Fate of [^{14}C]Diphenylamine in Stored Apples

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The nature and magnitude of residues in apples treated with [^{14}C]diphenylamine (DPA) was investigated following treatment and storage at reduced temperature. Additional apples were treated with a mixture of [^{14}C]DPA and deuterium-labeled DPA (D-10-DPA) for use in metabolite identification. Stored apples were sampled at 16 different intervals throughout a 40-week period. Total radioactive residue levels remained constant over the entire testing interval. The parent chemical and several metabolites were identified on the final harvest peel and pulp samples by quantitative and qualitative analytical methods. A majority of the terminal residue, which was confined largely in the peel, consisted of unmetabolized DPA. Residues in pulp, however, were largely composed of glycosyl conjugates of several hydroxylated diphenylamine (OH-DPA) metabolites. The major polar metabolite identified in stored apples was a glucose conjugate of 4-hydroxydiphenylamine (4-OH-DPA). Additional metabolites, characterized as glycosyl conjugates of 2-OH-DPA, 3-OH-DPA, 4-OH-DPA, or dihydroxy-DPA, were also detected along with their intact (i.e., nonconjugated) forms in apple pulp.

Keywords: *Apple scald; diphenylamine; metabolites; glucose conjugate*

INTRODUCTION

Superficial scald, a respiratory breakdown of the fruit cells, is one of the most common cold storage disorders of apples. This physiological abnormality, which can cause substantial economic losses to apple producers, can be controlled effectively by applying diphenylamine (DPA) to stored apples (Smock, 1955; Ginsburg, 1962). Its effectiveness proved so reliable that it rapidly was adopted as a standard commercial control for this disorder in many countries (Igle and D'Souza, 1989). Published reports indicated DPA to be an endogenous natural product in certain plants (Karawya and Wahab, 1984), but no published reports conclusively support its existence as a natural substance in apples. Harvey and Clark (1959) reported that about 90% of the DPA residues in apples can be accounted for in the outer 2−4 mm of the fruit. No DPA could be traced in the cortex (flesh) of the fruit at a depth greater than 1 mm. Other investigators found that 14 days after treatment, the distribution of DPA was confined mainly to the first millimeter layer of the treated fruit, with about 57% residing in the cuticle wax layer, 37% in the epidermis, and 6% in the hypodermis (Hanekom et al., 1976). During cold storage of fruit at −0.5 °C, DPA was degraded rapidly after 10 weeks of storage with 40% of the initial concentration still found in the fruit (Hanekom et al., 1976). Residue levels further dissipated to 27% of the initial concentration after 25 weeks of cold storage. Due to its postharvest application, DPA is considered a food additive by U.S. Food and Drug Administration (FDA). The federal residue tolerance level for DPA in apples is 10 ppm. In addition to being used for direct consumption, apples are also processed to produce cider and juice. The resulting byproduct, apple pomace, is used as a feed supplement for dairy cattle. Terminal DPA residues were analyzed in apples and processed products including cider and wet and dry pomace following DPA treatment at maximum labeled use rates (Johnson et al., 1996). Apples to be processed were treated at 10 times the maximum labeled use rates to enhance the detectability of DPA in cider. Initial DPA residues in whole apples were <10 ppm and progressively declined to <4 ppm under commercial controlled atmosphere (CA) storage. Only traces of DPA occurred in cider, but high concentrations were detected in both wet and dry pomace. DPA residues in cider and pomace also dissipated with time in CA storage. These results were consistent with the findings in another study, where the residue level of DPA in apples ranged from 0.4 to 3.8 ppm (Gutenmann et al., 1990). Residues in pomace ranged from 1.4 to 15.6 ppm, whereas those in cider were either nondetectable or less than 0.1 ppm. The oral LD_{50} for DPA in rats was 3000 mg/kg, indicating that the trace concentrations of DPA in the cider are probably of negligible health significance. In addition, when DPA was included at 5 ppm in the feed of a dairy cow, excretion occurred in the feces but was not found in milk or urine. About 50% of the dosed DPA was degraded by beef liver microsomes within 30 min (Gutenmann et al., 1990). Chronic toxicity studies performed on both male and female albino rats and male and female beagle dogs demonstrated at least a 100-fold margin of safety even when diphenylamine residues in apple were as high as 10 ppm (Booth, 1963). Although behavior of intact DPA during cold storage has been investigated, no research on degradation product formation in stored apples has been conducted. The objective of this study was to evaluate the distribution and nature of total diphenylamine residues, including related degradation products, in stored apples.

MATERIALS AND METHODS

Chemicals, Materials, and Solvents. Diphenylamine (DPA), uniformly labeled with ^{14}C on both rings of the

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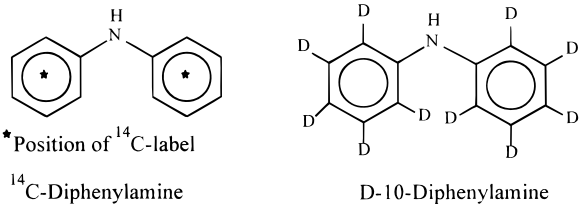
molecule, was used in this study. [^{14}C]DPA was obtained from Chemsyn Science Laboratories in Lexena, KS, and had a specific activity of 56.2 mCi/mmol (332.544 mCi/g) with a radiochemical purity of ≥98%. The dosing formulation was prepared by isotopically diluting [^{14}C]DPA with formulated Shield DPA 15% (EPA Registry No. 392-2, 15.43%, w/w; Shield Brite, Kirkland, WA). A 2.5% DPA emulsion was prepared by adding water. The specific activity of the isotopically diluted test compound was determined to be 4386 dpm/μg. The structure of the test substance is as follows:



^{14}C-Diphenylamine D-10-Diphenylamine

Another dosing formulation was prepared by isotopically diluting a sample of [^{14}C]DPA with a control formulation blank and deuterium-labeled DPA (D-10-DPA; >98% purity, Chemsyn Science Laboratories). A 2.5% aqueous D-10-DPA emulsion was prepared. The specific activity of the solution was determined to be 5414 dpm/μg. Originally it was intended to prepare a dosing solution containing [^{14}C]DPA with formulated Shield DPA 15% combined with D-10-DPA. The HPLC analysis of a mixture of [^{14}C]DPA and D-10-DPA, however, revealed that the retention times of the two components were significantly different, which made it difficult to isolate ^{14}C-labeled and deuterium-labeled metabolites in the same fraction. It was therefore decided to prepare two separate dosing formulations.

Unlabeled DPA, 3-OH-DPA, and indophenol were purchased from Aldrich Chemical Co., Inc. 4-Hydroxy-DPA, 2-OH-DPA, and 4,4′-di-OH-DPA were obtained from Ricerca, Inc. Scintillation cocktails were purchased from R. J. Harvey Instrument Corporation and Beckman Instruments, Inc. α-Amyloglucosidase, α-amylase, and pectinase (polygalacturonase) were purchased from Sigma Chemical Co. Novozym 249 was purchased from BioPacific Co. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was acquired from Supelco, Inc.

Test System. *Application of Test Chemical.* Red Delicious apples (*Malus punila*) averaging ~200 g in size were delivered from the Pennsylvania State University farm, Biglerville, PA. The apples were washed with running water, rubbed gently, dried, and treated with [^{14}C]DPA. Each apple was dipped into the 2.5% aqueous dosing solution for ca. 1−5 min at ambient temperature. Each treated apple was air-dried before being placed in storage. The storage cabinets (desiccator cabinets, Model H42058-0001, Bel-Art Products, NJ) were kept in a refrigerator at 0 ± 2 °C. Air within each cabinet was circulated and maintained at a relative humidity of 95 ± 5%. A total of 144 apples was dosed. Nine additional apples were dosed with an emulsion of deuterium-labeled DPA, which was fortified with [^{14}C]DPA at a nominal dose level of 50 ppm, and placed in the storage cabinet maintained at the same condition. The outgoing air flow from the storage cabinet was passed through a polymer trap prepared by packing ~3.2 g of Tenax-TA (35/60 mesh; Alltech, Deerfield, IL) into a plastic tube to trap any volatile components.

Sample Harvesting Schedule and Sample History. Eight apples were sampled at an interval of 3.5 h, 2 days, and 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, and 40 weeks posttreatment. At each storage interval, two apples were processed whole, while an additional six apples were peeled with a Dazey stripper (Model DVS-5) to generate peel and pulp samples. Each apple was washed with ~75 mL of methanol (MeOH) prior to processing. Pulp samples were first processed in liquid nitrogen and dry ice using a Hobart chopper to obtain a homogeneous mixture. Subsamples were further ground in a Tekmar analytical mill to obtain a more homogeneous sample prior to total radioactive residue (TRR) determination. Two whole apple and peel samples were frozen in liquid nitrogen and dry ice, and each sample was ground using the Tekmar analytical mill to yield a powder.

