Detection of Diphenylamine on Surfaces of Nontreated Apples (*Malus domestica* Borkh.)

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Anecdotal reports indicate that diphenylamine (DPA) may be endogenous in apples. To test this hypothesis, hexane extracts of apples were analyzed for residues of DPA by GC/MS. DPA was detected in samples of five cultivars that had been stored for 7−8 months at 0 °C. DPA concentration was approximately 0.1 μg g^{-1} (fresh weight). These fruit had not been treated with DPA before storage and were not stored with DPA-treated fruit. Detection of DPA on the walls of the storage rooms indicated possible contamination by airborne residues. DPA was also detected in freshly harvested commercially grown apples at three stages of development, in samples of organically grown apples, and in a sample of freshly harvested pears. All had detectable DPA residues at approximately 10% of the concentration detected in stored fruit. Analyses of the extracts using a HFBA derivatization procedure indicated detection of DPA in the presence of a coeluting interference. Therefore, whether or not DPA is endogenous to apples and pears remains uncertain. If so, concentrations can be presumed to be <0.01 μg g^{-1} of fresh weight of fruit. Results have significant implications when DPA residue measurements are interpreted.

Keywords: *Diphenylamine; apples; Malus domestica; superficial scald*

INTRODUCTION

Diphenylamine (DPA) is an antioxidant widely used to control development of the disorder "superficial scald" on apples during and following long-term storage at about 0 °C. Its effectiveness, first reported by Smock (1955), proved so reliable that it rapidly was adopted as a standard commercial control for this disorder in many countries (Ingle and D'Souza, 1989). It usually is applied as a dip or drench at 100−2000 mg kg^{-1} in water immediately before placement in storage. Residues decline rapidly on fruit stored at 0−3 °C. Huelin (1968) reported more than 50% reduction in 10 weeks and about 95% reduction in 30 weeks. It was concluded that volatilization accounted for only a small portion of this loss. DPA residue is confined predominantly to the surface 2−4 mm of fruit (Harvey and Clark, 1959), and 60% of it is located in the waxy cuticle (Huelin, 1968).

Due to its postharvest application, DPA is considered a food additive by the U.S. Food and Drug Administration (FDA). Its present tolerance level is 10 μg g^{-1} of fresh weight of fruit (Kupferman and Waelte, 1992). However, some countries do not permit sale of fruit with any detectable DPA residue, and within countries that allow DPA use, some markets will not accept fruit with detectable residue. As detection procedures have improved this has become a contentious issue.

Numerous anecdotal reports indicate that fruit with no record of DPA treatment often have detectable DPA residues, though at concentrations well below the FDA tolerance level. This has led to the conclusion that DPA is a natural product and is endogenous in apples. Karawya et al. (1984) reported that DPA was present in concentrations as high as 1% in Egyptian onions and green teas. They argued that DPA was a natural product and might be responsible for the putative anithyperglycemic activity of extracts prepared from these plant materials. There are no published reports of DPA as a natural product in apples. However, based on anecdotal information, a report from the Food and Agricultural Organization of the United Nations (FAO, 1984) states, "There is reasonable evidence that diphenylamine occurs naturally in apples although the level appears to be at or below 1 mg kg^{-1}."

We have conducted analyses of freshly harvested and stored apples by GC/MS to examine the questions of whether or not DPA is present in nontreated fruit and, if so, whether it is naturally occurring or is a result of contamination.

MATERIALS AND METHODS

Reagents. HPLC grade hexane was obtained from Fisher Scientific (Medford, MA); heptafluorobutyric anhydride (HFBA) and authentic DPA were from Aldrich Chemical Co. (Milwaukee, WI).

Fruit Samples. In April 1993, single 10-fruit samples of Red Delicious, Golden Delicious, McIntosh, Empire, and Cortland apples were taken at random from boxes of apples that had been stored at 0 °C for about 7 months. Fruit had been grown and stored at the University of Massachusetts Horticultural Research Center (HRC) under commercial conditions, and none had been treated with DPA. In August 1994, single 10-fruit samples were taken from McIntosh, Cortland, Empire, Red Delicious, and Golden Delicious apple trees growing under commercial conditions at the HRC. A Rhode Island Greening tree growing organically in a private yard in Leverett, MA, was also sampled. All of the August-harvest fruit were immature at the time of sampling. At the time of commercial harvest for each cultivar (September or October), a second 10-fruit sample was taken from each of these trees. In addition, a tree of Anjou pears was sampled, and the tree of McIntosh apples was sampled again when fruit were overly mature. In May 1994, a single 10-fruit sample was taken from each of two boxes of Red Delicious apples that had been grown and stored at the HRC. Both boxes of fruit came from the same

