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

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Abstract

A method was developed for extracting, identifying, and quantifying diphenylamine (DPA) derivatives in the peel of DPA-treated apples using gradient reversed-phase liquid chromatography with ultraviolet–visible absorption and atmospheric pressure chemical ionization detection (LC–UV–vis–APCI-MS). Compounds routinely analyzed using this method included hydroxylated, nitrosated, nitrated, and methoxylated diphenylamine derivatives. Analysis of peel treated with $0-8\,g\,L^{-1}$ DPA showed that peel DPA content was a limiting factor in derivative production and that recovery of most compounds over this range was linear.
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1. Introduction

Diphenylamine (DPA) is the antioxidant of choice for a diverse variety of applications including propellant stabilization and preservation of apple fruit quality. DPA is effectively employed for controlling a peel browning disorder, called superficial scald (hereafter referred to as "scald"), that renders fruit of a number of apple cultivars unmarketable [1,2]. For this purpose, DPA is generally applied following harvest and prior to cold storage in emulsion as a drench at concentrations of $1-2\,g\,L^{-1}$.

The efficiency of DPA for controlling scald is thought to result from its anti-oxidative utility [3,4]. Not only can DPA halt propagation of free radical cascades by donating the hydrogen from its secondary amine [5], but its aryl components can also directly interact with $^{\bullet}OH$ [6] or $^{\bullet}NO_2$ [7] forming primarily *ortho* and *para* hydroxyl or nitro adducts,

respectively. Reactions with $^{\bullet}NO$ purportedly result in the formation of *N*-nitrosodiphenylamine (NODPA) [8].

N-Nitrosation and C-nitration of DPA during storage of smokeless powders has been widely reported [7,8,9,10]. Metabolism of DPA in biological systems, including apple fruit, largely results in C-hydroxylation of one or both rings in the *ortho* and *para* positions [11–13] as well as lesser amounts of *meta* substituted derivatives in DPA treated apple fruit [13]. The presence of very low quantities of NODPA has also been reported in apple fruit [14]. Other DPA metabolites in apple fruit were detected but not identified [13].

Due to its use for treatment of foodstuff and its wide use as a propellant stabilizer and material antioxidant, qualitative and quantitative determination of DPA and DPA derivatives in these materials is of biological and environmental interest. Historically, chromatographic methods of separation of these components, including open column [9], thin-layer chromatography [11], gas chromatography/mass spectroscopy [15], capillary electrophoresis [16], high-pressure liquid chromatography–ultraviolet detection (HPLC–UV) [7],

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HPLC–UV–vis [8,12], and HPLC–electrospray ionization mass spectroscopy (ESIMS) [10] have been employed for this purpose. Improvements in column technology, thereby chromatographic resolution, and detection techniques have lead to increased prevalence of analyses of these compounds using methods developed for HPLC with more qualitative detection systems such as UV–vis or MS detection.

DPA derivative analysis of sample matrices such as smokeless powder are relatively simple and usually require little clean-up prior to analysis. Biological matrices, especially plant material, provide a much greater challenge, and, owing to the presence of large amounts of other components, require multiple clean-up and concentration steps prior to analysis of minor extractive constituents such as some of the minor DPA derivatives. Recent studies characterizing DPA metabolism by apple fruit have employed rigorous fractionation and concentration of the extract prior to HPLC analyses using either UV photolysis-chemiluminescence [14] or radioactive detection, GC–MS, and FAB-MS following application of ^{14}C-DPA to apple fruit [13].

The present study outlines a method that uses the advantages in selectivity, sensitivity, and identification provided by gradient reversed-phase HPLC–MS using an atmospheric pressure chemical ionization interface (APCI) coupled with UV–vis detection. This method was evaluated for qualitative and quantitative analysis of DPA derivatives in apple peel with minimal sample preparation.

2. Experimental

2.1. Chemicals

HPLC grade methanol (for extraction and sample preparation), GC Resolve grade methanol (for chromatography), hexanes, chloroform, formic acid, and acetic acid were purchased from Fisher Scientific (Pittsburg, PA, USA). Analytical standards: DPA, 9H-carbazole (CB), 3-methoxydiphenylamine (3MeODPA), 2-nitrodiphenylamine (2NO2DPA), 4-nitrodiphenylamine (4NO2DPA), *p-iso*butylhydratropic acid, rose bengal, methyl iodide (Sigma-Aldrich, St. Louis, MO, USA), NODPA, 4-hydroxydiphenylamine (4OHDPA), 3-hydroxydiphenylamine (3OHDPA) (TCI, Portland, OR, USA), *N*-phenyl-4-quinoneimine (NPPQ), and 4-methoxydiphenylamine (4MeODPA) were prepared in methanol as 1 mg mL^{-1} stock solutions.