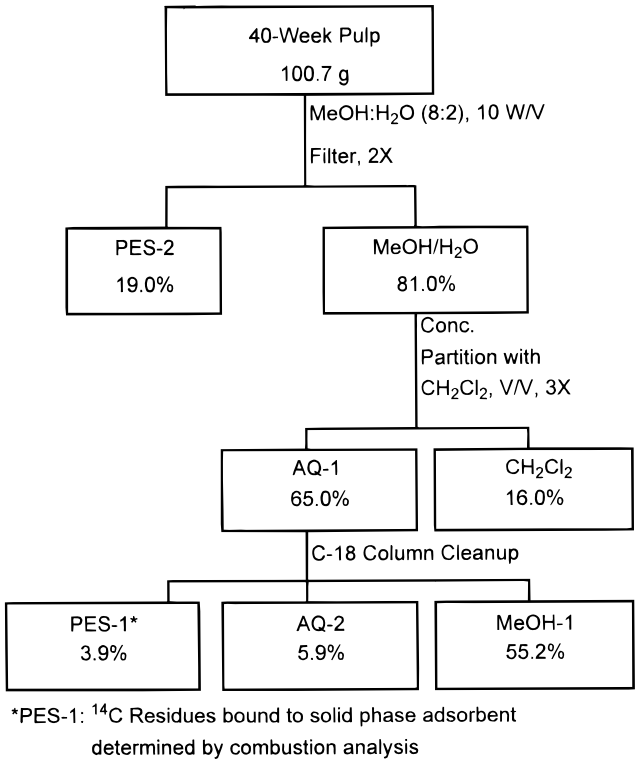


Figure 1. Extraction scheme for 40-week pulp.

Extraction and Fractionation of Pulp, Peel, and Whole Apple. Pulp, peel, and whole apple samples were initially extracted using a solvent mixture of methanol/water/chloroform (MeOH/H_2O/$CHCl_3$, 11:5:5). The $CHCl_3$ fraction was further partitioned with a mixture of acetonitrile (CH_3CN) and hexane (1:1, v/v). The percent distribution of TRR in each extracted fraction was obtained from each harvested sample. The extraction method was modified at the 16-week harvest interval. Each processed sample was extracted with a mixture of 10× MeOH/H_2O (8:2, v/v) and vacuum filtered. The procedure was repeated twice. The MeOH/H_2O extract was concentrated using a rotary evaporator to remove MeOH; extra care was taken to prevent loss of radioactive residue during the concentration. The remaining aqueous extract was partitioned three times with methylene chloride. Figure 1 illustrates the overall extraction and fractionation procedures used for the 40-week pulp samples. The aqueous fraction (AQ-1 in Figure 1) was purified using a C_{18} column (Baker-bond, J. T. Baker) prior to HPLC analysis. The aqueous fraction was loaded into the preconditioned C_{18} column, and the retained components were eluted with MeOH.

Hydrolyses of Postextraction Solids. *α-Amylase and α-Amyloglucosidase Hydrolyses.* The bound residue fraction from 40-week pulp or peel was mixed with phosphate buffer (pH ~6.5) and incubated at ambient temperature with α-amylase for 23 h. After incubation, the mixture was centrifuged to separate insoluble matter. The supernatant was stored in a refrigerator, and the remaining solids were suspended in acetate buffer (pH ~5.0) and incubated at ~55 °C with α-amyloglucosidase for about 64 h. The hydrolysis mixture was then centrifuged. The supernatant was combined with the initial supernatant obtained from the α-amylase hydrolysis, and the mixture was partitioned with EtOAc (1:1, v/v), three times, at three different pH levels. The EtOAc fractions obtained from partitioning at the three different pH levels were combined to yield a single EtOAc-1 fraction.

Novozym 249 Hydrolysis. The resulting PES fraction from 40-week pulp was mixed well with an acetate buffer (pH ~5.0). The sample was incubated with Novozym 249 at ~40 °C. After

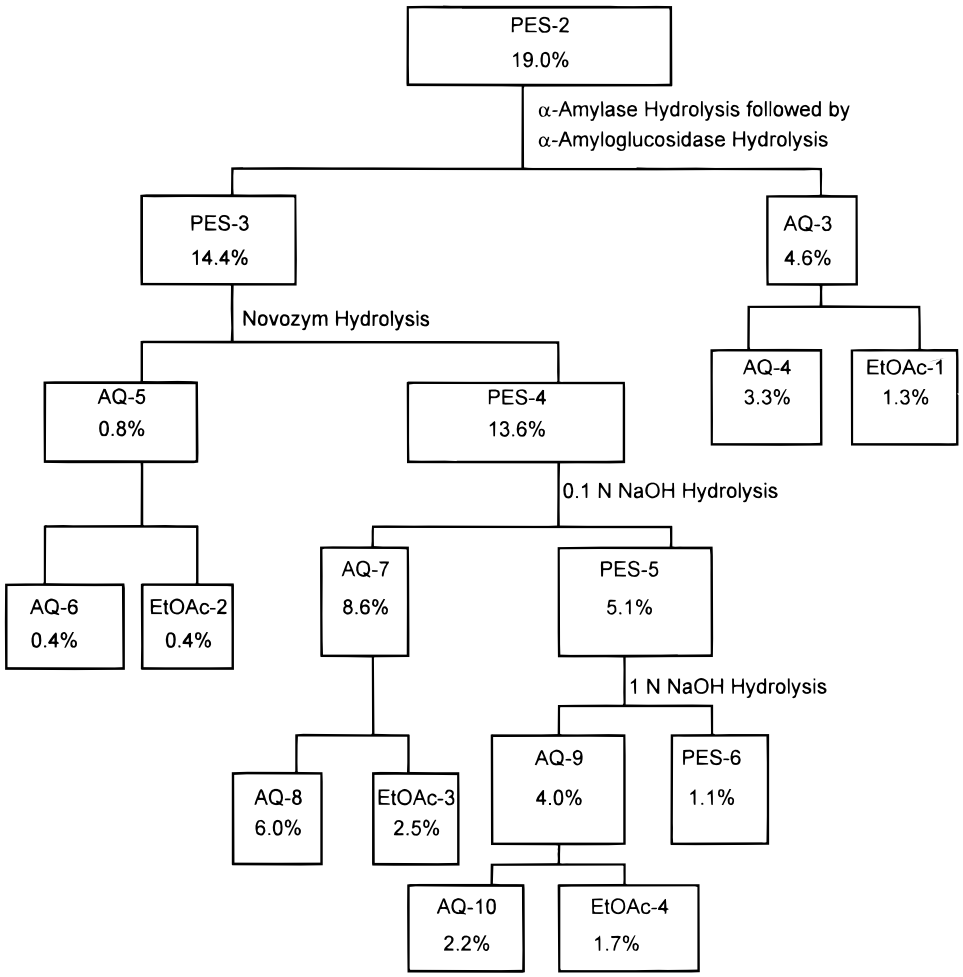


Figure 2. Fractionation of the bound residues in 40-week pulp.

incubation, the mixture was vacuum-filtered, and the filtrate was partitioned with EtOAc at three different pH levels to yield an EtOAc-soluble and an aqueous-soluble fraction.

Pectinase Hydrolysis. The resulting PES fraction from 40-week peel was mixed well with an acetate buffer (pH ~4.0) and incubated with pectinase at ~25 °C for 24 h. The hydrolysis mixture was centrifuged to yield an aqueous fraction and the PES-3 fraction. The supernatant was then partitioned with EtOAc (1:1, v/v) three times to yield an EtOAc-soluble fraction and an aqueous-soluble fraction.

Base Hydrolyses. The resulting PES fractions from 40-week pulp and peel were subjected to 0.1 N NaOH hydrolysis in a vacuum hydrolysis tube or in a three-necked, round-bottom flask under ambient conditions for 3 days. Each hydrolysis mixture was centrifuged to yield an aqueous fraction and insoluble solids. The aqueous hydrolysates were partitioned with EtOAc (1:1, v/v), three times at pH 4.5 and pH 1.5. The remaining PES fractions were placed into a three-necked, round-bottom flask, and ~20 mL of 1 N NaOH and porous boiling chips were added. One neck of the flask was attached to a nitrogen source, another to a water condenser, and the third to a vacuum pump. Air was purged from the system using a vacuum pump and was replaced with nitrogen for about 30 min. The sample was refluxed under nitrogen for approximately 24 h, cooled, and subsequently centrifuged to yield an aqueous fraction and insoluble solids. The supernatant was partitioned with EtOAc (1:1, v/v) three times at three different pH levels. The EtOAc fractions were combined to yield an EtOAc-soluble fraction. Figure 2 summarizes the extraction scheme for the PES fraction obtained from the 40-week pulp sample.

