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Relationship of Superficial Scald Development and α-Farnesene Oxidation to Reactions of Diphenylamine and Diphenylamine Derivatives in Cv. Granny Smith Apple Peel

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Cv. Granny Smith apple fruit, treated at harvest with aqueous emulsions containing diphenylamine (DPA) and DPA derivatives, were evaluated for the peel disorder superficial scald (scald) after 6 months of cold storage at 1 °C plus 0 or 7 days at 20 °C. Metabolism of these derivatives and α-farnesene oxidation were also evaluated after 6 months. Derivatives substituted at the para position prevented scald, but scald developed on fruit treated with derivatives substituted in the amino, ortho, or meta positions. The extent of scald control was also dependent on the chemical nature of the functional group used to derivatize DPA. Hydroxylation of DPA and DPA derivatives during storage was not associated with scald control. Methoxylated DPA derivatives produced during storage resulted from *O*-methylation of *C*-hydroxylated derivatives rather than *C*-methoxylation of DPA. *N*-Nitrosodiphenylamine provided partial scald control, possibly resulting from its degradation to DPA, indicating that the amino hydrogen of DPA may be crucial for scald control. Results suggest that functional group position and chemical properties both contribute to the efficacy of DPA derivatives for scald control.

KEYWORDS: ***Malus sylvestris* L. (Mill.) var. *domestica* Borkh. Mansf; scald; LCMS; antioxidant**

INTRODUCTION

Diphenylamine (DPA) is an arylamine antioxidant with a variety of uses, including superficial scald (henceforth called "scald") control in apple fruit (*1*). Scald is a physiological disorder characterized primarily by peel browning and, as severity increases, partial collapse of the first 5−6 layers of hypodermal cells resulting from oxidative injury (*2*, *3*). In susceptible cultivars, scald symptoms can develop following 2−4 months of cold storage and severity may increase after removal from storage. For scald control, commercial formulations containing DPA, in an aqueous emulsion, are applied following harvest and prior to cold storage as a drench at concentrations of 1−2 $g \cdot L^{-1}$.

Development of scald is highly dependent upon pre- and postharvest conditions. Scald incidence is influenced by cultivar susceptibility, fruit maturity at harvest, preharvest environment, fruit mineral content, light exposure, storage conditions, and ethylene levels in storage (*4*). Oxidation of the sesquiterpene α-farnesene produces conjugated trienes (CT) and 6-methyl-5-hepten-2-one (MHO), which are ostensibly related to scald development (*5*). DPA application inhibits CT and MHO formation (*6*, *7*), and application of either of these compounds provokes scald formation (*7*−*9*); therefore, the scald control conferred by DPA is purportedly a result of its efficiency as an antioxidant (*5*).

Antioxidants containing an amino group (*10*, *11*) provide the best scald protection, while antioxidants lacking an amino group, such as butylated hydroxytoluene (*12*), require much higher concentrations to be effective. The structure of DPA also enhances its antioxidative efficiency compared with some other antioxidants. The diaryl structure of DPA can protect the donor hydrogen on the secondary amine from hydrogen bonding, rendering DPA more active in a variety of media compared to antioxidants without this structure, regardless of its relative acidity (*13*). This structural relationship may be illustrated in apples where DPA provides better scald protection compared to *N*-benzylaniline and dibenzylamine (in order) (*10*). These compounds, although similar in structure to DPA, may present less steric hindrance to hydrogen bonding with solvents or solutes in this inter/intracellular environment due to greater distance between the aryl components and the secondary amine.

In addition to impeding radical cascades by donating its amino hydrogen (*14*), DPA can also react with active oxygen and active nitrogen species to form a variety of derivatives. Reactive oxygen and reactive nitrogen species (ROS and RNS, respectively), including $^{\bullet}OH$ and $^{\bullet}NO$, are present in plants (*15*, *16*). $^{\bullet}NO$ can react with O_2, producing $^{\bullet}NO_2$ (*17*). *C*-Hydroxylation of benzene or DPA with $^{\bullet}OH$, producing phenol (*18*) or

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4-hydroxydiphenylamine (*19*), respectively, have been reported. *N*-Nitrosation and *C*-nitration of DPA can result from reactions with •NO (*20*) and •NO_2 (*20*, *21*), respectively.

Extensive investigations involving storage of DPA-stabilized smokeless gun powders demonstrate the formation of *N*-nitrosodiphenylamine (NODPA), 2-nitrodiphenylamine (2NO2DPA), 4-nitrodiphenylamine (4NO2DPA), and various combinations of di- and trinitro combinations of DPA or NODPA resulting from degradation of this medium (*21*, *22*). Nitration in this substrate occurs predominantly at the ortho and para positions on one or both aryl rings while only nitrosation of the primary amine occurs. The reactions resulting in these derivatives are purportedly the primary means by which DPA stabilizes this medium (*20*, *21*).

Investigations of DPA metabolism in biological systems revealed the presence of 4-hydroxydiphenylamine (4OHDPA), 2-hydroxydiphenylamine (2OHDPA), and 4,4′-dihydroxydiphenylamine (4,4diOHDPA) (*23*). In apple fruit, DPA derivatives include 4OHDPA, 2OHDPA, 3-hydroxydiphenylamine (3OHDPA), 2,4-dihydroxydiphenylamine (2,4diOHDPA), indophenol (*24*), NODPA (*25*), 4-methoxydiphenylamine (4MeODPA), and 3-methoxydiphenylamine (3MeODPA) (*26*). In apples, 4OHDPA, 3OHDPA, and 2OHDPA are also present as glycosidic conjugates (*24*). 4OHDPA is typically the most abundant free DPA derivative in DPA-treated apples following storage (*24*, *26*). A description of mechanisms for DPA derivative formation in apples has yet to be developed.

Because the formation of these derivatives can result from reactions between DPA and ROS or RNS, the relative prevalence of individual derivatives may be indicative of the pervasiveness of various processes or conditions leading to generation of active species during apple fruit storage. This may include amelioration of disorders, like scald, purportedly resulting from oxidative stressors. For instance, the relative abundance of 4OHDPA and its glycosydic conjugates in DPA-treated apples could indicate a linkage between 4OHDPA presence and a mechanism by which DPA impedes scald formation. DPA "trapping" of otherwise harmful hydroxyl radicals could contribute to scald control, as is the case with smokeless powder stabilization where RNS are trapped by DPA.

