Study of Lability and Kinetics of the Main Carotenoid Pigments of Red Pepper in the De-esterification Reaction

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Different concentrations of potassium hydroxide in methanol were used to control the time required for complete de-esterification of the carotenoids present in paprika oleoresin, and the effectiveness of the overall process was determined. With concentrated solutions of potash, 20% and 30% (w/v), the reaction was completed in a shorter time but the overall process was less effective. Solutions of low concentration, 5% and 10% (w/v), enabled the kinetic study to be carried out and the rates of de-esterification for individual pigments to be compared. The effect that the fatty acid esterifying each carotenoid had on the reaction was deduced. The pigments esterified by long-chain fatty acids had a higher rate of de-esterification than those bonded with short-chain fatty acids.

Keywords: *Carotenoids; de-esterification; kinetics; lability*

INTRODUCTION

The pepper (*Capsicum annuum*) is one of the main sources of carotenoid pigments. Some of these, such as capsanthin and capsorubin, are synthesized only in this genus (Curl, 1962; Davies et al., 1970). During ripening, the pigment composition changes, the chloroplast pigments (chlorophylls and some carotenoids such as lutein and neoxanthin) disappear, while exclusively carotenoid chromoplast pigments are synthesized. As they are synthesized, they are esterified by fatty acids (FA) (Philip et al., 1971; Camara and Monéger, 1978; Mínguez Mosquera and Hornero Méndez, 1994).

The total or partial esterification of most carotenoids gives them a high stability against possible thermo- and photo-oxidation reactions (Daood and Biacs, 1986; Biacs et al., 1989). At the same time, and according to data in the literature (Gross, 1991; Mínguez Mosquera and Hornero Méndez, 1994), the different fatty acids that esterify the xanthophylls seem to have a discriminatory nature. Thus, capsanthin and capsorubin are esterified by short-chain, saturated fatty acids, such as lauric (12:0) and myristic (14:0) acids, while the yellow xanthophylls are esterified mainly by long-chain fatty acids, including unsaturated ones, such as oleic (18:1) and linoleic (18:2) acids.

The dehydrated products derived from pepper—paprika and oleoresin—are concentrates of carotenoid pigments that remain esterified for the most part; their color is an important physical quality. Consequently, the coloring capacity of the commercial derivatives of pepper depends on the carotenoid content they possess. With the aim of evaluating this attribute, rapid, reproducible methods have been developed to separate and quantify carotenoid pigments by HPLC. Because the carotenoid pigments may be esterified in many ways with the different fatty acids to which they bond, the direct extract of pigments must be de-esterified before the chromatographic analysis to reduce the number of chromatographic peaks and simplify quantification of the sample.

The literature includes different de-esterification methods, in which the first factor to consider is the concentration of the potassium hydroxide solution to be used. Moreover, this may be aqueous, methanolic, or ethanolic, and the concentration may vary from 10% to 60% (w/v) (Goodwin, 1976). The conditions under which the de-esterification is carried out may also vary. They may be at ambient temperature or under heating, with the consequent advantages and disadvantages: hot de-esterification requires less time, but the cold method does not affect the thermolabile carotenoids (Liaaen-Jensen, 1971).

In both cases, the addition of potassium hydroxide is necessary for the reaction to take place. The medium will thereby contain a high-energy reagent able to break the pigment—fatty acid bond. Since the nature of the fatty acid esterifying the pigment differs depending on the pigment to which it is bonded, different behaviors can be expected in the de-esterification reaction. In addition, some agent to which the pigments show lability may be liberated into the medium during the course of the reaction or once it is finished. According to Ittah et al. (1993), the potassium hydroxide present can cause deterioration of β-carotene during the process. Thus, it is necessary to know the concentration of potassium hydroxide and the minimum time needed for complete de-esterification of the intrinsic carotenoids of pepper without the induction of degradative reactions. When that is done, the kinetic study of the de-esterification reaction of pepper carotenoids can be considered.

EXPERIMENTAL PROCEDURES

Sample. The sample of oleoresin used in the kinetic study of de-esterification of carotenoids was supplied by the commercial company EVESA. The xanthophyll fraction was

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totally esterified. Fatty acid analysis, carried out according to the method of Mínguez Mosquera and Hornero Méndez (1994) showed no variation with respect to the literature data.

Standards. Standard solutions of β-carotene, β-cryptoxanthin, zeaxanthin, capsanthin, capsorubin, and violaxanthin were obtained using the method of Mínguez-Mosquera and Garrido-Fernández (1984) from the direct (non de-esterified) extract of the oleoresin. Silica gel plates, 60 GF_{254} (Merck, Darmstadt, Germany), 20 × 20 cm and 0.7 mm in thickness, were used for thin layer chromatography (TLC). Each standard was purified by rechromatographing with the same developer, repeating the operation until the required amount was accumulated. The solvent used in all standard solutions was ethanol.

