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Determination of ascorbic acid, dehydroascorbic acid and ascorbic acid-2-phosphate in infiltrated apple and potato tissue by high-performance liquid chromatography

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SUMMARY

A high-performance liquid chromatography procedure for the determination of ascorbic acid-2-phosphate (AAP), ascorbic acid (AA), and dehydroascorbic acid (DHAA) in raw apple and potato, treated with AAP and AA to prevent browning, was developed. These compounds were extracted with a mixture of mobile phase and 2.5% metaphosphoric acid and separated on an aminopropyl bonded-phase silica column. DHAA was determined as AA following reduction with dithiothreitol. The method was evaluated with spiked samples and found to be accurate and reproducible at concentrations as high as 0.9 m*M* AA or AAP.

INTRODUCTION

Recent studies of the control of enzymatic browning in fruits and vegetables have shown that ascorbic acid-2-phosphate (AAP), a stabilized source of ascorbic acid (AA), is a highly effective inhibitor of enzymatic browning in apple tissue[1] and may be applicable to potatoes and other commodities as well. Since this compound must undergo hydrolysis to AA *in situ* to be effective[2], and also since AA functions as a browning inhibitor, at least in part, by undergoing oxidation to dehydroascorbic acid (DHAA) in the course of reducing quinones to polyphenols[3], there is a need to measure all three species in treated samples to evaluate treatment effectiveness. Titrimetric methods, commonly used to determine AA in fruits and vegetables[4], are not sufficiently sensitive to measure residual AA concentrations in treated samples and do not respond to AAP, which is not a reducing agent. Wang *et al.*[5] described a high-performance liquid chromatography (HPLC) procedure for the determination of AA released from L-ascorbic-2-polyphosphate esters following phosphatase digestion. Doner and Hicks[6] employed an aminopropyl bonded-phase silica HPLC column for the determination of AA, or DHAA following reduction to AA with dithiothreitol (DTT). Our objective in the present study was to adapt this procedure to permit the determination of AAP without phosphatase treatment and to develop

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suitable extraction, clean-up and DTT reaction conditions for the analysis of apple and potato samples containing AAP, AA and DHAA.

EXPERIMENTAL

Chemicals and reagents

Reagent-grade chemicals and high-purity solvents were used except when specified otherwise. Aqueous solutions were prepared with glass-distilled water. The mobile phase comprised acetonitrile–0.05 *M* potassium dihydrogenphosphate (75:25). Aqueous solutions of AA (Mallinckrodt), DHAA (Aldrich) or AAP (provided by Professor Paul A. Seib, Kansas State University, Manhattan, KS, U.S.A.) were used as dips for apple and potato samples. These compounds were dissolved in mobile phase or a simulated sample extract comprising acetonitrile–aqueous 1.12% metaphosphoric acid (1:1) when used as standards for HPLC analyses. A 2.5% (w/v) solution of DTT (Sigma) in mobile phase was used for DHAA reduction.

HPLC procedure

The HPLC procedure of Doner and Hicks[6] was followed except that separations were carried out on a 25 × 0.46 cm I.D. Rainin 8-μm Dynamax-60A NH_2 column, and elution took place isocratically with mobile phase at 2 ml/min. The HPLC system consisted of a Rheodyne Model 7125 injector with a 20-μl sample loop, a Waters Model 6000A pump, a Waters Model 490 detector operated at 254 nm, and a Hewlett-Packard Model 3390A integrator.

Dipping procedure

Plugs were cut from 4–6 apples or potatoes with an electric cork borer, by using a 22-mm I.D. stainless-steel cutting tube, as described previously[7]. The plugs were submerged in aqueous solutions containing 56.8 m*M* AA, AAP or DHAA for 90 s and then drained in a plastic collander for about 30 s. The bottom of the collander was blotted carefully with paper toweling to remove adhering solution. Treated plugs were stored in a dry collander and covered with foil to minimize dehydration.

Sample extraction and clean-up

Plug samples weighing about 30 g were blended with 30 ml aqueous 2.5% metaphosphoric acid solution and 60 ml mobile phase for 2 min at high speed in a Waring Blendor jar. The homogenate was mixed with 2.4 g Celite analytical filter aid (Fisher) and filtered through Whatman No. 541 paper with suction. An additional 2.4-g portion of filter aid was mixed with the filtrate which was then refiltered through Whatman No. 50 paper with suction. Immediately prior to injection, aliquots of the second filtrate were cleaned up by passage through a C_{18} Sep-Pak cartridge (Waters), previously conditioned by flushing with acetonitrile, and an 0.45-μm nylon 66 membrane filter in a 13-mm plastic filter holder (Rainin). Cartridges could be re-used for as many as 12 samples with careful flushing between samples and overnight conditioning in acetonitrile. Samples to be analyzed for total AA were pretreated with DTT to reduce DHAA prior to clean-up. The pH of 25-ml aliquots of second filtrate was adjusted to 6 with 0.3 ml 10% sodium hydroxide, 1.0 ml DTT solution was added with stirring, and the reaction mixture was held at room temperature (*ca.* 20°C) for 30 min prior to clean-up and injection.