NPPQ was synthesized by exposing DPA in solution to light in the presence of the sensitizer-dye rose bengal. DPA (250 mg) was dissolved in 50 mL methanol, 10 mL dH$_2$O and 0.3 mL rose bengal solution (23.8 m*M*) added, and the solution placed under fluorescent white light at 22 °C for 16 h. Distilled water (10 mL) was then added and the mixture partitioned with chloroform. The chloroform phase was evaporated in vacuo and fractionated on a silica gel column using 90:10 then 80:20 hexanes/ethyl acetate as the mobile

phases. The solvent was dried off from the bright orange fraction containing pure NPPQ and the resulting orange-red powder stored under N$_2$ (g) at −20 °C.

Methylation of 4OHDPA was performed to prepare the 4MeODPA standard. 4OHDPA (0.4 g) was dissolved in 3 mL methyl iodide in a round bottom flask where light was eliminated. Following the addition of 10 mL 0.5 M NaOH, the mixture was refluxed for 2 h. Products were concentrated on a C$_{18}$ cartridge (Sep-pak, Waters Corp, Millford, MA, USA) and the concentrate eluted using 5 mL methanol. The concentrate was then purified through fractionation on a silica gel column with a 90:10 hexanes/ethyl acetate mobile phase after which fractions containing pure 4MeODPA were pooled, the solvents evaporated, the 4MeODPA recrystallized repeatedly, and stored in N$_2$ (g) at −20 °C.

2.2. Equipment

Samples were analyzed by injecting 0.5–10 μL into a Series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) controlled by Chemstation software (A.09.03) and equipped with a 5 μm Agilent Hypersil ODS (4.0 mm × 125 mm) reverse-phase column, a G1315B diode array detector (DAD), and a G1946D single quadrupole mass selective detector (MSD) using an APCI source. Following an initial 2 min hold at 50.0% methanol, a linear gradient of 50.0–99.8% methanol in aqueous 0.20% formic acid was executed in 25 min, followed by a final 5 min hold at 99.8% methanol. The column temperature and mobile phase flow rate were 20 °C and 0.5 mL min^{-1}, respectively. The eluate was first analyzed by the DAD and then the MSD. The DAD was adjusted to continuously monitor and record UV–vis spectra in the 230–700 nm range for the entire analysis.

The APCI spray chamber conditions were: Drying gas 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 maintained at 130 and 4000 V, respectively. The MSD was adjusted to monitor positive ions in the scanning mode, continuously monitoring and recording entire mass spectra within a 100–1000 *m/z* range, or selective ion monitoring (SIM) mode when compounds were identified and increased sensitivity was required.

Positive identification was achieved through comparison of extract constituents with the UV–vis and/or mass spectra of authentic standards as well as comparisons of retention times. Quantification of constituents was performed by comparison with known amounts of authentic standards added to the same volume (as added to each sample) of *p-iso*butylhydratropic acid, the internal standard.

2.3. Apple peel analysis

'Granny Smith' apples were obtained from a commercial orchard at commercial harvest in 2003. Apples were treated the day of harvest with 0, 1, 2, 4, or 8 g L^{-1} DPA (Shield

31%, Pace International, Seattle, WA), then placed in 1 kPa O_2, 1 kPa CO_2 controlled atmosphere (CA) storage. Storage chamber atmospheres were established within 60 h after harvest and monitored at 90 min intervals (Techni-Systems, Chelan, WA). Semi-static chamber atmospheres (purged only when atmosphere was adjusted) were maintained with N_2 generated from a membrane system (Permea, St. Louis, MO), compressed air and CO_2. Immediately upon removal following 6 months storage, scald incidence was recorded, then peel samples were collected to evaluate DPA metabolism. Apple peel was separated from the whole fruit using a potato peeler (three composite replicates containing peel from 6 fruit/lot), immediately frozen using N_2 (l), and stored at −80 °C until analysis.

