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Effect of amino acids on the chemical oxidation of olive *o*-diphenols in model systems

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Addition of certain amino acids (L-tryptophan, L-histidine, L-cysteine and L-cystine) to model solutions of caffeic acid and hydroxytyrosol (the two most characteristic *o*-diphenols of ripe olives) improved the dark colour obtained after oxidation. A detailed study carried out with L-cysteine and caffeic acid showed that, after an initial inhibitory effect, increasing concentrations of amino acid led to darker solutions. The same effect was also found in hydroxytyrosol solutions with increasing amounts (2–8 mM) of cysteine without lag phase, except for 10 mM level. Cysteine also produced darker colour with oxidised storage brine of ripe olives. These results open the door to a possible use of this amino acid as additive or aid in processing to produce darker and homogeneous ripe olives. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Two types of phenolic browning reaction can take place in certain foods: enzymic oxidation when polyphenol oxidase is present or, if the enzyme is removed or inactived, non-enzymic autoxidation (Singleton, 1987). These reactions generally result in a loss of nutritional value and the appearance of undesirable brown colours. However, such colours are desirable in some processed foods, for example, in black tea, chocolate, and ripe olives.

The quinones formed enzymatically or non-enzymatically under alkaline conditions are highly reactive substances and can polymerise with other quinones or combine with certain amino acids in food proteins, potentially producing a variety of different-coloured compounds. The reactions of chlorogenoquinone and caffeoquinone with peptides and proteins are likely to involve only the thiol, the terminal α-amino, and the lysine ϵ-amino groups (Pierpoint, 1969). Pierpoint (1966, 1969) also studied the formation of coloured compounds in the reaction between *o*-quinones and free amino acids while the interaction between the sulphydryl groups of cysteine and *o*-quinones has been demonstrated by other researchers (Cilliers and Singleton, 1990; Zhang and Dryhurst, 1994; Shen and Dryhurst, 1996). The reaction of the amino acids serine (Cabanes *et al.*, 1987), methionine (Igarashi and Yasui, 1985), lysine (Pierpoint *et al.*, 1977) and proline (Valero *et al.*, 1988) with *o*-quinones has been studied. Hurrell and Finot (1984), employing a model aqueous system of casein and caffeic acid, have investigated the nutritional implications of the reactions between proteins and oxidised phenolic acids.

The processing of ripe olives consists of successive treatments of olives with sodium hydroxide solutions (lyes) on three consecutive days and, during the intervals between these treatments, fruits are suspended in water through which air is bubbled. Throughout this operation olives darken progressively due to the oxidation of *o*-diphenols, hydroxytyrosol (3,4-dihydroxyphenylethanol) and caffeic acid (Brenes *et al.*, 1992). Different iron salts are added to fix the colour formed (Brenes *et al.*, 1995) and after 1 day at equilibrium, the product is canned and sterilised, as appropriate for a low-acid food (Fernández *et al.*, 1972). The whole process takes about 1 week. A shiny dark colour is not always obtained.

The oxidation reactions of olive *o*-diphenols, caffeic acid and hydroxytyrosol have been reported in model solutions in the presence and absence of metal cations (García *et al.*, 1996). The manganese ion significantly accelerated those reactions and also gave darker solutions.

The aim of this work was to determine the effect of different amino acids on the oxidation of olive *o*-diphenols in alkaline conditions, in order to accelerate these reactions and achieve darker solutions.

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MATERIALS AND METHODS

Chemicals

L-alanine (A), L-arginine hydrochloride (R), L-asparagine (N), L-aspartic acid (D), L-cysteine free base (C), L-cystine, L-glutamic acid (E), L-glutamine (Q), glycine (G), L-histidine monohydrochloride (H), hydroxy-L-proline, L-isoleucine (I), L-leucine (L), L-lysine monohydrochloride (K), L-methionine (M), L-phenylalanine (F), L-proline (P), L-serine (S), L-threonine (T), L-tryptophan (W), L-tyrosine (Y) and L-valine (V) were purchased from Sigma (St. Louis, MO). The symbol of the L-amino acids given in brackets is that used by Lenhinger (1972).

Caffeic acid was purchased from Fluka (Buchs, Switzerland). Hydroxytyrosol was prepared by alkaline hydrolysis of oleuropein (Extrasynthese, Genay, France) according to the method described by García *et al.* (1996).