Total Radioactive Residue (TRR) Analysis. Levels of radioactivity in solid samples (i.e., whole apples, pulp, peels, and PES) were determined by combusting subsamples in a Harvey OX-300 or OX-500 biological sample oxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ). $^{14}CO_2$ was trapped in Harvey carbon-14 cocktail, and the samples were analyzed using a liquid scintillation counter (Beckman Instruments, Inc.). Statistical correction for instrument recovery was performed using [14C]mannitol as a standard. Levels of radioactivity in liquid samples were determined by counting in 5 or 10 mL of Ready Value cocktail (Beckman Instruments, Inc.).

High-Performance Liquid Chromatography. HPLC instrumentation consisted of a Waters Model 712 autosampler (Waters, Milford, MA), a Waters 484 tunable absorbance detector, a Waters 600E multisolvent delivery system, and a Raytest Ramona-5 flow-through radioactivity monitor with a glass cell. A reversed-phase Supelcosil LC-18 column, 4.6 × 250 mm, 5 μm, was used. HPLC conditions (condition I) were as follows: solvent A, H_2O; solvent B, acetonitrile, flow rate, 1 mL/min; start at 100% A and hold for 3 min; linear increase to 60% B in 20 min, then to 80% B in 10 min, to 100% B in 3 min, and hold for 5 min. Radioactive peaks were detected by radioactive detector or by collecting the HPLC eluent at 0.5-min intervals and analyzing the fractions by LSC. A slightly different gradient condition and mobile phase (condition II) was used to obtain better resolution between 4-hydroxydiphenylamine (4-OH-DPA) and 3-hydroxydiphenylamine (3-OH-DPA): solvent A, H_2O; solvent B, MeOH, flow rate, 1 mL/min; start at 60% A; linear increase to 90% B in 25 min, then to 100% B in 2 min, and hold for 3 min. Another modified gradient condition (condition III) was employed to isolate an unknown metabolite (metabolite L).

Thin-Layer Chromatography (TLC). Thin-layer chromatography (TLC) was used for purification and qualitative purposes. Normal-phase silica gel plates (J. T. Baker, silica gel GF) were used. TLC plates that were used for purification

were developed twice in one dimension to 12 and/or 15 cm. Radioactive bands were scraped off, and the radioactivity was eluted with MeOH. The MeOH eluate was further analyzed by either TLC or HPLC. The solvent systems used during the study were as follows: solvent system 1, hexane/ethyl acetate/ammonium hydroxide (hexane/EtOAc/NH_4OH), 50:50:2; solvent system 2, hexane/acetone/acetic acid (hexane/acetone/HOAc), 50:50:5; solvent system 3, ethyl acetate/acetic acid/water (EtOAc/HOAc/H_2O), 92:8:4; solvent system 4, 1-butanol/acetic acid/water (*n*-BuOH/HOAc/H_2O), 45:30:30. Radiochromatograms were obtained by scanning the TLC plates using either an AMBIS radioanalytical imaging system (Automated Microbiology Systems, Inc.) or FUJIX BAS 1000 bio-imaging analyzer system (Fuji Photo Film Co., Ltd.). TLC plates were also visualized by exposure under short wavelength ultraviolet (UV) light.

Gas Chromatography/Radioactivity Monitoring (GC/RAM). GC instrumentation consisted of a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and a ^{14}C detector (Raga). An Rtx-1 column (Restek Corp., 15 mL × 0.25 mm i.d., 0.25 mm thick film) was used. The conditions were as follows: injector temperature, 230 °C; flame ionization detector, 280 °C; carrier gas, helium, ∼1.5 mL/min flow rate; makeup gas, hydrogen at ∼20 cm^3/min; oven temperature, 50 °C initial, 1 min hold at initial, to 300 °C at a rate of 5 °C/min, final hold for 5 min; ^{14}C detector; 3200 HV, 750 °C; reactor, hydrogen at ∼20 cm^3/min; counting gas, methane at ∼30 cm^3/min; split ratio, ∼3:7 (FID/^{14}C). GC/RAM was used for qualitative confirmation. Samples were preassayed by GC/RAM prior to GC/mass spectrometry (MS) to locate radioactive peaks of interest. A trimethylsilyl (TMS) derivative of the major unknown metabolite (metabolite L) was analyzed by GC/RAM prior to GC/MS analysis. The TMS derivative of metabolite L, obtained from D-10-DPA treated pulp and peel, was also subjected to GC analyses. TMS-derivatized mixtures prepared with eluates at R_t ∼22−24 min and ∼24−26 min were also subjected to GC/RAM analyses. A radioactive peak eluting at R_t 31.5 min by HPLC analysis of a pulp extract was also analyzed by GC/RAM under a similar condition except for the following: an Rtx-1 column (15 mL × 0.32 mm i.d., 0.5 mm thick film) was used; carrier gas, helium, ∼3 mL/min flow rate; oven temperature, 100 °C initial, 1 min hold at initial, to 300 °C at a rate of 10 °C/min, and a final hold for 5 min was employed.

Gas Chromatography/Mass Spectrometry (GC/MS). GC instrumentation consisted of a Hewlett-Packard 5790A gas chromatograph. An Rtx-1 column (15 mL × 0.25 mm i.d., 0.25 mm thick film, Restek Corp.) was used. Conditions were as follows: injector temperature, 230 °C; flame ionization detector, 280 °C; carrier gas, helium, ∼1.5 mL/min flow rate; oven temperature, 50 °C initial, 1 min hold at initial, to 300 °C at a rate of 5 °C/min, final hold for 5 min. A VG 7070 EQ mass spectrometer (VG analytical, Ltd.) was used in the electron impact (EI) mode at a scan range of 50−500 or 80−800 *m*/*z*. GC/MS analysis was used for qualitative identification of metabolites. GC/MS analysis of the ∼31.5-min HPLC eluate from pulp extract was conducted under the same conditions as described above except that a different oven temperature was used as follows: 100 °C initial, 1 min hold at initial, to 300 °C at a rate of 10 °C/min, and a final hold for 5 min.

Fast Atom Bombardment/Mass Spectrometry (FAB/MS). Isolated intact metabolite L from [^{14}C]DPA treated pulp and D-10-DPA treated pulp was subjected to fast atom bombardment, positive ion mode/mass spectrometry (FAB[+]/MS). The sample was dissolved in a "magic bullet" matrix, which was composed of a 3:1 mixture of dithiothreitol and dithioerythritol in methanol. Data were acquired in the continuum mode, and the signals were averaged over the entire analysis. The conditions used for the analysis were as follows: instrument, VG Analytical ZAB-TMS (VG Analytical, Ltd.); scan range, 50−1000 *m*/*z*; scan time, 2 s/decade, accelerating voltage, 8000.0 V; probe temperature, ambient; power supply, 8 kV at 2 mA, magnet 1 control, current; magnet 2 control, field; source gas, xenon.

Analyses of 4-OH-DPA and Major Unknown Metabolites. *Isolation of 4-Hydroxydiphenylamine.* 4-Hydroxydiphenylamine was isolated from the CH_2Cl_2 fraction of a pulp extract by HPLC using condition I, and the eluates at R_t ∼31−32 min were concentrated and subjected to gas chromatographic analyses. HPLC eluates were isolated from the D-10-DPA treated pulp sample in a similar manner.

Isolation of Metabolite L. Metabolite L (HPLC R_t ∼26.5 min) was isolated from the CH_2Cl_2 fraction and the MeOH-1 fraction from pulp and peel samples. The CH_2Cl_2 fraction was purified by preparative TLC in hexane/acetone/HOAc (50:50:4). Three major bands were scraped from each plate, and the silica gel was eluted with MeOH. The MeOH-1 fraction was purified by preparative TLC in EtOAc/HOAc/H_2O (92:8:4). Six major bands were scraped from each plate and eluted with MeOH. The band eluates were further purified by HPLC using condition I.

Isolation of Other Unknown Metabolites. The eluates at R_t ∼20−30 min were collected every minute by preparative HPLC using condition I. The eluates at R_t ∼22−24 min (metabolites I and J), ∼24−25 min (metabolite J), and 24−26 min (metabolite K) were each concentrated to dryness and redissolved with CH_3CN prior to TMS derivatization.

TMS Derivatization of Polar Metabolites. A portion of the purified metabolite L in acetonitrile was placed in a reaction vessel, mixed with BSTFA, and sealed with a Teflon-lined cap. The reaction mixture was then heated at ∼85 °C for 1 h. After it was cooled, the reaction mixture was concentrated to ∼10 μL using an N-Evap high-speed analytical evaporator. A similar procedure was applied to prepare a TMS derivative of the isolated metabolite L from the D-10-DPA treated pulp. TMS derivative mixtures were prepared using BSTFA with other isolated polar unknown metabolites (HPLC eluates at R_t ∼22−24 min [metabolites I and J], ∼24−25 min [metabolite J], and ∼24−26 min [metabolite K]).

