Influence of Washing Treatment and Storage Atmosphere on Phenylalanine Ammonia-Lyase Activity and Phenolic Acid Content of Minimally Processed Carrot Sticks

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2005

Impact Factor: 2.91 · DOI: 10.1021/jf049084b · Source: PubMed

4 AUTHORS, INCLUDING:

Reinhold Carle
Hohenheim University
392 PUBLICATIONS **10,290** CITATIONS

Influence of Washing Treatment and Storage Atmosphere on Phenylalanine Ammonia-Lyase Activity and Phenolic Acid Content of Minimally Processed Carrot Sticks

RALPH G. KLAIBER, SASCHA BAUR, ARJUNA KOBLO, AND REINHOLD CARLE*

Institute of Food Technology, Section Plant Foodstuff Technology, Hohenheim University, August-von-Hartmann-Strasse 3, D-70599 Stuttgart, Germany

The influence of washing uncut and shredded carrots (*Daucus carota* L. ssp. *sativa* var. Bangor) with chlorinated and ozonated water, respectively, as well as the storage of the produce under aerobic and anaerobic conditions, respectively, on PAL activity and synthesis of phenolic compounds have been evaluated on pilot plant scale. Inherent raw material inhomogeneity was compensated by pooling large sized samples, and frequent sampling ensured significant data. PAL activity was induced by processing and linearly increased throughout storage under aerobic conditions, whereas an anaerobic atmosphere resulted in a maximum activity peak at storage day 2−4. The accumulation of phenolic compounds showed good correlation with the kinetics of PAL activity. Although the influence of the washing treatments was weak, the use of chlorinated water for washing shredded carrots slightly delayed the onset of PAL activity. The phenolic content of the minimally processed carrots was dominated by trans and cis isomers of chlorogenic acid (∼95%). Additionally, the occurrence of *p*-coumaroylquinic acid (∼5%) and the novel finding of three dicaffeoylquinic acid isomers were reported. The synthesis of phenolic compounds was controlled, depending on storage atmosphere.

KEYWORDS: carrot (*Daucus carota* L.); phenylalanine ammonia-lyase (PAL); phenolic compounds; chlorogenic acid; minimal processing; washing treatment; storage atmosphere

INTRODUCTION

Consumption of minimally processed fruits and vegetables has increased as a response to the consumer demand for convenient and fresh produce in catering and retail branches. Among minimally processed vegetables, shredded carrots are of growing popularity. The purpose of minimal processing is to deliver to the consumer a freshlike product with an extended shelf life while ensuring food safety and maintaining sound nutritional and sensorial qualities (*1*).

Common industrial production of minimally processed carrots consists of cleaning, peeling, cutting, washing, and packaging (*2*, *3*). The main objective of washing aims at reducing the microbial load and removing debris as well as cellular fluids (*4*). Chlorine is commonly used as a sanitizer for horticultural products (*5*). Due to its high oxidizing potential, spontaneous decomposition, and low byproduct formation, ozonated water may represent an alternative disinfectant to ensure microbiological safety and quality of raw produce (*6*). In recent experiments, the implementation of a washing step with chlorinated water prior to shredding proved to be suitable for maintaining sensorial and microbial quality of minimally processed carrots (*7*). However, stress response reactions through processing, especially cutting operations, induce increased respiration rates, elevated phenylalanine ammonia-lyase (PAL) activity, and synthesis of lignin (*8*).

PAL (EC 4.3.1.5) catalyzes the first step of phenylpropanoid metabolism of higher plants, yielding the precursor of a large number of compounds such as lignins, flavonoids, coumarins, stilbenes, and benzoic acid derivatives, with diverse biological functions (*9*). In carrots, PAL is stimulated by abiotic and biotic stress, for example, as a response to wounding and ethylene, generally resulting in elevated levels of soluble phenolics (*10*, *11*). Phenolic compounds in fruits and vegetables are of great interest in many aspects. They contribute to the sensorial qualities of fruits and vegetables such as color, astringency, bitterness, and aroma (*12*). In stored and processed carrots, phenolics and 6-methoxymellein have been associated with bitter flavors (*13*−*15*). As a result of their antioxidant capacity, plant phenols may be able to prevent oxidative damage to vital biomolecules and are therefore hypothesized to be important in chronic disease prevention (*16*−*18*). Furthermore, phenolic compounds are of particular importance in plant defense mechanisms. They respond to phytopathogenic infections and promote healing processes in forming wound barriers such as lignin and suberin (*19*−*23*). Major phenols in carrots include chlorogenic acid, *p*-hydroxybenzoic acid, and various cinnamic acid derivatives (*15*, *24*).

While most research on physiological effects of processing and packaging was conducted on a laboratory scale (*24*−*26*),

* Corresponding author. Telephone: +49-711-459-2314. Fax: +49-711-459-4110. E-mail: carle@uni-hohenheim.de.

process-relevant data about commercial production of minimally processed carrots are scarce. However, for further optimization of postharvest produce management and prolongation of shelf life, detailed knowledge about the influence of washing treatments and packaging atmosphere on phenolic metabolism in tissues is indispensable.

The objective of the present study was to examine the kinetics of PAL activity and phenolic content during storage at 4 °C for 9 days as affected by washing treatments applying chlorinated and ozonated water, respectively. Furthermore, shredded carrots were packaged in films with different gas permeability to study the influence of aerobic and anaerobic metabolism on PAL activity and phenolic compounds. All trials of the present study were conducted on pilot plant scale, thus representing the stress impact of industrial production conditions. By pooling large sized samples inherent inhomogeneity of the raw material was compensated. Also, frequent sampling ensured significant data about PAL activity and synthesis of phenolic compounds throughout storage of the produce.

MATERIALS AND METHODS

Solvents and Reagents. Solvents and reagents were of analytical or HPLC grade. Milli-Q system (Millipore, Bedford, MA) ultrapure water was used for analytical purposes. 5-*O*-*trans*-Caffeoylquinic acid (t-CQA; chlorogenic acid), *p*-coumaric acid, and bovine serum albumin were purchased from Roth (Karlsruhe, Germany). Sodium hypochlorite solution (≥12% active chlorine), L-phenylalanine, and *trans*-cinnamic acid were from Fluka (Buchs, Switzerland); polyvinylpolypyrrolidone (Poliplasdone XL-10; PVPP) was supplied from Hedinger (Stuttgart, Germany). All other chemicals were obtained from VWR (Darmstadt, Germany).

Plant Material and Processing. Carrots (*Daucus carota* L. ssp. *sativa* cv. Bangor) were obtained from a local processor and stored in a dark cooling chamber up to 1 day at 4 °C prior to processing. All carrots were pre-cleaned, abrasion-peeled, and topped.

