, 80, 100, or 120 °C).

According to active complex theory, the enthalpy ($\Delta H^{\#}$) and entropy of activation and ($\Delta S^{\#}$) were determined by the Eyring equation:

$$\ln(k/T) = \frac{-\Delta H^{\#}}{RT} + \frac{\Delta S^{\#}}{R} + \ln\left(\frac{k_b}{h}\right)$$

where *k* is the rate constant at temperature *T*, k_b is the Boltzmann constant; *R* is the molar gas constant and *h* is the Planck constant. Therefore, $\Delta H^{\#}$ and $\Delta S^{\#}$ were estimated on the basis of linear

regression analysis of ln (k_i/T_{ij}) *versus* $1/T_{ij}$. The Gibbs free energy was estimated according to the Gibbs equation:

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$$

The pairs of $\Delta H^{\#}$ and $\Delta S^{\#}$ obtained were linearly correlated using the last equation. From which the isokinetic temperature (T_{isok}) and its corresponding Gibbs free energy (AG_{isok}) for the reaction could be estimated.

Calculations and Statistical Data Analysis. Data were expressed as the means ± SE. The data were analyzed for differences between means using one-way analysis of variance (ANOVA). The Brown & Forsythe test[30] was used as a post hoc comparison of statistical significance (*p* values < 0.05). Least-squares and non linear regression analysis were performed using Statistica 6.0 (StatSoft, Inc., 2001) and Statgraphics Centurion XV for Windows (Statpoint Technologies, Inc., 2005).

RESULTS AND DISCUSSION

Kinetic Study The qualitative carotenoid profile in the initial samples was typical of VOO[9, 31], with lutein and β-carotene as major carotenoids and violaxanthin, luteoxanthin, auroxanthin, neoxanthin, antheraxanthin, mutatoxanthin and β-cryptoxanthin as minor xanthophylls. The study of carotenoid thermal degradation in VOO has had to be separated into two sections, given the high amount of data. In the first stage of the study were reported the results for lutein, β-carotene and β-cryptoxanthin[24] and in this stage we are presenting the results concerning the 5,6-epoxide xanthophylls: neoxanthin, violaxanthin, antheraxanthin and their corresponding 5,8-furanoxide derivatives: neochrome, luteoxanthin and auroxanthin, and mutatoxanthin. **Table 1** shows the initial content of the pigments analyzed in this study for the high (N), medium (D), and low (J) pigmentation VOO matrices employed. The total pigment content includes chlorophylls and carotenoids as measured in this study and in previous ones[23,24].

Figure 1 shows the typical HPLC chromatograms for an olive oil pigment extract at three significant time points of the thermal degradation process studied: initial sample (t = 0 h), after 18 h

of heating at 120 ºC and after 42 h of heating at 120 ºC. The main peak is not numbered and corresponds to lutein, the thermal degradation of which has been studied in a previous work[24]. In the initial sample, there were 5,6-epoxy xanthophylls including neoxanthin (peak 1), neoxanthin isomer (peak 2), violaxanthin (peak 4) and antheraxanthin (peak 7), and 5,8-furanoid xanthophylls including luteoxanthin (peak 5), auroxanthin (peak 6) and mutatoxanthin (peak 8). **Figure 2** shows the structures of the studied carotenoids. The presence of 5,8-furanoxides already in the initial sample is due to the release of intracellular acid medium during the milling of olive fruit to obtain virgin olive oil because (1)- no 5,8-furanoxides have been found in olive fruits[17] and (2) it is known that acid conditions might induce the isomerisation of the 5,6-epoxide into a 5,8-furanoxide[32].

Three groups of xanthophylls were defined to study their evolution during heating, each group consisting of the 5,6-epoxy xanthophyll and its corresponding 5,8-furanoxide(s) (**Table 1**). The first group consisted of neoxanthin and neochrome (group I), the second group was made up of antheraxanthin and mutatoxanthin (group II) and the third and last group was formed by violaxanthin, luteoxanthin and auroxanthin (group III).

In each of this group, the initial percentage of 5,8-furanoxide xanthophylls were quite different. In group I, no 5,8-furanoxide xanthophyll was detected, in group II it represented between 23 and 46% of the carotenoids and in group III it exceeded 60%.

During heat treatment mentioned in Material and Methods, the concentration of 5,6- epoxy xanthophylls was gradually reduced (**Figure 1**), while changes in the corresponding 5,8-furanoxides were observed (**Figures 3-5**). Neochrome, mutatoxanthin and auroxanthin gradually increases over time, until they reached a maximum concentration (**Figures 3-5**). Then, they began to decline probably oxidized to colorless compounds. In contrast, the intermediate compound luteoxanthin maintained a gradual decrease in concentration from the start of treatment.

At maximum concentration, the highest percentage of 5,8-furanoxides comparing to epoxides were found in group III (luteoxanthin + auroxanthin) reaching values of up to 95%, followed by group II (mutatoxanthin), which ranged from 50% to 60%, and finally group I

(neochrome) which in no case exceeded 40%. For each group, the time required to achieve this maximum percentage of 5,8-furanoxides increased with decreasing temperature, and in all cases the highest time values corresponded to neochrome for all temperatures and matrices studied.

These results lead us to suggest the percentages of 5,8-furanoxide xanthophylls as chemical markers of heat treatment in a VOO. To support this claim, we will examine the experiment conducted at 120 °C in greater detail. **Table 2** shows the changes in the ratio of 5,6-epoxides to 5,8-furanoxides for the different groups of xanthophylls experienced during the heat treatment. This ratio decreased significantly during heat treatment and showed differences between groups. In the violaxanthin group (III), this relationship began at values < 1 in the initial sample and decreased to 0 (100% of 5,8-furanoxides) after 22h of heat treatment at 120 ºC. In the antheraxanthin group (II), the initial sample started with values > 1 but progressively decreased, reaching 0 after 22 h at 120 ºC. In the neoxanthin group (I) the relationship started at undefined values due to that the 5,8-furanoxide was not detected in the initial sample and decreased significantly during heat treatment, but in no case was less than 3. These results marked a difference compared to other groups of xanthophylls. Even a short time of heat treatment at 120 ºC (e.g. 1.5 h) was sufficient to decrease the initial 5,6 epoxide /5,8 furanoxide ratio in all groups, but this decrease was only significant mathematically for group I (neoxanthin). For the other groups of xanthophylls, no significant differences were observed for this ratio after 1.5 h of heat treatment since the values corresponding to the initial sample (**Table 1**) showed a wide range of variation between different VOO matrices. Therefore, the ratio neoxanthin/neochrome (or the percentage of neochrome) offers the best possibility to be used as a chemical marker of thermal treatment in VOO.

Similar losses of 5,6 epoxy xanthophylls after heat treatment have been described in other foods. Thermal effects were clearly observed on violaxanthin and anteraxanthin after pasteurization[33] and microwave heating[34] of orange juice. High losses of violaxanthin were also noted after cooking of pumpkin puree[35] and green vegetables[36] being more prone to degradation than β-carotene. There are also a few papers in which the isomerization of the epoxide function in

position 5,6 into a furanoxide function in position 5,8 is reported as a common reaction for the xanthophylls during thermal processing[13,37-39] but this is the first work where kinetic study is performed on this subject.

Zepka and Mercadante[40] studied the degradation compounds of carotenoids formed during heating of a simulated cashew apple juice. They also reported that the loss of total carotenoids was not compensated by those other isomers formed, indicating that isomerisation and oxidation to both coloured and no-colored compounds were the main reactions occurring during heating of carotenoids in aqueous-based and juice systems.

Based on the observed changes in the xanthophylls mentioned above, the kinetic models indicated in **Figure 6** were proposed. All kinetic models proposed involve consecutive reactions. The first reactions determine the formation of the 5,8-furanoxides and the final reactions determine the destruction of the chromophores resulting in the formation of non-colored compounds (nc).

Group I: In accordance with the mechanism proposed (**Figure 6**), neoxanthin (5,6-epoxide) leads to neochrome (5,8-furanoxide) and the last reaction leads to non-colored products.

The corresponding kinetic equations are expressed as follows:

$$V_{Neoxanthin} = -\frac{d[A]}{dt} = k_1[A]^n \quad [1]$$

$$V_{Neochrome} = \frac{d[B]}{dt} = k_1[A]^n - k_2[B]^n \quad [2]$$

$$V_{Colorless} = \frac{d[C]}{dt} = k_2[B]^n \quad [3]$$

[A]: concentration of neoxanthin; [B]: concentration of neochrome; [C]: concentration of non-colored products (nc); k_1 and k_2: rate constants for the various reactions; n: reaction order.