Enzyme Hydrolysis of Fractions Containing Polar Unknown Metabolites. A portion of unpurified metabolite L isolated from peel was mixed with 2 mg of α-amyloglucosidase in an acetate buffer (pH ∼5) and incubated at ∼55 °C for about 24 h. After incubation, the mixture was partitioned with EtOAc (v/v) three times to yield an EtOAc fraction and an aqueous (AQ) fraction. The EtOAc fraction was analyzed by TLC and HPLC. Other polar unknown metabolites were also subjected to enzyme hydrolyses using α-amyloglucosidase and/or α-amylase. HPLC eluates at R_t ∼23−24 (metabolite J), ∼24−25 (metabolite J), and ∼25−26 min (metabolite K) were each mixed with 20 mg of α-amylase in a phosphate buffer (pH ∼6.9) and incubated at ambient temperature for about 40 h. After incubation, each hydrolysis mixture was partitioned three times with CH_2Cl_2 (v/v), and each CH_2Cl_2 fraction was analyzed by 2-D TLC for aglycon characterization. HPLC eluates at R_t ∼20−21, ∼21−22, and ∼22−23 min were each mixed with 2 mg of α-amyloglucosidase in an acetate buffer (pH ∼5) and incubated at 55 °C for about 42 h. After incubation, each hydrolysis mixture was partitioned three times with CH_2Cl_2 (v/v). Each CH_2Cl_2 fraction was analyzed by 2-D TLC for characterization of each aglycon. The overall characterization flowchart for metabolite L is summarized in Figure 3.

RESULTS AND DISCUSSION

Total Radioactive Residues (TRR). During the 40-week storage period, 16 samplings were conducted on apples treated with the test substance isotopically diluted with [^{12}C]DPA. Two harvests, one at 154 days (22 weeks) and one at 266 days (38 weeks), were conducted on apples treated with the test substance isotopically diluted with D-10-DPA. The TRR levels in the MeOH washes and in each matrix (pulp, peel, and whole apple) were plotted against time (Figure 4). Although the radioactive residues were largely confined to peel, the radioactive residues in pulp gradually increased during storage. The recovered TRR in apples

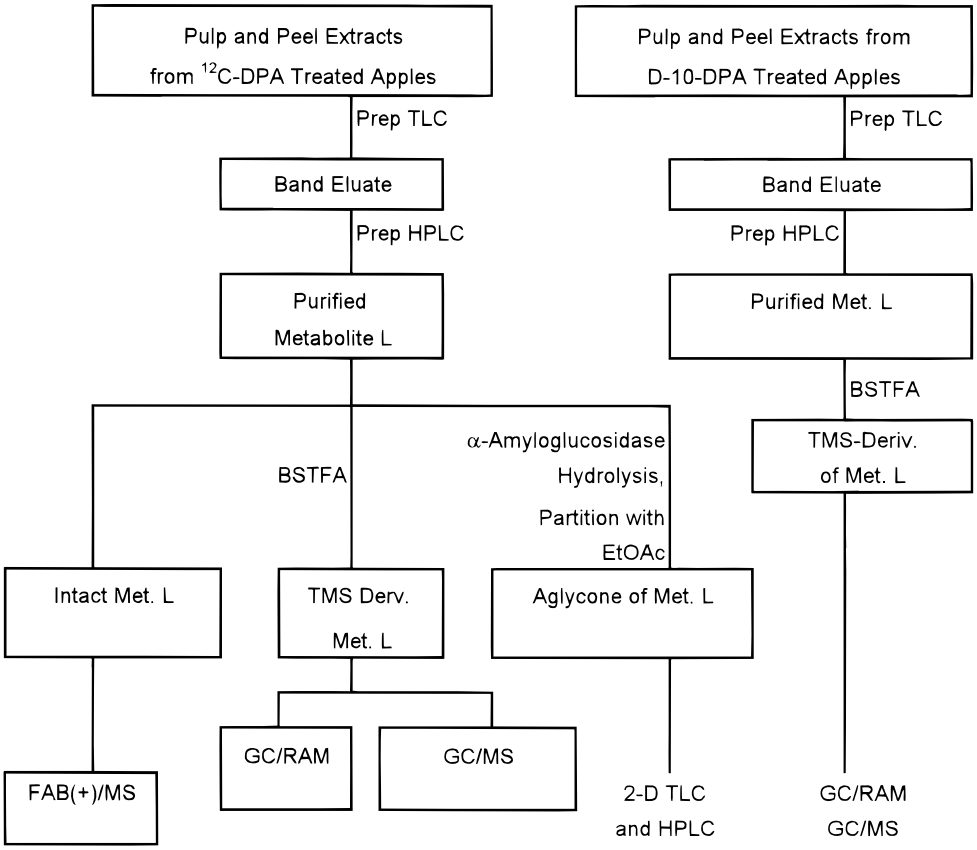


Figure 3. Metabolite L characterization summary.

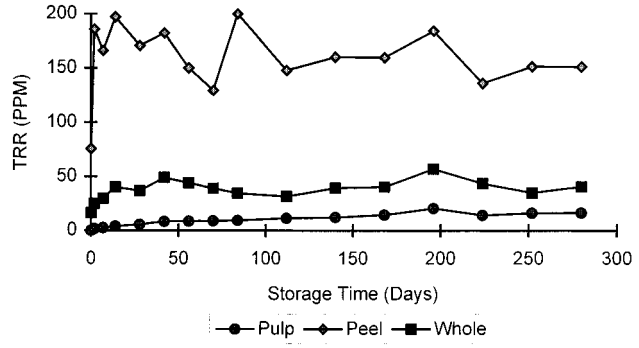


Figure 4. TRR levels recovered in MeOH washes and apples at various time intervals.

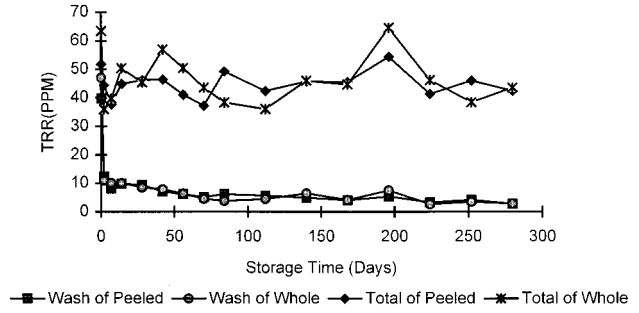


Figure 5. TRR values in each matrix at various time intervals.

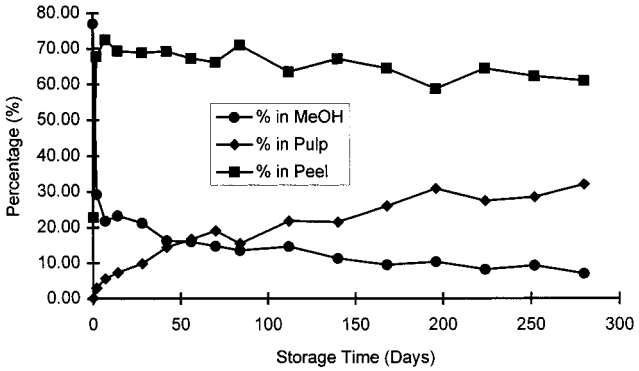


Figure 6. Schematic illustration of the percent TRR distribution in apple pulp, peels, and MeOH washes at various time intervals.

were constant throughout the storage period, ranging from 36 to 65 ppm, whereas washable residues gradually decreased to ~3% at the end of the storage period (Figure 5). The TRR data from pulp, peels, and MeOH washes expressed as percentages were plotted against time (Figure 6). Before storage and at each harvest, the weight of each apple was recorded to determine if there was any loss of apple weight during storage. During storage, apples showed a minimal loss of weight; the average weight loss of eight apples processed at each interval ranged from 0.27 to 8.05 g during the entire storage period. At the 12- and 40-week samplings, the Tenax-TA trap was disconnected from the storage setup and eluted with MeOH to measure the level of any volatile metabolites adsorbed onto the trap during storage. Results showed negligible amounts of radioactive residues in the trap eluates, indicating that insignificant amounts of radiocarbon were lost through volatilization under storage conditions.