Oxidation experiments

The experiments with caffeic acid and hydroxytyrosol were carried out with 50 ml of 1 mM of *o*-diphenols solution. This solution was buffered at pH 8 with 20 mM bis-tris propane buffer (Sigma), and different L-amino acids and L-cystine were added to reach 1 mM concentration, except in the specific experiments with L-cysteine where the concentration employed was variable: 2, 4, 8 and 10 mM. The mixture was stirred on a magnetic stirrer and incubated in a thermostatically controlled chamber at 30°C. In another experiment, L-cysteine at a concentration of 4 mM, was added when the *o*-diphenols had previously been oxidised.

Another experiment to study the effect of L-cysteine on oxidation of *o*-diphenol in olive brine was carried out. A storage brine (3 months) from ripe olive processing was diluted 1:3 with 20 mM bis-tris propane buffer solution to reach pH 8, and 4 mM L-cysteine was added. The mixture was incubated in a thermostatically controlled chamber at 30°C and stirred on a magnetic stirrer.

Colour of liquids

Absorption spectra of solutions were measured using a Hewlett–Packard Model 8450 UV-vis spectrophotometer. Samples (200 μl) were withdrawn at regular intervals throughout the incubation period and mixed with 800 μl of an 80 mM bis-tris propane buffer solution with the pH adjusted to 7. In the case of the brine experiment the dilution was 200 μl of samples with 10 ml of 80 mM bis-tris propane solution. Samples were measured with 1-cm path length.

The colour of liquids was expressed either as absorbance at 420 nm (Cilliers and Singleton, 1989) or in terms of the CIE L* a* b* parameters calculated from the absorption spectra (CIE, 1986).

HPLC analyses of *o*-diphenols

The method was the same as that described elsewhere (García *et al.*, 1996).

RESULTS AND DISCUSSION

Cilliers and Singleton (1989) showed a good correlation between the absorbance at 420 nm (A_{420}) and the caffeic acid disappearance at all pH and temperature levels assayed by them. Changes in 420 nm absorbance have also been used in this work to study the effect of the addition of different amino acids to model solutions of caffeic acid, hydroxytyrosol, and fermentation brines on the colour obtained. After 50 h of oxidation, the presence of amino acids in the model solutions gave rise to different absorbance values between caffeic acid and hydroxytyrosol (Fig. 1). In all the assayed conditions, A_{420} values were higher for solutions with caffeic acid than for those with hydroxytyrosol. As regards the effect of amino acids, valine, tyrosine, asparagine, aspartic acid, glutamic acid, lysine and arginine caused a certain decolourisation (lower A_{420}) compared with control, the effect being more pronounced in the caffeic

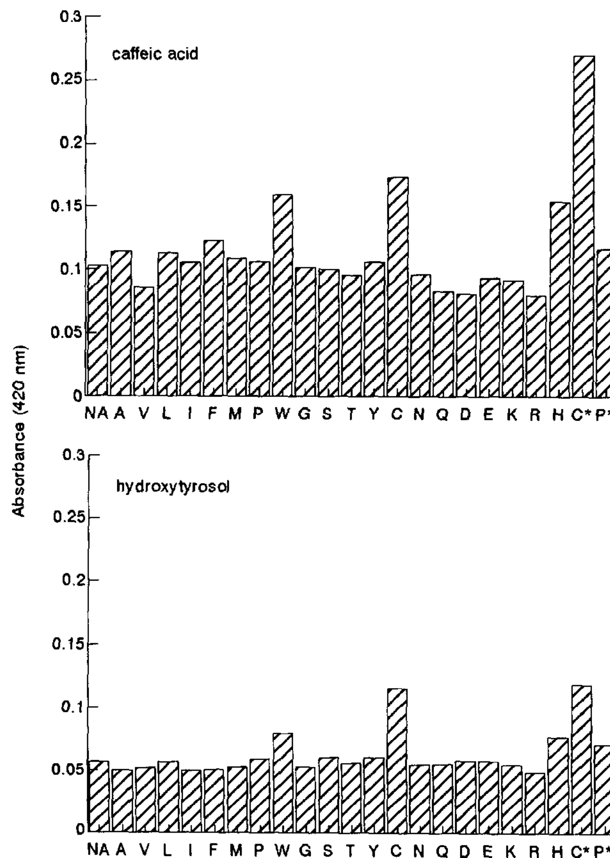

Fig. 1. Effect of different amino acids (1 mM) on the final colour (absorbance at 420 nm) of caffeic acid and hydroxytyrosol solutions (1 mM) oxidised for 50 h. See Materials and Methods for equivalences between symbols and amino acids. NA (non-addition of amino acids), C* (L-cystine), P* (hydroxy-L-proline).