DPA and DPA derivatives were extracted by adding 50 mL methanol and 200 μL of 0.282 mM *p-iso*butylhydratropic acid in methanol (internal standard) to approximately 25 g 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 80:20 methanol/dH_2O. The methanol was evaporated from the filtrate 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 partitioned three times with 50 mL chloroform. The epiphase was discarded, the chloroform fraction evaporated under reduced pressure, and the residue dissolved in 8 mL methanol. When fully dissolved, 2 mL deionized water was added. Relatively non-polar constituents were removed by passing this solution through a C_{18} cartridge (Sep-pak). Following the addition of 70 mL 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 isopropanol, evaporated under reduced pressure, and dissolved in 200 μL methanol.

2.4. Method validation

Experiments were performed to address levels and reproducibility of compound recovery as well as instrumental precision. Instrumental precision tests were performed using repeated analysis of authentic standard compounds. Spiked sample tests were executed as outlined above except a known quantity of authentic standard compounds, in addition to internal standard, were added to a set of apple peel (untreated with DPA) samples prior to homogenization. For the first test, 200 μL of a standard mix containing 2 μg 3OHDPA, NODPA, 4MeODPA, 3MeODPA, CB, 4NO2DPA, and 2NO2DPA was added. To represent the larger concentrations of 4OHDPA and DPA typically found in post-storage apple peel, 200 μL of a solution containing 400 μg of each of these compounds was added to a second sample of untreated apple peel prior to homogenization. Recoveries were calculated following adjustment of values according to internal standard recovery.

3. Results and discussion

The current method employs UV–vis spectral and mass selective detection to maximize sensitivity and qualitative identification of compounds. Previously, HPLC coupled with UV–vis detection [8] or ESI-MS [10] have been used to analyze DPA derivatives in explosives. None of these qualitative detection methods has been employed for analysis of DPA and DPA derivatives in apple peel where DPA derivatives containing multiple functional groups are present [13]. Kim-Kang et al. [13] used a variety of techniques including a combination of HPLC with UV and radioisotopic detection of ^{14}C-DPA and derivatives in addition to GC–MS and FAB-MS. This exhaustive approach identified many of the major components in peel and flesh of DPA-treated apples although requiring a more lengthy procedure to acquire similar data.

Due to variety in the chemical properties of these derivatives in apple peel, the current extraction and chromatographic methods were optimized to include simultaneous analyses of a wide variety of extract constituents while the detection method was optimized for sensitive DPA derivative detection. Recoveries of compounds from peel, spiked with authentic standards, are listed in Table 1. Recovery of

Table 1
Relative retention, compound recovery following internal standard correction from apple peel spiked with authentic standard, and assay precision during multiple standard injections

Compound	Relative retention		Recovery from spiked apple peel sample		Assay precision (%RSD) ($n \geq 3$)
	$t_R - t_M$ (min)	%RSD ($n \geq 3$)	%	%RSD ($n \geq 3$)	
4-Hydroxydiphenylamine	6.2	0.14	71.9[a]	19.4[a]	6.01[a]
N-Phenyl-4-quinoneimine	6.7	0.22			
3-Hydroxydiphenylamine	7.1	0.16	80.0	12.7	7.94
N-Nitrosodiphenylamine	11.3	0.37	100	4.49	6.86
4-Methoxydiphenylamine	12.3	0.30	64.2	4.33	5.74
9H-Carbazole	12.3	0.30	91.8	2.15	7.27
3-Methoxydiphenylamine	12.5	0.27	72.8	3.69	6.80
4-Nitrodiphenylamine	12.8	0.30	94.6	3.46	7.28
Diphenylamine	12.8	0.25	113	5.91	6.33
2-Nitrodiphenylamine	15.5	0.26	58.4	1.00	6.86