As previously described (*7*), two identical series were conducted in August (series 1) and October 2002 (series 2), respectively. Washing processes were conducted on a pilot plant scale with commonly used industrial equipment. The raw material was processed in 5 lines with different experimental arrangements. Batches consisting of 45 kg of uncut and shredded carrots, respectively, were treated as follows:

(I) shredding without washing (control); (II) pre-washing of uncut carrots in tap water (120 s, 4 °C); (III) pre-washing of uncut carrots in water containing 200 mg/L free chlorine (120 s, 4 °C); (IV) pre-washing of uncut carrots in water containing 1.3 mg/L ozone (120 s, 4 °C); (V) washing of shredded carrots in water containing 100 mg/L free chlorine (120 s, 4 °C).

Batches of uncut and shredded carrots, respectively, were submerged in 500 L of chlorinated, ozonated, and tap water, respectively, in a GEWA 2600 washing machine (Kronen, Willstätt, Germany) at 4 °C for 120 s. The pH was adjusted to tap water (∼8.0) using hydrochloric acid. The carrots were shredded (2 × 2 mm, 25−60 mm) with a GS-10 multipurpose belt cutting machine (Kronen, Willstätt, Germany). Adherent water and cellular fluids were removed using a spin-dryer (Turbo-K50 centrifuge, Kronen, Willstätt, Germany).

Since different films are used in the fresh-cut produce industry, 250 ± 2 g quantities of shredded carrots were packaged in 2 types of oriented polypropylene bags (195 × 250 mm; Amcor Flexibles, Bristol, UK) with the following oxygen and carbon dioxide transmission rates (OTR, CTR): film A (microperforated, 35 μm) OTR 12000 mL × m^{-2} × d^{-1} × atm^{-1}, CTR 13000 mL × m^{-2} × d^{-1} × atm^{-1}; film B (35 μm) OTR 1000 mL × m^{-2} × d^{-1} × atm^{-1}, CTR 4000 mL × m^{-2} × d^{-1} × atm^{-1}. After heat sealing using a R-25 packaging machine (Boss, Friedrichsdorf, Germany), samples were stored at 4 °C for a period of up to 9 days.

A sodium hypochlorite stock solution was used to prepare appropriate chlorine concentrations. Ozonated water was provided by an SWO-30 ozonizer equipped with a 400 L reservoir (Ozomatic, Baunatal, Germany) to ensure sufficient contact time between ozone gas and water, and connected with the GEWA 2600 washing machine (flow 130 L/min).

Headspace Gas Analysis. Prior to further sample preparation, oxygen and carbon dioxide concentrations within the bags were examined. Headspace gas samples were taken by means of a hypodermic needle through an adhesive septum previously fixed on the bags and measured using a Checkmate 9900 headspace analyzer (PBI Dansensor, Ringsted, Denmark). On each sampling day, 20 bags (days 2, 5, 7, and 9) and 8 bags (days 1, 3, and 4), respectively, for film A as well as 5 bags for film B (days 1−5, 7, and 9) were analyzed per processing line.

Sample Preparation. For each sample, the contents of 5 bags per treatment and day were pooled, frozen in liquid nitrogen, and comminuted with a Stephan universal machine UM 12 (Hameln, Germany) equipped with a cryogenic vessel. Aliquots were stored at −80 °C in sealed plastic containers prior to lyophilization in a Lyovac GT4 (Steris, Hürth, Germany). The freeze-dried material was stored at −30 °C. Prior to analysis, aliquots of the lyophilizate were ground to powder in a water-cooled Ika A10 laboratory mill (Staufen, Germany) to warrant maximum homogeneity.

Enzyme Extraction. PAL was extracted as described previously (*24*, *27*) with some modifications. Ground lyophilizate aliquots (150 mg), PVPP (150 mg), and calcium chloride dihydrate (75 mg, 0.5 mM) were mixed with 10 mL of 50 mM tris-(hydroxymethyl)aminomethane hydrochloride buffer (TRIS; pH 8.8) containing 1.5 mM 1,4-dithioerythritol to prevent oxidative damage to PAL (*24*). After stirring for 60 min at 4 °C, the homogenate was filtered through Schleicher & Schuell No. 5951/2 filter paper (Dassel, Germany), and the filtrates were centrifuged at 1500 × g for 10 min. Aliquots in the amount of 3 mL of the clarified supernatants were applied to Econo-Pac 10 DG desalting columns (Bio-Rad, Hercules, CA), preequilibrated with 20 mL of TRIS buffer, and subsequently eluted with 4 mL of TRIS buffer. The obtained enzyme extracts were kept on ice until performance of the kinetic PAL assays. Extractions were conducted in triplicate and analyzed separately for protein content.

Kinetic PAL Assay. PAL activity was determined according to the methods of refs *24* and *28* with some modifications. The reaction mixture contained a 200 μL aliquot of enzyme extract and 50 μL of L-phenylalanine (100 mM) in TRIS buffer (50 mM) as PAL substrate. After incubating the mixture at 37 °C for 30 min, 750 μL of methanol was added to stop the reaction. Reaction mixtures containing boiled enzyme extracts were used as reference. An aliquot of each reaction mixture was membrane-filtered (0.45 μm; Whatman Mini-UniPrep, Clifton, NJ) and analyzed for cinnamic acid by HPLC. For each enzyme extract, PAL activity was determined in duplicate.

HPLC Determination of PAL Activity. For the analysis of cinnamic acid, a Hewlett-Packard Series 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) was applied, consisting of an ALSTherm G1313A thermoautosampler, a G1312A binary gradient pump, a G1322A degasser, a ColComp G1316A column oven, and a G1315A diode array detection system. HP ChemStation software (Rev. A6.01) was used to analyze data. Separation was performed on an Aqua C_{18} RP column (150 mm × 3 mm i.d.) with a particle size of 3 μm (Phenomenex, Torrance, CA) fitted with a C_{18} ODS guard column (4 mm × 2 mm i.d.) and operated at 25 °C. Elution was performed isocratically using 0.5% acetic acid in aqueous acetonitrile (50%, v/v) as mobile phase. The injection volume was 3 μL, and the flow rate was set to 0.3 mL × min^{-1}. Chromatograms were recorded at 276 nm, and cinnamic acid was quantified by external standard calibration.

Spectrophotometric Determination of Protein Content. Protein content was determined according to Bradford (*29*) using bovine serum albumin as standard protein.

Extraction of Phenolic Compounds. Phenolic compounds were extracted as described previously (*30*) with some modifications. Ground lyophilizate (1 g) and 250 mg (1.4 mM) ascorbic acid were homogenized with 100 mL of aqueous methanol (70%, v/v) for 2 min using an Ultra-Turrax (Ika, Staufen, Germany). The homogenate was vacuum-

filtered through Schleicher & Schuell No. 595 filter paper (Dassel, Germany). To improve extraction of phenolic compounds, the filter residue was rinsed twice with 10 mL aliquots of the extraction solution. Methanol was removed under reduced pressure below 40 °C. The resulting aqueous solution plus a subsequent water rinse (2 × 10 mL) was acidified to pH 2 with hydrochloric acid and extracted three times with each 50 mL of ethyl acetate. The combined ethyl acetate fractions were dried using sodium dihydrogensulfate and passed through Schleicher & Schuell No. 5951/2 filter paper (Dassel, Germany). The organic solvent plus a subsequent 20 mL ethyl acetate rinse was evaporated to dryness below 40 °C, and the residue was dissolved in 5 mL of methanol. An aliquot was membrane-filtered (0.45 μm; Whatman Mini-UniPrep, Clifton, NJ) and analyzed by HPLC. For each sample, extraction of phenolic compounds was performed in duplicate.