acid solutions than in those of hydroxytyrosol. In contrast, methionine > leucine > hydroxy-proline > alanine > phenylalanine > histidine > tryptophan > cysteine > cystine produced darker caffeic acid solutions (higher A_{420} values). Hydroxy-proline > histidine > tryptophan > cysteine > cystine also caused darker hydroxytyrosol solutions.

Absorbance spectra in the range 400–700 nm did not exhibit any significant peak or shoulder, except for the oxidised solution of caffeic acid with added tryptophan, which showed a slight shoulder at 540–580 nm and black tones instead of reddish ones. This result is similar to that observed by Pierpoint (1969) after oxidation of chlorogenic acid in the presence of tryptophan.

Of the amino acids assayed, cysteine and cystine (a cysteine dimer) had the highest effect on darkening in both caffeic acid and hydroxytyrosol solutions. Cysteine has been widely studied as an inhibitor of the enzymic (Kahn, 1985) or chemical darkening in foods (Cilliers and Singleton, 1990; Zhang and Dryhurst, 1994; Shen and Dryhurst, 1996) although the effect depends on the amino acid/phenol ratio. In our experiments (Fig. 1), the presence of cysteine led to darker oxidised solutions, in agreement with the results reported by Pierpoint (1966). That author demonstrated that the enzymic browning was not inhibited when the cysteine/chlorogenic acid ratio was lower than 1.5; it was 1 in our experiments. Pierpoint (1966) also observed that cystine (in spite of being a cysteine dimer) never inhibited darkening in the concentration range studied, and that oxidation of chlorogenic acid and cystine solutions always led to dark brown colours. A similar result was observed in this work when oxidising caffeic acid and hydroxytyrosol in the presence of cystine. Thus, L-cysteine was chosen to continue the assays because it is on the GRAS (generally accepted as safe) list and is widely used in baking formulations (Friedman, 1996).

The UV-vis absorption spectra of a hydroxytyrosol solution oxidised in the presence of cysteine (Fig. 2) showed that the initial spectra (time 0 h) of hydroxytyrosol with and without added cysteine were similar and had only the characteristic peak of hydroxytyrosol at 280 nm. Thus, there was no increase in absorbance due to cysteine in the wavelength range studied (200–700 nm). The buffer solution used to maintain the pH did not interfere either, since its spectrum did not show any absorption above 250 nm.

After 68.5 h of oxidation, the hydroxytyrosol peak at 280 nm disappeared when cysteine was present, while it was observed in absence of cysteine. Furthermore, in the region 370–700 nm (vis), the spectrum corresponding to the oxidation in the presence of cysteine had higher values than that of hydroxytyrosol alone.

Presence of cysteine not only influenced colour development from hydroxytyrosol oxidation but also the kinetics of this reaction. This could be assumed from the spectral changes in Fig. 2, but it is clearly demonstrated in Fig. 3. Hydroxytyrosol oxidation rate was faster in