Consequently, if positions on DPA are already occupied by a functional group, the substitution could affect any reactions related to scald control. For example, the lack of the amino hydrogen for donation on NODPA impedes the capacity to curtail alkyl/alkoxy/peroxyl radical propagation, while *C*-nitration or hydroxylation may prevent crucial reactions between DPA and destructive ROS and RNS, which may play a role in this disorder.

The objective of this study was to use DPA derivatives substituted at various positions to examine the mechanism of scald inhibition conferred by DPA.

MATERIALS AND METHODS

Materials and Treatment of Apple Fruit with DPA and DPA Derivatives. Cv. Granny Smith [*Malus* sylvestris L. (Mill.) var. *domestica* Borkh. Mansf.] apple fruit (40 fruit/treatment), harvested in two consecutive years from a commercial orchard 1 month prior to commercial harvest, were submerged for 2 min in aqueous emulsions containing 2.0 g of DPA, 2NO2DPA, 4OHDPA, 3OHDPA, or NODPA dissolved in 2.25 mL·L^{-1} 2-propanol plus 8.0 mL·L^{-1} Triton X-100. In the second season, *N*-phenyl-4-quinoneimine (NPPQ), 4-methyldiphenylamine (4CH3DPA), and 4NO2DPA were also used, and additional treatment rates of 1.0 and 4.0 g·L^{-1} were added. Less NPPQ was obtained for these experiments; therefore, the NPPQ treatment rates were 0.5, 1, 2 g·L^{-1}. Untreated control fruit were treated similarly with solutions containing only 2-propanol and Triton X-100. Following treatment, fruit were allowed to dry at 22 °C, and then apples on fiberboard trays were placed in air storage at 1 °C. Apples were removed and scald incidence visually quantified after 6 months (day 0) and after an additional 7 days at 20 °C. Fruit peel was collected on day 0 using a potato peeler to assemble 3 composite samples (6 fruit/sample) for each treatment. Peel samples were frozen using N_2(l) and stored at −80 °C until extraction and analysis of DPA derivatives.

Extraction of DPA and DPA Derivatives. DPA and DPA derivatives were extracted by adding 50 mL of methanol and 200 μL of 0.282 mM *p*-isobutylhydratropic acid in methanol (internal standard) to approximately 25 g of frozen peel tissue and immediately homogenizing the mixture. After 20 min, the homogenate was vacuum filtered through Whatman #2 paper and the macerate washed twice with 50 mL of 80:20 methanol/deionized H_2O. The methanol was evaporated from the filtrate by using a rotary evaporation apparatus with a water bath set at 34 °C, acidified by adding acetic acid to approximately 0.4% (v/v), and then partitioned three times with 50 mL of chloroform. Acidification had no effect on the recovery of DPA and DPA derivatives and was performed to facilitate the recovery of certain acidic constituents, which were of interest but are not included in this report. The aqueous phase was discarded, the chloroform fraction evaporated using rotary evaporation, and the residue dissolved in 8 mL of methanol. When the residue was fully dissolved, 2 mL of deionized water was added. Relatively nonpolar constituents were removed by passing this solution through a C_{18} cartridge (Sep-pak, Waters, Milford, MA). Following the addition of 70 mL of deionized water to the eluate, the extract was again acidified by adding acetic acid to 0.4% and the analyte collected on another C_{18} cartridge. The analyte was eluted from the cartridge using 5 mL of 2-propanol, dried using rotary evaporation, and dissolved in 200 μL of methanol.

Reversed-Phase HPLC−UV/Vis−Atmospheric Pressure Chemical Ionization-Mass Spectroscopy (APCI-MS) Conditions. Samples were analyzed by injecting 0.5−10 μL into a Series 1100 HPLC system (Agilent Technologies, Palo Alto, CA) controlled by Chemstation software (A.09.03) and equipped with a 5 μm Agilent Hypersil ODS (4.0 × 125 mm) reverse-phase column, a G1315B diode array detector (DAD), and a G1946D single quadrupole mass selective detector (MSD) using an APCI source. Elution solvents used for a linear gradient were (A) 50:49.8:0.2 and (B) 99.8:0:0.2 methanol/deionized water/formic acid. The column temperature and mobile phase flow rate were 20 °C and 0.5 mL·min^{-1}, respectively. The mobile phase was comprised entirely of solvent A for the initial 2 min after sample injection, followed by a linear gradient of solvent A plus B until reaching 100% B at 25 min, and then remaining entirely solvent B until 40 min. The eluate was first analyzed by the DAD and then the MSD. The DAD continuously monitored and recorded spectra (200−700 nm) for the entire analysis.

The APCI spray chamber conditions were as follows: drying gas (N_2) flow = 5 L·min^{-1}, drying gas temperature = 350 °C, nebulizer pressure = 414 kPa, vaporizer temperature = 425 °C, and coronal discharge = 4 μA. The fragmentor and capillary potentials were 130 and 4000 V, respectively. The MSD was adjusted to monitor positive ions in the scanning mode, continuously monitoring and recording the entire mass spectra within a 100−1000 *m*/*z* range, or in selective ion monitoring (SIM) mode, when compounds were identified and increased sensitivity was required.

Positive identification of compounds was achieved through comparison of extract constituents with the UV−Vis and/or mass spectra of authentic standards as well as column retention comparisons. Quantification of constituents was performed by calculating a response factor using comparisons with known amounts of authentic standards. *p*-Isobutylhydratropic acid was used as an internal standard.

Analytical Standards. GC Resolve and HPLC grade methanol and HPLC grade hexanes, ethyl ether, formic acid, acetic acid, and 2-propanol were purchased from Fisher Scientific (Pittsburgh, PA). DPA, CB, 3MeODPA, 2NO2DPA, 4NO2DPA, 4-methyl-4′-hydroxydiphenylamine, and *p*-isobutylhydratropic acid were purchased from Sigma-Aldrich (St. Louis, MO). NODPA, 4OHDPA, 3OHDPA, and 4CH3DPA were purchased from TCI (Portland, OR). NPPQ and 4MeODPA were prepared as previously outlined (*26*). 2,6,10-Tri-

methyldodeca-2,7(*E*),9(*E*),11-tetraen-6-ol and α-farnesene standards were obtained as outlined below.