Distribution of ^{14}C Residues in Treated Pulp, Peel, and Whole Apple Samples. At each harvest, the peel, pulp, and whole apple samples were individually processed and extracted. Figure 1 shows an example of extraction and fractionation of the terminal

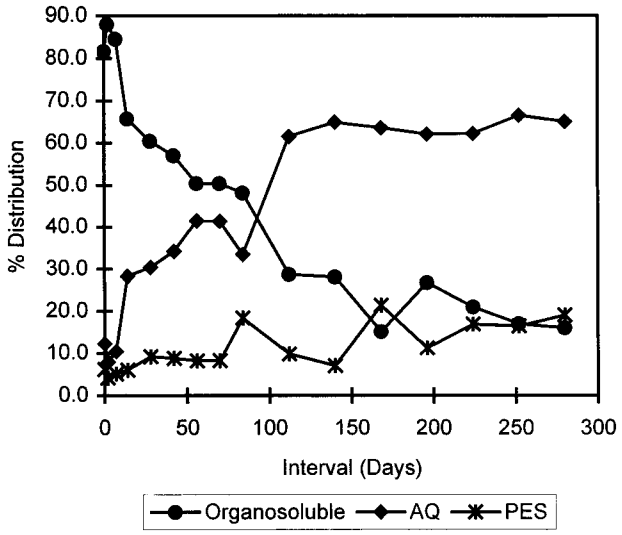


Figure 7. Percent distribution of TRR in each extracted fraction from pulp.

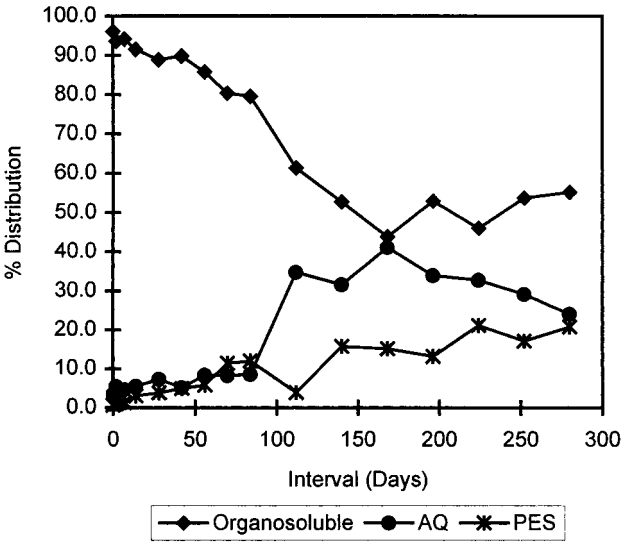


Figure 8. Percent distribution of TRR in each extracted fraction from peel.

40-week harvest pulp sample. Although in the earlier harvest, the majority of the TRR in pulp could be extracted into the organic fraction (above 75%), residues in the organic fraction significantly decreased to less than 60% after the 14-day harvest (Figure 7). The residues in the aqueous fraction, however, significantly increased from 8% (day 2) to 66% (40 weeks). The same pattern was observed in peel and whole apple samples, although the increases were not as great as those in pulp (Figures 8 and 9). The percent distribution of TRR as bound residues (PES) was low in all of the samples harvested, but this fraction also showed an increase in TRR from 4% (day 2) to 19% (40 weeks) in pulp PES from 0% (day 2) to 17% (40-week) in whole apple PES and from 1% (day 2) to 21% (40-week) in peel PES. The result indicated the formation of more polar products in apples during the storage.

Chromatographic Analyses of ^{14}C Residues in MeOH Washes, Pulp, and Peel. *MeOH Wash.* The MeOH washes from each harvest were analyzed either by normal-phase 2-D TLC or reversed-phase HPLC. Referenced standards were cochromatographed when applicable. The retention times (R_t) of the reference standards under HPLC condition I were as follows:

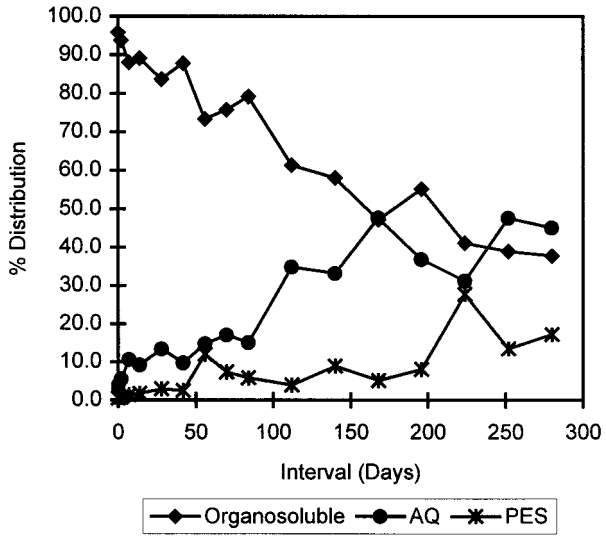


Figure 9. Percent distribution of TRR in each extracted fraction from whole apple.

DPA (37.32 min), 2-OH-DPA (34.15 min), 4-OH-DPA (31.48 min), 3-OH-DPA (31.82 min), 4,4′-dihydroxy-DPA (26.23 min), and indophenol (25.77 min). Diphenylamine (DPA), the parent compound, was the major product identified (87% in 40-week harvest sample), indicating that the majority of the apple surface residues were in the form of unchanged DPA. Four other metabolites were identified in the MeOH washes: 4-hydroxydiphenylamine (4-OH-DPA, 7%), 2-hydroxydiphenylamine (2-OH-DPA, 2%), a glycosyl conjugate of 3-OH-DPA (metabolite K, 2%), and *O*-glucose conjugate of 4-OH-DPA (metabolite J, 1%). Characterization of these conjugates will be discussed in the later section.

Pulp. Seven products found in the CH_2Cl_2 fraction from the pulp extraction of the 40-week harvest sample were DPA (11%), 4-OH-DPA (1%), metabolite J (<1%), metabolite K (~1%), metabolite L (1%), and metabolite M (1%) based on HPLC analysis (Figure 10). Diphenylamine was confirmed by 1-D TLC in comparison to the reference standard. 4-OH-DPA was confirmed by GC/MS. The mass spectrum of the same metabolite isolated from D-10-DPA treated pulp and peel showed the molecular ion of 9 atomic mass units higher than that isolated from [^{12}C]DPA treated apples. This confirmed that the compound is an aromatic ring-hydroxylated diphenylamine. Four unknown metabolites were designated as metabolites J−M, based on their HPLC retention times. The procedure for characterizing these metabolites will be discussed later. The AQ-1 fraction of the pulp from the 40-week harvest was applied on a C_{18} cartridge previously conditioned with MeOH and water. The retained components were eluted with MeOH. A majority of the TRR (85%) was found in the MeOH eluate (MeOH-1 fraction). Four metabolites detected in the MeOH-1 fraction from the 40-week pulp extraction were metabolites H (13%), I (12%), J (12%), and L (18%) based on the HPLC retention times. The metabolic profile of the AQ-2 fraction was also obtained by HPLC; six metabolites detected were ~1% each of metabolites B−F and I based on the HPLC R_t of each compound.

The PES fraction from the 40-week pulp sample was subjected to a series of hydrolyses including α-amylase, α-amyloglucosidase, Novozym 249 hydrolysis, mild base hydrolysis (0.1 N NaOH), and strong base hydrolysis

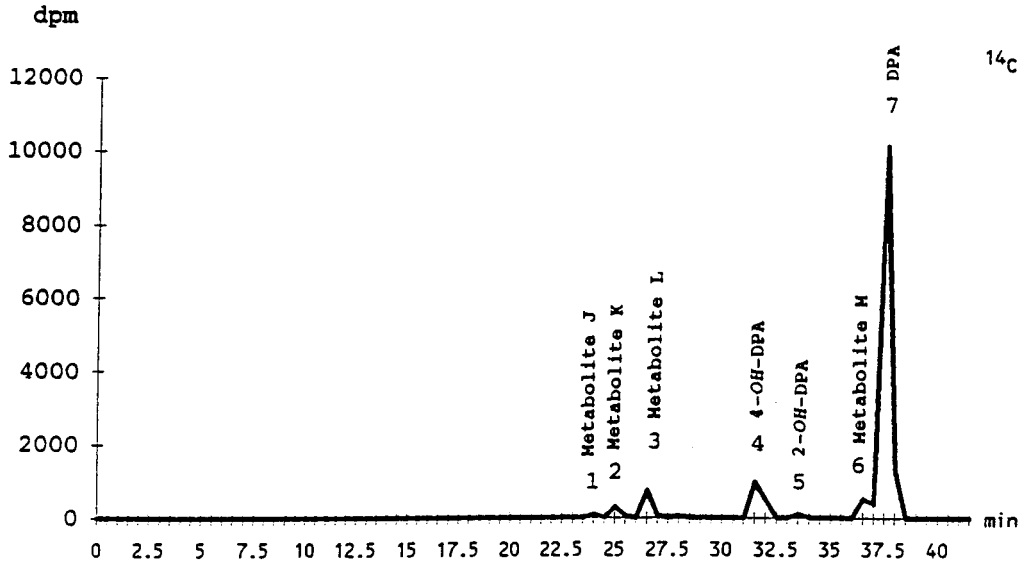


Figure 10. Reconstructed HPLC chromatogram of the CH_2Cl_2 fraction of 40-week pulp.