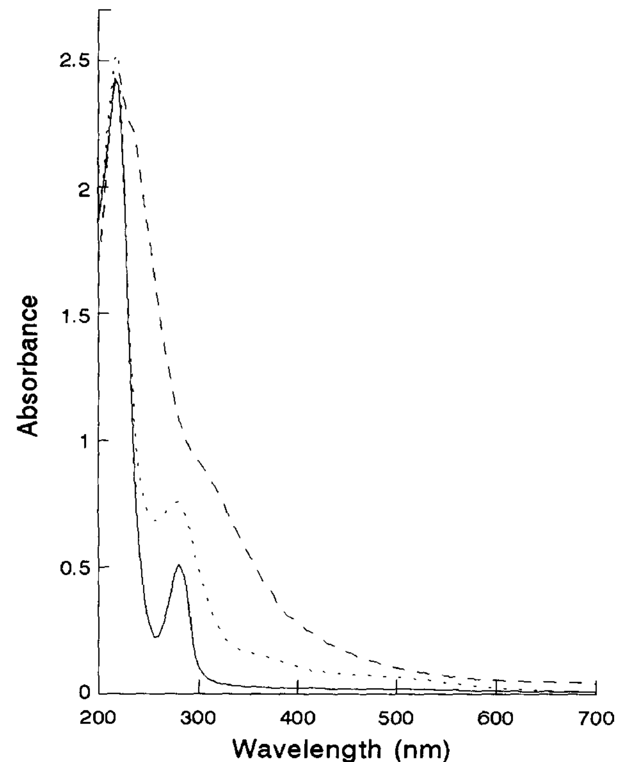

Fig. 2. UV-vis spectra of hydroxytyrosol (1 mM) at pH 7. Samples were measured at time 0 (-) and 68.5 h of oxidation with (- - -) and without (···) cysteine at a concentration of 4 mM.

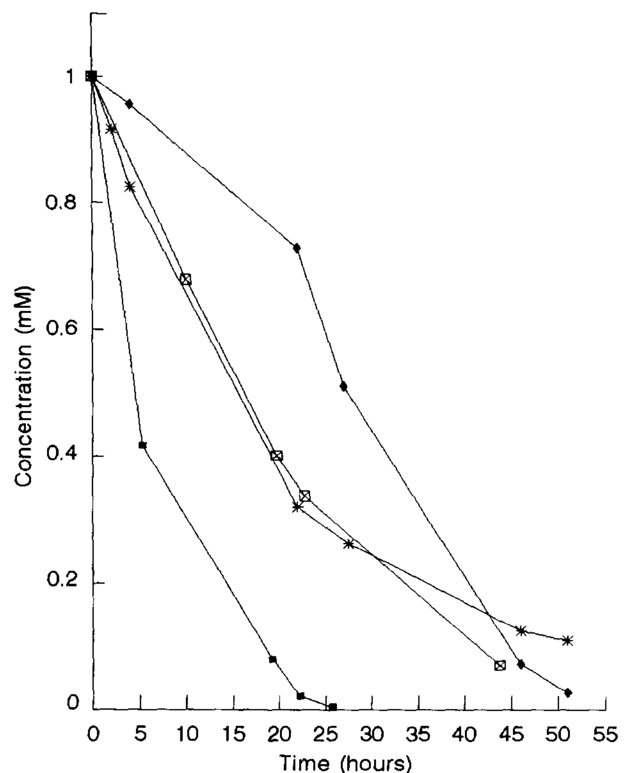

Fig. 3. Evolution of *o*-diphenol concentration as a function of oxidation time. Hydroxytyrosol with (■) and without (⊠) cysteine, caffeic acid with (◆) and without (*) cysteine. The amino acid was added at a concentration of 4 mM.

