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The Beneficial Effect of Citric and Ascorbic Acid on the Phenolic Browning Reaction in Stored Artichoke (*Cynara scolymus* L.) Heads

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ABSTRACT

Independent of the plant growing season artichoke heads stored at low temperature in closed polyethylene bags showed, with time, an increase in phenolics, especially caffeic acid. After 2 or 4 weeks, dependent on the storage temperature, the phenolic content decreased again while the pattern of changes in the phenolic level proved to be dependent on the harvesting period. Parallel with the increase in phenolic content, PAL activity increased but PPO activity remained almost constant or even decreased. Both citric and ascorbic acid were effective in improving the quality, as well as the shelf life, of the stored artichoke heads and by using either of the acids, an important delay of browning reaction could be noticed in the treated plant material.

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In order to better understand the nature of the important browning reaction in stored artichoke heads, the effects of citric and ascorbic acid on the phenolic metabolism, the PAL and PPO activity as well as on the iron/5-O-caffeoylquinic acid (IUPAC numbering) complexes, have been studied and the results obtained are discussed.

INTRODUCTION

The changes in phenolics occurring during the low temperature storage, in closed polyethylene bags, of fresh marketable artichoke heads have been reported in a previous paper (Lattanzio & Van Sumere, 1987). During such storage an important increase in phenolics, especially in soluble bound caffeic acid, was noticed.

However, in injured heads (internal blackening) a decrease in caffeic acid as well as several other phenolics has been recorded, and in badly injured heads (external symptoms of injury) the decrease was even more pronounced.

In addition, it is a well known fact that phenolic metabolism in plants can be enhanced under chill stress, and is also dependent on storage temperature (Lyons, 1973; Rhodes & Wooltorton, 1978; Rhodes *et al.*, 1981).

In this connection reference should be made to Henderson *et al.*, 1977; Graham & Patterson, 1982; Blankenship & Richardson, 1985; Siriphanic & Kader, 1985*a* and Sievwright & Shipe, 1986, who found that chlorogenic acid, as well as other phenolics, increased initially in several plant tissues injured by chilling. Subsequently, a gradual decrease in the amounts of the latter compounds, during storage of the plants at low temperature, occurred.

With regard to the above-mentioned increases in phenolics, it is also interesting to mention that certain enzymes, which are important for phenylpropanoid metabolism (e.g. phenylalanine ammonia lyase (PAL) and hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (CQT)), can be stimulated by low temperature treatments (Engelsma, 1970; Zucker, 1972; Lyons, 1973; Rhodes & Wooltorton, 1977; Siriphanic & Kader, 1985*b*). In some cases also polyphenoloxidase (PPO) may be activated (Siriphanic & Kader, 1985*a*; Mayer, 1987).

An increase in the activity of the above enzymes as well as in the level of the indicated phenolic components could further, in combination with temperature-dependent phase changes in the cellular membrane, contribute to the enzymic and non-enzymic browning processes.

In addition, phenolic browning reactions may, at least in part, also be caused by metal–polyphenol complexing and, in this connection, iron seems to be the commonest metal involved (Mathew & Parpia, 1971). Furthermore,

iron chelators like citric acid and ascorbic acid can affect plant tissue coloration (Hughes & Swain, 1962*a*, *b*; Vande Casteele *et al.*, 1981*a*; Subba Rao & Narasinga Rao, 1985) and this fact is most likely also relevant to artichoke heads which contain relatively high amounts of *o*-diphenols and iron (Lattanzio & Morone, 1979; Magnifico & Lattanzio, 1981). Therefore, this paper will give further information with regard to the possible role of the above-mentioned enzymes and phenolics during the browning processes in artichoke heads, while also additional information in relation to the role of citric acid and ascorbic acid, in improving both the quality and the shelf life of the stored artichoke heads will be discussed.

In order to prevent confusion it must be pointed out that quoted references use the pre-IUPAC nomenclature; this paper uses the IUPAC numbering system (1976).

MATERIALS AND METHODS

Plant material

Artichoke heads (cv. Catanese) of marketable quality were harvested at different periods during the plant growing season. Some of the heads were immediately analysed for phenolic compounds (reference values), while others were stored in closed polyethylene bags at 4°C. At different intervals, heads were taken and then analysed for their free and alkali-labile bound phenolic acids and apigenin and luteolin aglycone content (Lattanzio 1982; Lattanzio & Van Sumere, 1987). Heads were set 2 h with the cut stem (2 cm of length) in water (reference) and 1% citric acid or ascorbic acid solutions before storage. After imbibition, 2 or 3 g of solution by each head were absorbed.