From the balance of materials of all species, the concentration of colorless compounds over time is derived by the following equation:

$$[A]_0 + [B]_0 + [C]_0 = [A] + [B] + [C]$$

289 $[A]_0$: initial concentration of neoxanthin; $[B]_0$: initial concentration of neochrome; $[C]_0$: initial
290 concentration of nc. Concentrations [A]-[C] are those described for equations 1-3.
291 Solving the kinetic mechanism, assuming an order of 1 (n=1) and that all reactions are irreversible,
292 we get

293 $$[A] = [A]_0 \, e^{-k_1 \cdot t} \qquad [4]$$

294 $$[B] = \frac{k_1 [A]_0}{k_2 - k_1} \left[e^{-k_1 \cdot t} - e^{-k_2 \cdot t} \right] + [B]_0 \, e^{-k_2 \cdot t} \qquad [5]$$

295 *Group II:* The kinetic mechanism of group II is similar to group I. Thus, antheraxanthin (5,6-
296 epoxide) leads to mutatoxanthin (5,8-furanoxide), and this leads to non-colored products (**Figure**
297 **6**).

298 The corresponding kinetic equations are expressed as

299 $$V_{Antheraxanthin} = -\frac{d[A]}{dt} = k_3 [A]^n \qquad [6]$$

300 $$V_{Mutatoxanthin} = \frac{d[B]}{dt} = k_3 [A]^n - k_4 [B]^n \qquad [7]$$

301 $$V_{Colorless} = \frac{d[C]}{dt} = k_4 [B]^n \qquad [8]$$

302 [A]: concentration of antheraxanthin; [B]: concentration of mutatoxanthin; [C]: concentration of nc;
303 k_3 and k_4: rate constants for the different reactions; n: reaction order.
304 Using the material balance of all species, the next equation allows us to obtain the concentration of
305 colorless products over time:

306 $$[A]_0 + [B]_0 + [C]_0 = [A] + [B] + [C]$$

307 $[A]_0$: initial concentration of antheraxanthin; $[B]_0$: initial concentration of mutatoxanthin; $[C]_0$:
308 initial concentration of nc. Concentrations [A]-[C] are those described for equations 6-8.
309 Solving the kinetic mechanism, assuming an order of 1 (n=1) and that all reactions are irreversible,
310 we get

$$[A] = [A]_0 \, e^{-k_3 \cdot t} \quad [9]$$

$$[B] = \frac{k_3 [A]_0}{k_4 - k_3} \left[e^{-k_3 \cdot t} - e^{-k_4 \cdot t} \right] + [B]_0 e^{-k_4 \cdot t} \quad [10]$$

Group III: Violaxanthin differs from neoxanthin and antheraxanthin in its structure due to its two 5,6-epoxy groups. Therefore, the transformation of one of these epoxy groups into a 5,8-furanoid group leads to luteoxanthin. If the second epoxy group is transformed into 5,8-furanoid group, then this leads to auroxanthin. Accordingly, the proposed model (**Figure 6**) presents an additional consecutive reaction kinetic model from groups I and II described above. This further complicates the model and, consequently, its mathematical resolution.

The corresponding kinetic equations are expressed as follows:

$$V_{Violaxanthin} = -\frac{d[A]}{dt} = k_5 [A]^n \quad [11]$$

$$V_{Luteoxanthin} = \frac{d[B]}{dt} = k_5 [A]^n - k_6 [B]^n \quad [12]$$

$$V_{Auroxanthin} = \frac{d[C]}{dt} = k_6 [B]^n - k_7 [C]^n \quad [13]$$

$$V_{Colorless} = \frac{d[D]}{dt} = k_7 [C]^n \quad [14]$$

[A]: concentration of violaxanthin; [B]: concentration of luteoxanthin; [C]: concentration of auroxanthin; [D]: concentration of nc; k_5, k_6, and k_7,: rate constants for the different reactions; n: reaction order.

The next equation allows us to obtain the concentration of colorless products over time:

$$[A]_0 + [B]_0 + [C]_0 + [D]_0 = [A] + [B] + [C] + [D]$$

$[A]_0$: initial concentration of violaxanthin; $[B]_0$: initial concentration of luteoxanthin; $[C]_0$: initial concentration of auroxanthin; $[D]_0$: initial concentration of nc. Concentrations [A]-[D] are those described for equations 11-14.

Resolving the kinetic mechanism, assuming an order of 1 (n=1) and that all reactions are irreversible, we get

$$[A] = [A]_0 \, e^{-k_5 \cdot t} \quad [15]$$

$$[B] = \frac{k_5 [A]_0}{k_6 - k_5} \left[e^{-k_5 \cdot t} - e^{-k_6 \cdot t} \right] + [B]_0 \, e^{-k_6 \cdot t} \quad [16]$$

$$[C] = k_5 k_6 [A]_0 \left[\frac{e^{-k_5 \cdot t}}{(k_7 - k_5)(k_6 - k_5)} - \frac{e^{-k6 \cdot t}}{(k_7 - k_6)(k_6 - k_5)} + \frac{e^{-k_7 \cdot t}}{(k_7 - k_5)(k_7 - k_6)} \right] \quad [17]$$
$$+ \frac{k_6 [B]_0}{(k_7 - k_6)} \left[e^{-k_6 \cdot t} - e^{-k_7 \cdot t} \right] + [C]_0 e^{-k_7 \cdot t}$$

In accordance with the proposed kinetic equations 4, 5, 9, 10 and 15-17, and by nonlinear regression analysis of the experimental data, the rate constants for each of the proposed reactions in the mechanisms were estimated. For treatment of the high-pigmentation matrix (sample N) at 120ºC, **Figures 3-5** show the concentration changes found and the regression estimated. **Table 3** shows the values for the estimated rate constants, together with the standard error and determination coefficient (R^2) for each reaction studied. The determination coefficients obtained showed a good fit of the experimental data to the equations proposed and demonstrate that the first-order mechanism is appropriate for describing the thermal degradation of neoxanthin, antheraxanthin and violaxanthin in VOO.

Studies describing the kinetics of carotenoids degradation in fruit- and vegetable-based products are rather limited although this information would be very useful and industrially relevant for predicting changes in bioactive compounds during processing and shelf life of these foods[18]. In those studies, analysis of kinetic data also suggested a first-order model to describe degradation of carotenoids in green table olives[41], paprika oleoresins[19], citrus juice[13], carrot puree[18] and oils enriched with β-carotene and lutein[21].

In general, all kinetic constants doubled or tripled for each 20ºC increase in temperature, demonstrating a marked effect of temperature in reaction rates, similar to other carotenoids in

VOO[24]. However, this effect was lower than that found in the thermal degradation of chlorophyll compounds in VOO[23].

The rate constant estimated for neoxanthin isomerisation was significantly lower than that of antheraxanthin and violaxanthin (**Table 3**), in all temperatures and matrices, suggesting that neoxanthin has a relatively greater heat resistance. This result partly agrees with Fratianni et al.[34], who found that violaxanthin was the most unstable compound followed by antheraxanthin.

Chemical structures of the carotenoids significantly affects thermal stability. In group I, the ratio of rate constants between neoxanthin and neochrome was < 1 in all cases (average 0.4±0.1 of four temperatures and samples studied), indicating that the 5,8-isomer degradation into nc products is the preferred reaction. This explains why maximum concentration of 5,8-furanoxide (neochrome) does not exceed that of its predecessor 5,6 epoxide (neoxanthin) at any point in the heat treatment.

In contrast, in the other two xanthophyll groups, the 5,8-furanoxide formation reaction was always preferred. In group II, the rate constant of mutatoxanthin formation (k_3) was always higher than its degradation to colorless products (k_4). Similarly, in group III, the formation rate constants 5,8-furanoxides (luteoxanthin from violaxanthin (k_5) and auroxanthin from luteoxanthin (k_6)) were always higher than the rate constant of the final degradation reaction of auroxanthin to colorless products (k_7).

Thermodynamic Study. The Arrhenius model and transition state theory were used to determine the influence of temperature on the reaction rates. **Table 4** displays the values estimated for the thermodynamic parameters (entropy, enthalpy, activation energy and Gibbs free energy), with their respective standards errors for each matrix and reaction analysed.

To study the effect of matrix type on the reaction mechanism, we compared the thermodynamic parameters estimated in the three types of VOO. In general, no significant differences were found in the parameters $\Delta S^{\#}$ and $\Delta H^{\#}$ characterising the reactions of isomerisation and degradation of xanthophylls (t-test $P \leq 0.05$) (**Table 4**). These results enable all the matrices to be considered a single reaction medium. An exception is the isomerisation reaction of luteoxanthin

to auroxanthin for which significant differences in the corresponding thermodynamic parameters (E_a, $\Delta S^{\#}$, $\Delta H^{\#}$) were found in matrix J, suggesting a slight effect of the matrix in this case. Also, differences in the activation energy were found in matrix D, N and D for the degradation of neoxanthin, neochrome and mutatoxanthin, respectively.

With respect to the estimated values for activation energy of isomerisation reactions, higher values were found in xanthophylls with a single epoxide group (neochrome and mutatoxanthin) than in those with two epoxy groups (luteoxanthin and auroxanthin), in all matrices studied. Mathematically, this can be interpreted as follows: a temperature increase produces a greater increase in the rate constant for 5,6-monoepoxy-compounds degradation; that is, a smaller temperature change is needed to form 5,8-monofuranoxy-compounds more rapidly.