Synthesis and Mass Spectral Identification of 4-Nitro-4′-hydroxydiphenylamine and 2-Nitro-4′-hydroxydiphenylamine. 4-Nitro-4′-hydroxydiphenylamine and 2-nitro-4′-hydroxydiphenylamine were prepared from 4-nitroaniline or 2-nitroaniline and hydroquinone following the method of Schneider (1899). Following the reaction, the mixture was partitioned with chloroform, the chloroform phase was collected and evaporated, and the residue was dissolved in hexanes and purified using a silica gel column with 90:10 hexanes/ethyl acetate as the mobile phase. Pure fractions containing 4-nitro-4′-hydroxydiphenylamine or 2-nitro-4′-hydroxydiphenylamine were collected, and the solvent was evaporated. 4-Nitro-4′-hydroxydiphenylamine: APCIMS *m*/*z* 231 ([M + H]$^+$, 100), 214 (18), 184 (15). 2-Nitro-4′-hydroxydiphenylamine: APCIMS *m*/*z* 231 ([M + H]$^+$, 100), 213 (18), 196 (35), 168 (7.0).

Extraction of α-Farnesene and Conjugated Trienes. Oxidation of α-farnesene was analyzed in the second season. On the day apples were removed from 6 months of cold storage, peel disks were prepared using a 16.6 mm (diameter) cork borer, and accessory tissue was removed using a razor blade. Disks were immediately frozen using N_2(l) and stored at −80 °C until extraction and analysis. Disks were extracted by submerging three disks per replication (1 disk/fruit; 3 replications/treatment) in 4 mL of hexanes contained in a 30-mL test tube. After 10 min, disks were removed and discarded and the hexanes evaporated under a stream of N_2(g). The resulting residue was weighed and then dissolved in 200 μL of absolute methanol with the aid of an ultrasonic bath filled with room-temperature water. The extract was then filtered prior to analysis.

To obtain α-farnesene and CT for purification and identification, epicuticular constituents were extracted from 110 mature cv. Granny Smith apples following 6 months of storage in air at 1 °C by dipping each apple in approximately 500 mL of hexanes for 30 s. Additional hexanes were added as necessary to ensure the appropriate volume was maintained. The resulting extract was passed through a column containing 20 g of silica gel (100−200 mesh) and the eluate collected. Hexanes were evaporated from the eluate under reduced pressure, and the remaining waxy residue was washed three times with 25 mL of methanol and sonicated, and the undissolved material was removed by centrifugation. The remaining waxy substances were removed by passing the compiled methanol extract through a C_{18} cartridge. The methanol was evaporated under reduced pressure, and then the α-farnesene remaining was stored until used in a tube purged with Ar(g) prior to sealing.

The most abundant CT was isolated by eluting the adsorbed contents of the 20-mL silica gel column (see above) with 100 mL of 90:10 hexanes/ethyl ether. Solvents were removed under reduced pressure, and the waxy residue was washed three times with 10 mL of methanol and sonicated, and the undissolved material was removed by centrifugation. The remaining waxy substances were removed by passing the compiled methanol extract through a C_{18} cartridge, and the methanol was removed under reduced pressure. The residue was dissolved in 3 mL of methanol and transferred to the preabsorbant layer of a silica-coated (L6KDF, 250 μm) thin-layer chromatography plate and separated using 5:1 hexanes/ethyl ether as the mobile phase. The most abundant CT had a response factor of 0.26 and was removed from the plate by scraping the solid phase off, dissolving it in methanol, and filtering the mixture prior to analysis.

Identification of α-farnesene and the CT standards was made using gas chromatography−electron impact mass spectroscopy (GC−EI-MS) and HPLC−UV/vis−APCI-MS. HPLC−UV/vis−APCI conditions are described above. GC−EI-MS analyses were performed using a Hewlett-Packard 5890 Series 2 gas chromatograph equipped with a 5971 EI-MS detector and an Agilent Ultra 2 capillary column (25 m × 0.2 mm × 0.33 μm film thickness). Pure standards were injected into an inlet maintained in the splitless mode at 250 °C and samples were eluted using a temperature gradient starting at 100 °C, held for 1 min, raised to 280 °C at 15 °C/min, and held at the final temperature for 1 min. The transfer line temperature was maintained at 280 °C and the filament voltage at 70 eV, and positive ions from *m*/*z* 30−300 were monitored continuously. Quantification of α-farnesene and CT standards was performed using optical density measurements of the purified standard to calculate concentration based on published molar extinction coefficients, ϵ_{232} = 27 740 (*34*) and ϵ_{269} = 42 500 (*30*), respectively.

Spectral characteristics of α-farnesene were as follows: EIMS *m*/*z* 204 (M$^+$, <1), 189 (1.4), 161 (3.4), 147 (1.5), 123 (34), 119 (37), 107 (43), 93 (100), 79 (45), 69 (62), 55 (52). The observed EIMS was confirmed as α-farnesene by spectral comparison with entries in the NIST 2002 GC-MS library and similarity to that obtained by Whitaker et al. (*32*): APCIMS *m*/*z* 205 ([M + H]$^+$, 100), 149 (10.2), 123 (6.5); UV−vis 232 nm (100, peak), 262 (baseline). Spectral characteristics of the most abundant CT were as follows: EIMS *m*/*z* 220 (M$^+$, <1), 202 (2.2), 187 (3.3), 162 (52), 159 (19), 147 (15), 137 (33), 133 (15), 119 (42), 107 (31), 105 (41), 95 (95), 93 (94), 91 (62), 79 (54), 69 (77), 55 (100); APCIMS *m*/*z* 203 ([M + H]$^+$ − H_2O, 100), 147 (11), 123 (8.6); UV−vis 222 nm (7.2, valley), 259−264 (76−78, shoulder), 269 (100, peak), 276 (80, valley), 281 (81, peak), 304 (baseline).

Statistical Analyses. Single rate comparisons of scald incidence were analyzed using *z*-statistics to separate means. Regression analyses of scald protection provided by DPA and DPA derivatives using multiple application rates were performed using the Logistic procedure provided in the SAS software package (Version 8.0, SAS institute, Cary, NC). ANOVA and mean separation procedures, using Fisher's LSD analysis, were performed on peel DPA derivative constituents, α-farnesene, and CT data also using the SAS software package.

RESULTS AND DISCUSSION

Scald Control Efficiency of DPA and DPA Derivatives. In both seasons, 2 g·L^{-1} DPA and 4OHDPA provided similar levels of scald control through 6-month cold storage plus 0 or 7 days of ripening at 22 °C (Figures 1 and 2). The same amount of NODPA provided some scald control, but less than that of DPA and 4OHDPA in both seasons. Fruit treated at the same rate with 4CH3DPA and NPPQ, which were only tested in the second season, exhibited significantly less scald than the control fruit after 6 months. 3OHDPA and 2NO2DPA, in both seasons, and 4NO2DPA, in the second season, did not control scald at 2 g·L^{-1}.