Separation of Phenolic Compounds. The same Hewlett-Packard Series 1100 HPLC system as described for PAL activity determination was utilized for the analysis of phenolic compounds. Separation was performed using a Synergy C_{18} Hydro-RP column (150 mm × 3 mm i.d.) with a particle size of 4 μm (Phenomenex, Torrance, CA) fitted with a C_{18} ODS guard column (4 mm × 2 mm i.d.) and operated at 25 °C. The mobile phase consisted of A: 5% formic acid in water (v/v) and B: 5% formic acid in methanol (v/v). The gradient program was as follows: 12% B to 27% B (18 min), 27% to 45% B (18 min), 45% to 60% B (4 min), 60% B (3 min), 60% to 12% B (3 min), and 12% B (4 min). The injection volume for all samples was 3 μL, and the flow rate was set to 0.3 mL × min^{-1}. Chromatograms were recorded at 280, 320, and 330 nm (peak width 0.05 min); spectra were recorded from 200 to 450 nm (interval 0.5 nm).

Identification and Quantification of Phenolic Compounds. Phenolic compounds were identified by means of retention time, UV spectrophotometric analysis, and mass spectrometry. LC-MS analysis was performed with an HPLC system (Agilent, Waldbronn, Germany) identical to the system described above. Mass spectrometry was performed on an Esquire 3000 plus ion trap mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ionization (ESI) interface operated in negative and positive mode. Bruker Daltonics DataAnalysis 3.1 software was used to analyze data. Mass spectrometric data were acquired in full scan mode over the *m*/*z*-range of 50−800. Sensitivity of the mass spectrometer was optimized using t-CQA as standard. The optimal tuning parameters for negative (ESI−) and positive (ESI+) electrospray ionization were found to be 4.00 kV for capillary, 60 eV for cone, a dry gas temperature of 365 °C, and a nebulizing gas pressure of 40 psi. Collision-induced dissociation spectra were obtained with a fragmentation amplitude of 1.0 V (MS^n).

Hydroxycinnamic acid derivatives were quantified by external standard calibration with t-CQA (chlorogenic acid) at 330 nm with the exception of 5-*O*-*cis*-caffeoylquinic acid (c-CQA) which was analyzed at 320 nm. Quantification of *O*-*p*-coumaroylquinic acid was performed by external standard calibration with *p*-coumaric acid at 320 nm.

Data Analysis. Linear regression fittings were analyzed using SigmaPlot version 7.0 (SPSS Science, Erkrath, Germany). Data were subjected to analysis of variance (ANOVA) using SigmaStat version 2.03 (SPSS Science, Erkrath, Germany) separately for each series. In the case of significant differences, individual washing procedures and packaging films, respectively, were compared using the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Evolution of Package Atmosphere. A modified atmosphere within the bags was formed by respiration of shredded carrots. As previously reported by Klaiber et al. (*7*), an equilibrium modified atmosphere (EMA) was reached after 3 to 4 days of refrigerated storage. Oxygen levels varied depending on washing treatment and packaging film. Similar trends were obtained for the control (line I, no washing) and all pre-washing treatments of uncut carrots (lines II−IV), respectively. In both series, equilibrium oxygen concentrations in film A bags ranged from 8 to 12% (lines I−IV) and from 14 to 15% for washed, shredded carrots (line V), respectively. In bags made of film B, anaerobic conditions (<1% oxygen) were attained on days 2 to 4. Carbon dioxide levels showed inverse correlation. **Figure 1** illustrates the evolution of the gas compositions during storage of packaged, shredded carrots in series 2, considering lines I, III, and V as examples. Surprisingly, when shredded carrots were washed with chlorinated water (line V), significantly lower respiration rates were obtained. Since the degree of wounding was identical for all processed carrots, reduced respiration may be ascribed to inhibitory effects of chlorinated water on plant metabolism and microbial activities.

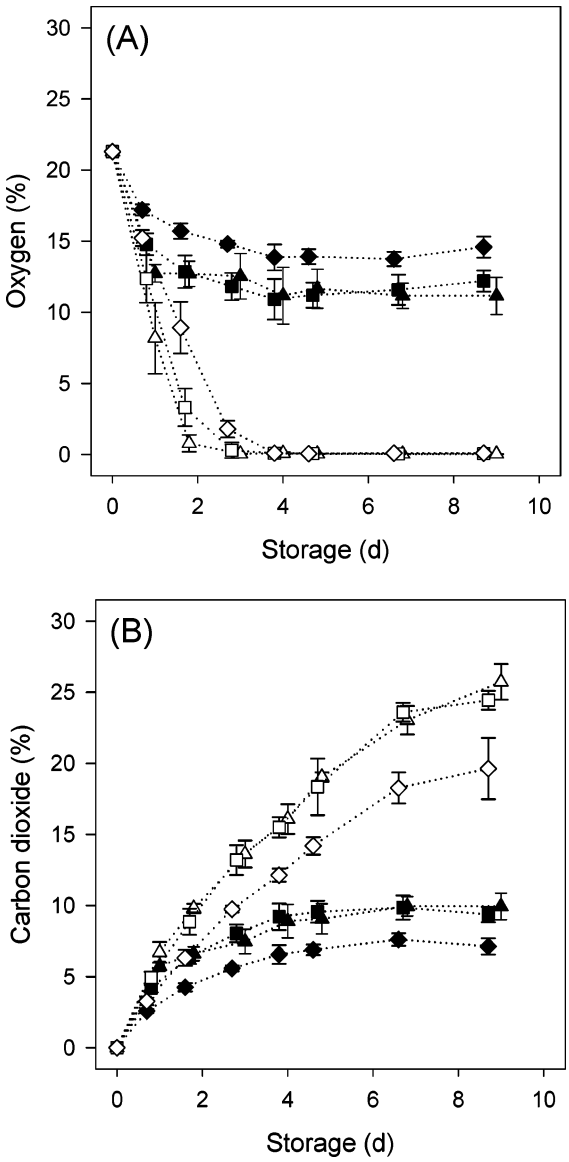


Figure 1. Effects of washing treatment and packaging film on oxygen (A) and carbon dioxide (B) concentrations within bags of shredded carrots during storage at 4 °C. Values represent the mean of 4–18 bags (± standard deviation) from series 2. Closed symbols, film A; open symbols, film B. (▲/△), line I, control, shredding without washing; (■/□), line III, pre-washing uncut carrots with chlorinated water; (◆/◇), line V, washing shredded carrots with chlorinated water.