In all cases, values for the $T\Delta S^{\#}$ term were negative (due to the negative values of entropy); however, enthalpy values ($\Delta H^{\#}$) were positive, as were the Gibbs free energy values ($\Delta G^{\#}$), making the reactions nonspontaneous.

Isokinetic ratio. The isokinetic ratio was studied along the same lines as previous studies[23, 24], to determine whether there were changes in the reaction mechanisms (first case) or whether some specific step in the mechanism had greater importance under our different experimental conditions (VOO matrices with high, medium and low pigmentation) (second case).

The isokinetic effect (or isoequilibrium) is defined as the intersection point between the straight Arrhenius (or van't Hoff) lines that show the thermodynamics of a series of similar reactions or reactions in various media[42]. This cut-off point is the isokinetic temperature at which reactions take place at identical rates. Specifically, the experiments study the same reaction taking place in various oily matrices. Thus, we are with the second case: a greater importance of a particular step in the mechanism.

To study the existence of an isokinetic ratio among oily matrices, the Arrhenius straight lines obtained for each of the three oily matrices studied were represented together. The study was

repeated for each of the reactions including the mechanism for thermal degradation of neoxanthin, antheraxanthin and violaxanthin. No isokinetic ratio was found for any of them.

Figure 7 shows the example of the violaxanthin isomerisation reaction. We could not conclude that there was an isokinetic ratio as the Arrhenius straight lines for the three samples (N, D and J) did not present any common cut-off points. These straight lines are almost parallel, but are also very close to one another (all points lie within the same interval of confidence). Consequently, all points can be explained by a single Arrhenius line, so that the reaction mechanism is not affected at any stage by different pigment content in the oily matrix. This same result was observed for the other reactions studied.

They are, therefore, isoenthalpic and isoentropic straight lines. This observation is consistent with the thermodynamic parameters (**Table 4**), which do not show significant differences (t-test $P \leq 0.05$) between the various oily matrices. Thus, there is no isokinetic ratio, and one can conclude that the type of oily matrix does not affect the isomerisation reaction mechanisms of neoxanthin, antheraxanthin, violaxanthin and luteoxanthin, and the degradation reactions of neochrome, mutatoxanthin and auroxanthin during any of its steps. Consequently, the thermodynamic parameters characterised here can be extrapolated to any type of VOO matrix.

The isokinetic effect can also be considered in a series of similar reactions, as in the case of the degradation of neochrome, mutatoxanthin and auroxanthin to form colorless products, and in the case of isomerisation of neoxanthin, antheraxanthin and violaxanthin to 5,8-furanoids. The average values of the rate constants obtained in the three VOO matrices studied were used to obtain the Arrhenius straight lines (**Figures 8** and **9**).

In the first case (**Figure 8**), the confidence intervals of Arrhenius straight lines for mutatoxanthin and neochrome overlap (100% of data between confidence limits), whereas the overlap is lower with the confidence intervals of auroxanthin straight lines (50% of data within confidence limits). This results in two straight lines which are cut at an isokinetic temperature of 383K (±15) and indicates the same isomerisation mechanism, but affected by the temperature

change in one or another of its steps. Thus, at temperatures below the isokinetic temperature, the formation of colorless products from auroxanthin is the most rapid, followed by mutatoxanthin and neochrome respectively. At temperatures above isokinetic, the formation from neochrome is the most rapid, followed by mutatoxanthin and auroxanthin respectively.

In the second case (**Figure 9**), the three lines were considered independent because the level of overlap was less than in the previous case (100% of violaxanthin and antheraxanthin data were found only within their respective confidence limits). Thus, the lines intersect in pairs, leading to three isokinetic temperatures. One of these, the intersection of neoxanthin and antheraxanthin, takes place at high temperature (>1000K), well above the boiling point of olive oil. The other two isokinetic temperatures are below the boiling point of olive oil, 450K (≈177 °C) for the intersection of violaxanthin and neoxanthin and 403K (≈130 °C) for violaxanthin and antheraxanthin. The isomerisation mechanism of these pigments is the same, but some of the mechanism steps are influenced by temperature (the influence of temperature is similar for neoxanthin and antheraxanthin, and very different from violaxanthin). Therefore, above the isokinetic temperature, isomerisations are faster in xanthophylls with an epoxide group (antheraxanthin and neoxanthin), whereas at lower temperatures the isomerisation of violaxanthin with two epoxide groups is preferred.

Compensation Effect. A kinetically compensated system requires that the various thermodynamic parameters obtained for the same reaction in different environments define an isokinetic line. This theoretical line includes all of the various kinetic and thermodynamic coordinates of a single reaction, with the isokinetic temperature (T_{iso}) being the line slope and the increase in Gibbs free energy of all reactions at the T_{iso} being the intercept, according to:

$$\Delta H^{\#} = T_{iso}\Delta S^{\#} + \Delta G^{\#}$$

There are some papers describing degradation reactions of carotenoids in different reaction media and reporting the existence an isokinetic line defined by thermodynamic parameters and its application in stability prediction studies[43, 44].

Liu and Guo[45] demonstrated that the compensation effect and the isokinetic effect are not necessarily synonymous as had been previously thought, and that the existence of one does not imply the existence of the other. Errors are inevitable in experiments and the data used are therefore estimators of the corresponding variables. Consequently, it is possible that the real values are not correlated, although their estimators are. This would be the case for the so-called false compensation effect. Krug et al.[46] proposed that the straight line in the plane ΔH versus ΔS was only a manifestation of the statistical pattern of the compensation, and that this hypothesis can be ruled out if the estimation of the line slope is sufficiently different from the harmonic temperature (T_{hm}), defined as:

$$T_{hm} = \frac{n}{\sum_{i=1}^{n} \frac{1}{T_i}}$$

Liu and Guo[45] proposed a method for distinguishing the real compensation effects from the false ones, based on the graphical representation of experimental values of enthalpies and entropies with their error bars in the $\Delta H^{\#}$ versus $\Delta S^{\#}$ plane.

To apply this study to our experimental data, the linear regressions $\Delta H^{\#}$ versus $\Delta S^{\#}$ were estimated for each of the reactions. **Table 5** shows the values obtained for the line slope (T_{iso}) and the corresponding determination coefficients (R^2). An isokinetic line was obtained in all cases (R^2>0.95), except in violaxanthin isomerisation. However, by comparing the estimated isokinetic temperature and the T_{hm} under study conditions (362K), we deduced that the compensation effect could only be true for the degradation of neoxanthin, luteoxanthin, auroxanthin and antheraxanthin, for a series of similar reactions that involved the isomerisation of neoxanthin, violaxanthin and antheraxanthin, and for the degradation of neochrome, auroxanthin and mutatoxanthin to colorless products. Finally, applying the error bar method proposed by Liu and Guo (2001) showed that there is no true compensation effect in these cases, except for the group of reactions of neochrome, auroxanthin and mutatoxanthin to colorless products (**Figure 10B**).

The degradation of 5,6-epoxy xanthophylls in VOO during heat treatment followed first-order kinetics. The analysis of the 5,8-furanoxide compounds (reaction intermediates) that appear during the thermo-degradation of neoxanthin, antheraxanthin and violaxanthin to colorless products has established that the degradation process is not simple, and takes place in several consecutive elemental steps. The marked effect of temperature on the reaction mechanism was revealed. The thermal stability varied among carotenoids and was greater for neoxanthin but was significantly affected by changes in their chemical structure. A true kinetic compensation effect exists only for the case of similar reactions in the degradation of neochrome, mutatoxanthin and auroxanthin to colorless products.

No significant effect of the oily medium on the reaction mechanisms of any of these xanthophylls have been found from the isokinetic study, which compared kinetic and thermodynamic parameters determined in the three VOO matrices of different pigment content (high, medium, and low). The thermodynamic parameters characterised in this study could therefore be applied to any type of VOO matrix yielding a mathematical model developed from activation energies, which predict xanthophylls degradation and 5,8-furanoxide formation if the time–temperature profile of the processing method is known. Reaction conditions similar to those used in the soft deodorisation of VOO (1.5h at 120ºC) are sufficient to increase the percentage of 5,8-furanoxides, decreasing the natural 5,6-epoxide/ 5,8-furanoxide ratio. This criterion was significant for neoxanthin/neochrome ratio and could be proposed as a chemical marker of heat treatment in VOO.

ACKNOWLEDGMENT

We thank Sergio Alcañiz-García for his technical assistance.

503 **REFERENCES**

504 (1)Krinsky, N.I. The biological properties of carotenoids. *Pure Appl. Chem.* **1994**, *66*, 1003-1010.

505 (2)Goodwin, T. W. The Biochemistry of the Carotenoids, Vol. I. Plants, 2nd ed. Chapman and Hall,
506 New York, NY. **1980**.