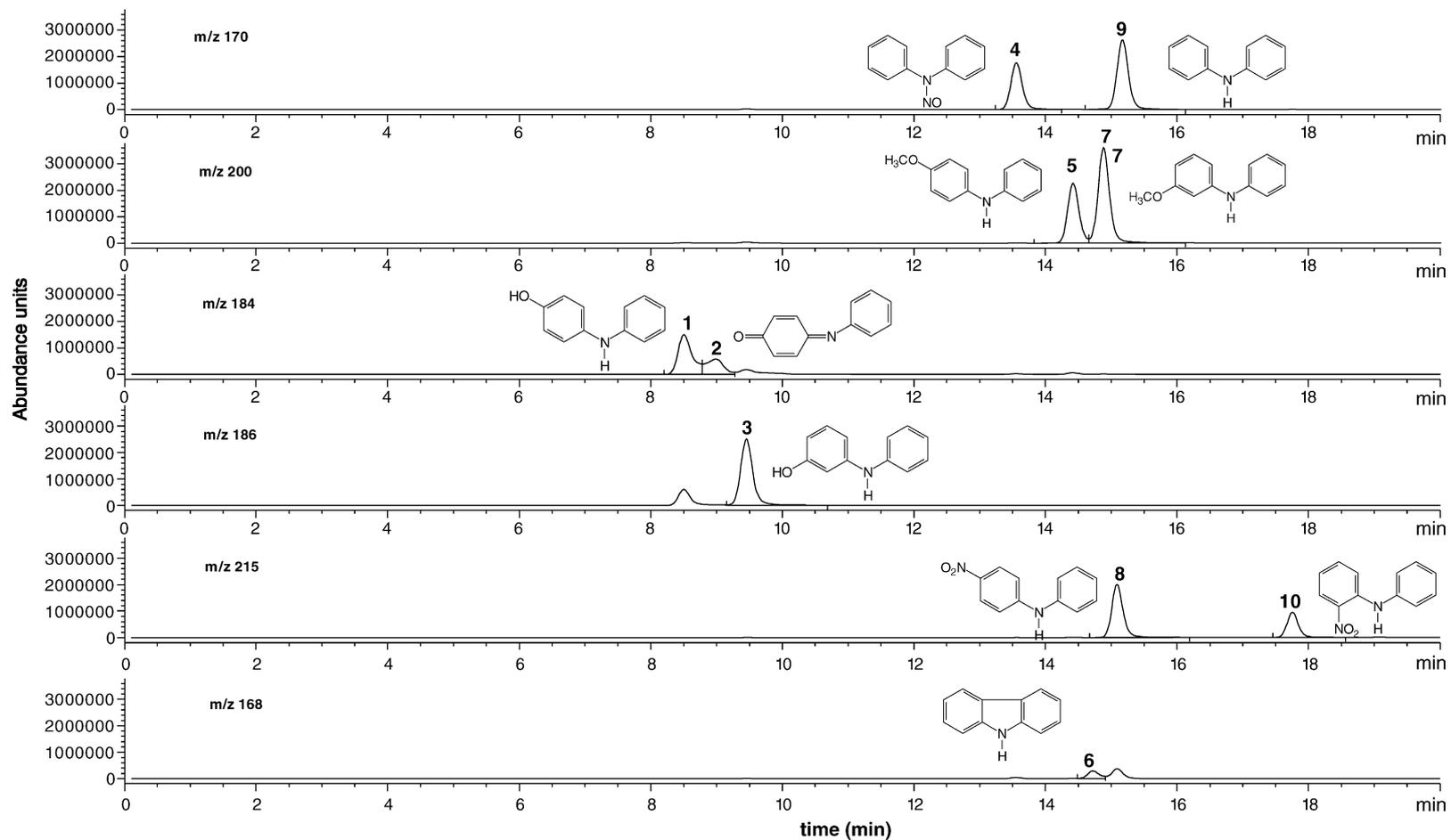[a] Values for combined 4-hydroxydiphenylamine and *N*-phenyl-4-quinoneimine peaks.

Fig. 1. Extracted chromatograms of analysis of DPA and DPA derivatives. Samples were analyzed by HPLC–UV–vis–MS. DPA derivatives analyzed were: (1) 4-hydroxydiphenylamine (4OHDPA); (2) *N*-phenyl-4-quinoneimine (NPPQ); (3) 3-hydroxydiphenylamine (3OHDPA); (4) *N*-nitrosodiphenylamine (NODPA); (5) 4-methoxydiphenylamine (4MeODPA); (6) 9H-carbazole (CB); (7) 3-methoxydiphenylamine (3MeODPA); (8) 4-nitrodiphenylamine (4NO2DPA); (9) diphenylamine (DPA); (10) 2-nitrodiphenylamine (2NO2DPA).

more or less polar compounds was generally less efficient than NODPA or DPA which were recovered at a similar level to that of the internal standard. Variability in recovery was highest for 4OHDPA and 3OHDPA possibly due to increased error related to phase and C_{18} extraction limitations.