Influence of Storage Atmosphere and Washing Treatment on PAL Activity. Although different raw material of the identical cultivar was used, almost the same PAL activities for both series were obtained. Therefore, PAL activity kinetics of series 2 is shown as an example (**Figure 2**). Prior to processing, carrots showed negligible PAL activities of ∼0.13 nkat/g fresh weight (FW). In both series, PAL activity of samples packaged

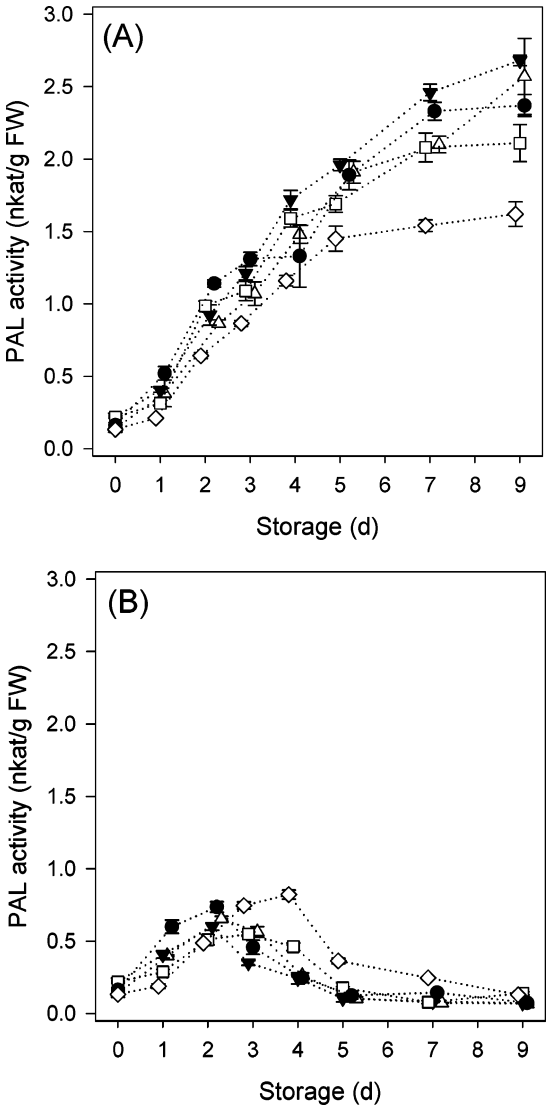


Figure 2. Effect of washing treatments on PAL activity of shredded carrots packaged in film A (A) and film B (B) during storage at 4 °C. Values represent the mean of 6 measurements (± standard deviation) from series 2. (△), line I, control, shredding without washing; (▼), line II, pre-washing uncut carrots with tap water; (□), line III, pre-washing uncut carrots with chlorinated water; (●), line IV, pre-washing uncut carrots with ozonated water; (◇), line V, washing shredded carrots with chlorinated water. FW, fresh weight.

in film A linearly increased throughout storage (determination coefficient for linear regression fitting: $r^2 > 0.94$ for days 0−5) and reached 2.4−2.7 nkat/g FW at day 9 for lines I, II, and IV. For pre-washing (line III) and washing of shredded carrots (line V) with chlorinated water, activities were 1.8−2.1 and ∼1.6 nkat/g FW at day 9, respectively. PAL activity of samples packaged in film B exhibited a maximum of 0.57−0.74 nkat/g FW at days 2−4 and decreased afterward to the initial level. Washing shredded carrots with chlorinated water (line V) resulted in significantly lower (film A) and delayed onset of PAL activity (film B), respectively. This observation was in good accordance with the concomitant elevated equilibrium oxygen level in film A bags and the slower oxygen consumption in film B bags, respectively, indicating inhibitory effects of chlorine on plant metabolism. For all series, extracted protein contents of carrots were nearly constant throughout storage (data not shown).

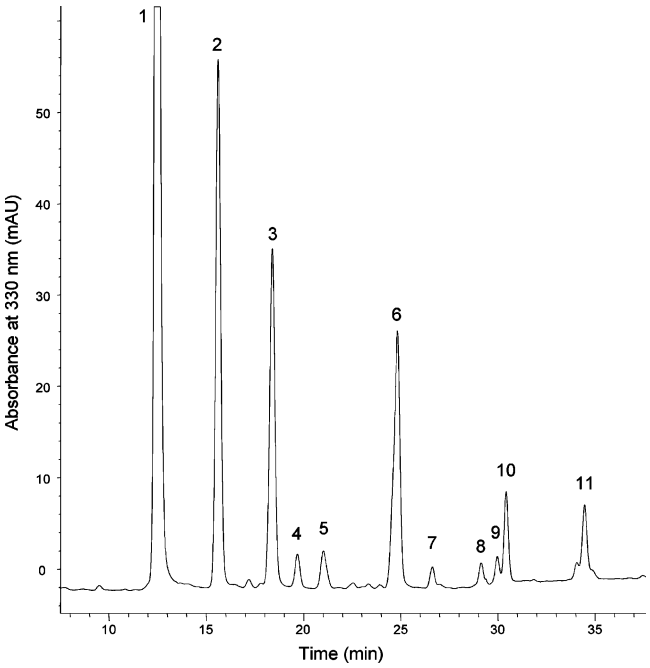
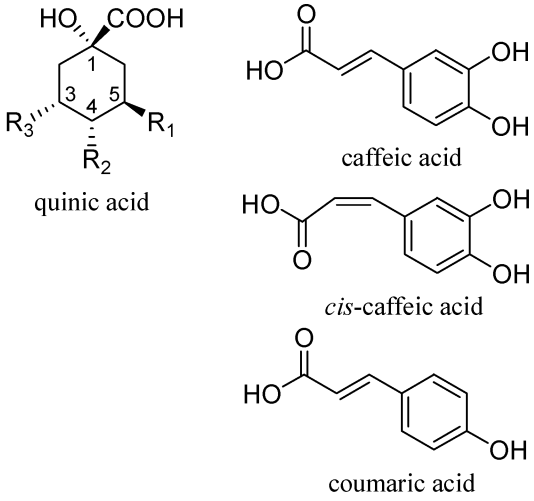


Figure 3. HPLC profile of phenolic compounds in shredded, stored carrots (330 nm). **1** 5-*O-trans*-caffeoylquinic acid, **2** 5-*O-cis*-caffeoylquinic acid, **3** *O-trans-p*-coumaroylquinic acid, **4** not identified, **5** tentatively identified as *O-cis-p*-coumaroylquinic acid, **6** unidentified caffeoylester, **7** not identified, **8**–**10** di-*O*-caffeoylquinic acids, **11** not identified.