In the second season, additional treatments containing concentrations of 1 and 4 g·L^{-1} confirmed the efficiency of DPA, 4OHDPA, NODPA, and 4CH3DPA for scald control, albeit at differing levels (Table 1). Scald control using 4NO2DPA improved at the 4 g·L^{-1} rate. NPPQ applied at the 2 g·L^{-1} rate was effective for scald control.

Metabolism of DPA and DPA Derivatives. As already mentioned, treatment of apple fruit with DPA followed by long-term storage can result in the presence of varying levels of 4OHDPA, 4MeODPA, 3OHDPA, 3MeODPA, and NODPA. Evaluation of the DPA and DPA derivative content of peel tissue sampled from DPA-treated apples during both seasons corroborated most previous findings (Table 2).

Diphenylamine was present at varying concentrations in fruit from all treatments. It was most prevalent in DPA-treated fruit, less in NODPA-treated fruit, and very low in fruit receiving the other treatments. Small quantities of DPA were also recovered from untreated fruit. The presence of small amounts of DPA in untreated apple fruit has been previously reported and may result from pre- or postharvest contamination or some yet unidentified metabolic event (*27*). NODPA was converted to DPA in appreciable quantities and subsequently into many of the derivatives typically found in DPA-treated fruit. This indicates that DPA formed following NODPA treatment may actually confer the lesser degree of scald control observed. Degradation of NODPA to DPA and its subsequent transformation has previously been reported in mammalian systems (*28*).

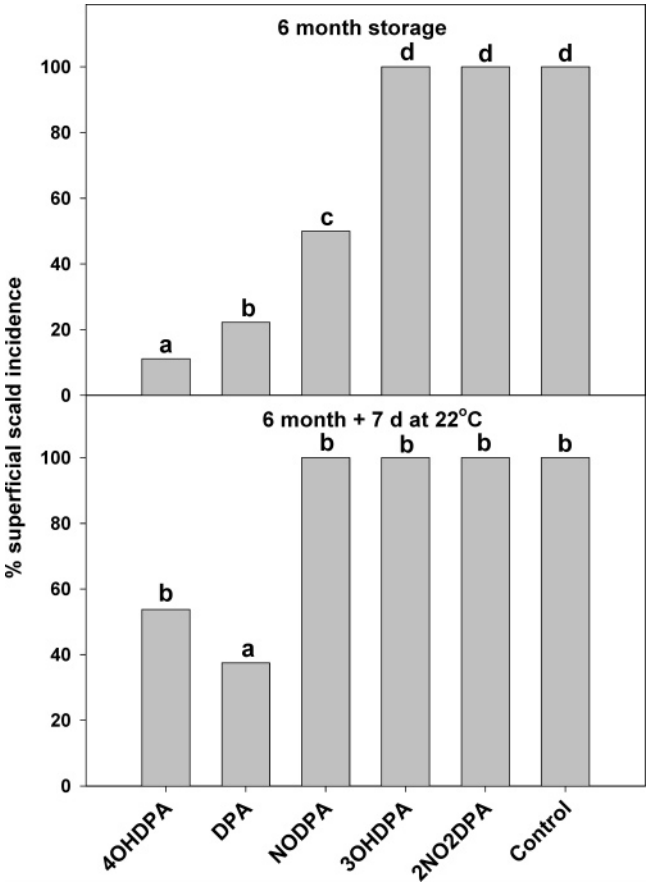

Figure 1. Season 1 scald incidence following 6-month storage at 1 °C, either immediately after removal or after a 7 day (at 20 °C) poststorage ripening period. Cv. Granny Smith apple fruit were treated with aqueous emulsions containing 2 g·L^{-1} DPA and DPA derivatives at harvest. Different lower case letters denote significant differences between means according to *z*-statistics ($n = 40$; $\alpha = 0.05$).

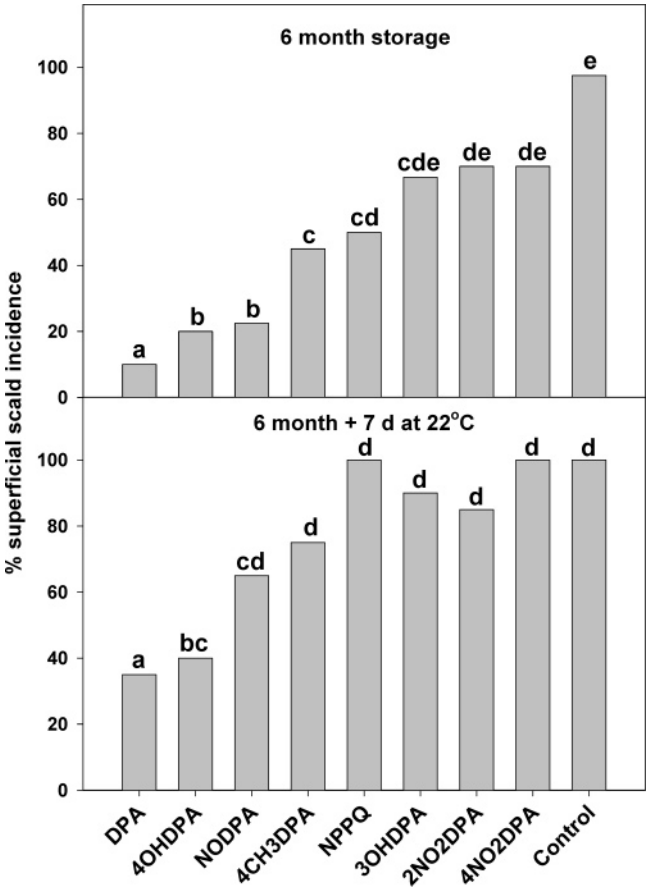

Figure 2. Season 2 scald incidence following 6-month storage at 1 °C, either immediately after removal or after a 7 day (at 20 °C) poststorage ripening period. Cv. Granny Smith apple fruit were treated with aqueous emulsions containing 2 g·L^{-1} DPA and DPA derivatives at harvest. Different lower case letters denote significant differences between means according to *z*-statistics ($n = 40$; $\alpha = 0.05$).

The partial control of scald provided by NODPA and its conversion to DPA during storage suggests that the amino position may be important in the process by which DPA prevents injury that results in development of scald symptoms. Additional studies using *N*-alkylated DPA derivatives are needed for further validation of this assertion.