507 (3)Meléndez-Martínez, A.J.; Vicario, I.M.; Heredia, F.J. Carotenoid pigments: structural and
508 physicochemical considerations. *Arch. Latinoam. Nutr.* **2007**, *57*, 109–117.

509 (4) Britton, G.; Liaaen-Jensen, S.; Pfander, H. *Carotenoids. Volume 5: Nutrition and health*. Basel,
510 Switzerland: Birkhäuser Verlag. **2009**.

511 (5) Castenmiller, J.J; West, C.E. Bioavailability and bioconversion of carotenoids. *Annu. Rev. Nutr.*,
512 **1998**, *18*, 19-38.

513 (6) Granado-Lorencio, F.; Olmedilla-Alonso, B.; Herrero-Barbudo, M.C.; Blanco-Navarro, I.;
514 Pérez-Sacristán, B.; Blázquez-García, S. In vitro bioaccessibility of carotenoids and tocopherols
515 from fruits and vegetables. *Food Chem*. **2007**, *102*, 641-648.

516 (7) Bermúdez, B.; Pacheco, Y.; López, S.; Abia, R.; Muriana, J.G. Digestion and absoption of olive
517 oil. Grasas y Aceites. **2004**, *55*, 1-10.

518 (8) International Olive Oil Council (IOC). Trade Standard Applying to Olive Oils and Olive-
519 Pomace Oils. **2009.** Madrid: IOC/T.15/NC nº 3/Rev. 4. Available online at
520 http://www.internationaloliveoil.org /downloads/NORMAEN1.pdf

521 (9) Gandul-Rojas, B.; Mínguez-Mosquera, M.I. Chlorophyll and carotenoid composition in virgin
522 olive oils from various spanish olive varieties. *J. Sci. Food Agric*. **1996**, *72*, 31-39.

523 (10) Rao, A.V.; Rao, L.G. Carotenoids and human health. *Pharmacol. Res.*, **2007**, *55*, 207–216.

524 (11) Rodriguez-Amaya, D.B. *A guide to carotenoid analysis in foods*. International Life Sciences
525 Institute (ILSI) Press, Washington, **1999**.

526 (12) Oliver, J.; Palou, A. Chromatographic determination of carotenoids in foods. J. *Chromatogr A*,
527 **2000**, 881, 543–555.

528 (13) Dhuique-Mayer, C.; Tbatou, M.; Carail, M.; Caris-Veyrat, C.; Dornier M.; Amiot, M.J. Thermal
529 degradation of antioxidant micronutrients in Citrus juice: Kinetics and newly formed compounds.
530 *J. Agric. Food Chem.* **2007**, *55*, 4209-4216.
531 (14) Meléndez-Martínez, A.J.; Vicario, I.M.; Heredia, F.J. Geometrical isomers of violaxanthin in
532 orange juice. *Food Chem*, **2007**, 104, 169–175.
533 (15) Lee, H.S.; Coates, G.A.; Effect of thermal pasteurization on Valencia orange juice color and
534 pigments. *Lebensm. Wiss. Technol.* **2003**, *36*, 153-156.
535 (16) Gallardo-Guerrero, L.; Roca, M.; Mínguez-Mosquera, M.I. Distribution of chlorophylls and
536 carotenoids in ripening olives and between oil and alperujo when processed using a two-phase
537 extraction system. *J. Am. Oil Chem. Soc.* **2002**, *79*,105-109.
538 (17) Mínguez-Mosquera, M.I.; Gandul-Rojas, B.; Garrido-Fernández, J.; Gallardo-Guerrero, L.
539 Pigments present in virgin olive oil. *J. Am. Oil. Chem. Soc*. **1990**, *67*, 192-196.
540 (18) Lemmens, L.; De Vleeschouwer, K.; Moelants, K.R.N.; Colle, I.J.P.; Van Loey, A.M.;
541 Hendrickx, M.E. Beta-Carotene Isomerization Kinetics during Thermal Treatments of Carrot
542 Puree. *J. Agric. Food Chem.* **2010**, *58*, 6816-6824**.**
543 (19) Pérez-Gálvez, A.; Jarén-Galán, M.; Mínguez-Mosquera, M.I. Effect of high-temperature
544 degradative processes on ketocarotenoids present in paprika oleoresins. *J. Agric. Food Chem.* **2000**,
545 *48*, 2966-2971.
546 (20) Henry L.K., Catignani G., & Schwartz S. Oxidative degradation kinetics of lycopene, lutein, and
547 9-cis and all-trans beta-carotene. *J. Am. Oil Chem. Soc.* **1998**, *75*, 823-829**.**
548 (21) Achir, N.; Randrianatoandro, V.A.; Bohuon, P.; Laffargue, A.; Avallone, S. Kinetic study of
549 beta-carotene and lutein degradation in oils during heat treatment. *Eur. J. Lipid Sci. Technol.* **2010**,
550 *112*, 349-361.
551 (22) Mancebo-Campos, V.; Fregapane, G.; Salvador, M.D. Kinetic study for the development of an
552 accelerated oxidative stability test to estimate virgin olive oil potential shelf life. *Eur. J. Lipid Sci.*
553 *Tech.* **2008**. *110*, 969-976.

554 (23) Aparicio-Ruiz, R.; Mínguez-Mosquera, M.I.; Gandul-Rojas, B. Thermal Degradation Kinetics
555 of Chlorophyll Pigments in Virgin Olive Oils. 1. Compounds of Series *a*. *J. Agric. Food Chem.*
556 **2010**, *58*, 6200-6208.
557 (24) Aparicio-Ruiz, R.; Mínguez-Mosquera, M.I.; Gandul-Rojas, B. Thermal Degradation Kinetics of
558 Lutein, β-carotene and β-cryptoxanthin in Virgin Olive Oils. *J. Food Comp. Anal*. **2011**, *24*, 811-
559 820.
560 (25) Mínguez-Mosquera, M.I.; Gandul-Rojas, B.; Gallardo-Guerrero, L. Rapid method of
561 quantification of chlorophylls and carotenoids in virgin olive oil by HPLC. *J. Agric. Food Chem*.
562 **1992**, *40*, 60-63.
563 (26) Khachik, F.; Beecher, G. R.; Whittaker, N. F. Separation, Identification, and quantification of the
564 Major carotenoid and Chlorophyll Constituents in Extracts of Several Green Vegetables by Liquid
565 Chromatography. *J. Agric. Food Chem.* **1986**, *34*, 603-616.
566 (27) Walalí-Loudiyi, D.; Chimitah, M.; Loussert, R.; Mahhou, A.; Boulouha, B. Morphologic and
567 physiologic characters of olive tree clones from Picholine Marroqui variety. *Olivae*, **1984**, *3*, 26-
568 31.
569 (28) Hornero-Méndez, D.; Gandul-Rojas, B.; Mínguez-Mosquera, M.I. Routine and sensitive SPE-
570 HPLC method for quantitative determination of pheophytin *a* and pyropheophytin *a* in olive oils.
571 *Food Research. Int*. **2005**, *38*, 1067-1072.
572 (29) Van Boekel, M.A.J.S. Kinetic Modeling of Food Quality: A Critical Review. *Comprehen. Rev.*
573 *Food Sci. Food Safe*. **2008**, *7*, 144-158.
574 (30) Brown M.B.; Forsythe A.B. Robust tests for the equality of variances, *J. Am. Statistical Assoc.*
575 **1974**, *69*, 364–367.
576 (31) Gandul-Rojas, B.; Roca M.; Mínguez-Mosquera, M.I. Use of chlorophyll and carotenoid
577 pigment composition to determine authenticity of virgin olive oil. *J. Am. Oil. Chem. Soc.* **2000**, *77*,
578 853-858.