PAL activity during storage was mainly influenced by the oxygen content in the package. Whereas film A packaged carrots showed continuous increase in PAL activity throughout storage, the induction pattern of film B packaged samples exhibited a maximum activity at days 2−4. When oxygen was depleted, PAL activity declined to negligible values. The monitored rise of PAL activity in shredded carrots was supposed to be due to de novo enzyme synthesis. Analogous behavior was previously reported for potato (wound-induced) and strawberry (ripening) (*31*−*33*). Since enzyme activity decreased rapidly, the authors assumed the involvement of a PAL inactivation system that caused an enzyme activity loss likewise reported previously for some plant tissues (*34*, *35*).

The findings of the present study are consistent with previous studies, where a 10-fold increase in PAL activity within 48 h after wounding followed by constant activity on this level throughout storage (EMA: 11−14% oxygen, <1% carbon dioxide) was observed for carrot sticks washed with chlorinated water (100 mg/L) (*21*, *25*). In a further study with samples exclusively stored in air, a higher increase in PAL activity up to day 3 followed by an abrupt decline until day 7 was reported (*24*). When shredded carrots were stored in oriented polypropylene bags with low oxygen permeability, similar changes of PAL activity were monitored; however, activities remained very low. Since sampling intervals of up to 4 days were applied in this study, the virtual maximum PAL activity remained indefinite.

Phenolic Compounds in Minimally Processed Carrots. The chromatographic separation of phenolic compounds extracted from stored, shredded carrots is presented in **Figure 3**. Corresponding UV maxima and mass spectrometric data of phenolic compounds are shown in **Table 1**. The predominant component was identified as the trans isomer of 5-*O*-caffeoylquinic acid (**1**; t-CQA; chlorogenic acid). Further phenolic compounds were recognized as 5-*O-cis*-caffeoylquinic acid (**2**; c-CQA), *O-p*-



Compound	R_1	R_2	R_3
quinic acid	OH	OH	OH
5-*O*-*trans*-caffeoylquinic acid (chlorogenic acid)	caffeic acid	OH	OH
5-*O*-*cis*-caffeoylquinic acid (*cis*-chlorogenic acid)	*cis*-caffeic acid	OH	OH
3,5-di-*O*-caffeoylquinic acid	caffeic acid	OH	caffeic acid
4,5-di-*O*-caffeoylquinic acid	caffeic acid	caffeic acid	OH
3,4-di-*O*-caffeoylquinic acid	OH	caffeic acid	caffeic acid
O-*p*-coumaroylquinic acid	*p*-coumaric acid	OH	OH

Figure 4. Structures of quinic acid and 6 quinic acid esters.

Table 1. HPLC–PDA and Mass Spectrometric Data of Phenolic Compounds in Shredded, Stored Carrots[a]

	compound	MS $[M-H]^-$	MS^2 $[M-H]^-$	UV maximum (PDA) λ [nm]
1	5-*O*-*trans*-caffeoylquinic acid	353	191	326, 303sh, 247
2	5-*O*-*cis*-caffeoylquinic acid	353	191	316, 246
3	*O*-*trans*-*p*-coumaroylquinic acid	337	191	316, 300sh
4	not identified	335	179, 135	328, 303sh
5	*O*-*cis*-*p*-coumaroylquinic acid[b]	337	191	305
6	unidentified caffeoylester	365	203, 185	328, 303sh, 247
7	not identified	319	163	312, 301sh
8	di-*O*-caffeoylquinic acid isomer	515	353, 191	326, 302sh, 246
9	di-*O*-caffeoylquinic acid isomer	515	353, 191	326, 304sh, 246
10	di-*O*-caffeoylquinic acid isomer	515	353, 191	328, 303sh, 246
11	not identified	527	365	328, 303sh, 247

[a] PDA = photodiode array detection, sh = shoulder. [b] Tentatively identified.

coumaroylquinic acid (**3**; CoQA), as well as 3 di-*O*-caffeoylquinic acid isomers (**8**−**10**; diCQA) (**Figure 4**).

In the ESI−, compounds **1** and **2** showed the same molecular ion $[M-H]^-$ at *m*/*z* 353 in accordance with the molecular formula $C_{16}H_{18}O_9$. Their molecular ions $[M-H]^-$ yielded a single peak at *m*/*z* 191 in the MS^2 event corresponding to deprotonated quinic acid. Using ESI+, both compounds showed a $[M+H]^+$ ion at *m*/*z* 355 which fragmented into an ion at *m*/*z* 163 in the MS^2 event, thus following the typical pattern after positive ionization of the carbonyl oxygen (*36*). Compound **1** coeluted with t-CQA used as standard and exhibited the same UV characteristics with a maximum at 326 nm. Compared with t-CQA, compound **2** had a similar ESI^-/MS spectrum but showed a hypsochromic shift to an UV maximum at 316 nm, indicating compound **2** as c-CQA. These data are in good agreement with those previously published for eggplant (*37*).

MS spectra of compounds **3** and **5** yielded a molecular ion $[M-H]^-$ at *m*/*z* 337 ($C_{16}H_{18}O_8$). In ESI^-/MS^2, a peak at *m*/*z* 191 corresponding to deprotonated quinic acid was attained. In the ESI+, compound **3** showed a $[M+H]^+$ ion at *m*/*z* 339 yielding one fragment at *m*/*z* 147 in the MS^2 event. UV and MS data of this compound corresponded with those of CoQA. Analogous to compounds **1** and **2**, a hypsochromic shift from 316 nm (compound **3**) to 305 nm (compound **5**) was observed, suggesting **5** to be the cis isomer of CoQA.

The collision-induced dissociation mass spectra of compounds **8**, **9**, and **10** are given in **Figure 5**. All compounds had an identical $[M-H]^-$ ion at *m*/*z* 515 in ESI^- corresponding to the sum formula of diCQA ($C_{25}H_{24}O_{12}$), which has been previously found in carrots (*15*, *24*). The molecular ions $[M-H]^-$ of the caffeoylquinic acid derivatives yielded peaks at *m*/*z* 353 ($[M-H]^-$ of t-CQA) in the MS^2 event and *m*/*z* 191 ($[M-H]^-$ of deprotonated quinic acid) in the MS^3 event. Analysis in ESI^+ mode resulted in $[M+H]^+$ molecular ions at *m*/*z* 517 fragmenting into ions at *m*/*z* 355 and *m*/*z* 163 in the MS^2 and MS^3 events, respectively. The observed neutral losses,each of 162 u, corresponded to the loss of caffeic acid units. While 3,4- and 3,5-di-*O*-caffeoylquinic acid were previously described in carrots