4OHDPA, which provided some scald protection without conversion to DPA, was recovered in substantial quantities in DPA- and 4OHDPA-treated fruit and also in NODPA-treated fruit, although only in the second season. Small quantities of 4OHDPA were also recovered from NPPQ-treated fruit. NPPQ and 4OHDPA are closely related compounds that can readily interconvert when redox conditions are appropriate (*29*), and as a consequence, the 4OHDPA and NPPQ peaks were combined for chromatographic quantification because it was not possible to determine if NPPQ was an artifact of the extraction or chromatographic procedure (*26*). 3OHDPA was only recovered in 3OHDPA-, DPA-, and NODPA-treated fruit. Considerably less 4OHDPA and 3OHDPA were recovered from fruit treated with these compounds than was typical for the other treatments, possibly due to glycosylation of these hydroxylated derivatives (*24*). Glycosides of *C*-hydroxylated DPA derivatives (*26*) were not evaluated in this study. 4NO2DPA and 2NO2DPA were only recovered from apples to which the compounds were previously applied.

Formation of Methoxylated DPA Derivatives. Substantial amounts of 4MeODPA formed in fruit treated with DPA, lesser amounts in apples treated with 4OHDPA and NODPA, and a trace in fruit treated with NPPQ. Given that DPA, NPPQ, and NODPA were all converted in some degree to 4OHDPA and that the quantities of 4MeODPA loosely reflect those of recovered 4OHDPA, especially in season 2, *O*-methylation of 4OHDPA, rather than *C*-methoxylation of DPA, is the most likely scenario for methoxylated DPA synthesis in this system. This is particularly apparent when considering that apples treated with 3OHDPA contained 3MeODPA but not detectable amounts of 4MeODPA. 3MeODPA recovered from DPA-treated fruit

Table 1. Scald Control Efficiency of DPA and DPA Derivatives Applied to Cv. Granny Smith Apples and Evaluated after 6-Month Storage at 1 °C

	scald incidence (%)				regression analyses[a]	
treatment	0 g·L^{-1}	1 g·L^{-1}	2 g·L^{-1}	4 g·L^{-1}	linear	quadratic
diphenylamine	98	52	10	0.0	*	
4-hydroxydiphenylamine	98	50	20	10	*	*
N-nitrosodiphenylamine	98	75	22	15	*	*
4-methyldiphenylamine	98	42	45	20	*	*
N-phenyl-4-quinoneimine[b]	98	100	95	50		
3-hydroxydiphenylamine	98	90	67	82	*	*
2-nitrodiphenylamine	98	95	70	82	*	*
4-nitrodiphenylamine	98	85	70	48	*	

[a] An asterisk indicates significant fit ($p \leq 0.05$; $n = 40$). [b] Treatments containing 0.5, 1, and 2 g·L^{-1} of NPPQ were applied.

Table 2. Diphenylamine Derivatives Recovered (ng·g^{-1}) from Peel of Cv. Granny Smith Apple Fruit Treated with 2 g·L^{-1} of Various Diphenylamine Derivatives and Stored for 6 Months at 1 °C during Season 1 (A) and Season 2 (B)

(A) Season 1

treatment	DPA	4OHDPA[a]	3OHDPA	NODPA	2NO2DPA	4MeODPA	3MeODPA
control	2.54	ND[b]	ND	ND	ND	ND	ND
diphenylamine	2280	966	4.23	1.62	ND	295	ND
4-hydroxydiphenylamine	7.44	61.8	ND	ND	ND	175	ND
3-hydroxydiphenylamine	7.59	ND	314	ND	ND	ND	38.6
N-nitrosodiphenylamine	1920	1.25	13.4	1980	ND	142	ND
2-nitrodiphenylamine	160	ND	ND	ND	4320	ND	ND
LSD[c] ($p \leq 0.05$; $n = 3$)	121	203	51.6	73.4	600	60.0	5.18

(B) Season 2

treatment	DPA	4OHDPA[a]	3OHDPA	NODPA	2NO2DPA	4NO2DPA	4MeODPA	3MeODPA
control	2.90	ND	ND	ND	ND	ND	ND	ND
diphenylamine	5340	3370	2.67	2.40	ND	ND	308	1.85
4-hydroxydiphenylamine	2.70	522	0.150	ND	ND	ND	142	ND
3-hydroxydiphenylamine	12.3	ND	858	ND	ND	ND	ND	143
N-nitrosodiphenylamine	3640	342	12.0	421	ND	ND	194	ND
2-nitrodiphenylamine	2.10	ND	ND	ND	3830	ND	ND	ND
4-nitrodiphenylamine	26.9	ND	ND	ND	ND	5370	ND	ND
4-methyldiphenylamine	2.90	ND	ND	ND	ND	ND	ND	ND
N-phenyl-4-quinoneimine	16.8	14.2	ND	ND	ND	ND	23.6	ND
LSD ($p \leq 0.05$; $n = 3$)	914	707	93.0	215	206	144	47.8	36.5

[a] Values reflect combined peaks for 4-hydroxydiphenylamine and *N*-phenyl-4-quinoneimine. [b] ND = not detected. [c] LSD = least significant difference evaluated using Fisher's least significant difference analysis.

may simply reflect minor quantities of 3OHDPA derived in those fruit.

Hydroxylated Derivatives of 4NO2DPA and 2NO2DPA. While derivatives typically recovered from DPA-treated apples were not detected in fruit treated with 4NO2DPA and 2NO2DPA, there was evidence of some similar derivatization processes. Derivatives of both 4NO2DPA and 2NO2DPA containing hydroxyl groups in the 4′ position were detected and identified. Additionally, other compounds with similar molecular masses and CI mass spectra were found but not identified, indicating the presence of other hydroxylated derivatives of 4NO2DPA and 2NO2DPA. Even though derivatives hydroxylated in the para position formed in appreciable amounts, these compounds provided less or no scald protection at the treatment concentrations applied. 4CH3DPA provided some scald control even though the corresponding *p*-hydroxylated derivative, 4-methyl-4′-hydroxydiphenylamine, was not detected in fruit treated with this compound. This result provides additional evidence that these hydroxylation reactions have no readily apparent relationship to scald control.