Employment of APCI-MS allowed for highly sensitive detection of DPA derivatives in the SIM mode, regardless of peak resolution, and identification of derivatives using mass spectra obtained from the total ion chromatogram (TIC) in the scan mode. For example, linear regression of the calibration curve generated for 0.3–1200 ng NODPA using the SIM mode provided the equation $Y = 2.11 \times 10^5 X - 2.04 \times 10^5$ (where Y = peak area and X = amount NODPA) with $R^2 = 0.9994$. Detection limits were determined using authentic standards dissolved in methanol. The minimum detection limit for NODPA was 20 pg assuming a $S/N = 5$. This detection limit is considerably lower than the 2 ng ($S/N = 3$) reported by Mathis and McCord [10] for quantifying a similar compound using extracted ion chromatograms from LC–ESIMS TIC. Adjustments were made to the injection volume to assure that measured values fell within the linear portion of the calibration curves. Instrument precision was sufficient where relative compound retention deviated less than 0.38% and peak area of multiple injections of authentic standard deviated between 5.5 and 8.0% in all cases.

Resolution of compounds with similar peaks in their mass spectra was sufficient so as not to interfere with quantification and qualitative identification of these compounds

(Fig. 1). Further qualitative identification using UV–vis spectra was used for only the most abundant compounds in samples due to co-elution with other photoactive extract components (Table 2). Retention and spectral comparisons with purchased or synthesized authentic standards provided final qualitative identification. Utilization of this method not only facilitated quantification of DPA derivatives, but also a wide variety of other apple peel constituents not reported here.

The present study confirms the presence of previously reported as well as novel DPA derivatives in apple peel. Kim-Kang et al. [13] reported large quantities of DPA, 4OHDPA and the glucosidic conjugate, and smaller quantities of 2OHDPA, 3OHDPA, 2,4-dihydroxydiphenylamine (2,4-diOHDPA) and their glycosidic conjugates. Small amounts of NODPA have also been reported in the peel of DPA treated apples [14]. Using the current method, the range of DPA derivatives found in the peel of DPA treated apples has been expanded to include 4MeODPA and 3MeODPA. Other related compounds, including 4NO2DPA, 2NO2DPA, and 9H-carbazole were included in the instrumental optimization although they were not detected in these samples.

The *m/z* of the major mass spectral peaks of identified DPA derivatives indicate most compounds remained intact following ionization. For 4MeODPA, 4NO2DPA, and 2NO2DPA, CI molecular ions ($[M+H]^+$) with some fragmentation were detected. Absence or greatly reduced abundance of the molecular ion occurred following the ionization of NODPA and 4OHDPA.

Table 2

Elution order, CI mass spectral, and UV–vis spectral characteristics used for identification of diphenylamine and diphenylamine derivatives using LC–UV–vis–APCI-MS with an Agilent Hypersil ODS column and a methanol gradient

Peak	Rel. RT $t_R - t_M$ (min)	Compound	CI mass spectra (*m/z*) (relative abundance, fragment identification)	UV–vis spectra [wavelength (nm) (relative intensity, spectral feature[a])]
1	6.2	4-Hydroxydiphenylamine	186 (35.3, $[M+H]^+$), 184 (100[b])	282 (100,p), 352 (bl)
2	6.7	*N*-Phenyl-4-quinoneimine	184 (100, $[M+H]^+$)	265 (100,p), 282–288 (84–85,sh), 360 (5.1,v), 450 (20,p), 582 (bl)
3	7.1	3-Hydroxydiphenylamine	186 (100, $[M+H]^+$)	250 (27,v), 282 (100,p), 334 (bl)
4	11.3	*N*-Nitrosodiphenylamine	170 (100, $[(M-NO)H+H]^+$)	257 (68,v), 294 (100,p), 370–390 (5.7–4.1,sh), 422 (bl)
5	12.3	4-Methoxydiphenylamine	200 (100, $[M+H]^+$), 123 (6.7, $[M+H]^+-Ar$), 108 (5.5, $[M+H]^+-ArNH$)	284 (100,p), 352 (bl)
6	12.3	9H-carbazole	168 (100, $[M+H]^+$)	234 (97,p), 252 (100,v), 256 (42,p), 269 (9.0,v), 292 (36,p), 306 (6.1,v), 323 (8.3,p), 331 (6.5,v), 335 (6.8,p), 349 (bl)
7	12.5	3-Methoxydiphenylamine	200 (100, $[M+H]^+$)	250 (25,v), 284 (100,p), 334 (bl)
8	12.8	4-Nitrodiphenylamine	215 (100, $[M+H]^+$), 198 (18, $M-[O+H]^+$), 168 (6.2, $M-[NO_2+H]^+$)	256 (51,p), 306 (6.8,v), 398 (100,p), 518 (bl)
9	12.8	Diphenylamine	170 (100, $[M+H]^+$)	247 (21,v), 284 (100,p), 334 (bl)
10	15.5	2-Nitrodiphenylamine	215 (100, $[M+H]^+$), 197 (11, $M-[OH+H]^+$), 180 (23, $M-([OH+H]^+-OH)$)	240 (71,v), 259 (100,p), 328 (3.9,v), 431 (47,p), 566 (bl)