The water content was determined by drying the plant material in a forced-draft oven at 105°C.

Extraction of plant material

For the qualitative and quantitative determination of the phenolics, the plant material was first homogenized with hot MeOH–EtOH (1:1) and then refluxed under nitrogen (Vande Casteele *et al.*, 1981*a*). After the centrifugation, the solution was first concentrated under vacuum and then partitioned to give fraction A (free phenolics), fraction B (carbonate-soluble, alkali-labile bound phenolics) and fraction C (carbonate insoluble, alkali-labile bound phenolics). Fraction D was obtained from the residue insoluble in MeOH–EtOH after basic hydrolysis, acidification and ether extraction

(full details of this procedure can be found in the paper by Vande Casteele *et al.*, 1981*b*).

All fractions were qualitatively and quantitatively analyzed for phenolics by HPLC. For flavonoid aglycone analysis, the hydrolyses were carried out under nitrogen with 2M HCl (see Lattanzio & Van Sumere, 1987).

HPLC analysis of phenolic compounds

HPLC analysis was performed with a Perkin-Elmer Series 4 liquid chromatograph, which was equipped with a 3600 Data Station, a fluorimetric detector LS-3 and a spectrophotometric photodiode array 1040 Hewlett Packard, coupled with a HP-85 computer and a HP-9121 disk drive. The pilot signal to the spectrophotometric detector was set at 325 nm and the fluorimetric detector at 265 nm (excitation) and 345 nm (emission).

An analytical Merck (Darmstad, GFR) column (250 mm × 4 mm) prepacked with LiChrosorb RP-18 (7 μ) was used throughout this work. The solvent system consisted of A:MeOH and B:acetic acid–water (5/95; v/v). The elution profile was: 0–25 min 15–40% A in B; 25–30 min 40% A in B (isocratic); 30–45 min 40–63% A in B; 45–47 min 63% A in B (isocratic); 47–51 min 63–99% A in B.

The flow rate was always 1 ml/min and at the start the column pressure was 15 MPa. Samples of 6 μl were applied to the column by means of a 6 μl loop valve.

More information about the identification of the compounds, and the correction of the results obtained can be found in the previous paper (Lattanzio & Van Sumere, 1987).

Extraction of PPO and PAL activities

Plant material was macerated in a blender for 10 min with (2·5 ml/g) cold acetone (−15°C). The suspension was centrifuged and the residue was twice more homogenized with cold acetone. For PPO extraction, the dried acetone powder was stirred for 20 min with 0·02M K-acetate buffer at pH 5·7 (5 ml/g original tissue wt) and the suspension kept overnight (Vande Casteele *et al.*, 1981*a*) and then centrifuged giving rise to a supernatant (crude extract) (Vande Casteele *et al.*, 1981*a*). For PAL extraction, 0·1M Na-borate buffer at pH 8·8, instead of K-acetate buffer, was employed (Zucker, 1965). In each case the supernatant (crude extract) was used for the determination of the PPO and PAL activities.

HPLC assay of PPO activity

This assay is only acceptable in the absence of chlorogenase (Van Sumere & Parmentier, 1964; Parmentier & Van Sumere, 1964). Since such an enzyme

did not prove to be present in artichoke heads, PPO activity was assessed as follows.

After 5 min at 37°C the reaction mixture (1 ml of 5-*O*-caffeoylquinic acid (5-*O*-CQA, according to the IUPAC (1976) nomenclature) 0·01M in K-phosphate buffer 0·15M pH 6·0 + 1 ml of 0·15M K-phosphate buffer pH 6·0 + 3 μl of PPO (crude extract)) was treated with 3 μl of 6N HCl and the residual 5-*O*-CQA was extracted with diethylether. Subsequently the diethylether layer was rapidly dried under low vacuum and redissolved in 500 μl of MeOH. This latter solution was then injected in a liquid chromatograph and HPLC analysis was carried out as previously described (Lattanzio & Van Sumere, 1987). One peak only, corresponding to the residual 5-*O*-CQA, was found in the chromatogram. Its isomers and/or caffeic acid were absent.

Inhibition of PPO activity

1% citric acid and 1% ascorbic acid were prepared in either 0·15M K-phosphate buffer at pH 6·0 or 0·6M K-phosphate buffer, pH 6·0. In a second set of HPLC assays (see above) the latter solutions were employed instead of the original K-phosphate buffer. When using K-phosphate buffer 0·15M, in combination with either 1% citric acid or 1% ascorbic acid the pH of the medium dropped respectively to 3·7 and 4·5. However, when the phosphate concentration was increased to 0·6M the final pH of the reaction mixture remained at 6·0. PPO activity was not affected by this increased ionic strength.