579 (32) Davies, B.H. Carotenoids. In Goodwin TW (ed), Chemistry and biochemistry of plant pigments,
580 2nd ed, vol 2, Academic Press, London, **1976**, 38-165.
581 (33) Lee, H.S.; Coates, G.A.; Effect of thermal pasteurization on Valencia orange juice color and
582 pigments. *Lebensm. Wiss. Technol.* **2003**, *36*, 153-156.
583 (34) Fratianni, A.; Cinquanta, L.; Panfili, G. Degradation of carotenoids in orange juice during
584 microwave heating. *Food Sci. Tech.* **2010**, *43*, 867-871.
585 (35) Provesi, J.G.; Dias, C.O.; Amante, E.R. Changes in carotenoids during processing and storage of
586 pumpkin puree. *Food Chem.* **2011**, *128*, 195-202.
587 (36) De Sá, M.C.; Rodriguez-Amaya, D.B. Optimization of HPLC quantification of carotenoids in
588 cooked green vegetables – Comparison of analytical and calculated data. *J. Food Comp. Anal.*,
589 **2004**, *17*, 37–51.
590 (37) Suzuki, Y.; Shioi, Y. Identification of chlorophylls and carotenoids in major teas by high-
591 performance liquid chromatography with photodiode array detection. *J. Agric. Food Chem.* **2003**,
592 *51*, 5307-5314.
593 (38) Mezadri, T.; Perez-Galvez, A.; Hornero-Mendez, D. Carotenoid pigments in acerola fruits
594 (Malpighia emarginata DC.) and derived products. *Eur. Food Res. Technol.*, **2005**, *220*: 63-69.
595 (39) Cano, M.P.; Marin, M.A. Pigment composition and color of frozen and canned kiwi fruit slices.
596 *J. Agric. Food Chem.* **1992**, *40*, 2141-2146.
597 (40) Zepka, L.Q.; Mercadante, A.Z. Degradation compounds of carotenoids formed during heating of
598 a simulated cashew apple juice. *Food Chem.* **2009**, *117*, 28-34.
599 (41) Mínguez-Mosquera, M.I.; Gandul-Rojas, B. Mechanism and kinetic of the degradation of
600 carotenoids during the processing of green table olives**.** *J. Agric. Food Chem.* **1994**, *42*, 1551-1554.
601 (42) Karpinski, Z.; Larsson, R. On the isokinetic effect of neopentane hydrogenolysis over metal
602 catalysts. *J. Catal*., **1997**, *168*, 532-537.

(43) Jarén-Galán, M.; Pérez-Gálvez, A.; Mínguez-Mosquera, M.I. Prediction of decoloration in Paprika oleoresins. Aplication to studies of stability in thermodinamically compensated systems. *J. Agric. Food Chem.* **1999**, *47*, 945-951.

(44) Sánchez, A.M.; Carmona, M.; Ordoudi, S.A.; Tsimidou, M.Z.; Alonso, G.L. Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark. *J Agric Food Chem.* **2008**, *56*, 1627-1637.

(45) Liu, L.; Guo, Q.X.; Isokinetic Relationship, Isoequilibrium Relationship, and Enthalpy-Entropy Compensation. *Chem. Rev*. **2001**, *101*, 673-695.

(46) Krug, R.R.; Hunter, W.G.; Grieger, R.A.; Enthalpy-entropy compensation: 1.Some fundamental statistical problems associated with the analysis of Van't Hoff and Arrenhius data. 2. Separation of the chemical from the statistical effect. *J. Physical Chem*. **1976**, *80*, 2335-2351.

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT-EU, Spanish and European Government) and FEDER (CE) in the Project AGL-2007-66139-C02-01 and by the Junta de Andalucía (AGR 148-2009-10).

618 **FIGURE CAPTIONS**

619 **Figure 1.** HPLC profile of xanthophylls from virgin olive oil (sample N), at initial sample (t = 0 h),
620 and after 18 h and 42 h of heating at 120 ºC. Detection was by absorption at 450nm. Peaks: 1,
621 neoxanthin; 2, neoxanthin isomer; 3, neochrome; 4, violaxanthin; 5, luteoxanthin; 6, auroxanthin; 7,
622 antheraxanthin; 8, mutatoxanthin.

623 **Figure 2.** Structures of carotenoids.

624 **Figure 3.** Evolution of concentration-time of neoxanthin (□) and neochrome (○) in VOO (sample N)
625 during 42 h at 120 ºC, and corresponding fits (—) to the mathematical model developed in this study
626 (eqs. 4-5).

627 **Figure 4.** Evolution of concentration-time of antheraxanthin (○) and mutatoxanthin (□) in VOO
628 (sample N) during 42 hours at 120 ºC, and corresponding fits (—) to the mathematical model
629 developed in this study (eqs. 9-10).

630 **Figure 5.** Evolution of concentration-time of violaxanthin (○), luteoxanthin (□) and auroxanthin (◇)
631 in VOO (sample N) during 42 hours at 120 ºC, and corresponding fits (—) to the mathematical
632 model developed in this study (eqs. 15-17).

633 **Figure 6.** Kinetic mechanisms for thermal degradation pathway of neoxanthin (A), antheraxanthin
634 (B) and violaxanthin (C) in VOO.

635 **Figure 7.** Arrhenius plot for 5,6-epoxide/5,8-furanoxide isomerization of violaxanthin in VOO oil
636 samples studied (N,○; D,□; J,◇). Confidence intervals (95%).

637 **Figure 8.** Arrhenius plot for a series of similar reactions: neochrome (○—), mutatoxanthin (□- -) and
638 auroxanthin (◇···) degradation to colorless in VOO. (average values from the three samples (N, D,
639 J); confidence intervals 95%).

Figure 9. Arrhenius plot for a series of similar reactions: neoxanthin (○–), antheraxanthin (□ -) and violaxanthin (◇···) 5,6-epoxide/5,8-furanoxide isomerisation reaction in VOO. (average values from the three samples (N, D, J); confidence intervals, 95%).

Figure 10. Graphic representation of $\Delta H^{\#}$ versus $\Delta S^{\#}$ by error bars method[45]: (A) false compensation effect for the group of 5,6-epoxide/5,8-furanoxide isomerization reactions of neoxanthin, violaxanthin and antheraxanthin; (B) true compensation effect for the group of degradation reactions of neochrome, auroxanthin and mutatoxanthin to noncolored products.

Table 1. Initial Content for Xanthophyll Compounds and Total Pigments in Virgin Olive Oils[a].

	Group I[b]			Group II			Group III				Total
Sample[c]	Neox.[d]	Neoc.	Ratio	Anther.	Muta.	Ratio	Violax.	Luteo.	Auro.	Ratio	Pigments[e]
N	1.00±0.04	0.00±0.00	ud[f]	0.84±0.01	0.30±0.00	2.80	0.80±0.01	1.40±0.01	0.55±0.02	0.41	36.91±0.55
D	0.73±0.01	0.00±0.00	ud	0.67±0.01	0.20±0.00	3.35	0.40±0.01	0.66±0.00	0.33±0.07	0.40	28.23±0.80
J	0.25±0.00	0.00±0.00	ud	0.12±0.00	0.10±0.00	1.20	0.15±0.00	0.18±0.00	0.07±0.00	0.60	15.30±0.26

[a]Data, expressed as µmol/kg, represent the mean value ± SD for three determinations. CV≤3.5%. [b]Each group consisting of the 5,6-epoxy xanthophyll and its corresponding 5,8-furanoxide(s). Ratio is 5,6-epoxy/5,8-furanoxide(s). [c]The sample codex corresponds to the harvest date of the olive fruits used to obtain the virgin olive oils studied, November (N), December (D), January (J). [d]Neox. , Neoxanthin; Neoc., Neochrome; Anther., Antheraxanthin; Muta., Mutatoxanthin; Violax., Violaxanthin; Luteo., Luteoxathin and Auro., Auroxanthin. [e]Total chlorophyll and total carotenoid pigments. [f]ud, undefined.

Table 2. Ratios between isomers 5,6-epoxide/5,8-furanoxide by groups[a].

Time (h)	Neox/Neoc	Violax/Luteo+Auro	Anther/Muta
0	ud	0.41	2.83
1.5	16.92	0.29	1.35
3	9.57	0.21	0.86
4.5	7.63	0.15	0.62
6	5.86	0.11	0.47
7.5	5.22	0.09	0.38
9	4.78	0.07	0.31
10	4.57	0.06	0.27
14	4.11	0.03	0.18
18	3.90	0.02	0.13
22	3.82	0.00	0.00
26	3.74	0.00	0.00
30	3.98	0.00	-
34	3.49	0.00	-
38	3.52	-	-
42	3.61	-	-

[a]Neox, Neoxanthin; Neoc, Neochrome; Anther, Antheraxanthin; Muta, Mutatoxanthin; Violax, Violaxanthin; Luteo, Luteoxathin and Auro, Auroxanthin; ud, undefined.

Table 3. Rate Constants (k) and Determination Coefficients (R^2) Estimated for the Kinetic Mechanism of the Thermal Degradation of Neoxanthin, Antheraxanthin and Violaxanthin in VOO.