Linearity and recovery experiments

Standard curves were run for solutions of AA or AAP in simulated sample extract at concentrations between 0.114 and 1.14 m*M* to determine the linear range of the procedure. Initial recovery experiments were carried out by adding aliquots of AA or DHAA solution to blender jars prior to the extraction of apple or potato samples. The recovery of AA and AAP from various apple cultivars and Russet potatoes was determined in replicated trials by spiking freshly prepared extracts with these compounds at concentrations of 0.28, 0.57, and 0.85 m*M* and comparing the HPLC peak areas with those obtained for unspiked samples and solutions of AA or AAP in simulated sample extract. The AA content of spiked samples was corrected for endogenous AA prior to the recovery calculation.

RESULTS AND DISCUSSION

HPLC separation of AA and AAP

Typical chromatograms for the separation of AA and AAP on the aminopropyl column are shown in Fig. 1. No interfering peaks were seen on chromatograms for unspiked apple or potato samples (other than endogenous AA). The retention time of AAP could be reduced by gradient elution, increasing the proportion of buffer to 50%. However, isocratic separation was found to be satisfactory within the time frame of the experiments conducted in this study.

Linearity of standard curves

Standard curves for AA and AAP in simulated sample extract in the concentration range 0.114–1.14 m*M* are shown in Fig. 2. A linear relationship between peak area and concentration was obtained at concentrations as high as 0.9 m*M*. Slopes of standard curves were similar for AA and AAP and were consistent from trial to trial.

Recovery of AA, AAP and DHAA

Initial studies of AA recovery from Granny Smith apple plugs, spiked with 333 mg/kg AA, yielded values in excess of 95%. Recovery experiments carried out with

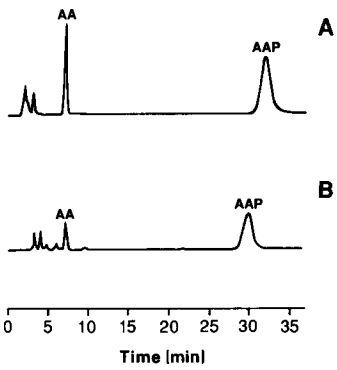

Fig. 1. Chromatograms showing the separation of ascorbic acid (AA) and ascorbic acid-2-phosphate (AAP) on an aminopropyl bonded-phase silica column. (A) Granny Smith apple plugs, 2.5 h after dipping; and (B) Russet Burbank potato plugs, immediately after dipping, each dipped in 56.8 m*M* AAP for 90 s.

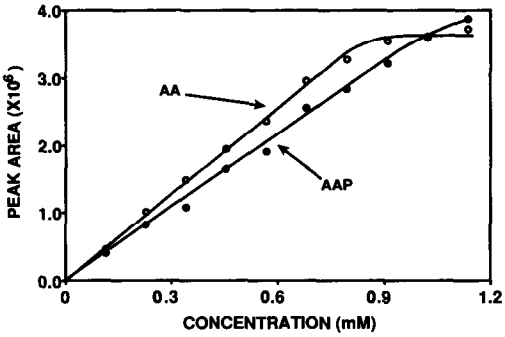

Fig. 2. Standard curves for ascorbic acid (AA) and ascorbic acid-2-phosphate (AAP) in acetonitrile–1.12% metaphosphoric acid (1:1).

spiked apple and potato extracts demonstrated near quantitative recovery of both AA and AAP at concentrations of 0.28, 0.57 and 0.85 m*M* (Table I). The slopes of concentration *vs.* peak area curves were similar for standards and spiked extracts and slightly lower for AA, compared to AAP. Thus, the accuracy of the determination appears to be independent of commodity and AA or AAP concentration, within the range likely to be encountered in treated samples.

DHAA recovery was at least 92% in spiked apple and potato samples (Table II). Neutralization of metaphosphoric acid in sample extracts is essential if quantitative recovery of DHAA is to be obtained. DHAA recovery was only 14% in unneutralized extract (pH 2.9) and 45% at pH 4. These results indicate that by analyzing samples, both with and without DTT reduction, one can follow the oxidation of AA to DHAA due to quinone reduction and other oxidation reactions.