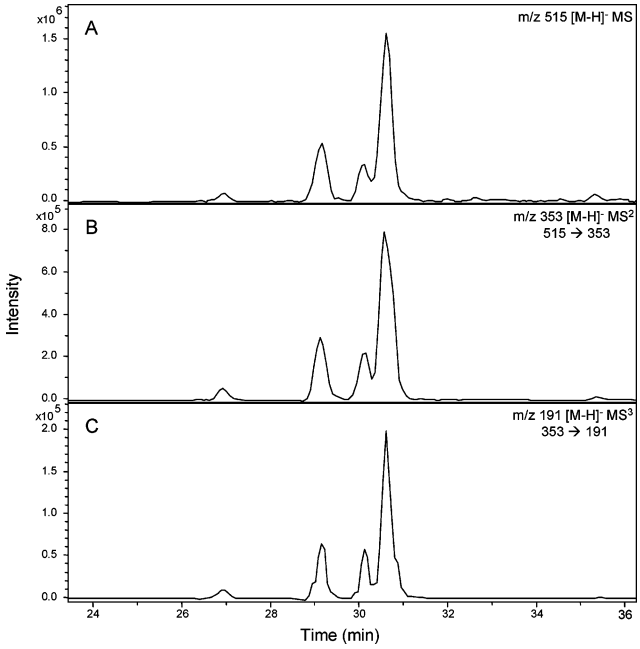


Figure 5. LC-ESI-/MS^n collision-induced dissociation analysis of dicaffeoylquinic acid isomers (compounds **8**, **9**, and **10**) in carrot extract. Ion traces of A: MS *m*/*z* 515, B: MS^2 *m*/*z* 353, and C: MS^3 *m*/*z* 191.

(*11*, *15*, *27*, *38*, *39*), to the best of our knowledge, this is the first report of three di-*O*-caffeoylquinic isomers in shredded carrots.

Compounds **4**, **6**, **7**, and **11** could not yet be identified. However, compounds **6**, **7**, and **11** displayed identical fragmentation patterns and equally exhibited neutral loss of 162 u of the molecular ion $[M-H]^-$ corresponding to the loss of caffeic acid. Furthermore, in the ESI^-, compound **6** yielded peaks at *m*/*z* 179 and *m*/*z* 135 in the MS^2 event representing the typical fragmentation pattern of caffeic acid. Therefore, these compounds were preliminarily identified as caffeic acid derivatives.

Although *p*-hydroxybenzoic acid has been previously reported in carrot tissue (*27*, *39*, *40*), its occurrence could not be confirmed in the present study. This may be ascribed to different carrot cultivars as well as growth and storage conditions.

Influence of Storage Atmosphere and Washing Treatment on Phenolic Compounds. In both series, total chlorogenic acid (TCQA = t-CQA + c-CQA) was found to be predominant throughout storage, representing ~95% of all identified phenolic compounds. The amount of CoQA was ~5% of the identified phenolics. diCQA was detected in noticeable concentrations up to 0.25 mg/100 g fresh weight at day 9 in series 1 and at days 7 and 9 in series 2, respectively. As an example, contents of TCQA and CoQA in series 1 during storage are shown in **Figure 6** and **Figure 7**, respectively.

Phenolic contents of shredded carrots were mainly determined by the storage atmosphere, whereas the influence of different washing treatments was negligible. For film A packaged carrots, a linear increase of TCQA ($r^2 > 0.95$) throughout storage was monitored for all processing lines. In series 1, TCQA contents of all processed carrots initially ranged from 0.2 to 0.5 mg/100 g FW and rose to 3.9−4.3 mg/100 g FW after storage for 7 days. Similar trends were observed for series 2; however, significantly higher concentrations increasing from 1.2 ± 0.3 mg/100 g FW (day 0) to 9.6 ± 0.8 mg/100 g FW (day 7) were found. In both series, TCQA concentration of processed carrots at day 0 corresponded with that of the raw material. Initial TCQA contents of film B packaged carrots increased from 0.4 ± 0.1 mg/100 g FW to 1.2 ± 0.1 mg/100 g FW (series 1) and from 1.2 ± 0.2 mg/100 g FW to 2.2 ± 0.2 mg/100 g FW (series 2) at day 3, respectively, and subsequently remained constant until day 9. Similar effects were observed for CoQA contents in shredded carrots packaged in film A and film B varying between 3 and 6% of the corresponding TCQA concentrations.

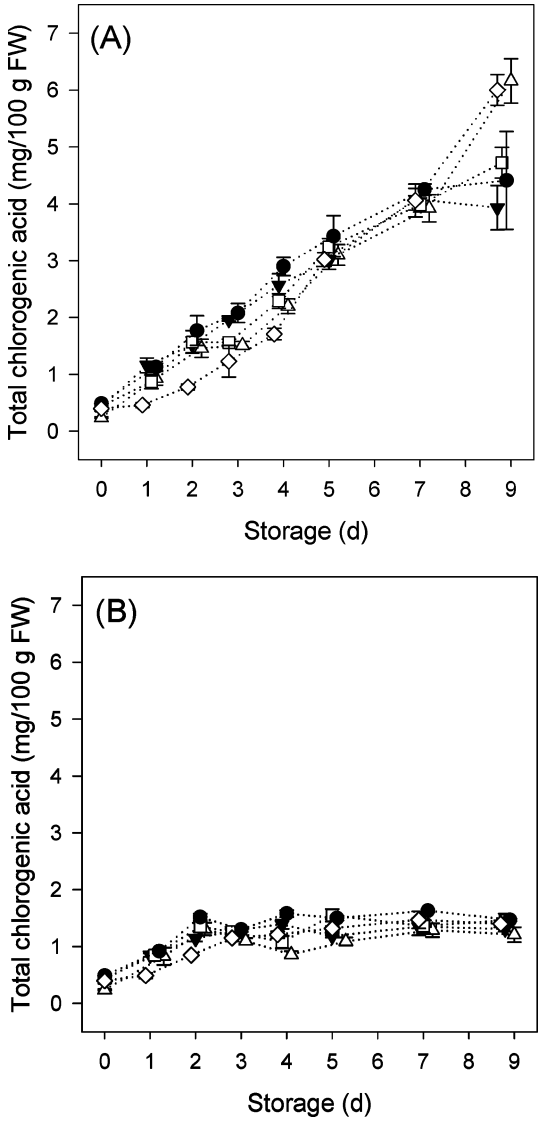


Figure 6. Effect of washing treatments on total chlorogenic acid content of shredded carrots packaged in film A (A) and film B (B) during storage at 4 °C. Values represent the mean of 2 measurements (± standard deviation) from series 1. (△), line I, control, shredding without washing; (▼), line II, pre-washing uncut carrots with tap water; (□, line III, pre-washing uncut carrots with chlorinated water; (●, line IV, pre-washing uncut carrots with ozonated water; (◇), line V, washing shredded carrots with chlorinated water. FW, fresh weight.

In both series, differences in TCQA contents of carrots packaged in film A and film B, respectively, were statistically significant ($p \leq 0.05$) at days 3−9. Regardless of the packaging film, TCQA content of line V (washing shredded carrots with chlorine) in both series was significantly lower ($p \leq 0.05$) at day 2 when compared to all other treatments (lines I−IV).