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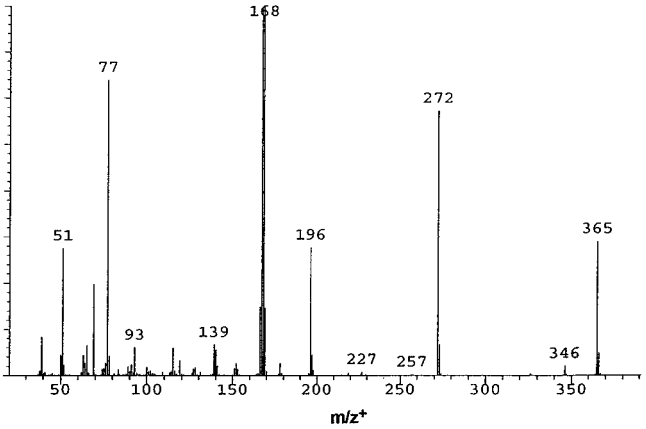

Figure 1. Electron impact (70 eV) mass spectrum of heptafluorobutyric anhydride derivative of diphenylamine.

tree. One box had been dipped in 2 g kg^{-1} DPA water for 20 s before storage. The second box was not with treated with DPA.

Extraction of DPA Residues. Shortly after sample collection, fruit were weighed and then individually and in sequence dipped and periodically rotated in 100 mL of hexane for 3 min. The small volume of hexane that evaporated was replaced and the extract stored at −10 °C until GC/MS analysis. A blank sample of hexane was carried through with each analysis.

Storage Room Surface Samples. In May 1993, two 0.1 m^2 areas in each of three cold storage rooms at the HRC were wiped vigorously with cotton balls held with solvent-rinsed stainless steel tweezers. The cotton balls had been pre-extracted with hexane and air-dried. DPA residues were extracted from surface wipe samples by immersing a cotton ball in 10 mL of hexane for 30 min, after which the hexane was drained and diluted to volume. Extracts were stored at −10 °C until analysis.

Derivatization. Selected extracts obtained from unstored fruit, the pear sample, and DPA-treated and untreated Red Delicious apples were treated with HFBA to form the HFB derivative of DPA. Quantitation was by the method of standard addition. The procedure was according to that of Allen and Young (1980) with some modification. Five milliliters of extract was placed in each of four test tubes. To two was added 0.2 mL of 0.5 μg mL^{-1} authentic DPA in hexane. This was followed by addition of 5 mL of 5% aqueous sodium hydroxide to all tubes. The tubes were then incubated in a 70 °C water bath for 3 min and cooled to room temperature. Two milliliters of the organic phase was transferred into another tube and 20 μL of HFBA was added. The mixture was heated at 70 °C for 3 min to form the DPA−HFB derivative and then cooled under running water. One milliliter of distilled−deionized water was added to each tube, and the mixture was shaken for 1 min to hydrolyze any excess anhydride. Then 1 mL of 5% aqueous ammonia was added and shaken for another minute to extract all of the heptafluorobutyric acid into the aqueous phase. The hexane phase was reserved for analysis.

GC/MS Analysis. All extracts were analyzed using a Hewlett-Packard 5989A gas chromatograph/mass spectrometer system. The GC oven was fitted with a 30 m HP-5 (Hewlett-Packard, Avondale, PA) fused silica capillary column, 0.25 mm (i.d.) and 0.25 μm film. All injections were in splitless mode at 250 °C. The GC column was directly coupled to the ion source through an interface maintained at 280 °C. The helium carrier gas head pressure was fixed at 100 kPa with the oven temperature profile as follows: 60 °C, hold 1 min, to 300 °C at 10 °C/min. The final temperature was maintained for 6 min. The mass spectrometer was operated in the selected ion mode. The ion monitored for DPA analysis (m/z^+ 169) was observed to be the base peak and molecular ion of the electron impact DPA spectrum. HFB−DPA derivative ions monitored in separate analyses were m/z^+ 365, 272, and 168. These were some of the most abundant ions observed in a full-scan