Control of α-Farnesene Oxidation by DPA and DPA Derivatives. UV−vis spectral characteristics of the most abundant CT were typical of those observed previously for CTs with absorbance maxima of 259, 269, and 281 (*30*−*32*). There were also numerous minor compounds detected with similar UV−vis spectra, albeit different APCI mass spectra. Because concentrations of these minor compounds largely reflected those of the most abundant CT, identification and quantification of the most abundant CT was the primary focus. The EI mass spectrum for the most abundant CT in this study is similar to that reported by Whitaker et al. (*32*), who tentatively identified it as 2,6,10-trimethyldodeca-2,7(*E*),9(*E*),11-tetraen-6-ol, in agreement with Rowan et al. (*31*), who also reported this to be the most abundant form in cv. Granny Smith apple peel. The APCI mass spectrum shows a major peak at *m*/*z* 203 ($[M + H]^+ - H_2O$), further corroborating this identification.

Increased CT content reflected elevated scald incidence, however, there was no significant difference in α-farnesene content among different treatments (Figure 3). DPA treatment reduces CT formation (*6*, *33*), although there is disagreement over exactly how this happens. DPA purportedly can lessen α-farnesene levels (*34*, *35*) by reducing ethylene production and cellular respiration (*35*, *36*). Ethylene can stimulate α-farnesene production (*37*, *38*). However, Whitaker (*33*) reported DPA treatment resulted in delayed α-farnesene production with no change in the maximum level of α-farnesene, while CT formation was significantly reduced following both air and low O_2 storage. This is in agreement with the present study, indicating that prevention of scald development by DPA is not caused by decreased α-farnesene synthesis.

Mechanism for Scald Control by DPA. As already mentioned, reactions between DPA and various radical species in other systems can lead to the formation of many of the derivatives found in DPA-treated apple peel. These include reactions with $^{\bullet}OH$ forming 4OHDPA (*19*) as well as $^{\bullet}NO$ and $^{\bullet}NO_2$ producing NODPA (*20*). Also, like those found in apple peel, *C*-hydroxylated derivatives ensuing from these reactions are most likely to be substituted in the para position with negligible substitutions occurring in the inactivating meta position as well as the sterically hindered ortho position (*39*).

However, the formation of these derivatives through reactions with ROS and RNS does not preclude the possibility of enzyme-catalyzed syntheses of many of these derivatives. For instance, endogenous oxygenases may catalyze the formation of hydroxylated DPA derivatives. A mutant bacterial cytochrome P450, altered so that it recognizes a variety of aryl substrates, including DPA, catalyzed the synthesis of 4OHDPA exclusively from DPA (*40*). None of these reactions have been reported in plants and the specificity of this one, in particular, fails to account for the 3OHDPA and 2OHDPA present in DPA-treated apple. *O*-Methylation reactions, which appear to produce 4MeODPA and 3MeODPA, occur widely in plants by activity of *O*-methyltransferases. These enzymes catalyze the transfer of methyl groups from methyl carriers to a wide variety of substrates, including *C*-hydroxylated phenolics (*41*), which are

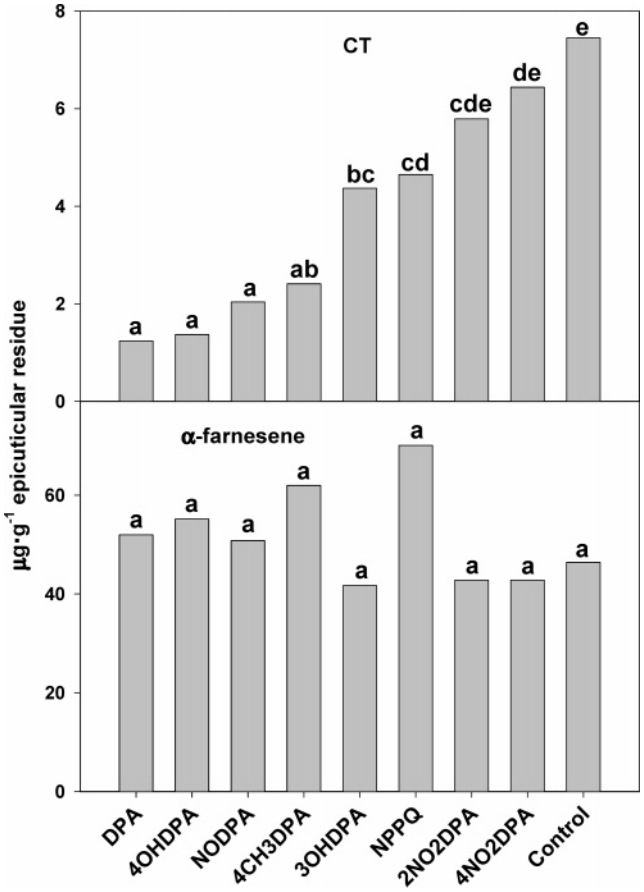

Figure 3. Season 2 epicuticular residue conjugated trienol and α-farnesene content following 6-month storage at 1 °C. Epicuticular residue was extracted by submerging peel disks in hexanes. Cv. Granny Smith apple fruit were treated with aqueous emulsions containing 2 g·L^{-1} DPA and DPA derivatives at harvest. Different lower case letters denote significance differences between means according to Fisher's least significance difference test ($n = 3$; $p \leq 0.05$).

abundant in apple fruit (*42*, *43*) and contain structural components similar to *C*-hydroxylated DPA.

These results augment the present knowledge of DPA metabolism in apple fruit during storage. However, even though these results suggest that a variety of radical species are formed in cv. Granny Smith apples during storage, a clear association between one or more radical species or reactions and scald development is not readily apparent. The position on DPA where functional groups are added does not reconcile differences in scald control efficiency depicted in this study. For instance, 4OHDPA and, to a lesser degree, 4CH3DPA provided protection from scald while the DPA derivatives substituted in other positions did not. This suggests that vacancy of the meta and ortho position is crucial to the scald control function of DPA. However, 4NO2DPA provided lesser control than the other derivatives at the same application rates, implying that specific chemical properties of the various functional groups, such as propensity for electron withdrawal, is also relevant to the scald control mechanism of DPA and its derivatives.