These findings are in good agreement with previous studies (*27*, *39*), obtaining high accumulation of phenolic compounds when shredded carrots were stored in air, whereas carbon dioxide levels of >30% or the absence of oxygen led to strongly diminished phenol concentrations as discussed for PAL activi-

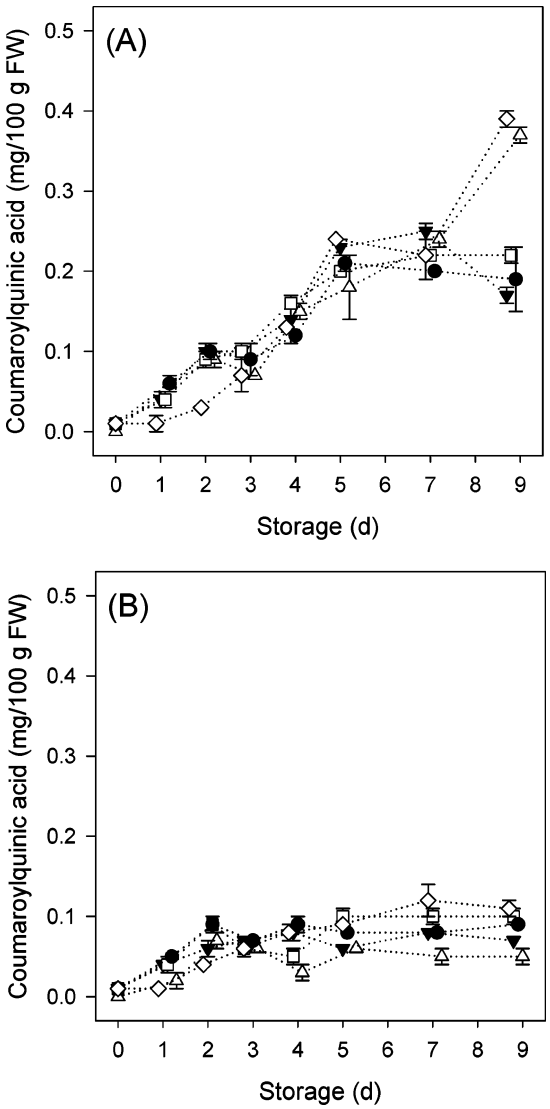


Figure 7. Effect of washing treatments on *p*-coumaroylquinic acid content of shredded carrots packaged in film A (A) and film B (B) during storage at 4 °C. Values represent the mean of 2 measurements (± standard deviation) from series 1. (△), line I, control, shredding without washing; (▼), line II, pre-washing uncut carrots with tap water; (□), line III, pre-washing uncut carrots with chlorinated water; (●), line IV, pre-washing uncut carrots with ozonated water; (◇), line V, washing shredded carrots with chlorinated water. FW, fresh weight.

ties. The higher phenolic contents obtained in series 2 may be ascribed to different factors, for example, growth and storage conditions, maturity at harvest, and elevated lignification.

The accumulation of TCQA and CoQA correlated well with the respective PAL activity, giving evidence of the strong impact of storage atmosphere on phenolic contents of shredded carrots. For shredded carrots packaged in film A, sufficient oxygen levels throughout storage supported PAL activity. Consequently, synthesis of phenolic compounds in shredded carrots continued throughout product shelf life. PAL activity of film B packaged carrots reached maximum values at days 2−4 and ended after oxygen depletion, resulting in almost constant phenolic levels thereafter. There has been controversial discussion about correlation of PAL activity and phenolic contents in minimally processed carrots. Whereas Stodolak et al. (*26*) did not find a correlation, Babic et al. (*27*) reported good consistence between increase in PAL activity and accumulation of phenolic compounds. Also, for potato and transgenic tobacco, almost linear correlation between PAL activity and chlorogenic acid content was found (*33*, *41*). As reported previously, severe off-odor and off-flavor formation due to anaerobic metabolism occurred in film B bags after a 3 day storage (*7*). Therefore, potential differences in flavor related to phenolic content could not be observed.

In conclusion, phenolic content in shredded carrots was perfectly controlled by the application of a modified storage atmosphere. Accumulation of phenolics was effectively suppressed by storage under ultralow oxygen conditions (<1%). However, adverse effects on sensory properties (off-odor/off-flavor) due to anaerobic respiration must be considered (*7*). Additionally, using aerobic storage atmosphere, antimicrobial effects of phenolic compounds on shelf life extension and their nutritional quality may play a beneficial role.

ABBREVIATIONS USED

c-CQA, 5-*O*-*cis*-caffeoylquinic acid; CoQA, *O*-*p*-coumaroylquinic acid; diCQA, di-*O*-caffeoylquinic acid; EMA, equilibrium modified atmosphere; ESI−, negative electrospray ionization; ESI+, positive electrospray ionization; FW, fresh weight; i.d., inner diameter; PAL, phenylalanine ammonia-lyase; PDA, photodiode array detector; PVPP, polyvinylpolypyrrolidone; t-CQA, 5-*O*-*trans*-caffeoylquinic acid (chlorogenic acid); TCQA, total chlorogenic acid; TRIS, tris-(hydroxymethyl)-aminomethane hydrochloride.

ACKNOWLEDGMENT

The authors thank Vita Holding GmbH (Reutlingen, Germany) for supply of the plant material and Amcor Flexibles Europe (Bristol, United Kingdom) for the generous gift of packaging material. We are also grateful to Klaus Mix for excellent technical assistance. This research project was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AiF and the Ministry of Economics and Labor, Project No.: AiF-FV 12817 N.

LITERATURE CITED

(1) Vasconcellos, J. A. Regulatory and safety aspects of refrigerated minimally processed fruits and vegetables: a review. In *Minimally processed fruits and vegetables*; Alzamora, S. M., Tapia, M. S., López-Malo, A., Eds.; Aspen Publishers: Gaithersburg, 2000; pp 319−343.

(2) Varoquaux, P.; Wiley, R. C. Biological and biochemical changes in minimally processed refrigerated fruits and vegetables. In *Minimally processed refrigerated fruits and vegetables*; Wiley, R. C., Ed.; Chapman and Hall: New York, 1994; pp 226−268.

(3) Ahvenainen, R. Minimal processing of fresh produce. In *Minimally processed fruits and vegetables*; Alzamora, S. M., Tapia, M. S., López-Malo, A., Eds.; Aspen Publishers: Gaithersburg, 2000; pp 277−290.

(4) Simons, L. K.; Sanguansri, P. Advances in the washing of minimally processed vegetables. *Food Aust.* **1997**, *49*, 75−80.

(5) Beuchat, L. R. Surface disinfection of raw produce. *Dairy Food Environ. Sanit.* **1992**, *12*, 6−9.

(6) Kim, J.-G.; Yousef, A. E.; Dave, S. Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Prot.* **1999**, *62*, 1071−1087.

(7) Klaiber, R. G.; Baur, S.; Magel, L.; Hammes, W. P.; Carle, R. Quality of shredded, packaged carrots as affected by different washing treatments. *J. Food Sci.* **2004**, *69*, SNQ161−166.

(8) Bolin, H. R.; Huxsoll C. C. Control of minimally processed carrot (*Daucus carota*) surface discoloration caused by abrasion peeling. *J. Food Sci.* **1991**, *56*, 416−418.