		120 ºC			100 ºC			80 ºC			60 ºC		
Reaction[a]	**Sample[b]**	**k^c x 10^3 (h^{-1})**	**SE**	**R^2**	**k^c x 10^3 (h^{-1})**	**SE**	**R^2**	**k^c x 10^3 (h^{-1})**	**SE**	**R^2**	**k^c x 10^3 (h^{-1})**	**SE**	**R^2**
D. Neoxanthin	N	44.91a	1.21	0.99	10.99a	0.14	1.00	3.66a	0.29	0.95	0.74a	0.03	0.97
k_1	D	48.74b	1.05	1.00	17.48b	0.70	1.00	4.47b	0.16	0.99	0.97b	0.02	0.99
	J	60.59c	1.32	0.99	15.46c	0.49	0.98	9.17c	0.30	0.99	0.99b	0.03	0.98
D. Neochrome	N	213.47d	2.48	0.99	51.18d	0.79	0.99	10.40d	0.38	0.94	1.31c	0.06	0.99
k_2	D	228.23e	3.20	0.99	81.93e	1.30	0.97	13.53e	0.37	0.96	1.68d	0.05	0.99
	J	182.67f	2.87	0.99	63.51f	1.20	0.96	27.04f	0.53	0.99	1.38e	0.11	0.93
D. Antheraxanthin	N	228.23g,e	3.50	1.00	141.74g	5.30	0.97	10.79g,d	0.27	1.00	6.86e	0.13	1.00
k_3	D	182.69h,f	1.57	1.00	96.99h	1.34	1.00	8.91h,c	0.24	1.00	5.87f	0.07	1.00
	J	152.48i	2.28	1.00	77.99i	1.15	1.00	15.05i	0.39	1.00	4.91g	0.27	0.97
D. Mutatoxanthin	N	178.92j	2.98	1.00	111.68j	3.11	0.97	8.61j,c,h	0.21	0.95	4.55h,g	0.05	0.99
k_4	D	144.82k	1.46	1.00	69.15k	1.06	0.99	7.78k	0.19	0.94	4.70i,g	0.05	0.99
	J	131.86l	1.23	0.99	51.64l,d	0.20	1.00	14.94l,i	0.26	0.99	3.10j	0.06	0.96
D. Violaxanthin	N	257.95m	3.65	1.00	170.17m	4.86	0.99	109.76m	1.47	1.00	54.06k	0.50	1.00
k_5	D	289.35n	2.67	1.00	204.92n	6.43	0.99	93.67n	1.61	1.00	63.80l	0.92	1.00
	J	267.38o	1.56	1.00	165.10o	0.64	1.00	90.27n,o	1.74	1.00	54.36k	0.32	1.00
D. Luteoxanthin	N	191.42p	2.91	1.00	150.27p,g	3.52	0.98	89.92o	0.60	1.00	35.09m	0.11	1.00
k_6	D	226.70q,e,g	1.23	1.00	187.17q	5.29	0.98	73.86p	1.24	1.00	40.34n	0.18	1.00
	J	195.52p	0.91	1.00	140.76r,g	0.93	1.00	70.58q	0.85	1.00	43.98o	0.28	1.00
D. Auroxanthin	N	145.85r	1.43	0.98	119.36s	1.40	0.98	68.60r,p,q	6.37	1.00	21.46p	0.04	1.00
k_7	D	155.69s,i	1.41	0.99	142.22t,g,q	2.56	0.96	53.82s	0.60	0.99	23.82q	0.06	1.00
	J	135.91t	0.33	1.00	110.37u,j	0.62	0.99	51.68s	1.55	1.00	17.04r	0.05	1.00

[a]Reactions according to the kinetic mechanism shown in **Figure 6**: D, degradation; [b]S, Sample codex as in Table 1; [c]Values are obtained from a minimum of 16 experimental data points analyzed in duplicate; SE, standard error; At each temperature, different letters between rows indicate significant differences ($p \leq 0.05$).

Table 4. Thermodynamic parameters[a] for the thermodegradation reaction of xanthophyll compounds in Virgin Olive Oil.

Reaction[b]	Sample[c]	$\Delta S^{\#}$ [(cal/mol·K)]	SE[d]	$\Delta H^{\#}$(kcal/mol)	SE	E_a(kcal/mol)	SE	$\Delta G^{\#}_{298}$(kcal/mol)	SE
Neoxanthin	N	-38.74	1.07	16.75	0.39	17.79	0.12	28.30	0.39
Neoxanthin	D	-39.18	0.71	16.41	0.26	17.10	0.12*	28.09	0.26
Neoxanthin	J	-39.59	3.69	16.11	1.33	17.84	0.21	27.91	0.33
Neochrome	N	-23.84	0.93	21.30	0.34	22.16	0.06*	28.40	0.34
Neochrome	D	-24.40	2.16	20.91	0.78	21.63	0.32	28.18	0.78
Neochrome	J	-27.75	5.47	19.69	1.98	21.56	0.38	27.96	1.98
Antheraxanthin	N	-36.31	5.50	16.29	1.99	15.51	0.62	27.11	1.99
Antheraxanthin	D	-38.24	4.99	15.76	1.80	15.14	0.42	27.16	1.80
Antheraxanthin	J	-40.71	2.02	14.88	0.73	15.08	0.35	27.01	0.73
Mutatoxanthin	N	-35.02	5.18	16.96	1.87	16.25	0.66	27.39	1.87
Mutatoxanthin	D	-39.54	4.27	15.46	1.54	14.99	0.31*	27.24	1.54
Mutatoxanthin	J	-39.21	1.08	15.60	0.39	16.49	0.17	27.29	0.39
Violaxanthin	N	-62.04	0.31	6.17	0.11	6.84	0.07	24.66	0.11
Violaxanthin	D	-62.16	0.54	6.02	0.19	6.73	0.15	24.54	0.19
Violaxanthin	J	-61.74	0.37	6.28	0.13	6.93	0.06	24.68	0.13
Luteoxanthin	N	-60.59	1.24	6.89	0.45	7.66	0.37	24.95	0.45
Luteoxanthin	D	-59.79	1.54	7.05	0.56	7.89	0.46	24.87	0.56
Luteoxanthin	J	-62.94	0.76*	6.02	0.27*	6.59	0.18*	24.77	0.27
Auroxanthin	N	-58.48	1.79	7.89	0.65	8.80	0.53	25.31	0.65
Auroxanthin	D	-58.76	2.44	7.68	0.88	8.67	0.71	25.19	0.88
Auroxanthin	J	-57.03	2.20	8.49	0.79	9.43	0.59	25.48	0.79

[a] $\Delta S^{\#}$, activation entropy; $\Delta H^{\#}$, activation enthalpy; Ea, activation energy, $\Delta G^{\#}$, Gibbs free energy; [b] Reactions according to the kinetic mechanism showed in **Figure 6**; [c] S, Sample codex as in **Table 1**; [d] SE, standard error; *, Indicate significant differences for a parameter between different samples ($p \leq 0.05$).

Table 5. Isokinetic Temperature (T_{isok}) and Determination Coefficients (R^2) Estimated by Leffer´s Compensation Law ($\Delta H^{\#}_i = \Delta H^{\#}_0 + \beta \Delta S^{\#}$) for the Thermal Degradation Reactions of Xanthophylls in Virgin Olive Oil.

Reaction[a]	β[c]	**SE**	R^2
D. Neoxanthin	751.8	14.4 [d]*	0.99
D. Neochrome	394.1	40.6	0.99
D. Violaxanthin	591.1	248.9	0.89
D. Luteoxanthin	338.2	35.0*	0.99
D. Auroxanthin	448.9	44.1*	0.99
D. Antheraxanthin	322.8	23.9*	0.99
D. Mutatoxanthin	327.9	74.9	0.99
Group of reactions[b]			
Neox, violax and anther degradation reactions	424.1	16.1*	0.99
Neoc, auro and muta degradation reactions	385.7	3.7*	1.00

[a]Reactions according to the kinetic mechanism showed in **Figure 6**; D, degradation.[b]Group of reactions. Neox, Neoxanthin; Neoc, Neochrome; Anther, Antheraxanthin; Muta, Mutatoxanthin; Violax, Violaxanthin; Luteo, Luteoxathin and Auro, Auroxanthin.[c] $\beta = T_{isok}$. [d]*Indicates significant differences ($p \leq 0.05$) with the mean harmonic temperature (T_{hm}) = 362K.

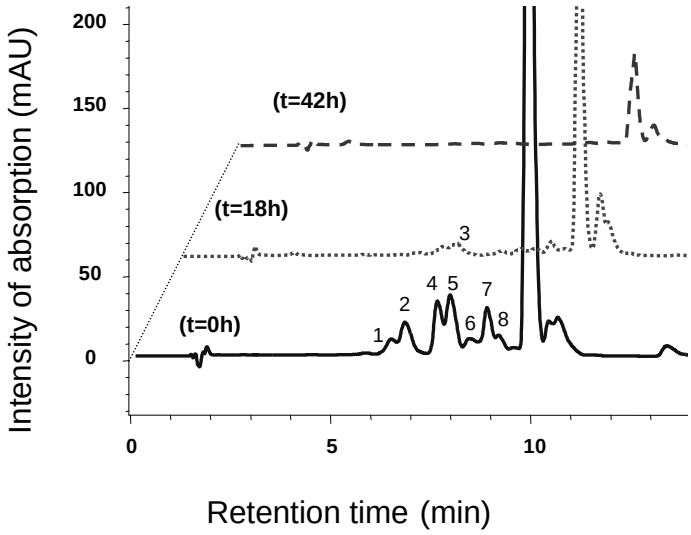

Intensity of absorption (mAU)
200
150
100
50
0
(t=42h)
(t=18h)
(t=0h)
1
2
3
4
5
6
7
8
0
5
10
Retention time (min)