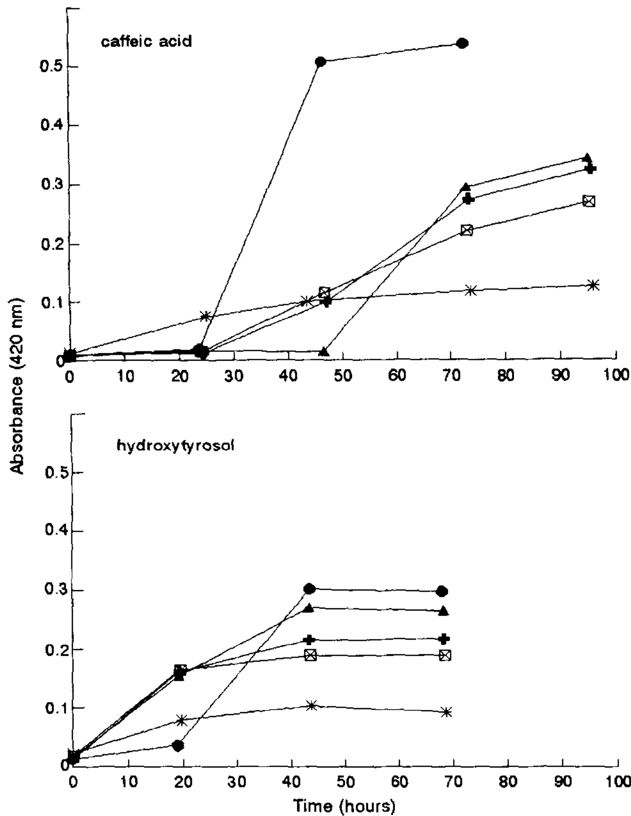

Fig. 4. Evolution of colour (absorbance at 420 nm) during the chemical oxidation of *o*-diphenols in presence of various L-cysteine concentrations: 0 mM (*), 2 mM (⊠), 4 mM (+), 8 mM (▲), 10 mM (●).

presence of cysteine during the whole reaction time, causing total disappearance of this *o*-diphenol after 25 h oxidation. In absence of cysteine, the residual hydroxytyrosol remained higher throughout the period studied and could be quantified even after 45 h of oxidation.

In contrast, cysteine had an inhibitory effect, during the first 25 h, on the caffeic acid oxidation. Then, there was an increase in the rate, which led practically to disappearance of caffeic acid after 50 h. This behaviour is in accordance with that found by Cilliers and Singleton (1990) when oxidising model solutions of caffeic acid in the presence of cysteine at pH 10 and 20°C.

The different behaviour of caffeic acid and hydroxytyrosol in the presence of cysteine did not change when using increasing concentrations of amino acid (Fig. 4). In the caffeic solutions, there was an appreciable lag period in the darkening of solutions, ranging from 20 to 45 h reaction time. Similar results were observed by Cilliers and Singleton (1990) during the oxidation of caffeic acid in an alkaline medium, and by Kahn (1985) when studying the effect of diverse cysteine concentrations on the colour formed by enzymic oxidation of dopamine. After the lag phase, there was a progressive darkening, and the final darkness of the solutions was proportional to the cysteine levels used. However, the highest level assayed (10 mM) led to the shortest lag period and a markedly dark colour.

With respect to hydroxytyrosol (Fig. 4), presence of cysteine in the range studied (2–8 mM) always proportionally increased the darkness of final solutions. There was no lag phase except for the highest level assayed (10 mM) of the nitrogenous compound, which showed a delay in the darkening of solutions of about 20 h although, after continuing the oxidation, the solution achieved the highest value in absorbance.

Results have demonstrated the important role played by cysteine in the oxidation of caffeic acid and hydroxytyrosol, but nothing has been deduced with respect to the mechanism of action. However, it has been observed that the addition of the amino acid after *o*-diphenol oxidation (Fig. 5) produced a certain increase (which was higher in the caffeic acid solution) in absorbance, although colours were markedly less intense than those obtained when the amino acid was added before oxidation. This means that cysteine has an active role in the oxidation reactions, leading to the formation of darker and, possibly, different polymers than those formed in its absence.

Storage brines of ripe olives usually have the same content in phenolics as fruits, which is why they were