High-Performance Liquid Chromatography (HPLC). Samples were analyzed by HPLC using a quaternary pump (Waters model 600E) with a UV−vis diode detector (DAD) (model 996). The apparatus was fitted with an injection valve (Rheodyne model 7125) with a loop of 20 μL. The column employed was a C_{18} packed with Spherisorb ODS 2 (5 μm, 25 cm × 4 mm i.d.), supplied by Hewlett-Packard. A precolumn (1 cm × 4 mm i.d.) of the same packing was used to protect the main column.

The carotenoid pigments were monitored and quantified using the method previously designed by Mínguez Mosquera and Hornero Méndez (1993). This method uses the reverse-phase technique with a binary gradient comprising acetone−water at a flow rate of 1.5 mL/min. Quantification was performed using β-apo-8′-carotenal (Sigma Chemical Co., St. Louis, MO) as internal standard for calibration, and detection was performed at 450 nm.

Pigment Lability to Potassium Hydroxide Solutions. Known volumes of potassium hydroxide solutions in ethanol at concentrations of 5%, 10%, 20%, and 30% were mixed with aliquots of each standard solution of pigment at the ratio of 1:1 (v/v). The two reagents taking part in the de-esterification reaction, pigment and potassium hydroxide, were put into contact. The initial concentrations of each solution mixture were 5.1 μg/mL of capsanthin, 3.0 μg/mL of capsorubin, 3.3 μg/mL of violaxanthin, 2.9 μg/mL of β-carotene, 2.5 μg/mL of zeaxanthin, and 2.1 μg/mL of capsolutein. The possible losses due to contact with the potassium hydroxide solutions were detected by measuring absorbance with a UV−vis spectrophotometer (Hewlett-Packard model 8452A) at 5-min intervals for 1 h.

Monitoring the De-esterification Reaction. The pigment de-esterification reaction was carried out at ambient temperature, under green light, by dissolving ∼0.025 g of oleoresin in 50 mL of ethyl ether in a decanting funnel. The de-esterification was verified using four concentrations (5%, 10%, 20%, and 30% w/v) of potassium hydroxide in methanol, adding 25 mL of solution in each case and monitoring the reaction time. The solution mixture was gently shaken. The reaction times controlled were 1, 2, 3, 5, 7, and 10 min if the 20% and 30% (w/v) solutions were used and 5-min intervals if the solutions employed were 5% and 10% (w/v).

Each time point represents an analysis in quadruplicate.

Once the reaction time was reached, in each case the reaction was stopped by the addition of 200 mL of NaCl solution (10% w/v) so two phases were formed. The possible pigmentation in the aqueous phase was recovered. Internal standard solution was added for the subsequent quantification by HPLC.

Kinetic Study of the De-esterification Reaction. The kinetic study of the de-esterification was carried out on the basis of the following general reaction:

$$\text{pigment}-\text{FA} + \text{OH}^{\ominus} \rightarrow \text{pigment} + \text{FA}^{\ominus}$$

The potassium hydroxide breaks the pigment−fatty acid bond, and the hydroxyl group is incorporated into the pigment. The fatty acid remains joined by ionic bond to the K^+ ion. If the pigment is joined to more than one fatty acid, the reaction that takes place is essentially the same. The nature of the fatty acid, that is, whether it is long or short chain, affects the development of the reaction, as shown below.

The rate of de-esterification is expressed by the equation

$$-\mathrm{d}(C_{\mathrm{pe}})/\mathrm{d}t = kC_{\mathrm{pe}}^{\alpha}C_{\mathrm{OH}^{\ominus}}^{\beta} \quad (1)$$

where C_{pe} and $C_{\mathrm{OH}^{\ominus}}$ are the concentrations of esterified pigment and potassium hydroxide, respectively, α and β are the corresponding orders of reaction, and k is the kinetic constant. As the concentration of esterified pigment can be measured over time, the method of excess components is employed to determine the kinetic parameters; an excess of potassium hydroxide is added, so that its concentration remains constant throughout, and the rate of de-esterification is expressed by the equation

$$-\mathrm{d}(C_{\mathrm{pe}})/\mathrm{d}t = k'C_{\mathrm{pe}}^{\alpha} \quad (2)$$

where $k' = k(C_{\mathrm{OH}^{\ominus}})^{\beta}$, both constant, is the pseudospecific rate constant. Thus, the change in concentration of esterified pigment with reaction time enables the order of reaction and the kinetic constant to be obtained. The concentration of esterified pigment, expressed as percentage remaining, is calculated from the difference between the concentration of totally de-esterified pigment and the concentration of de-esterified pigment obtained in each time data analysis.

