

## Determination of L-Ascorbyl 6-Palmitate in Bread Using Reverse-Phase High-Performance Liquid Chromatography (HPLC) with Electrochemical (EC) Detection

TSUI-HWA TRACY HUNG, PAUL A. SEIB, and KARL J. KRAMER

### ABSTRACT

L-Ascorbyl palmitate (AP) was stable for up to 5 hr at 25°C in an extracting medium consisting of 90% dimethylsulfoxide (DMSO) with 0.12% metaphosphoric acid and 0.05% L-ascorbic acid (AA). When the DMSO-acid medium was used with reverse-phase, high-performance liquid chromatography (HPLC) to determine 0.6-4.8 mg AP/g dry dough or bread, AP was base-line resolved on the chromatograms. Reverse-phase HPLC with electrochemical detection was also used to obtain base-line resolution of AA extracted from bread using 3% aqueous metaphosphoric acid. Fresh bread after cooling 1 hr retained 50.7% of AP and 58.1% of AA added at the dough stage (150 mg of AP or 63.8 mg of AA per 100g flour). During storage of the bread in polyethylene bags at 25°C, AA was lost approximately three times faster than AP. AA could not be detected in the bread after 10 days, whereas 30% AP remained. Bread baked with 0.15% AP in the formula retained 9% of the adult RDA for vitamin C after 3 days of bagged storage, and 6% RDA after 10 days; in a nitrogen atmosphere the bread retained 90% AP after 5 days.

### INTRODUCTION

L-ASCORBYL 6-PALMITATE (AP) has useful properties in breadmaking; it strengthens dough, increases loaf volume, improves crumb grain and replaces shortening (Hoseney et al., 1977; Koch et al., 1987). Furthermore, AP (0.38%, in flour), like sodium stearoyl 2-lactylate (SSL), softens bread crumb more effectively than a mixture of distilled monoglycerides (0.5%) and shortening (3%) (Koch et al., 1987).

Commercial samples of L-ascorbyl 6-palmitate can be readily incorporated into bread dough by gently premixing AP with vegetable oil or plastic shortening, then adding the premix to the other ingredients. When adding a water-dispersion of AP directly to the mixer, high speed mixing of the slurry is required to disperse the agglomerated particles of AP. Without dispersion in oil or high-speed mixing, AP added with the other ingredients at the mixer gives dark specks on the bread crust and is not fully effective as a dough conditioner (Koch et al., 1987).

Mauro et al. (1979) used high-performance liquid chromatography (HPLC) with UV detection to assay AP in bread. Those workers reported approximately 80% retention of AP in a no-shortening pup-loaf when 0.5% sodium L-ascorbyl 6-palmitate (based on flour) was added to dough. Electrochemical detection (EC) of L-ascorbic acid (AA) and several of its derivatives is known to be approximately five times more sensitive than UV (Brunt and Bruins, 1979; Grün and Loewus, 1983). Furthermore, in crude extracts, EC detection may yield fewer chromatographic peaks caused by interfering substances than UV detection. The object of this study was to develop an improved assay for AP in bread using HPLC with EC. The

new assay procedure was used to compare the stability of AP to that of L-ascorbic acid (AA) in freshly made and aged bread.

### MATERIALS & METHODS

#### Materials

L-Ascorbyl 6-palmitate (AP, m.p. 116-117°C) was a gift from Hoffmann-La Roche, Inc., Nutley, NJ. L-Ascorbic acid (AA), HPLC-grade methanol and sodium acetate were from Fisher Scientific, St. Louis, MO. Octyltriethylammonium phosphate (Q8), the ion-pairing agent, was from Regis Chemical Co., Morton Grove, IL. All other chemicals were reagent grade. Bread flour (protein 11.85% on a 14% m.b.) was a commercial product of Ross Milling Co., Wichita, KS; the flour contained no additives. Soy oil was Hain brand (Hain Pure Food Co., Los Angeles, CA).

#### HPLC

The HPLC system consisted of a 6000A solvent delivery system (Waters Associates, Inc., Milford, MA), an electrochemical detector (Model LC-4, Bioanalytical System, West Lafayette, IN) with a glassy carbon working electrode and a type TL-5A flow cell, a Rheodyne loop injector (0.02 mL, Alltech Associates, Inc., Deerfield, IL), and an integrating recorder (Model C-R3A Chromatopac, Shimadzu Co., Tokyo). AP was determined with a reverse-phase column (50 × 4.6 mm, stainless steel, Supelcosil LC-18, particle size 5 μm; Supelco, Inc., Bellefonte, PA). AA was determined also on a reverse-phase column (Alltech C-18, 250 × 4.6 mm, particle size 5 μm). The analytical columns were protected by a precolumn with a 40 × 4.6 mm ODS-10 cartridge (Bio-Rad Laboratories, Richmond, CA). The mobile phase used to determine AP was a 77/23 (V/V) mixture of methanol and 80 mM acetate buffer (pH 5). The mobile phase for AA assay contained 80 mM acetate buffer (pH 4.2), 0.1 mM disodium ethylenediamine tetraacetate, and 1 mM octyltriethylammonium phosphate. Eluting solvents were degassed by an ultrasonic generator (Fisher Sonic Dismembrator, Model 300, Fisher Scientific, St. Louis, MO), and the flow rates were set at 2 and 1 mL/min, respectively, for AP and AA determinations. The columns were maintained at 25°C and the potential of the detector was set at + 0.72V vs a Ag/AgCl reference electrode. Quantitative determinations of AP were made by comparing integrated areas of known amounts of the standard compound, whereas those of AA were done by comparing peak height values.