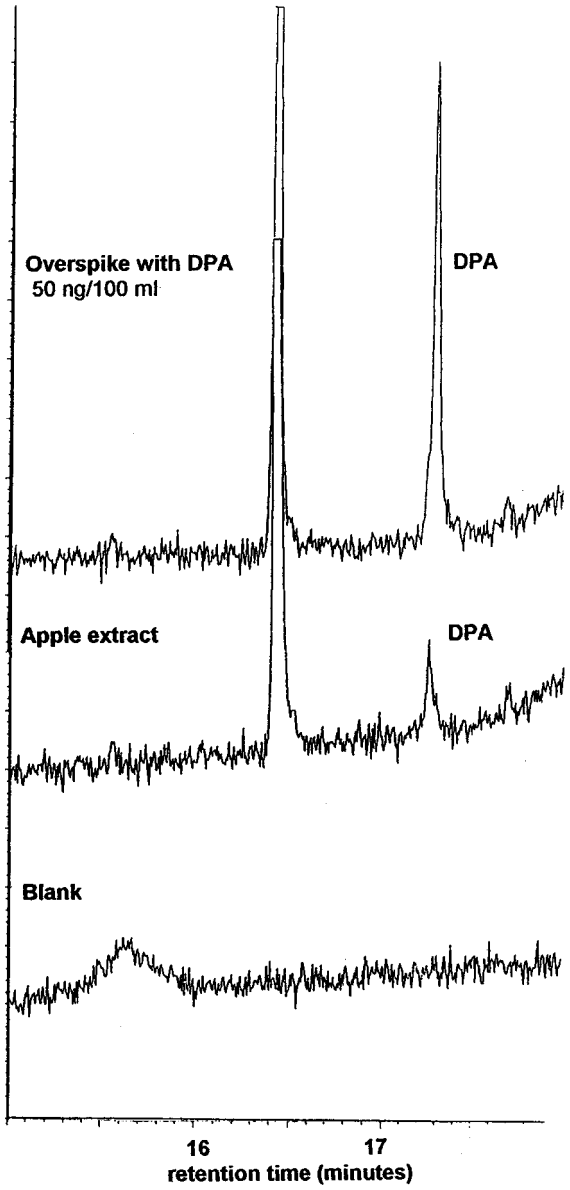

Figure 2. Ion (m/z^+ 169) current chromatograms.

spectrum (Figure 1), with the molecular ion m/z^+ 365 and the base peak m/z^+ = 168. All dwell times were 60 ms, giving 20−30 scans per GC peak.

RESULTS

In the analysis of underivatized hexane-extracted DPA, a single symmetric peak corresponding to the retention time of authentic DPA was observed. Figure 2 provides the ion current (m/z^+ 169) chromatograms of a blank, an apple (Cortland) extract, and the same extract overspiked with DPA.

Samples of Red Delicious, Golden Delicious, McIntosh, Cortland, and Empire apples that had been stored at 0 °C for 7−8 months all contained detectable amounts of DPA, ranging from 0.03 to 0.13 μg g^{-1} of fruit. Results are summarized in Table 1. Fruit size varied among cultivars but did not consistently influence measurements. DPA contamination in the storage facility was verified by analysis of cotton wipes (Table 2).

The fruit analyzed after 7−8 months, mentioned above, were stored in room 1, which produced the highest DPA residues among the three rooms tested. Although few DPA-treated fruit had been kept in these rooms in 1993, all rooms had contained treated fruit in

Table 1. Concentration of DPA on Nontreated Stored Apples

cultivar	DPA concn (μg g^{-1})	cultivar	DPA concn (μg g^{-1})
Red Delicious	0.03	Empire	0.13
Golden Delicious	0.13	Cortland	0.10
McIntosh	0.03		

[a] DPA concentration expressed as fresh weight of the apples.

Table 2. DPA on HRC Apple Storage Room Surfaces

room[a]	wall[b]	DPA (μg m^{-2})
1	1	13.1
	2	7.0
4	1	1.8
	2	2.5
5	1	0.4
	2	0.4

[a] Room 1 contained fruit stored in 0 °C air. Room 4 had fruit stored at 3% O_2 and 5% CO_2 at 3 °C. Room 5 had fruit stored at 2% O_2 and 2% CO_2 at 0 °C. Each room has a capacity of about 2500 bushels of fruit. At the time of sampling, rooms 4 and 5 had been emptied and room 1 contained only a few hundred bushels. [b] Wall 1 was a side wall and wall 2, the inside of the door.

Table 3. Concentration of DPA in Freshly Harvested Nontreated Fruit

	DPA concn[a,b] (μg g^{-1})	
cultivar	immature	mature
Red Delicious	0.003	0.003
Golden Delicious	0.004	0.001
McIntosh	0.002	0.002[c]
		0.001[d]
Empire	0.007	0.002
Cortland	0.002	0.001
Rhode Island Greening	0.003	0.002
Anjou pear		0.001

[a] Presence of DPA confirmed by HFBA derivatization. [b] Concentration in fruit by fresh weight. [c] Harvested Sept 22 (mature). [d] Harvested Oct 7 (overly mature).

previous years. Room 5 had the greatest volume of treated fruit in previous years, and it produced the lowest residues among wall wipes.