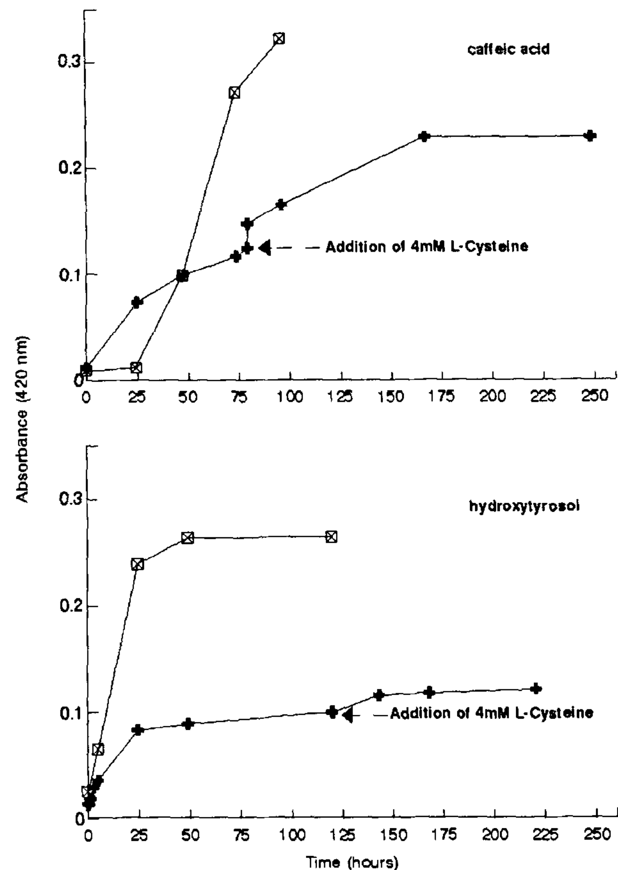

Fig. 5. Effect of the moment of addition of L-cysteine (4 mM) on the final colour of model solutions. L-cysteine was added before (⊠) or after (+) chemical oxidation of *o*-diphenols (1 mM).

Table 1. Values of L* a* b* parameters in the model solutions employed. The oxidations were carried out in the presence and absence of cysteine (4 mM)

Sample	Time (h)	Cysteine (mM)	L*	a*	b*
Caffeic acid	0[a]	–	99.36	−0.10	0.59
Caffeic acid	96	0	95.16	1.03	6.87
Caffeic acid	96	4	90.06	−1.05	15.65
Hydroxytyrosol	0[a]	–	98.59	0.23	0.87
Hydroxytyrosol	68	0	96.30	1.49	5.12
Hydroxytyrosol	68	4	93.47	−2.23	13.08
Brine	0[a]	–	99.06	−0.41	1.50
Brine	214	0	95.54	0.45	6.55
Brine	214	4	94.83	−0.71	7.52

[a]At initial time the spectra with and without cysteine were the same.

used to study darkening during oxidation with and without added cysteine (Fig. 6). The brine used in our experiments had hydroxytyrosol 3.5 mM, and cysteine at 4 mM was added (a ratio of about 0.87:1). The solution containing cysteine developed a darker colour (higher A_{420}) throughout the oxidation period, and differences after 50 h oxidation were comparable to those observed in the model solutions of hydroxytyrosol with added cysteine (ratio 1:1) (Fig. 1).

Colour changes in all the model solutions due to the oxidation process were also characterised by CIE L* a* b* parameters (Table 1). Lightness (L*) decreased with oxidation, and the effect was greater in the presence of cysteine. The a* parameter increased for those solutions containing only *o*-diphenols, meaning that the polymers had in these cases higher reddish tones; a* decreased when oxidation was carried out in the presence of cysteine, indicating a higher participation of greenish tones. Finally, b* was always higher in the oxidised solutions and in the presence of cysteine (higher yellowish tones).

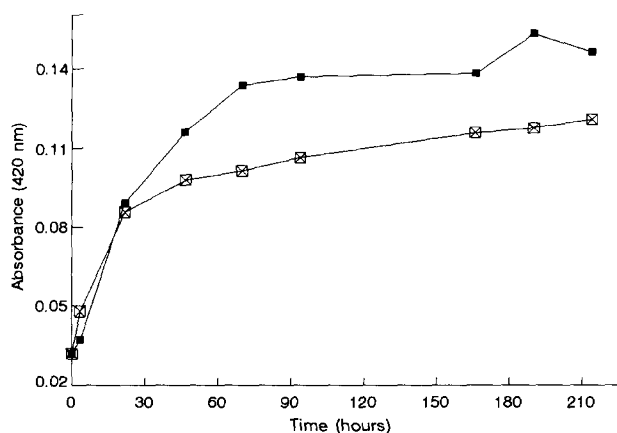

Fig. 6. Evolution of brine colour (absorbance at 420 nm) as a function of oxidation time. Brine with (■) and without (⊠) cysteine, at a concentration of 4 mM.

CONCLUSION

Results obtained in this work have demonstrated that some amino acids can contribute to a darker colour in model solutions containing the most characteristic *o*-diphenols of ripe olives: hydroxytyrosol and caffeic acid. In particular, cysteine increased the oxidation rate of hydroxytyrosol and led to darker colours in model solutions of caffeic acid, hydroxytyrosol, and storage brine of ripe olives. These findings can open the door to a possible use of this amino acid during any step of ripe olive processing, particularly in the darkening phase to produce a more intense and homogeneous dark colour in the final product.

ACKNOWLEDGEMENTS

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