HPLC assay of PAL activity

One millilitre of phenylalanine, 0·01M (prepared in 0·1M borate buffer, pH 8·8) + 1 ml Na-borate buffer, pH 8·8, + 0·5 ml crude extract were incubated at room temperature overnight (Zucker, 1965). The reaction was stopped with one drop of 6N HCl, and cinnamic acid produced was extracted with diethylether and then analysed by HPLC. One unit (U) of activity was defined as the amount of enzyme that forms 1 nmol of cinnamic acid per hour in the standard assay.

Iron-chelating activity of citric and ascorbic acids

Assays on the complexing activity of iron (ferrous and ferric ions) were carried out in 0·02M K-acetate buffer at pH 6·0. Solutions containing 5-*O*-CQA, iron and citric or ascorbic acids (at various molar ratios), in 5 ml of K-acetate buffer, were kept at 35°C for 1 h and then analyzed by HPLC for their residual (free) 5-*O*-CQA content. The same solutions, without iron, were

used as blanks. (Solvent system: A = MeOH; B = 0·02M K-acetate buffer, pH 5·7. The elution profile was identical to the one used for the HPLC-analysis of phenolics.) The same test solution was also assayed colorimetrically, at 580 nm, by means of a Perkin-Elmer LC-55 spectrophotometer.

RESULTS AND DISCUSSION

Artichoke heads harvested in the winter months can easily be stored in good conditions for 2 weeks at 20°C and for 2 months at 4°C (Lattanzio & Van Sumere, 1987). However, the shelf life diminishes remarkably for heads harvested during the spring months, but treatment with a 1% citric acid solution delayed the browning reaction in a very pronounced way (untreated and treated samples showed, respectively, 75% and 15% internal blackening).

In Table 1 the effect of a 1% citric acid solution on the phenolic metabolism in healthy artichoke heads is represented. From these results it

TABLE 1

Changes of Phenolic Compounds (mg/100 g dry wt.) in Healthy (Not Discolored) Artichoke Heads, Stored 23 Days in Different Conditions. (Harvesting Date: January)

Phenolic compound	*Fresh marketable*	*Stored at room temperature*		*Stored at 4°C*	
		Untreated	*1% Citric acid*	*Untreated*	*1% Citric acid*
Fraction A: Free					
Caffeic acid	—	—	—	—	—
Apigenin	—	—	—	—	—
Luteolin	—	—	—	—	—
Fraction B: Soluble bound					
Vanillic acid	0·9	0·5	1·0	1·0	0·4
Syringic acid	5·3	7·5	6·5	14·9	2·9
p-Coumaric acid	2·8	5·2	5·0	3·1	2·3
Caffeic acid	2 581·2	5 270·0	2 958·2	3 637·0	3 111·8
Ferulic acid	5·1	26·7	3·9	18·3	27·0
Apigenin	31·0	48·2	49·5	44·5	18·0
Luteolin	36·2	66·9	67·3	78·3	26·1
Fraction D: Insoluble bound					
Vanillic acid	0·4	0·6	0·9	1·1	0·3
Syringic acid	0·9	2·2	2·0	5·9	1·4
p-Coumaric acid	5·7	9·0	6·0	11·3	9·8
Caffeic acid	562·8	1 409·5	873·1	1 143·9	873·1
Ferulic acid	0·1	0·1	0·2	0·3	+

TABLE 2

Effect of Citric Acid on Phenolic Content (mg/100 g dry wt.) and Quality of Artichoke Heads Stored 18 Days Both at Room Temperature and 4°C. (Harvesting Date: April).