Figure 1

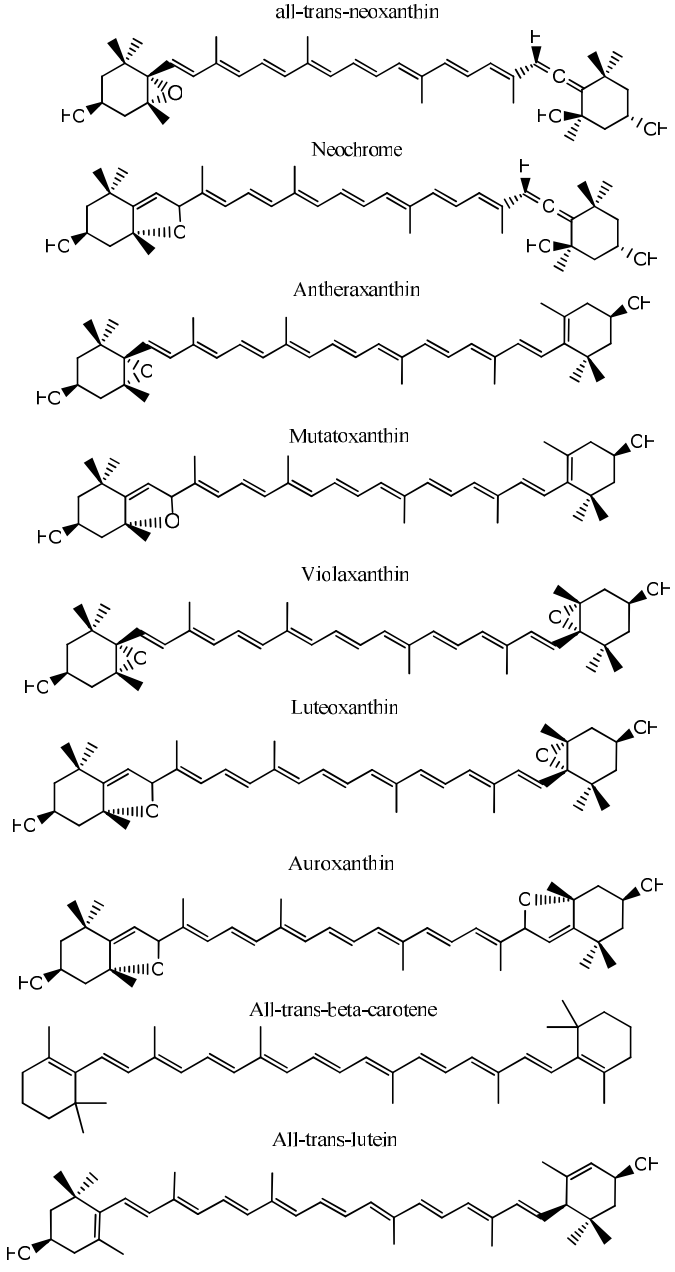

all-trans-neoxanthin
Neochrome
Antheraxanthin
Mutatoxanthin
Violaxanthin
Luteoxanthin
Auroxanthin
All-trans-beta-carotene
All-trans-lutein

Figure 2

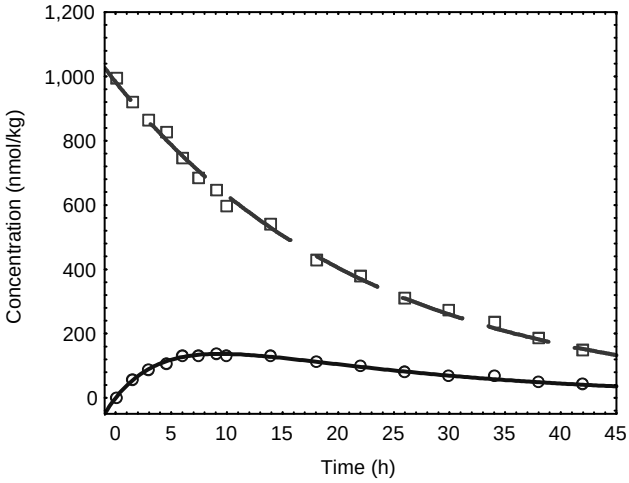

1,200
1,000
800
600
400
200
0
Concentration (nmol/kg)
0
5
10
15
20
25
30
35
40
45
Time (h)

Figure 3

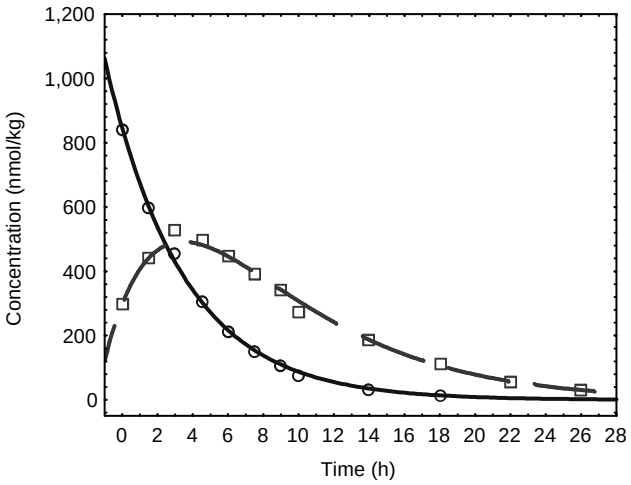

1,200
1,000
800
600
400
200
0
Concentration (nmol/kg)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (h)

Figure 4

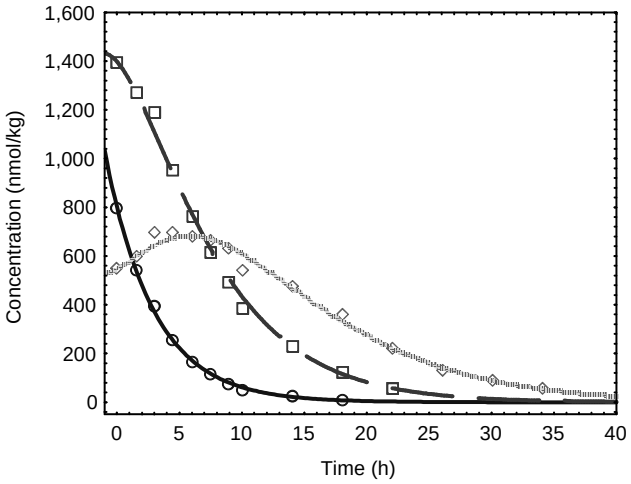

1,600
1,400
1,200
1,000
800
600
400
200
0
Concentration (nmol/kg)
0
5
10
15
20
25
30
35
40
Time (h)

Figure 5

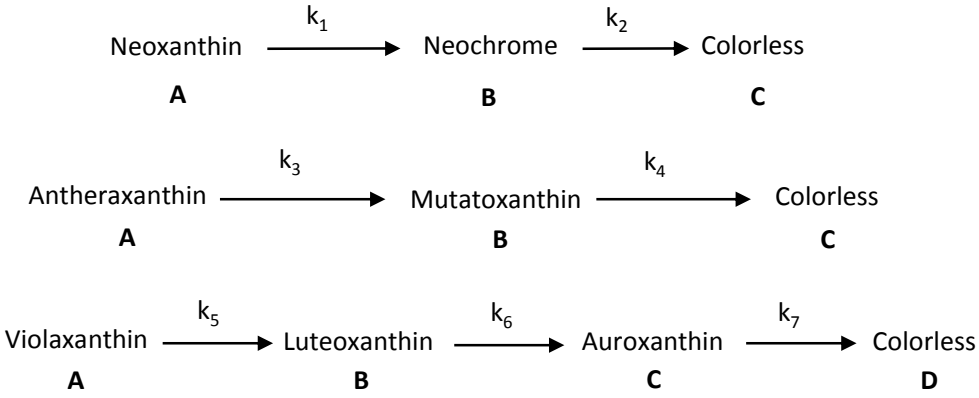

Neoxanthin
k1
Neochrome
k2
Colorless
A
B
C
Antheraxanthin
k3
Mutatoxanthin
k4
Colorless
A
B
C
Violaxanthin
k5
Luteoxanthin
k6
Auroxanthin
k7
Colorless
A
B
C
D