(9) Hahlbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347−369.
(10) Sarkar, S. K.; Phan, C. T. Effect of ethylene on the qualitative and quantitative composition of the phenol content of carrot tissues. *Physiol. Plant.* **1974**, *30*, 72−76.
(11) Aubert, S.; Babic, I.; Amiot, M. J.; Nguyen-the, C. Les composés phénoliques marqueurs de la qualité de carottes conservées au froid. *Acta Hort.* **1993**, *354*, 201−214.
(12) Tomás-Barberán, F. A.; Espín, J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853−876.
(13) Sondheimer, E. Bitter flavor in carrots. III. The isolation of a compound with spectral characteristics similar to hydrocarbon extracts of bitter carrots. *Food Res.* **1957**, *22*, 206−209.
(14) Cantwell, M.; Yang, S. F.; Rubatzky, V.; Lafuente, M. T. Isocoumarin content of carrots as influenced by ethylene concentration, storage temperature and stress conditions. *Acta Hort.* **1989**, *258*, 523−534.
(15) Sarkar, S. K.; Phan, C. T. Naturally occurring and ethylene induced phenolic compounds in the carrot root. *J. Food Prot.* **1979**, *42*, 526−534.
(16) Macheix, J. J.; Fleuriet, A.; Billot, J. The main phenolics of fruit. In *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990; pp 1−98.
(17) Hollman, P. C. H. Evidence for health benefits of plant phenols: local or systemic effects? *J. Sci. Food Agric.* **2001**, *81*, 842−852.
(18) Duthie, G. G.; Duthie, S. J.; Kyle, J. A. M. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutr. Res. Rev.* **2000**, *13*, 79−106.
(19) Lewis, B. G.; Davis, W. P.; Garrod, B. Wound-healing in carrot roots in relation to infection by *Mycocentrospora acerina*. *Ann. Appl. Biol.* **1981**, *99*, 35−42.
(20) Garrod, B.; Lewis, B. G.; Brittain, M. J.; Davies, W. P. Studies on the contribution of lignin and suberin to the impedance of wounded carrot root tissue to fungal invasion. *New Phytol.* **1982**, *90*, 99−108.
(21) Howard, L. R.; Griffin, L. E.; Lee, Y. Steam treatment of minimally processed carrot sticks to control surface discoloration. *J. Food Sci.* **1994**, *59*, 356−358, 370.
(22) Rhodes, J. M.; Wooltoron, L. S. C. The biosynthesis of phenolic compounds in wounded plant storage tissues. In *Biochemistry of wounded plant tissues*; Kahl, G., Ed.; Walter de Gruyter & Co: Berlin, 1978; p 243.
(23) Hoffman, R.; Heale, J. B. Cell death, 6-methoxymellein accumulation, and induced resistance to *Botrytis cinerea* in carrot root slices. *Physiol. Mol. Plant Pathol.* **1987**, *30*, 67−75.
(24) Babic, I.; Amiot, M. J.; Nguyen-the, C.; Aubert, S. Changes in phenolic content in fresh ready-to-use shredded carrots during storage. *J. Food Sci.* **1993**, *58*, 351−356.
(25) Howard, L. R.; Griffin, L. E. Lignin formation and surface discoloration of minimally processed carrot sticks. *J. Food Sci.* **1993**, *58*, 1065−1067, 1072.
(26) Stodolak, B.; Leja, M.; Mareczek, A. Some aspects of metabolism of phenolics in carrot root slices. *Food Agric. Environ.* **2003**, *1*, 88−94.
(27) Babic, I.; Amiot, M. J.; Nguyen-the, C.; Aubert, S. Changes in phenolic content in fresh ready-to-use shredded carrots during storage. *J. Food Sci.* **1993**, *58*, 351−356.
(28) Mitra, A.; Mayer, M. J.; Mellon, F. A.; Michael, A. J.; Narbad, A.; Parr, A. J.; Waldron, K. W.; Walton, N. J. 4-Hydroxycinnamoyl-CoA hydratase/lyase, an enzyme of phenylpropanoid cleavage from *Pseudomonas*, causes formation of C_6-C_1 acid and alcohol glucose conjugates when expressed in hairy roots of *Datura stramonium* L. *Planta* **2002**, *215*, 79−89.
(29) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(30) Hisaminato, H.; Murata, M.; Homma, S. Relationship between the enzymatic browning and phenylalanine ammonia-lyase activity of cut lettuce, and the prevention of browning by inhibitors of polyphenol biosynthesis. *Biosci. Biotech. Biochem.* **2001**, *65*, 1016−1021.
(31) Hyodo, H. Wound-induced phenylalanine ammonia-lyase in potato tuber tissue. *J. Biochem.* **1976**, *79*, 277−282.
(32) Cheng, G. W.; Breen, P. J. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J. Amer. Soc. Hort. Sci.* **1991**, *116*, 865−869.
(33) Cantos, E.; Tudela, J. A.; Gil, M. I.; Espín, J. C. Phenolic compounds and related enzymes are not rate-limiting in browning development of fresh-cut potatoes. *J. Agric. Food Chem.* **2002**, *50*, 3015−3023.
(34) Creasy, L. L. The role of enzyme inactivation in the regulation of synthetic pathways: a case history. *Physiol. Plant.* **1987**, *71*, 389−392.
(35) Blankenship, S. M.; Unrath, C. R. PAL and ethylene content during maturation of Red and Golden Delicious apples. *Phytochemistry* **1988**, *27*, 1001−1003.
(36) Fang, N.; Yu, S.; Prior, R. L. LC/MS/MS characterization of phenolic constituents in dried plums. *J. Agric. Food Chem.* **2002**, *50*, 3579−3585.
(37) Whitaker, B. D.; Stommel, J. R. Distribution of hydroxycinnamic acid conjugates in fruit of commercial eggplant (*Solanum melongena* L.) cultivars. *J. Agric. Food Chem.* **2003**, *51*, 3448−3454.
(38) Alasalvar, C.; Grigor, J. M.; Zhang, D.; Quantick, P. C.; Shahidi, F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *J. Agric. Food Chem.* **2001**, *49*, 1410−1416.
(39) Babic, I.; Amiot, M. J.; Nguyen-the, C. Changes in phenolic content in fresh ready-to-use shredded carrots during storage. *Acta Hort.* **1993**, *343*, 123−128.
(40) Schnitzler, J.-P.; Madlung, J.; Rose, A.; Seitz, H. U. Biosynthesis of *p*-hydroxybenzoic acid in elicitor-treated carrot cell cultures. *Planta* **1992**, *188*, 594−600.
(41) Howles, P. A.; Sewalt, V. J. H.; Paiva, N. L.; Elkind, Y.; Bate, N. J.; Lamb, C.; Dixon, R. A. Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol.* **1996**, *112*, 1617−1624.

Received for review June 7, 2004. Revised manuscript received September 30, 2004. Accepted November 15, 2004.

JF049084B