To test the possibility that DPA measured on the fruit after storage was the result of contamination from DPA residues in storage, fruit of the same cultivars were extracted directly after harvest. Immature fruit all tested positive for DPA, with concentrations ranging from 0.002 to 0.007 μg g^{-1} of fruit (Table 3). Rhode Island Greening fruit grown organically in a private yard also tested positive for DPA. Repeat measurements on samples from the same trees at the time of commercial harvest yielded similar concentrations of DPA in hexane extracts. Extracts from McIntosh fruit harvested at the overripe stage and a sample of mature Anjou pears also contained much less DPA than did stored fruit.

To confirm DPA measurements, an HFBA derivatization procedure was used. Characteristic ions were observed to coelute with authentic HFB−DPA. However, the ratio of ion abundances observed for untreated samples differed from ratios obtained with authentic HFB−DPA and HFB−DPA from a DPA-treated Red Delicious sample. As indicated in Table 4, the m/z^+ 168, 272, 365 ratio for the samples was 1.0:0.3:0.2, whereas HFB−DPA gave 1.0:0.6:0.3. This suggests a possible misassignment of the peak or a coeluting interference. If a coeluting interference is assumed, then reducing the ion abundance of the base peak (m/z^+ 168) by half would give ion ratios similar to what we measured. In turn, this would reduce concentration estimates in nontreated fruit by 50%.

Table 4. Ion Abundances of HFB-Derivatized DPA

	m/z^+		
sample	168	272	365
authentic DPA (0.02 ng)			
peak area	2460	1417	967
ratio to m/z^+ 168	1.00	0.58	0.39
DPA-treated apple[a]			
peak area	644566	89365	182805
ratio to m/z^+ 168	1.00	0.60	0.28
untreated apple[a]			
peak area	69209	23834	12241
ratio to m/z^+ 168	1.00	0.34	0.18

[a] Red Delicious apples stored at 0 °C in air for 8 months at the HRC.

DISCUSSION

These results indicate that DPA or a DPA-like substance was present on surfaces of freshly harvested apples and pears that had not been treated with DPA. It was present at about the same concentration in immature, mature, and overly mature fruit despite the fact that fruit fresh weight tripled between immature and mature stages. It is unlikely that this substance could have been introduced from a contaminant in another material being applied to apples during their commercial care since the Rhode Island Greening tree received no care and was located many miles from any commercial orchard.

Fruit that had been stored for 7−8 months at 0 °C contained much higher levels of DPA than freshly harvested fruit. The increase during storage may have resulted from contamination within the storage room, since DPA was present on the storage room walls (Table 2). Galantini et al. (1992) also detected DPA on storage surfaces as well as in the air within apple storage rooms. However, it seems unlikely that the magnitude of our observed increase could be accounted for solely by deposition of airborne residues. The surface area of fruit contained in a storage room greatly exceeds the area of the walls, floor, cooling system, and bins, and the residues that we measured on surfaces in the storage rooms were not large. Galantini et al. (1992) found at least 15 times as much DPA in the air within rooms filled with DPA-treated as within rooms filled with nontreated fruit. At the HRC where our studies were conducted, in the year prior to our sample collection (1993) only a small quantity of DPA-treated fruit were present in the room from which we sampled stored fruit. Thus, we conclude that at least part of the increase may have been through endogenous production.

Whether or not the residue detected was truly DPA, however, is not clear from the results. The derivatized products of authentic DPA and the extract from DPA-treated Red Delicious apples had approximately the same ratio among the three most prominent ions in selected ion monitoring GC/MS (Table 4). In contrast, the extracts from nontreated stored fruit produced an alternate ion ratio, suggesting that these samples contained something other than DPA or a mixture of DPA and some other compound that coeluted with DPA. Further research is needed to clarify this observation. We also recognize that at the very low levels detected, postharvest contamination of unstored fruit was possible and needs further examination.

Despite this uncertainty, our results have important practical significance. Some countries and some mar-

kets practice zero-tolerance for DPA residue on apples. Our results demonstrate that a DPA signal in a fruit extract does not necessarily indicate DPA treatment or DPA contamination of the fruit. Every measurement that we made on fruit extracts produced a positive DPA signal, whether fruit were stored or freshly harvested and whether the fruit were grown conventionally or organically. It seems clear that a zero-tolerance for DPA with highly sensitive analyses is inappropriate. Rather, judgments should be based on the magnitude of the DPA "residue" on fruit.

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