[a] abbreviations of UV–vis spectral features: p = peak, v = valley, sh = shoulder, bl = baseline.
[b] A-2 peak may result from oxidation of 4-hydroxydiphenylamine to *N*-phenyl-4-quinoneimine during APCI.

Post-column thermal de-nitrosation of NODPA (MW = 198 amu), resulting from the high vaporizer temperature employed for APCI, is evident in its mass spectrum where 170 m/z ($[(M-NO)H + H]^+$) is the most abundant ion and the 199 m/z ($[M + H]^+$) is absent. It would be expected that the thermal decay would result in a diphenylamidogen radical with MW = 168 and therefore a CI molecular ion with 169 m/z. However, following the thermal decay of NODPA, the diphenylamidogen is likely reduced forming DPA during or prior to CI resulting in the 170 m/z fragment upon ionization. The molecular ion of NODPA is present when using ESI [10] where heat vaporization is not required. Because the thermal decay of NODPA occurs post-column, resolution of DPA and NODPA was not impacted. In earlier studies utilizing GC for DPA and DPA derivative analysis, thermal decay may explain why there was a failure to detect nitrosated and nitrated DPA, since gas chromatographic techniques can result in pre-column thermal degradation of these compounds as with nitrosated compounds like NODPA [17]. Sporadic presence of these compounds in DPA treated apples may have also resulted in lack of detection as reported amounts were quite low (Table 3).

The abundance of the molecular ion was also reduced in the CI mass spectrum of 4OHDPA. 4OHDPA (MW = 185 amu) can be readily oxidized resulting in the formation of NPPQ (MW = 183 amu) [18] (Fig. 2). This oxidation may result in the high abundance of 184 m/z $[M + H]^+$ revealed in the spectrum of 4OHDPA indicating spray chamber conditions may be conducive to this reaction. 4OHDPA and NPPQ are resolved using these chromatographic conditions. The oxidation of 4OHDPA may also occur during the extraction procedure, potentially explaining the small amounts of resolved NPPQ which were detected in our samples. Since this compound may be an artifact, the area under NPPQ and 4OHDPA peaks were added together for the quantification of 4OHDPA/NPPQ. 3OHDPA did not display this property.

Some derivatives reported by Kim-Kang et al. [13] were either not detected or excluded from this report. Analyses of the glycosidic conjugates of the hydroxylated derivatives were omitted and would require additional glycosidic hydrolysis of the aqueous phase following chloroform partitioning. There was mass spectral evidence of the presence of 2OHDPA and monomeric glycosides of 4OHDPA but full identification of either compound was not pursued. Additionally, dihydroxylated DPA derivatives did not partition into chloroform from water, rendering the detection of 2,4-diOHDPA unfeasible using this method.

DPA derivative production by apples treated with increasing concentrations of DPA was evaluated following 6 months CA storage. Small amounts of DPA were detected in the untreated fruit (Table 3). Traces of DPA in untreated apples have been previously reported, although its origins are unknown [19]. 4OHDPA, NODPA, 4MeODPA, 3MeODPA, and DPA peel content increased linearly with DPA application concentration. Second order increases in 4MeODPA,

Table 3
DPA and DPA derivative content (ng g^{-1} fresh weight) and standard deviation (SD) of 'Granny Smith' apple peel sampled from fruit treated with increasing rates of DPA at harvest then stored for 6 months in 1 kPa O_2, 1 kPa CO_2 at 1 °C