A process where the amino hydrogen is donated, requiring subsequent resonance stabilization of the diphenylamidogen radical, may be affected by substitutions in certain positions. Such a mechanism is indicated by the decreased scald control efficiency of NODPA when compared to DPA. In meta- and ortho-substituted derivatives, such as 2NO2DPA, 3OHDPA, and 4NO2DPA, the ability to form stabile resonance species may be compromised by adverse electronic delocation effects. Sugihara et al. (*44*) suggest a cyclic mechanism, similar to the model for DPA inhibition of hydrocarbon oxidation outlined by Boozer and Hammond (*14*), for DPA-mediated lipid peroxide quenching in red blood cells. Diphenylamidogen radicals can rapidly react with peroxide radicals, forming diphenylnitric oxide radicals (*45*, *46*). Appel et al. (*28*) found evidence of diphenylnitric oxide radicals as well as its quenched, although unstable, product, *N*-hydroxydiphenylamine, in rat and hamster cell cultures treated with DPA. However, as in our report, *N*-hydroxydiphenylamine was not found in cv. Granny Smith apples treated with DPA (*24*). In this fashion, donation of the amino hydrogen to prevent radical cascades provoked by α-farnesene oxidation may be the primary mechanism by which DPA ameliorates scald formation, as reflected by the close association of CT occurrence and scald incidence in this study.

Additional processes related to DPA metabolism, or their interplay, may also affect scald control efficiency. Changes in polarity, resulting from addition of functional groups, may also affect DPA scald control efficiency by changing intracellular location of the derivative, potentially making it more available where needed to prevent scald. In this way the conversion of DPA to 4OHDPA, the major DPA derivative formed in apple, may "target" the molecule to specific tissue regions by making it more water soluble or available for glycosylation. Also, the *O*-methylation process portrayed in this study may be associated with scald control; however, no more specific hypotheses can be developed using current knowledge.

Conclusions. Functional group position and characteristics of DPA derivatives greatly affected each compound's ability to control scald. DPA derivatives containing substitutions in the para position controlled scald to varying degrees, while derivatives substituted in the meta and ortho positions afforded no protection at the concentrations used. Scald protection imparted by NODPA was likely related to its degradation to DPA, suggesting that the presence of an amino hydrogen is crucial to the efficiency of DPA in preventing scald. Hydroxylation of 4NO2DPA and 2NO2DPA occurred but afforded little coincident scald control. The evidence provided supports the likelihood that production of 4MeODPA and 3MeODPA results from methylation of 4OHDPA and 3OHDPA, respectively. The degree of oxidation of α-farnesene reflects the extent of scald incidence.

These results support a cyclic scald control mechanism that relies primarily on amino hydrogen donation and subsequent stabilization and quenching of the diphenylamidogen radical. While accumulation of many DPA derivatives occurs, the results do not support a mechanism similar to the purported role of DPA in propellant stabilization, where DPA derivatization prevents propellant degradation. In this way, the efficiency of scald control displayed by each of these derivatives may simply relate to their ability to prevent the oxidation of α-farnesene, assuming conjugated triene generation is a cause of scald and not merely a symptom. However, studies revealing the presence of these derivatives in DPA-treated fruit following different durations of storage, under different storage conditions, and different treatments known to reduce scald may reveal other relationships between their presence and scald incidence.

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LITERATURE CITED

(1) Smock, R. M. A comparison of treatments for control of the apple scald disease. *Proc. Am. Soc. Hortic. Sci.* **1957**, *69*, 91−100.

(2) Bain, J. M. A. A histological study of the development of superficial scald in Granny Smith apples. *J. Hortic. Sci.* **1956**, *31*, 234−238.

(3) Bain, J. M.; Mercer, F. V. The submicroscopic cytology of superficial scald, a physiological disease of apples. *Aust. J. Biol. Sci.* **1963**, *16*, 442−449.

(4) Ingle, M.; D'Souza, M. C. Physiology and control of superficial scald of apples: A review. *HortScience* **1989**, *24*, 28−31.

(5) Whitaker, B. D. Oxidative stress and superficial scald of apple fruit. *HortScience* **2004**, *39*, 933−937.

(6) Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples V. Oxidation of α-farnesene and its inhibition by diphenylamine. *J. Sci. Food Agric.* **1970**, *21*, 44−48.

(7) Mir, N.; Perez, R.; Beaudry, R. M. A poststorage burst of 6-methyl-5-hepten-2-one (MHO) may be related to superficial scald development in 'Cortland' apples. *J. Am. Soc. Hortic. Sci.* **1999**, *124*, 173−176.

(8) Whitaker, B. D.; Saftner, R. A. Temperature-dependent autoxidation of conjugated trienols from apple peel yields 6-methyl-5-hepten-2-one, a volatile implicated in induction of scald. *J. Agric.* Food Chem. **2000**, *4*, 2040−2043.

(9) Rowan, D. D.; Hunt, M. B.; Fielder, S.; Norris, J.; Sherburn, M. S. Conjugated triene oxidation products of α-farnesene induce symptoms of superficial scald on stored apples. *J. Agric. Food* Chem. **2001**, *49*, 2780−2787.

(10) Huelin, F. E. Superficial scald, a functional disorder of stored apples. II. Promoters and inhibitors. *J. Sci. Food Agric.* **1964**, *15*, 227−236.

(11) Hardenburg, R. E.; Anderson, R. E. Postharvest chemical, hot-water, and packaging treatments to control apple scald. *Proc. Am. Soc. Hortic. Sci.* **1965**, *87*, 93−99.

(12) Gough, R. E.; Shutak, V. G.; Olney, C. E.; Day, H. Effect of butylated hydroxytoluene (BHT) on apple scald. *J. Am. Soc. Hortic. Sci.* **1973**, *98*, 14−15.

(13) MacFaul, P. A.; Ingold, K. U.; Lusztyk, J. Kinetic solvent effects of hydrogen atom abstraction from phenol, aniline, and diphenylamine. The importance of hydrogen bonding on their radical-trapping (antioxidant) activities. *J. Org. Chem.* **1996**, *61*, 1316−1321.

(14) Boozer, C. E.; Hammond, G. S. Molecular complex formation in free radical reactions. *J. Am.* Chem. Soc. **1954**, *76*, 3861−3862.

(15) Van Breusegem, F.; Vranová, E.; Dat, J. F.; Inzé, D. The role of active oxygen species in plant signal transduction. *Plant Sci.* **2001**, *161*, 405−414.

(16) Wojtaszek, P. Nitric oxide in plants: To NO or not to NO. *Phytochemistry* **2000**, *54*, 1−4.

(17) Bonner, F. T.; G. Stedman. The chemistry of nitric oxide and redox-related species. In *Methods in Nitric Oxide Research*; Feelisch, M., Stamler, J. S., Eds.; John Wiley and Sons: New York, 1996.

(18) Balakrishnan, I.; Reddy, M. P. Mechanism of reaction of hydroxyl radicals with benzene in the γ radiolysis of the aerated aqueous benzene system. *J. Phys. Chem.* **1970**, *74*, 850−855.