TABLE I

RECOVERY OF ASCORBIC ACID (AA) AND ASCORBIC ACID-2-PHOSPHATE (AAP) FROM SPIKED APPLE AND POTATO EXTRACTS

Sample	*Compound*	*Slope*[a]		*Recovery*[b] *(%)*		
		Standard curve	*Spiked extracts*	*Level of spiking (mM)*		
				0.28	*0.57*	*0.85*
Granny Smith apple	AA	4.30	4.08	102	95	95
	AAP	5.10	4.99	100	103	97
Red Delicious apple	AA	3.97	4.26	97	121	104
	AAP	4.99	5.07	127	108	109
Golden Delicious apple	AA	4.00	4.27	94	96	100
	AAP	5.09	5.38	95	100	102
Russet potato	AA	3.97	3.87	97	91	98
	AAP	4.42	5.13	103	116	112

[a] For peak area ($\times 10^6$) *vs.* concentration (m*M*); correlation coefficients for regression > 0.99.
[b] Based on 2–4 replicate determination for each standard and spiked extract.

TABLE II

RECOVERY OF DEHYDROASCORBIC ACID (DHAA) FROM SPIKED APPLE AND POTATO PLUGS AND SIMULATED EXTRACTS

Sample	*DHAA added*[a] *(μg/ml)*	*Total ascorbic acid (TAA) found*		
		Endogenous (μg/ml)	*Spiked (μg/ml)*	*Recovery*[b] *(%)*
Winesap apple, pH 2.9	50.6	3.3	10.5	14
Simulated extract, pH 4	50.6	–	22.8	45
Simulated extract, pH 5	50.6	–	50.2	99
Granny Smith apple[c]	35.7	4.9	40.2	99
Red Delicious apple[c]	35.7	3.3	36.3	92
Russet potato[c]	36.6	27.3	62.9	97

[a] Calculated as μg ascorbic acid/ml sample extract.

[b] Recovery $= \frac{(\text{TAA spiked} - \text{TAA endogenous})}{\text{DHAA added}} \cdot 100.$

[c] Adjusted to pH 6.

TABLE III

PRECISION OF HPLC DETERMINATION OF ASCORBIC ACID (AA) AND ASCORBIC ACID-2-PHOSPHATE (AAP) IN APPLE AND POTATO PLUGS

Sample	*Treatment*	*Compound determined*	*Peak area*		*Coefficient of variation*
			Mean ± S.D.	*n*	
Standard	0.57 m*M* AA	AA	953 500 ± 77 360	9	8.1
Golden Delicious	Spiked with 0.57 m*M* AAP	AAP	2 735 700 ± 104 800	8	3.8
Red Delicious	Dipped in 56.8 m*M* AAP, held 48 h	AA	1 790 100 ± 41 300	7	2.3
		AAP	1 060 800 ± 85 700	7	8.1
Russet potato	Dipped in 56.8 m*M* AAP, held 24 h	AA	2 423 600 ± 111 300	4	4.6
		AAP	359 400 ± 51 400	4	14.3

TABLE IV

ASCORBIC ACID-2-PHOSPHATE (AAP), ASCORBIC ACID (AA), AND DEHYDROASCORBIC ACID (DHAA) IN INFILTRATED GRANNY SMITH APPLE PLUGS

Apple plugs infiltrated with 56.8 m*M* AAP solution and stored at *ca.* 20°C.

Time after dipping (h)	*Concentration (μmol/100 g)*		
	AAP	*AA*	*DHAA*
0.1	218.5	10.5	6.1
5	111.5	171.0	30.9
24	9.7	241.5	10.4
48	0	165.4	21.5

Precision of method

Examples of the precision of the HPLC method are shown in Table III. Typically, coefficients of variation were less than 10%, except when residual concentrations of AA or AAP were small, as in the Russet potato sample. With this degree of precision, the HPLC method is suitable for determinations of AA or AAP uptake in treated samples or for investigations of AAP hydrolysis and subsequent oxidation following treatment.

Application of HPLC method to infiltrated samples

Data in Table IV show the application of the HPLC method to Granny Smith apple plugs, infiltrated with AAP by dipping in a 56.8 m*M* solution for 90 s and then stored at *ca.* 20°C for 48 h. It can be seen that the AAP concentration in treated plugs decreased almost to zero within 24 h while the AA concentration increased, presumably due to AAP hydrolysis by endogenous acid phosphatase. The DHAA concentration in the infiltrated plugs was relatively small throughout the storage period. An investigation of the uptake and fate of AAP in infiltrated fruits and vegetables will be reported elsewhere.

AAP might be used as a stable form of Vitamin C in various food or feed products, cosmetics or pharmaceuticals[2]. With minor modification, the HPLC procedure described herein would be applicable to the evaluation of these systems as well as infiltrated fruits and vegetables.

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