Table 1. Sumary of Metabolite Distribution in DPA-Treated Apples

	distribution within each separated fraction			distribution expressed as percentages of peeled whole apples			
metabolite ID	pulp (%)	peel (%)	MeOH wash (%)	pulp (%)	peel (%)	MeOH wash (%)	total (%)
Phase I Metabolites							
diphenylamine (DPA)	12.3	50.5	87.2	3.9	30.8	6.1	40.8
2-hydroxydiphenylamine (2-OH-DPA)	0.5	1.6	1.7	0.2	1.0	0.1	1.3
3-hydroxydiphenylamine (3-OH-DPA)	1.0	ND[a]	ND	0.3	ND	ND	0.3
4-hydroxydiphenylamine (4-OH-DPA)	3.5	7.2	7.7	1.1	4.4	0.5	6.0
Phase II Metabolites							
glucose conjugate of 4-OH-diphenylamine (metabolite L)	20.7	12.7	1.3	6.6	7.8	0.1	14.5
oligosaccharide conjugate of dihydroxyDPA (metabolite H)	14.1	1.0	ND	4.5	0.6	ND	5.1
oligosaccharide conjugate of 2-OH-diphenylamine (metabolite I)	15.6	0.4	ND	5.0	0.2	ND	5.2
oligosaccharide conjugate of 4-OH-diphenylamine (metabolite J)	14.4	8.6	ND	4.6	5.3	ND	9.9
oligosaccharide conjugate of 3-OH-diphenylamine (metabolite K)	4.0	1.6	2.0	1.3	1.0	0.1	2.4
Unknowns							
polar unidentified (AQ-1)	3.9	ND	ND	1.2	ND	ND	1.2
polar unknowns	8.2	13.0	ND	2.6	8.0	ND	10.6
nonpolar unknowns	0.7	0.5	ND	0.2	0.3	ND	0.5
bound residues	1.1	2.9	ND	0.4	1.7	ND	2.1
total	100.0	100.0	100.0	32.0	61.0	7.0	100.0

[a] ND, not detected.

(1 N NaOH). (See Figure 2.) Each hydrolysis yielded 4-OH-DPA, 3-OH-DPA, DPA, and many other polar metabolites in each hydrolysate. The overall metabolite distribution in the pulp sample from the 40-week harvest is summarized in Table 1.

Peel. Four metabolites identified in the CH_2Cl_2 fraction from the peel extraction of the 40-week sample were as follows: DPA (49%), 4-OH-DPA (3%), 2-OH-DPA (2%), and metabolite L (2%) based on their HPLC retention times as compared to available reference standards. DPA was confirmed by 1-D TLC. 4-Hydroxydiphenylamine was confirmed by cochromatography with reference standards using a different gradient condition (HPLC condition II). The AQ-1 fraction of the pulp from the 40-week harvest was purified using C_{18} column chromatography. A majority of the TRR (81%) was found in the MeOH eluate (MeOH-1 fraction). The remaining radioactive residue was found in the AQ-2 fraction (5% of TRR in peel). Three metabolites were detected in the MeOH-1 fraction from the 40-week peel extraction: 4-OH-DPA (3%), metabolite J (6%), and metabolite L (10%) based on their HPLC retention times. The metabolic profile of the AQ-2 fraction was also obtained by HPLC; seven metabolites identified were ~1% each of metabolites A, D, E, F, G, H, and I based on the R_t of each compound.

The bound residue fraction (PES) from the 40-week peel was subjected to a series of hydrolyses including α-amylase, α-amyloglucosidase, pectinase hydrolysis, mild base hydrolysis (0.1 N NaOH), and strong base hydrolysis (1 N NaOH). Each hydrolysis yielded 4-OH-DPA, 2-OH-DPA, DPA, and many other polar metabolites in the hydrolysate. The overall metabolite distribution in the peel sample from the 40-week sampling is summarized in Table 1.

Isolation and Characterization of Unknown Metabolites. *Metabolite L (Glucoside Conjugate of 4-Hydroxy-DPA).* Metabolite L was isolated and purified by preparative TLC and HPLC. Isolated metabolite L was converted to a TMS derivative using BSTFA. The TMS derivative was subjected to GC by both GC/FID/RAM and GC/MS (Figures 11 and 12). The GC chromatogram of the TMS derivative by FID is almost identical with the mass ion chromatogram of the same

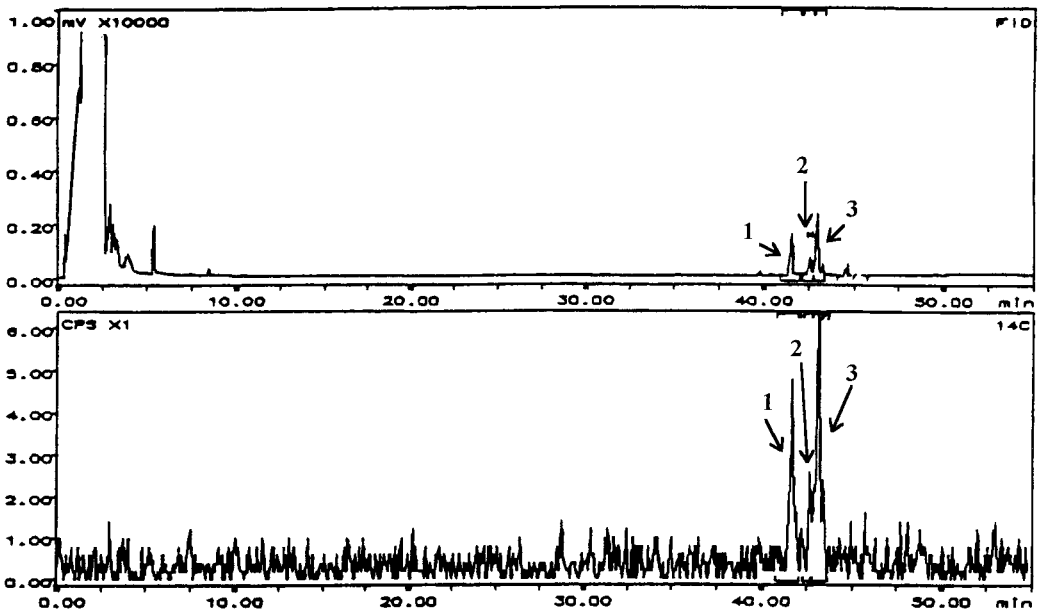


Figure 11. GC radiochromatogram of the TMS derivative of metabolite L.

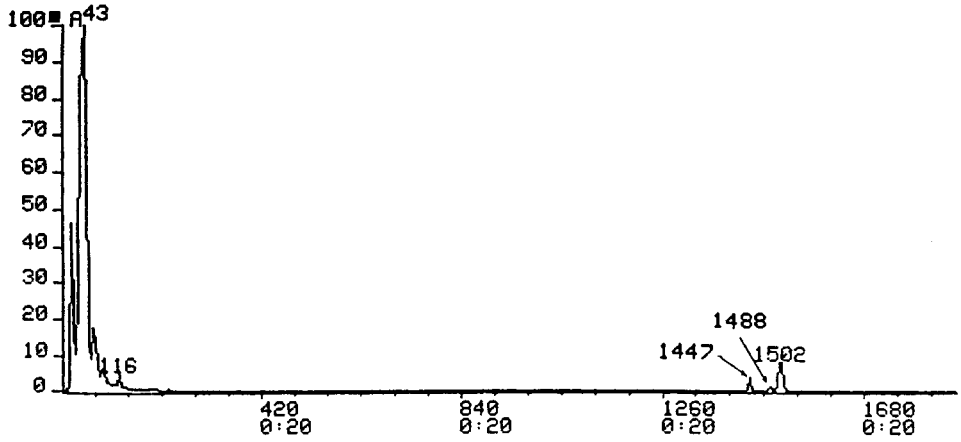


Figure 12. Mass ion chromatogram of the TMS derivative of metabolite L.

sample. Three major radioactive peaks were seen in the radioactive trace. Mass spectra from scan 1447, 1448, and 1502, which corresponded to the GC radioactive region of interest, were almost identical with each other and were identified as a glycosyl (i.e., glucose) conjugate of hydroxy-DPA. They could be TMS derivatives of a glycosyl conjugate of OH-DPA with different degrees of silylation. Figure 13 contains the mass spectrum of scan 1502. The spectrum showed the base peak at *m/z* 185, which indicated the possibility of hydroxylated DPA (OH-DPA). The remaining mass fragmentation pattern was similar to that of TMS derivatives of sugars. The TMS-derivatized molecular ion was observed at *m/z* 635. (The *N*-TMS-derivatized molecular ion, *m/z* = 707, may be unstable.) The mass ions at *m/z* 450 and 361 were derived from a tetra-TMS-monosaccharide moiety. The mass ion at *m/z* 531 represents the loss of TMS−OCH_3 from molecular ion 635. The mass fragmentation of TMS-derivatized metabolite L is illustrated in Figure 14.