(19) Schmidt, K. H.; Bromberg, A.; Meisel, D. Oxidation of diphenylamine by OH radicals and excitation of the diphenylamino and OH adduct radicals. *J. Phys. Chem.* **1985**, *89*, 4352−4357.

(20) Lussier, L. S.; Gagnon, H.; Bohn, M. A. On the chemical reactions of diphenylamine and its derivatives with nitrogen dioxide at normal storage temperature conditions. *Propellants, Explosives, Pyrotechnics* **2000**, *25*, 117−125.

(21) Espinoza, E. O'N.; Thornton, J. I. Characterization of smokeless gunpowder by means of diphenylamine stabilizer and its nitrated derivatives. *Anal. Chim. Acta* **1994**, *288*, 57−69.

(22) Schroeder, W. A.; Malmberg, E. W.; Fong, L. L.; Trueblood, K. N.; Landerl, J. D.; Hoerger, E. Chromatographic investigations of smokeless powder. *Indust. Eng. Chem.* **1949**, *41*, 2818−2827.

(23) Alexander, W. E.; Ryan, A. J.; Wright, S. E. Metabolism of diphenylamine in the rat, rabbit, and man. *Food Cosmet. Toxicol.* **1968**, *3*, 571−579.

(24) Kim-Kang, H.; Robinson, R. A.; Wu, J. Fate of [^{14}C]diphenylamine in stored apples. *J. Agric. Food Chem.* **1998**, *46*, 707−717.

(25) Lillard, T. J.; Hotchkiss, J. H. *N*-nitrosodiphenylamine in diphenylamine treated apples. In *Nitrosamines and Related N-nitroso Compounds;* ACS Symposium Series 553; Loeppky, R. N., Michejda, C. J. Eds.; American Chemical Society: Washington, DC, 1994; pp 358−360.

(26) Rudell, D. R.; Mattheis, J. P.; Fellman, J. K. Evaluation of diphenylamine derivatives in apple peel using gradient reversed-phase liquid chromatography with ultraviolet−visible absorption and atmospheric pressure chemical ionization mass selective detection. *J. Chromatogr. A.* **2005**, *1081*, 202−209.

(27) Bramlage, W. J.; Potter, T. L.; Ju, Z. Detection of diphenylamine on surfaces of nontreated apples (*Malus domestica* Borkh.). *J. Agric. Food Chem.* **1996**, *44*, 1348−1351.

(28) Appel, K. E.; Görsdorf, S.; Scheper, T.; Ruf, H. H.; Rühl, C. S.; Hildebrandt, A. G. Metabolic denitrosation of diphenylnitrosamine: A possible bioactivation pathway. *J. Cancer Res. Clin. Oncol.* **1987**, *113*, 131−136.

(29) Leedy, D. W.; Adams, R. N. Anodic oxidation pathways of aromatic amines. IV. Diphenylamine systems in aqueous solution. *J. Am. Chem. Soc.* **1970**, *92*, 1646−1650.

(30) Anet, E. F. L. J. Autoxidation of α-farnesene. *Aust. J. Chem.* **1969**, *22*, 2403−2410.

(31) Rowan, D. D.; Allen, J. M.; Fielder, S.; Spicer, J. A. Identification of conjugated triene oxidation products of α-farnesene in apple skin. *J. Agric. Food Chem.* **1995**, *43*, 2040−2045.

(32) Whitaker, B. D.; Solomos, T.; Harrison, D. J. Quantification of α-farnesene and its conjugated trienol oxidation products from apple peel by C_{18}−HPLC with UV detection. *J. Agric. Food* Chem. **1997**, *45*, 760−765.

(33) Whitaker, B. D. DPA treatment alters α-farnesene metabolism in peel of 'Empire' apples stored in air or 1.5% O_2 atmosphere. *Postharv. Biol. Technol.* **2000**, *18*, 91−97.

(34) Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples. IV. Effect of variety, maturity, oiled wraps and diphenylamine on the concentration of α-farnesene in the fruit. *J. Sci. Food Agric.* **1968**, *19*, 297−301.

(35) Du, Z.; Bramlage, W. J. Roles of ethylene in the role of conjugated trienes in superficial scald development on stored apples. *J. Am. Soc. Hortic. Sci.* **1994**, *118*, 807−813.

(36) Lurie, S.; Klein, J.; Ben-Arie, R. Physiological changes in diphenylamine-treated 'Granny Smith' apples. *Israel J. Bot.* **1989**, 38, 199−207.

(37) Watkins, C. B.; Bramlage, W. J.; Cregoe, B. A. Superficial scald of 'Granny Smith' apples is expressed as a typical chilling injury. *J. Am. Soc. Hortic. Sci.* **1995**, *120*, 88−94.

(38) Gong, Y.; Tian, M. S. Inhibitory effect of diazocyclopentadiene on the development of superficial scald in Granny Smith apple. *Plant Growth Regul.* **1998**, *26*, 117−121.

(39) Smith, M. B.; March, J. *March's Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*. 5th ed.; John Wiley & Sons: New York, 2001.

(40) Bell, S. G.; Rouch, D. A.; Wong, L. Selective aliphatic and aromatic carbon−hydrogen bond activation catalysed by mutants of cytochrome $P450_{cam}$. *J. Mol. Catal. B.* **1997**, *3*, 293−302.

(41) Poulton, J. E. Transmethylation and demethylation reactions in the metabolism of secondary plant products. In *The Biochemistry of Plants: A Comprehensive Treatise*; Stumpf, P. K., Conn, E. E., Eds.; Academic Press: London, UK, 1981; Vol. 7, pp 667−723.

(42) Lancaster, J. E. Regulation of skin color in apples. *Crit. Rev. Plant Sci.* **1992**, *10*, 487−502.

(43) Schieber, A.; Keller, P.; Carle, R. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *J. Chromatogr. A* **2001**, *910*, 265−273.
(44) Sugihara, T.; Rao, G.; Hebbel, R. P. Diphenylamine: An unusual antioxidant. *Free Rad. Biol. Med*. **1993**, *14*, 381−387.
(45) Thomas, J. R. The identification of radical products from the oxidation of diphenylamine. *J. Am. Chem. Soc.* **1960**, *82*, 5955−5956.
(46) Thomas, J. R.; Tolman, C. A. Oxidation inhibition by diphenylamine. *J. Am. Chem. Soc.* **1963**, *84*, 2930−2935.

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