The order of the reaction is deduced using the integral method. This method uses a trial-and-error procedure to find reaction order. The order is supposed and eq 2 is integrated. If the order assumed is correct, the appropriate plot (determined from this integration) of the concentration−time data should be linear (Fogler, 1992).

Reagents. *Solvents* included acetone, ethanol, ethyl acetate, diethyl ether, light petroleum ether, hexane, and methanol (all of ACS grade). For LC, acetone (LC grade) and deionized water were used.

Chemical products included NaCl, KOH, and Na_2SO_4 (all of ACS grade).

RESULTS AND DISCUSSION

The experiments carried out to test the lability of each pigment to the solutions of potassium hydroxide assayed showed that the absorbance of the mixture of standard pigment and potassium hydroxide was constant during the hour monitored as maximum time for the de-esterification reaction. This fact allows one to discard the lability of the carotenoid pigments, including that of β-carotene to the solutions of potassium hydroxide.

The use of high or low concentrations of potassium hydroxide to verify the de-esterification reaction led to notable differences in the results. With the solution of 30% potassium hydroxide, complete de-esterification was verified at 7 min, while a reaction time of 40 min was necessary when the solution was 5%. The rapid de-esterification occurring in the former case was noteworthy, with capsolutein and zeaxanthin reaching 50% de-esterification in the first minute of reaction, which made monitoring difficult.

Furthermore, at the end of the reaction with concentrated potassium hydroxide solutions (20% and 30%), there was a greater transference of pigmentation to the aqueous phase, meaning a higher number of recoveries and manipulations of the sample because of the extra washing of the ether phase necessary to remove the potassium hydroxide not consumed in the reaction. Table 1 shows the percentage of de-esterified xanthophylls present in the oleoresin during the course of the reaction as a result of the treatment with the aforementioned solutions of potassium hydroxide. As can be seen, the short times necessary and the speed at which

Table 1. Time Evolution of the De-esterification Reaction of the Main Oleoresin Pigments with 20% and 30% (w/v) KOH solution

	% of de-esterified pigment				
reaction time (min)	capsorubin	violaxanthin	capsanthin	capsolutein	zeaxanthin
20% KOH solution					
1	22.0 ± 1.0	19.9 ± 0.8	18.4 ± 0.7	36.1 ± 0.7	27.5 ± 0.9
2	28.1 ± 0.8	29.2 ± 1.0	29.9 ± 0.9	40.1 ± 1.1	35.7 ± 1.0
3	27.4 ± 1.3	30.6 ± 1.2	29.6 ± 1.4	39.4 ± 1.4	36.2 ± 1.5
5	42.2 ± 1.4	38.4 ± 1.3	39.6 ± 1.1	42.9 ± 0.8	43.2 ± 1.2
7	80.5 ± 0.9	79.4 ± 1.4	85.0 ± 0.48	89.7 ± 1.2	88.6 ± 0.9
10	100	100	100	100	100
30% KOH solution					
1	30.6 ± 0.6	30.5 ± 0.8	29.5 ± 0.8	55.1 ± 1.0	44.4 ± 0.9
2	70.5 ± 0.9	73.8 ± 1.1	75.2 ± 0.6	86.0 ± 1.3	87.2 ± 0.8
3	72.4 ± 1.1	72.7 ± 1.2	77.0 ± 1.0	89.2 ± 0.9	87.3 ± 0.8
5	80.1 ± 0.7	73.3 ± 1.0	81.8 ± 0.8	95.5 ± 0.9	92.3 ± 1.2
7	100	100	100	100	100

the reaction takes place prevent the obtaining of trustworthy data for a proper kinetic study, due partly to the speed of the reaction and partly to the difficulty in recovering the coloring matter.

Despite the fact that our "low-concentration" solutions required longer de-esterification times, they needed fewer washings (the excess of potassium hydroxide was practically removed in the first washing) and did not produce pigmentation transference to the aqueous phase, so that the reaction could be monitored. With potassium hydroxide concentrations of 5% and 10% (w/v), the best fit of the experimental data in both cases gave first-order kinetics, as seen in Figure 1. The correlation coefficients and kinetic parameters calculated from the model are shown in Table 2.