The same metabolite isolated from D-10-DPA treated apples was also subjected to TMS derivatization, followed by GC/FID/RAM and GC/MS analyses. The GC profiles obtained by GC/FID/RAM and GC/MS were similar to those obtained from [^{12}C]DPA treated samples. The mass spectrum of two major radioactive peaks showed the same mass fragmentation pattern as those from [^{12}C]DPA treated samples with a molecular ion at an increment of 9 mass units (*m/z* at 644), indicating that hydroxylation and conjugation were in the aromatic ring. The spectrum is shown in the bottom of Figure 13.

Metabolite L isolated from [^{12}C]DPA samples was subjected to FAB(+)/MS; the mass spectrum, which showed a distinctive $(M + H)^+$ ion at *m/z* 348, confirmed that the compound is a monosaccharide glycosyl conjugate of OH-DPA (addition of one sugar = 162).

Metabolite L was also isolated from the MeOH-1 fraction of the peel (40-week harvest) and subjected to α-amyloglucosidase hydrolysis. The hydrolyzed radioactivity was extracted into the EtOAc fraction. Cochromatography of the EtOAc fraction with 4-OH-DPA by both HPLC and 2-D TLC confirmed the aglycon of metabolite L as 4-OH-DPA. In addition, because the fraction was hydrolyzed by an enzyme that is specific for hydrolysis of α-1,4 and α-1,6 linkages between glucose units in polysaccharides, the metabolite was therefore confirmed as a glucose conjugate of 4-OH-DPA.

Polar Metabolites H−K. Several polar metabolites in addition to metabolite L were isolated from apple pulp by preparative HPLC. GC/FID/RAM chromatography

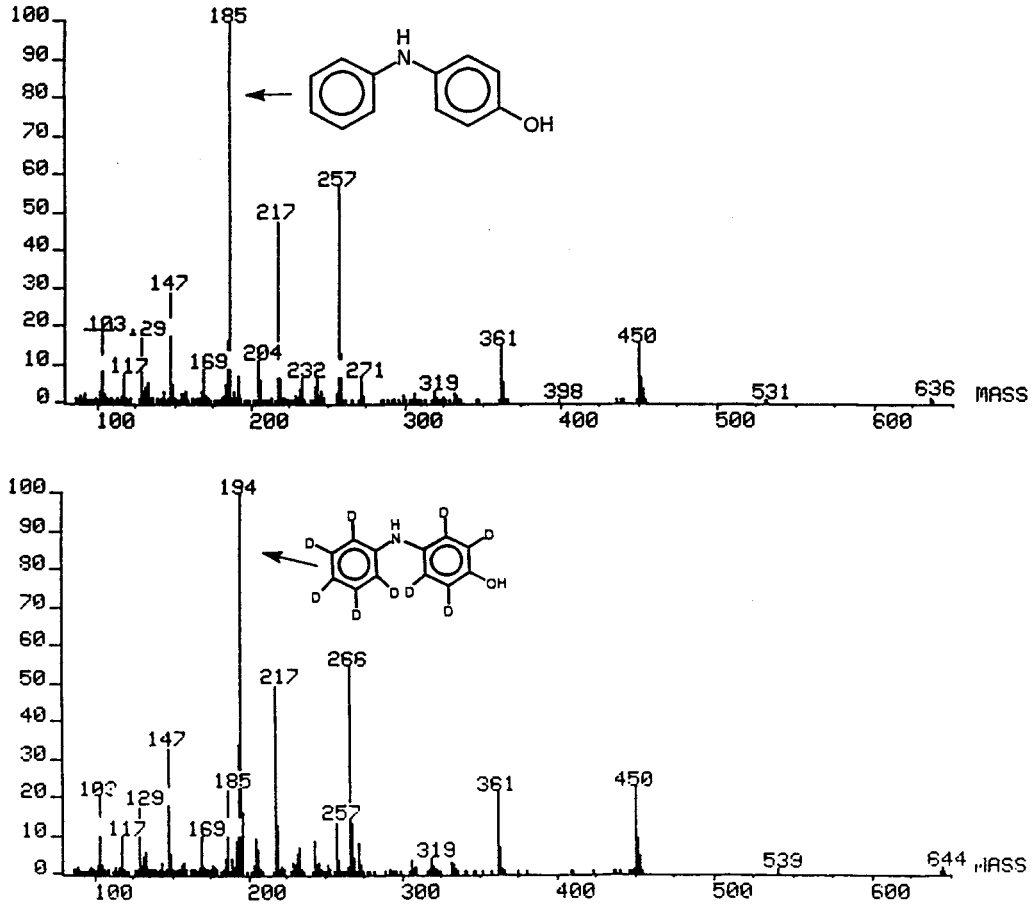


Figure 13. Mass spectrum of the TMS derivative of metabolite L.

of a TMS derivatized mixture of metabolites I and J (HPLC R_t ~22−24 min) revealed a radioactive peak at R_t ~24.63 min, which was identified by GC/MS as a TMS ether of hydroxy-DPA ($M^+ = 257$). The presence of this type of product was inconsistent with the chromatographic behavior of nonconjugated hydroxylated DPA metabolites, which were generally observed at a later retention time (greater than 31 min) under the HPLC conditions employed. Its presence was probably due to an in situ breakdown during isolation or derivatization of the conjugate metabolites. TMS-derivatized mixtures of other isolated polar metabolite fractions failed to provide any meaningful mass spectrometry results.

To better characterize and identify the unknown metabolites, each isolated polar fraction was subjected to enzyme hydrolysis using either α-amylase or α-amyloglucosidase. The hydrolysate from each fraction was partitioned with CH_2Cl_2 and analyzed by 2-D TLC. Figure 15 shows a representative TLC chromatogram obtained from the α-amyloglucosidase hydrolysate of an HPLC-isolated fraction at R_t ~20−21 min (metabolite H). The major aglycon in the fraction was an unknown with a TLC R_f value close (although not identical) to those of 4,4′-dihydroxy-DPA and indophenol reference standards, suggesting that it could be positional isomer of 4,4′-dihydroxy-DPA. The structure of metabolite H was proposed as a glycosyl conjugate of 2,4′-dihydroxy-DPA based on the observation that the second most prevalent position of aromatic hydroxylation in the apple was at the 2-position. TLC analysis of the hydrolysate of the R_t ~21−22-min HPLC eluate revealed a mixture of two polar compounds; one was close to the TLC origin, and the other was shown to be the aglycon of metabolite H. The polar compound close to the origin could have been a reaction byproduct of the dihydroxy-DPA formed during sample workup. The hydrolysate of another fraction (HPLC R_t ~22−23 min, metabolite I) contained 2-OH-DPA as the major aglycon, along with the same two polar unknown compounds detected in prior fractions. The hydrolysate of another fraction (R_t ~23−24 min, metabolite J) contained 4-OH-DPA as the major aglycon and 3-OH-DPA as the minor aglycon by TLC chromatographic comparison with appropriate reference standards. The hydrolysate of another HPLC fraction (HPLC R_t ~24−26 min), designated as metabolite K, showed a similar profile to that of the hydrolysate of metabolite J, whose major aglycon was 4-OH-DPA. The fractions also contained 3-OH-DPA as a minor component. On the basis of the above results, metabolite H was presumed to be a glycosyl conjugate of a dihydroxylated-DPA. Metabolite I was considered to be a glycosyl conjugate of 2-OH-DPA. Metabolite J was proposed to be a glycosyl conjugate of 4-OH-DPA. Metabolite K was proposed to be a glycosyl conjugate of 3-OH-DPA. The sugar portions of these metabolite conjugates could have been either monosaccharide and/or oligosaccharides.