Compounds	0 g L^{-1} DPA		1 g L^{-1} DPA		2 g L^{-1} DPA		4 g L^{-1} DPA		8 g L^{-1} DPA		Regression analyses	
	ng g^{-1}	SD	ng g^{-1}	SD	ng g^{-1}	SD	ng g^{-1}	SD	ng g^{-1}	SD	Linear	Quadratic
4-Hydroxydiphenylamine/N-phenyl-4-quinoneimine**	ND		397	(193)	5300	(2200)	9410	(1840)	20900	(5180)	*	NS
3-Hydroxydiphenylamine	ND		4.29	(0.911)	5.75	(5.14)	10.1	(1.39)	11.3	(14.6)	NS	NS
N-Nitrosodiphenylamine	ND		0.796	(0.300)	2.28	(0.488)	2.54	(0.157)	4.67	(0.659)	*	NS
4-Methoxydiphenylamine	ND		22.3	(6.38)	85.7	(29.3)	89.3	(14.8)	118	(11.3)	*	*
3-Methoxydiphenylamine	ND		0.488	(0.119)	2.72	(0.433)	3.86	(0.0942)	5.86	(0.866)	*	*
Diphenylamine	19.0	(16.8)	2960	(519)	13000	(2140)	18400	(575)	28100	(3150)	*	*

Analysis using LC–APCI-MS with an Agilent Hypersil ODS column and a methanol gradient followed a preparatory method involving aqueous/methanolic extraction, partitioning into chloroform, and solid phase (C18) extraction and concentration. ND = not detected. NS = not significant.
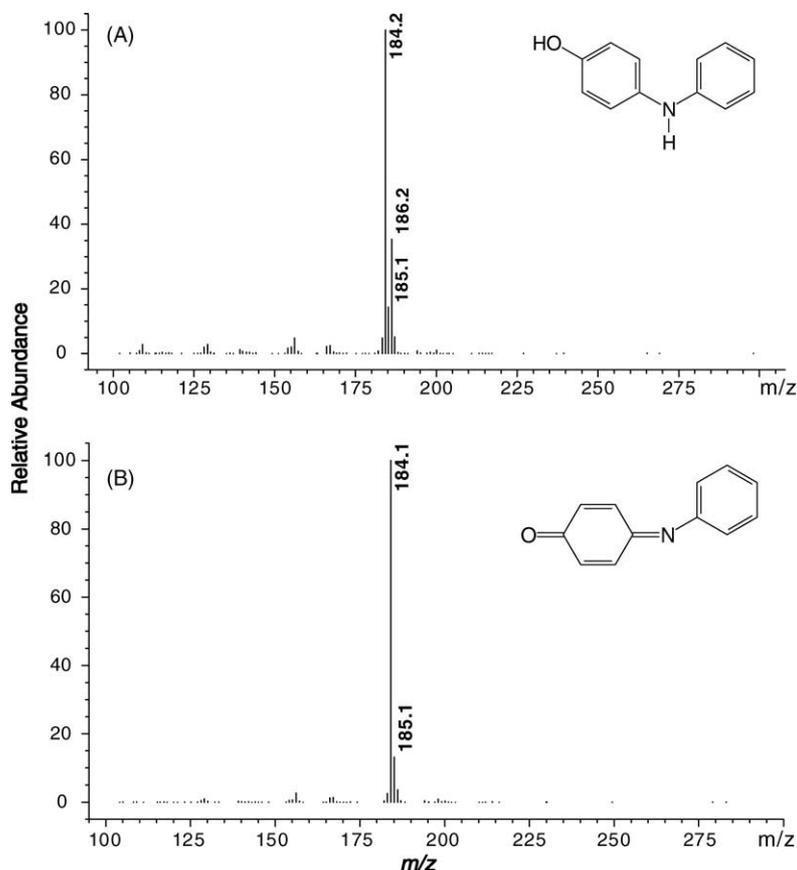* Significant fit ($n = 3$; $p \leq 0.05$).
** Values for combined peaks.

Fig. 2. Positive ion APCI mass spectra of (A) 4-hydroxydiphenylamine (4OHDPA) and (B) N-phenyl-4-quinoneimine (NPPQ).

3MeODPA, and DPA with DPA application concentration were also significant. 3OHDPA did not increase with DPA concentration. These results indicate that, within the range tested, peel DPA content is a limiting factor in derivative production, and that recovery and analyses of these compounds in the ranges recorded in this study are valid. As expected, variance in amounts was considerably higher than those reported for the spiked, untreated apple peel samples due to biological variability.