Figure 6

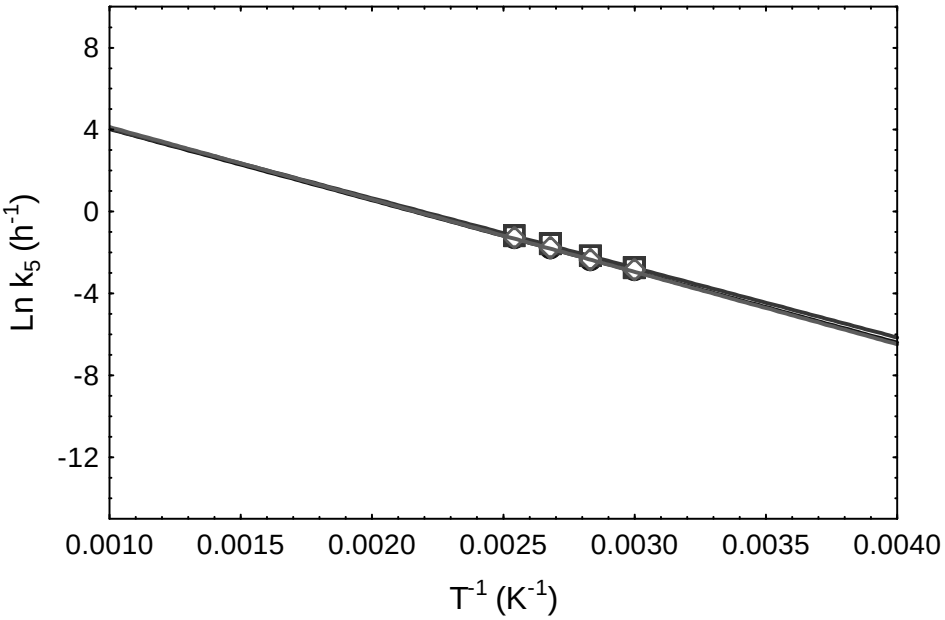

8
4
0
-4
-8
-12
Ln k_5 (h^{-1})
0.0010
0.0015
0.0020
0.0025
0.0030
0.0035
0.0040
T^{-1} (K^{-1})

Figure 7

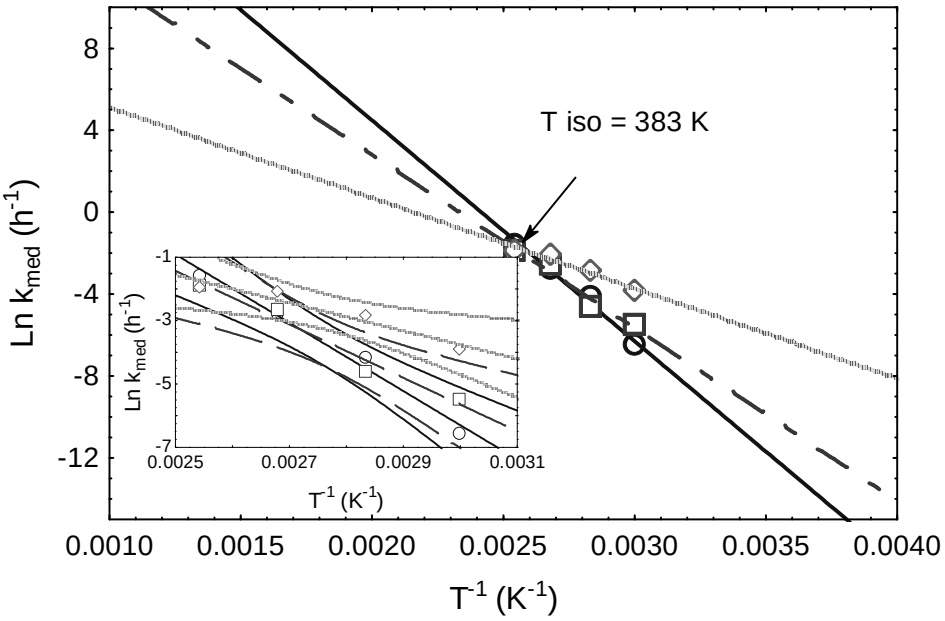

T iso = 383 K
Ln k_med (h^-1)
T^-1 (K^-1)
8
4
0
-4
-8
-12
0.0010
0.0015
0.0020
0.0025
0.0030
0.0035
0.0040
-1
-3
-5
-7
0.0025
0.0027
0.0029
0.0031

Figure 8

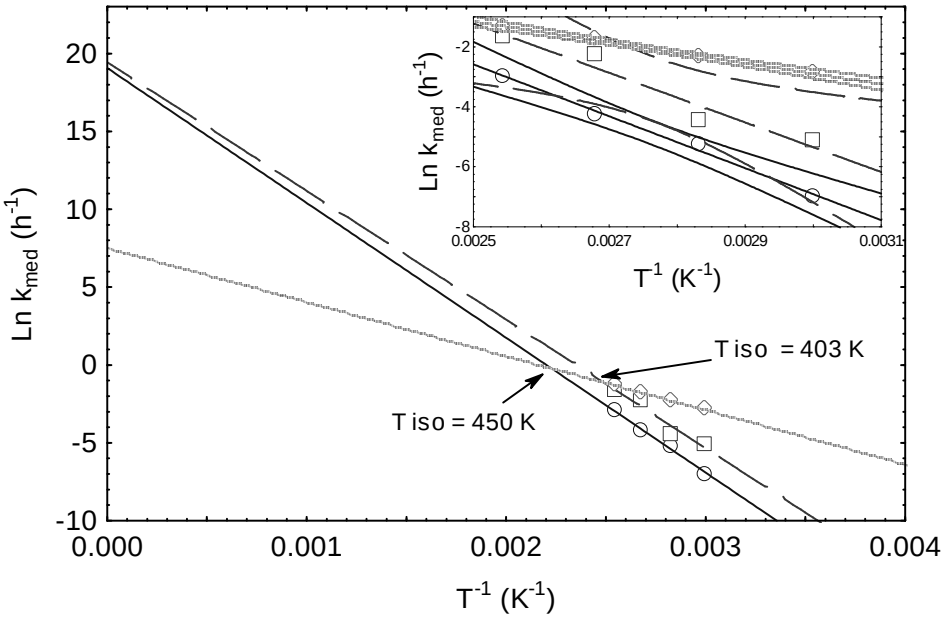


Figure 9

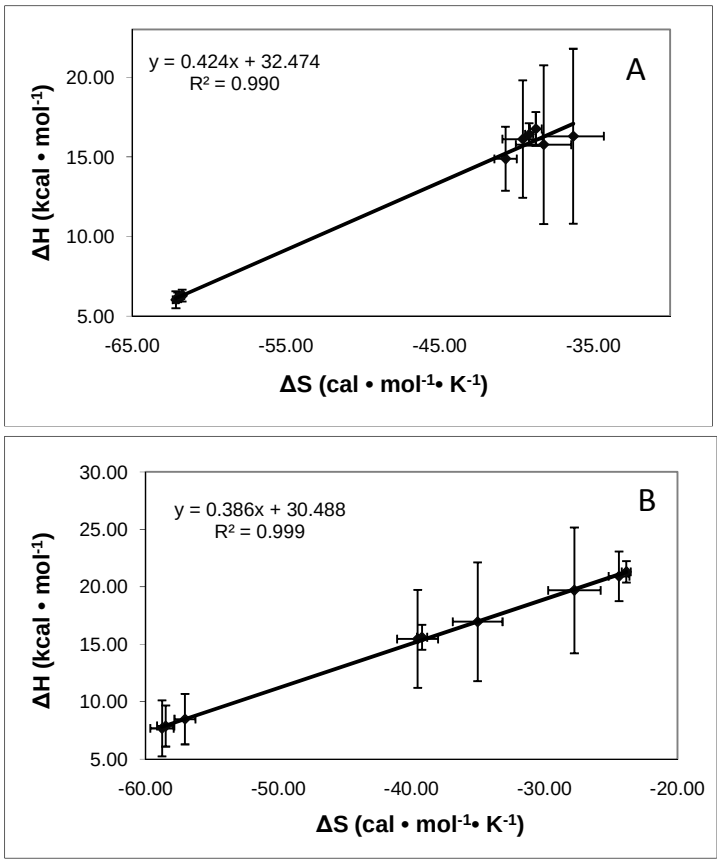
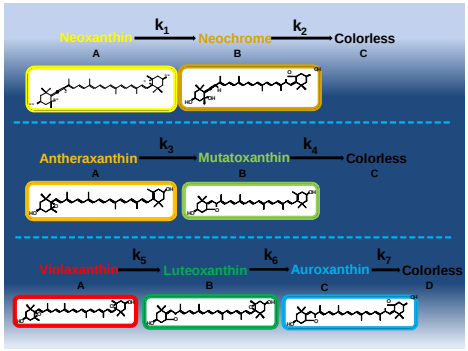

y = 0.424x + 32.474
R² = 0.990
A
ΔH (kcal • mol-1)
20.00
15.00
10.00
5.00
-65.00
-55.00
-45.00
-35.00
ΔS (cal • mol-1• K-1)
y = 0.386x + 30.488
R² = 0.999
B
ΔH (kcal • mol-1)
30.00
25.00
20.00
15.00
10.00
5.00
-60.00
-50.00
-40.00
-30.00
-20.00
ΔS (cal • mol-1• K-1)

Figure 10

TOC GRAPHIC


Neoxanthin
A
k1
Neochrome
B
k2
Colorless
C
Antheraxanthin
A
k3
Mutatoxanthin
B
k4
Colorless
C
Violaxanthin
A
k5
Luteoxanthin
B
k6
Auroxanthin
C
k7
Colorless
D