Phenolic compound	*Fresh marketable*	*Stored at room temperature*		*Stored at 4°C*	
		Untreated (Internal blackening)	*1% Citric acid (Healthy)*	*Untreated (External symptoms of injury)*	*1% Citric acid (Healthy)*
Fraction A: Free					
Caffeic acid	—	—	—	2·7	—
Apigenin	—	—	—	23·4	—
Luteolin	—	—	—	23·8	—
Fraction B: Soluble bound					
Vanillic acid	0·9	0·5	0·5	0·7	1·2
Syringic acid	5·3	2·8	3·2	2·0	7·0
p-Coumaric acid	2·8	3·0	1·9	2·4	1·9
Caffeic acid	2 581·2	1 754·0	2 831·4	1046·1	2 922·8
Ferulic acid	5·1	4·2	4·2	9·5	5·3
Apigenin	31·0	10·3	16·7	+	+
Luteolin	36·2	13·0	20·2	+	+
Fraction D: Insoluble bound					
Vanillic acid	0·4	0·3	0·3	0·8	0·6
Syringic acid	0·9	0·4	0·5	1·0	1·5
p-Coumaric acid	5·7	11·9	6·7	8·7	5·9
Caffeic acid	562·8	428·7	576·1	472·9	661·7
Ferulic acid	0·1	+	+	1·3	+

follows clearly that citric acid stabilizes the latter metabolism and this in its turn results in an improvement of the quality of the stored heads. Indeed, as can be seen from Table 2, the heads treated with citric acid proved always to be of better quality than the untreated ones.

From the same Table it follows further that samples with external visible symptoms of injury also showed the presence of free caffeic acid, apigenin and luteolin. Presumably, the presence of the latter phenolics in the free state must be ascribed to hydrolytic phenomena, which undoubtedly run parallel with the increasingly overall damaging effect on the plant tissues.

Similar protective effects can also be noticed when 1% ascorbic acid solutions are used. However, in practice citric acid is much cheaper than ascorbic acid and for this reason citric acid treatments are also more acceptable.

Figure 1 shows the effects of both 1% citric acid and 1% ascorbic acid

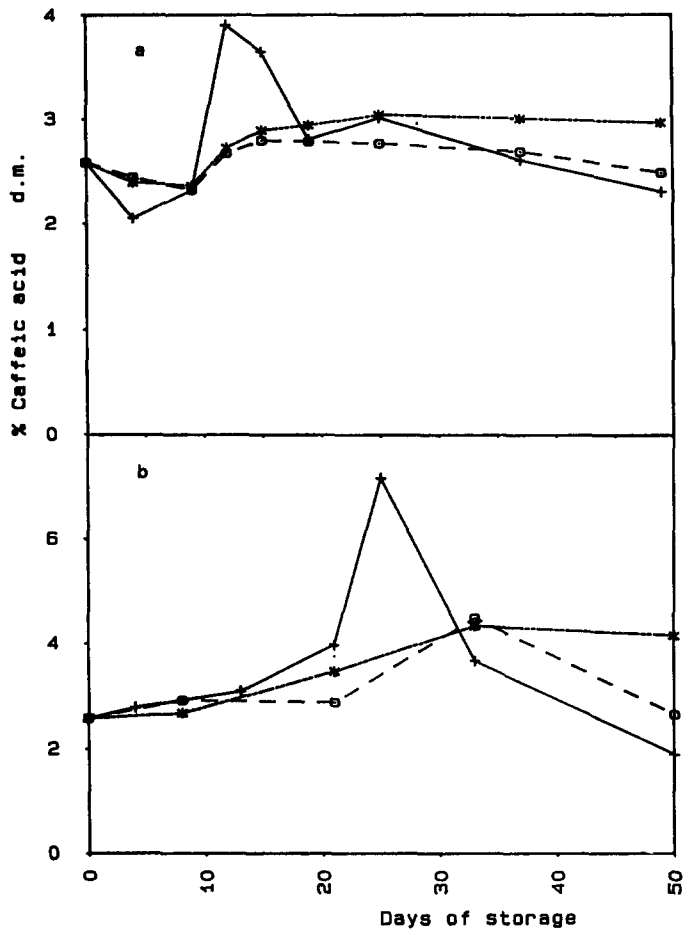

Fig. 1. Changes in caffeic acid during the storage at 4°C in untreated (+———+), 1% citric acid treated (○-----○) and 1% ascorbic acid treated (*— —*) heads. (a) Harvesting date: February. (b) Harvesting date: December.

solutions on the metabolism of the soluble alkali-labile bound caffeic acid in stored artichoke heads.

This compound is the most abundant phenolic component. It probably originates mainly from the mono- and dicaffeoylquinic acids. In MeOH:EtOH extracts of fresh artichoke heads 4 monocaffeoylquinic acid and 6 dicaffeoylquinic isomers were found to occur. Amongst the monocaffeoylquinic isomers the relative abundances were 81% 5-*O*-caffeoylquinic acid, 14% 4-*O*-caffeoylquinic acid, 3% 3-*O*-caffeoylquinic acid and 2% 1-*O*-caffeoylquinic acid, while amongst the dicaffeoylquinic acids the distribution was, respectively: 41% 1,5-*O*-dicaffeoylquinic acid,

21% 3,4-*O*-dicaffeoylquinic acid, 17% 3,5-*O*-dicaffeoylquinic acid, 11% 4,5-*O*-dicaffeoylquinic acid, 7% 1,4-*O*-dicaffeoylquinic acid and 3% 1,3-*O*-dicaffeoylquinic acid.