While the mechanism of DPA derivative synthesis in apple fruit remains unevaluated, reactions between reactive oxygen species and/or reactive nitrogen species and DPA may result in their formation. Aryl compounds, including benzene [20] and DPA [21], can react with $^\bullet$OH forming mainly *para* hydroxylated derivatives in the case of DPA. Reactions between $^\bullet$NO and $^\bullet$NO$_2$ can result in *N*-nitrosation or C-nitration, respectively [7,8]. Reactions catalyzed by endogenous oxygenases, such as cytochrome P450, may drive hydroxylated DPA biosynthesis [22]. All of these reactions, including less explicable ones, such as C-methoxylation to form 4MeODPA, require additional investigation to discern the exact reactants and processes leading to their formation in apple fruit. Elucidation of these processes may indicate mechanisms by which DPA interaction with reactive and/or radical species can provide scald control or result from other unique biological processes.

4. Conclusions

Identification and quantification of minute quantities of DPA and DPA derivatives is essential for characterization and monitoring of these compounds as well as understanding the various mechanisms by which DPA confers its antioxidative protection in biological systems. Using LC–DAD–APCI-MS, DPA and many DPA derivatives were identified and picogram quantities routinely quantified from peel of DPA treated apple fruit. Additionally, CB, 4NO2DPA, and 2NO2DPA can be quantified using the same method. Given its validity for evaluating these compounds in this complex sample matrix, this method is likely to be useful for analyses of other biological systems. Providing the formation of these compounds may result from reactions with biologically significant chemical species or metabolic processes, monitoring derivative formation during physiological events that are ameliorated by DPA may help elucidate the function of oxidative stress in those phenomena.

References

[1] R.M. Smock, Proc. Am. Soc. Hort. Sci. 69 (1957) 91.

[2] M. Ingle, M.C. D'Souza, Hortscience 24 (1989) 28.

[3] F.E. Huelin, I.M. Coggiola, J. Sci. Food Agric. 21 (1970) 44.

[4] B.D. Whitaker, Postharv. Biol. Tech. 18 (2000) 91.

[5] C.E. Boozer, G.S. Hammond, J. Am. Chem. Soc. 76 (1954) 3861.

[6] M.B. Smith, J. March, March's Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, Fifth ed., John Wiley and Sons Inc., New York, 2001, p. 916.

[7] E.O'N. Espinoza, J.I. Thornton, Anal. Chim. Acta 288 (1994) 57.

[8] L.S. Lussier, H. Gagnon, M.A. Bohn, Propell., Explos., Pyrotech. 25 (2000) 117.

[9] W.A. Schroeder, E.W. Malmberg, L.L. Fong, K.N. Trueblood, J.D. Landerl, E. Hoerger, Indust. Eng. Chem. 41 (1949) 2818.

[10] J.A. Mathis, B.R. McCord, J. Chromatogr. A 988 (2003) 107.

[11] W.E. Alexander, A.J. Ryan, S.E. Wright, Food Cosmet. Toxicol. 3 (1965) 571.

[12] K.E. Appel, S. Görsdorf, T. Scheper, H.H. Ruf, C.S. Rühl, A.G. Hildebrandt, J. Cancer Res. Clin. Oncol. 113 (1987) 131.

[13] H. Kim-Kang, R.A. Robinson, J. Wu, J. Agric. Food Chem. 46 (1998) 707.

[14] T.J. Lillard, J.H. Hotchkiss. In: Nitrosamines and Related *N*-Nitroso Compounds, ACS Symposium Series, 553, 1994, p. 358.

[15] K. Tatsumi, H. Yamada, S. Kitamura, Arch. Biochem. Biophys. 226 (1983) 174.

[16] D.M. Northrop, D.E. Martire, W.A. MacCrehan, Anal. Chem. 63 (1991) 1038.

[17] Agency for Toxic Substances and Disease Registry, Public Health Service, US Department of Health and Human Services. in: Toxicological Profile for *N*-Nitrosodiphenylamine, 1993, p. 55.

[18] D.W. Leedy, R.N. Adams, J. Am. Chem. Soc. 92 (1970) 1646.

[19] W.J. Bramlage, T.L. Potter, Z. Ju, J. Agric. Food Chem. 44 (1996) 1348.

[20] M.P. Balakrishnan, Reddy, J. Phys. Chem. 74 (1970) 850.

[21] K.H. Schmidt, A. Bromberg, D. Meisel, J. Phys. Chem. 89 (1985) 4352.

[22] S.G. Bell, D.A. Rouch, L. Wong, J. Mol. Catal. B 3 (1997) 293.