Summary of DPA Metabolite Distribution in Pulp and Peel. Apple pulp and peel contained a variety of identifiable oxidative (i.e., hydroxylated) degradates that reflected the lability of the parent chemical and verified its utility as an antioxidant and preservative. Intact diphenylamine remained the primary contributor (41% in Table 1) to the apple total terminal residue after 40 weeks of cold storage. The

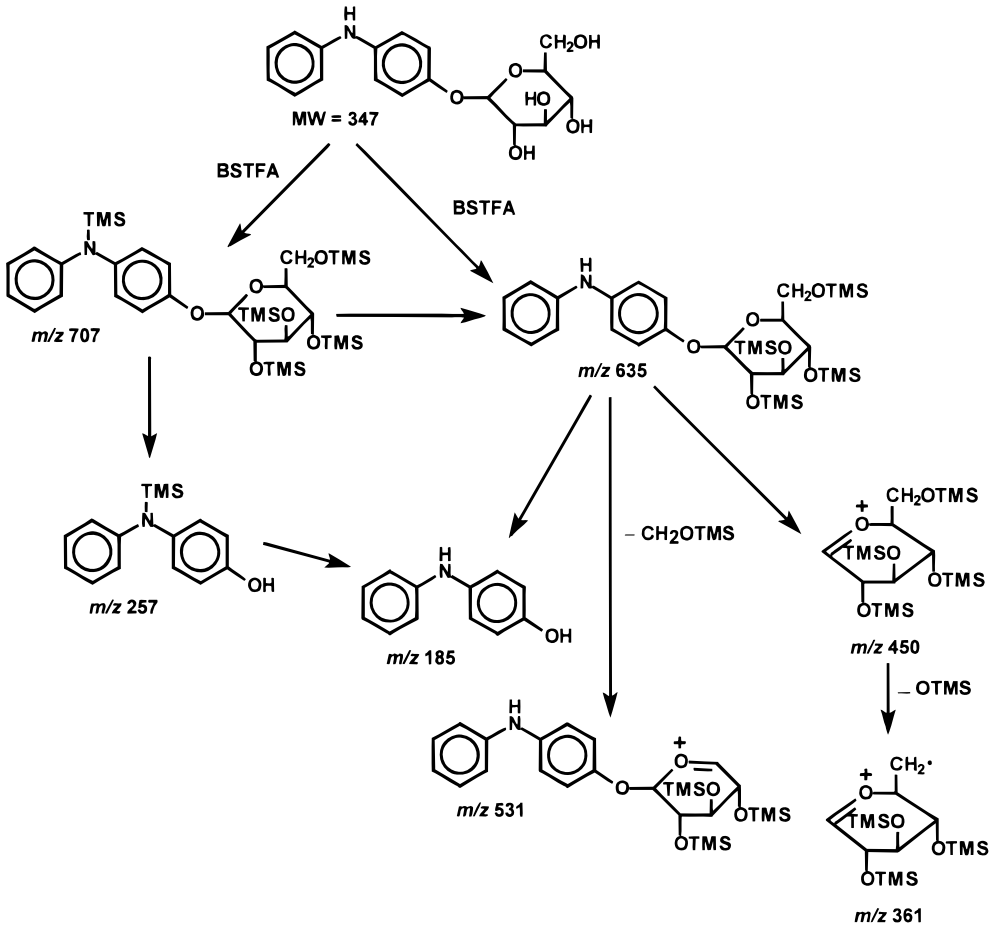


Figure 14. Mass fragmentation pattern of the TMS derivative of metabolite L.

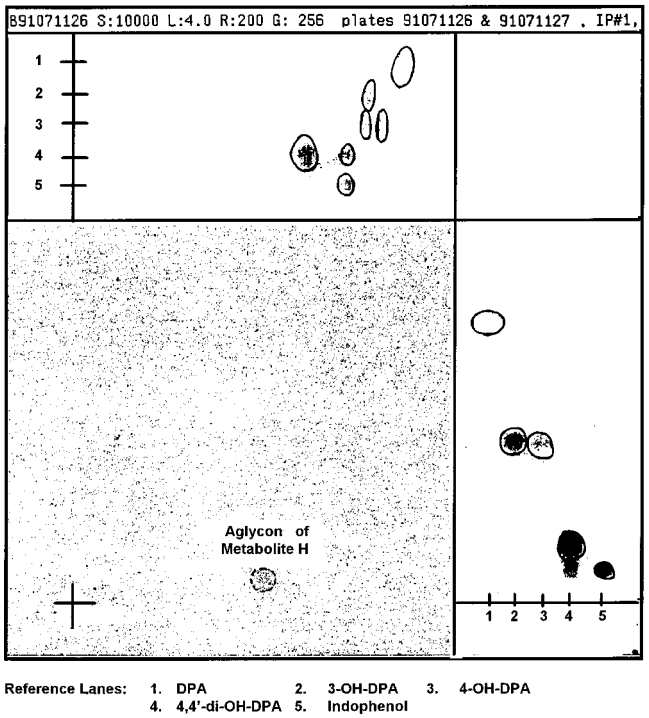


Figure 15. TLC radiochromatogram of the α-amyloglucosidase hydrolysate of metabolite H.

major metabolite or degradate was confirmed to be a monohydroxylated product, 4-OH-DPA. This degradate was present in its free form and as a variety of monomeric and oligomeric glycosyl conjugates. An additional monohydroxylated metabolite, namely, 2-OH-DPA, was also detected in a nonconjugated form and in a glycosidic conjugate form. 4-Hydroxydiphenylamine and a dihydroxylated diphenylamine metabolite, thought to be a positional isomer of 4,4′-dihydroxydiphenylamine (proposed as 2,4′-dihydroxydiphenylamine), were also present as mono- or oligosaccharide conjugates. Finally, other minor metabolites and/or degradates were formed, either via plant degradation or in situ during the extraction, isolation, and analytical processes. Given their net contribution to the total apple residue, parent chemical and 4-hydroxydiphenylamine were the principal "marker residues". The percent distributions of the aforementioned metabolites in MeOH washes, peels, and pulp are summarized in Table 1. The percent distributions of metabolites detected in pulp, peel, and MeOH wash expressed as percentages of peeled whole apples are also summarized in Table 1. As presented in Results and Discussion, pulp, peel, and MeOH wash comprised 32%, 61%, and 7% of the TRR recovered from the peeled whole apples. Diphenylamine, hydroxydiphenylamine, and conjugates of hydroxydiphenylamine-related metabolites accounted for 86% of the TRR in the peeled whole apples.

Proposed Metabolic Pathways. The proposed metabolic pathways of diphenylamine (DPA) in stored apples are illustrated in Figure 16. Three potential pathways could be summarized from this investigation. The first major pathway was proposed as ring hydroxylation at three different positions, namely, 2, 3, and 4, and subsequent conjugation with glucose, oligosaccharides, or other biomolecules. The second major pathway

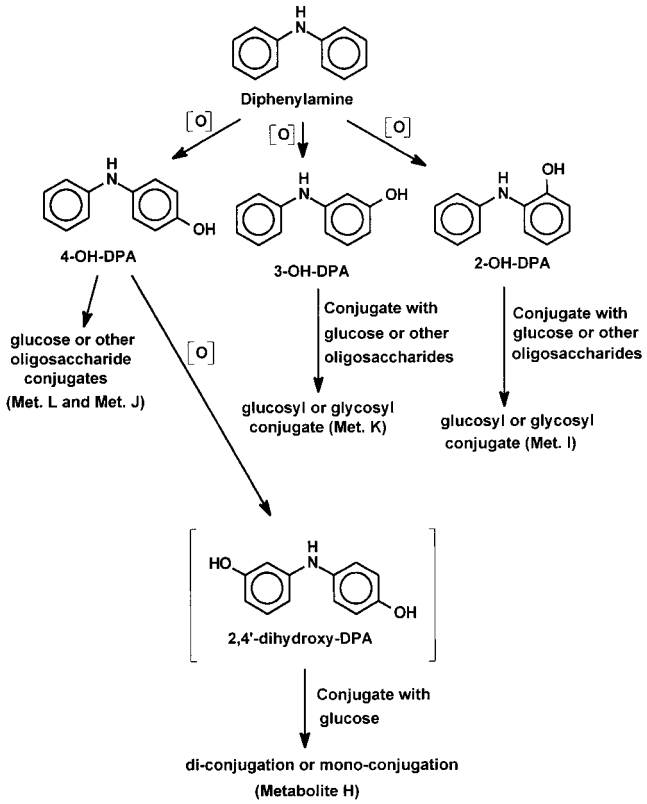


Figure 16. Proposed metabolic pathways of diphenylamine in stored apples.

was proposed as dihydroxylation at another ring of the monohydroxydiphenylamine. Finally, the third major pathway was proposed as further conjugation of dihydroxydiphenylamine with glucose at one or both of the hydroxyl groups and then further conjugation with biomolecules.

CONCLUSION

Following its application to apples, diphenylamine exhibits the ability not only to migrate from the site of treatment (i.e., surface of the fruit) but also to transform readily under cold storage conditions. The parent chemical is initially converted, presumably by both biological and abiotic processes, to a number of phase I (free, nonconjugated) hydroxylated products, including 2-hydroxydiphenylamine, 3-hydroxydiphenylamine, 4-hydroxydiphenylamine, and a dihydroxylated degradate. The phase I hydroxylated degradates or metabolites then undergo a phase II metabolic process (i.e., conjugation), which results in the formation of glucosidyl or glycosidyl conjugates. The conjugation process is extended to a third level, which is manifested by the formation of hydroxydiphenhylamine-related carbohydrate biopolymers that are incorporated into compartments of the cell wall.

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