Figure 1 indicates further that the level of caffeic acid tended to increase with storage to a certain maximum and then decrease again. The time of the occurence of the peak being dependent upon the harvesting time (Fig. 1(a) and 1(b)). Figure 1 shows further that, at each of the two different harvesting dates, the caffeic acid content was markedly lowered by either the citric acid or ascorbic acid treatments. In addition, both treatments showed, from a qualitative as well as a quantitative point of view, almost the same effect, although ascorbic acid seemed to be the most effective in lowering the phenolic content during the final days of the storage period.

Furthermore, it has been generally noticed that the increasing phenolics are good substrates for browning reactions, although only exceptionally has a corresponding increase in PPO activity been found to occur (Siriphanic & Kader, 1985*a*). During our experiments, PPO activity either remained constant or even decreased with storage. Moreover, the PPO activity extracted from the treated samples (be it with citric or ascorbic acid) represented only about 30% of that of the untreated heads. Most likely this decrease in PPO activity must be ascribed to the enzymic copper-chelating activity of both acidic compounds (Mayer, 1987) and not to a pH effect because both the untreated and treated heads shows a pH value between 6·0 and 6·2. Baruah & Swain (1953) also suggested that ascorbic acid affects the specific structure of the enzyme in which copper is involved. They proved that the PPO from potato undergoes a marked denaturation in the presence of vitamin.

On the other hand, PAL activity was (and this in agreement with the changes in caffeic acid) enhanced during the cold storage of the artichoke heads (see also Engelsma, 1970; Zucker, 1972; Camm & Towers, 1973). Indeed, in untreated heads PAL activity increased about five-fold (from 5·01 to 23·89 U/(mg protein)) during the first days of storage, whereafter the activity decreased again to a low level. In the latter connection it may be of interest to refer to work performed by Engelsma (1970), whose findings suggest that cold shocks could well release PAL from a preexisting but inactive PAL protein inhibitor complex. Anyhow, low temperature induction of PAL-activity could well explain the corresponding changes in caffeic acid which were, as already mentioned above, always observed in untreated heads.

In stored heads treated with either citric or ascorbic acid, the increase in PAL activity was slower and much less pronounced (respectively 17·13 and 15·99 U/mg protein) than in the corresponding controls. Furthermore, although the pattern of changes in PAL activity seems to correlate rather

well with the metabolism of caffeic acid (and this both in untreated and treated heads), the relationship between PAL activity and citric acid or ascorbic acid treatment is not so readily explainable, and further research with regard to this problem is required.

In order to understand better the exact nature of the organoleptic and commercially so important browning phenomenon several experiments were performed. First of all the effect of either 1% citric or 1% ascorbic acid on PPO activity, obtained from artichoke heads, was investigated.

As demonstrated with both polarographic (Ysi (Yellow Springs Instruments Co.)) and HPLC techniques, crude artichoke extracts can via their PPO activity (especially between pH 5·7 and 7·0 (pH optimum)) readily oxidize 5-*O*-CQA. The enzyme(s) showed further only a low activity with pyrocatechol and caffeic acid, while the activity with dicaffeoyl quinic acids was very faint (see also Lattanzio *et al.*, 1987). When the pH value was adequately controlled (0·6M buffer) citric acid (1%) had no inhibitory effect, whereas ascorbic acid (1%) did. This observation is not easily reconciled with the observation that citric acid treatment inhibits discoloration *in vivo*.

Therefore the influence of both acids on internal blackening has been further investigated along other routes. Indeed, non-enzymatic browning reactions may also occur in plant tissues (Vande Casteele *et al.*, 1981*a*). In this respect both the presence of relatively high amounts of caffeic acid derivatives (amongst them 5-*O*-CQA and iron (10 mg/100 g dry matter)) in the artichoke heads must be stressed. It can therefore not be excluded that a variable amount of 5-*O*-CQA (and/or other caffeoyl derivatives) in the plant tissues is slowly and chemically oxidized through the catalytic action of iron ions (Vande Casteele *et al.*, 1981*a*). If this is the case then the action of both citric and ascorbic acid could, at least to a large extent, be explained by their iron-complexing properties. It is indeed quite possible that, dependent on the ratios of *o*-diphenolics to (ferrous and or ferric) iron ions, more or less intense and also more or less rapid colorations could be produced by complex formation. Very useful information on the 5-*O*-CQA–iron complex and the effect of some organic ligands on the latter may be found in papers by Hughes & Swain (1962*a*, *b*).