The values of the kinetic constant depending on pigment enable the xanthophylls to be divided into two groups. The first comprises zeaxanthin and capsolutein, which have values for the parameter k' of between 1.2- and 2-fold higher than the other xanthophylls. This makes it necessary to shorten the control times in the first period of the reaction. These two yellow pigments are esterified by long-chain, unsaturated fatty acids. The red pigments, capsanthin and capsorubin, and violaxanthin are included in the other group, in which the rate of de-esterification is shorter, as indicated by the values of the constants, which are lower than those of the first group but very similar one to another. These xanthophylls are esterified by short-chain, saturated fatty acids. The effect of the esterifying fatty acids on the kinetic behavior of the pigments is thus shown to be a higher or lower rate of de-esterification of carotenoids. The presence of different functional groups in the structure of the different pigments can also have a certain effect: capsolutein with one epoxide group inside the ring of β-inone, zeaxanthin with two hydroxyl groups in its structure, violaxanthin with two epoxide groups, and capsanthin and capsorubin with one and two ketone groups, respectively, may modulate the reaction rate via the distinct polarity conferred on the pigment by the presence or absence of these groups, leading in each case to a greater or lesser effectiveness of the alkaline treatment.

At the same time we want to point out that whatever the solution of potassium hydroxide used in the assays, the same final concentration was achieved for each pigment, the same as occurs with the carotenoid standards, ruling out a destructive effect of this reagent on the carotenoids assayed, including β-carotene.

Evaluating the parameters of reaction time necessary to complete the de-esterification reaction and the num-

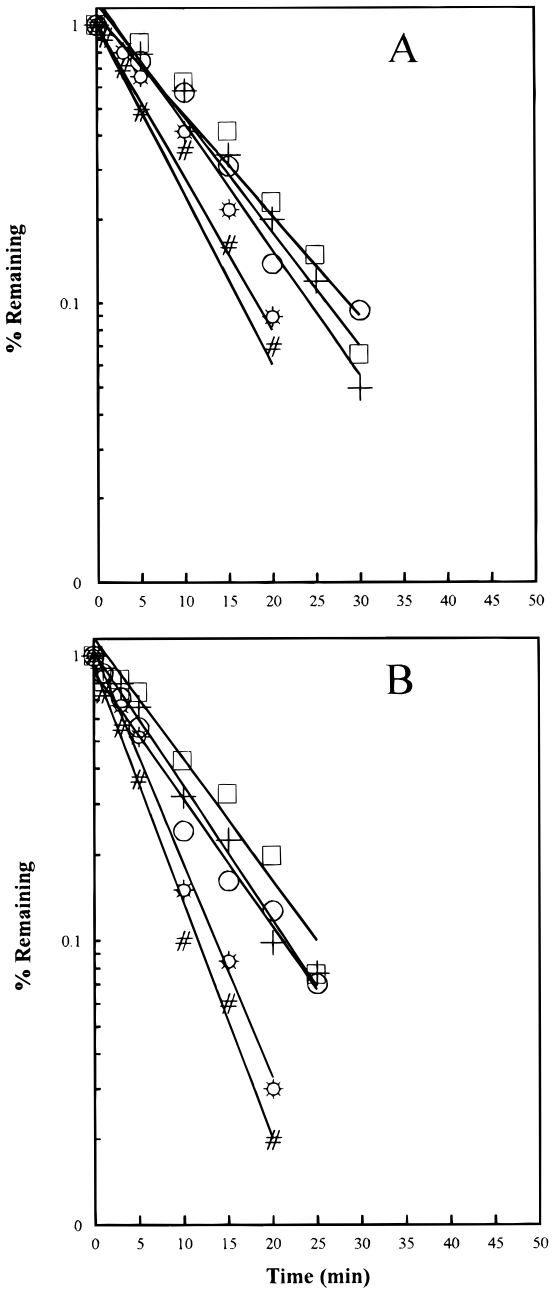

Figure 1. First-order kinetics and their corresponding fits of capsorubin (□), capsanthin (+) violaxanthin (○), capsolutein (#), and zeaxanthin (⊙) in the de-esterification reaction with (A) 5% (w/v) and (B) 10% (w/v) KOH.

Table 2. Pseudospecific Rate Constants and Correlation Coefficients (CC) of the Main Pigments in the De-esterification Reaction as a Function of the KOH Concentration Used

		5% (w/v) KOH		10% (w/v) KOH	
pigment	reaction order[a]	$k' \pm$ SD ($\times 10^{-3}$, min^{-1})	CC	$k' \pm$ SD ($\times 10^{-3}$, min^{-1})	CC
capsorubin	1	91 ± 8	0.98	97 ± 8	0.98
violaxanthin	1	86 ± 6	0.98	106 ± 6	0.99
capsanthin	1	100 ± 7	0.98	111 ± 4	0.99
zeaxanthin	1	119 ± 10	0.99	173 ± 11	0.99
capsolutein	1	129 ± 10	0.99	195 ± 14	0.99

[a] First order: ln (% remaining) $= 4.605 - k't$.

ber of washings to remove the excess of potassium hydroxide, we consider the 10% (w/v) solution to be the most appropriate for complete de-esterification of the oleoresin without induction of degradative reactions in the pigments comprising it.

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