In our experiments, 5-*O*-CQA proved to be completely complexed, after 1 h at 35°C, when a molar ratio of 5-*O*-CQA (ferrous) iron 1:2 was employed. The colour of the complex changed at pH 6·0 from blue to green–blue with increasing ferrous iron concentrations. However, by adding citric acid to the reaction mixture an almost complete disappearance of the colour could be observed. By means of HPLC analysis of the residual non-complexed 5-*O*-CQA, it was further shown that with a (ferrous) iron–citric acid ratio of 1:5, 5-*O*-CQA was only complexed for 13%, while with a ratio of 1:10 the totality of the free 5-*O*-CQA (proving a complete dissolution of the complex) could be recovered.

In a second set of assays ferric-iron was used. At pH 6·0 the colour of the 5-*O*-CQA–(ferric)iron complex was again blue and the intensity of the coloration increased when the 5-*O*-CQA–(ferric)iron changed from now 1:1 to 2:1 ratio. After addition of citric acid to the complex in a ratio (ferric)iron: citric acid, 1:10, again a complete disappearance of the colour took place and after HPLC analysis all the 5-*O*-CQA proved to be in the free form.

As to the effect of ascorbic acid on the 5-*O*-CQA–iron complex, the colorimetric results were not in agreement with the chromatographic ones. The latter proved that the ascorbic acid complexing activity was lower than the citric acid. Indeed, it is possible that a variable amount of the 5-*O*-CQA may be chemically oxidized by oxygen under the catalytic action of iron. There are conditions when ascorbate can contribute to oxidative damage by reducing Fe(+3) to Fe(+2). This results in the production of oxyradicals by the Haber–Weiss cycle (Mahoney & Graf, 1986). HPLC analysis measured the overall effect by determining the residual non-complexed 5-*O*-CQA.

CONCLUDING REMARKS

All the results in connection with the effects of citric acid and ascorbic acid on PPO activity, PAL activity and the 5-*O*-CQA–iron complex formations, seem to indicate that the browning phenomena in stored artichoke heads may be both of enzymic and non-enzymic nature, with most probably the latter reaction being the dominant one. In fact, PPO activity did not increase during the storage of the artichoke heads and in some cases even a decrease in the oxidase activity was observed.

The stabilizing effect of both acids on the metabolism of the phenolics is more difficult to explain. However, it is quite possible that at the start of browning phenomena, which are not due to handling or any other mechanical damage, PAL activity may play an important role. Indeed, this enzyme may be responsible for the increase in the phenolic and especially the 5-*O*-CQA level since a direct quantitative relationship between the latter enzyme activity and the amount of caffeoylquinic derivative, synthesized from endogenous substrates, has been described (Zucker, 1965). Such a relationship would further mean that PAL activity may be a rate-controlling factor in the synthesis of 5-*O*-CQA. CQT, another important enzyme in the 5-*O*-CQA biosynthetic pattern, does not control the production of the latter compound (Rhodes & Wooltorton, 1978). It seems therefore possible to speculate that cellular PAL (Jones, 1984), stimulated by low temperature and low ethylene levels, could be responsible for a biosynthetic increase in the level of phenolics, being the reaction driven by ATP produced by respiration. Since 5-*O*-CQA and other phenolics are mainly synthesized in the chloroplasts (Alibert & Boudet, 1984) where also 'the bulk of the iron

cell' (Price, 1968) exists, the iron-chelating ability of the increased *o*-diphenols could well cause browning reactions. The iron-chelating activity of citric or ascorbic acid may, in turn, antagonize such a discoloration and as a result the quality of the stored heads may be much better.

In addition, or even alternatively, low temperature-induced toxic oxygen forms (radicals or superoxide forms) could react with membranous lipidic components, resulting in an intracellular decompartmentalization (Turc *et al.*, 1986; Mayer, 1987). If the latter speculation holds true then the positive effect of citric and ascorbic acid could also, to a certain extent, be due to their *in vivo* action on PPO activity. The latter hypothesis is further partly supported by the low PPO activity found in heads treated with either citric or ascorbic acid.

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