#### Stability of the sodium salt of AP in phosphate buffer (pH 7) at 48°C

Sodium L-ascorbyl 6-palmitate (SAP) was prepared as reported by Hoseney et al. (1977). In a repetition of the extraction method used by Mauro et al. (1979), SAP (250 mg) was dissolved in methanol (25 mL), and a 1 mL aliquot was added to 49 mL of 0.1M phosphate buffer (pH 7) containing sodium dodecyl sulfate (250 mg) and 6 mg of α-amylase (*Bacillus subtilis*, Sigma Chemical Co., St. Louis, MO). The mixture was shaken gently in a water bath at 48°C, and 0.02 mL of the mixture was injected directly into the chromatograph at various times.

#### Breadmaking

Pup-loaves were made according to the procedure of Finney and Barmore (1943). The full-formula dough contained the following in-

*Authors Hung and Seib are affiliated with the Dept. of Grain Science & Industry at Kansas State Univ., Manhattan, KS, 66506. Author Kramer is affiliated with the Dept. of Biochemistry at Kansas State University and the U.S. Grain Marketing Research Laboratory, USDA-ARS, Manhattan, KS 66502.*

redients: flour, 100g (14% m.b.); water, 66g; sucrose, 6g; nonfat dry milk, 4g; soy oil, 2g; instant dry yeast, 0.72g; sodium chloride, 1.5g; enzyme-active malt, 0.5g; and potassium bromate, 1 mg. Immediately prior to mixing, AP (150 mg per dough) was preblended with soy oil (2g) and the mixture added to the flour. In loaves baked with added L-ascorbic acid, AA (63.8 mg per dough) was added immediately prior to mixing as a freshly prepared aqueous solution (5 mL of a 1.28% solution). Doughs were mixed to optimum in a vertical pin mixer, fermented (90% RH) for 180 min at 30°C, and proofed 55 min at 30°C. One dough containing AP was freeze-dried after proofing, whereas all others were baked 24 min at 218°C. After baking, loaf volume and weight were recorded. Loaves were cooled 1 hr under ambient room conditions and stored for 0, 1, 3, 5, and 10 days at 25°C in polyethylene bags (0.038 mm thickness). Immediately after storage, the bread was freeze-dried, ground in a Waring Blendor and then stored at -20°C until assay. The moisture (AACC, 1983) of all pup-loaves in this study was 31-33%, whereas that of freeze-dried bread was 1.5-4%. Prior to assay, the bread was ground with a mortar and pestle to pass a U.S. No. 60 wire-mesh screen.

#### Determination of AP

A standard curve of AP was constructed as follows. AP stock solutions were prepared by dissolving AP (15, 22.5, 30, 60 and 120 mg) in a mixture of dimethylsulfoxide (DMSO, 45 mL) and freshly prepared 30% aqueous metaphosphoric acid (5 mL) containing L-ascorbic acid (AA, 600 mg). An aliquot (2 mL) of an AP stock solution was diluted to 50 mL with 90% aqueous DMSO, and the mixture stirred 10 min at 25°C. An aliquot (2 mL) of the 90% DMSO solution was made to 10 mL with methanol. After mixing, a small amount of white precipitate formed. The mixture was centrifuged (15,000 rpm, Eppendorf Model 5412 Microcentrifuge, Brinkmann Instruments Inc., Westbury, NY), and 0.02 mL of the clear supernatant was injected into the chromatograph.

The precipitate was examined to determine whether it contained AP. The precipitate was mixed with an equal volume of methanol, the mixture centrifuged and the methanol extract examined by UV spectroscopy and thin-layer chromatography on silica gel. The thin-layer plate was developed with a mixture CHCl<sub>3</sub> and CH<sub>3</sub>OH (4:1 V/V) and visualized by spraying with 50% aqueous sulfuric acid and charring on a hot plate.

The recovery of AP added to blank bread was done as follows. Blank bread was baked with 2% soy oil but no AP. An aliquot (2 mL) of an AP stock solution prepared as described in the previous paragraph, was added to 1g of freeze-dried bread. Then, 48 mL of 90% DMSO in water was added, and the mixture stirred on a magnetic stir plate at 25°C for 0.5-5 hr. After different extraction periods, an aliquot (2 mL) was pipetted into a volumetric flask (10 mL) and made to volume with methanol. The addition of four volumes of methanol to the DMSO-acid extract precipitated starch and other polymers. Centrifugation gave a clear alcoholic supernatant in which AP was soluble (Cort, 1974). The clear supernatant (0.2 mL), which contained 48, 72, 96, 192, or 384 ng of AP, was injected into the chromatograph.

Bread was baked from dough containing 150 mg of AP per 100g flour. After 0, 1, 3, 5, and 10 days storage in polyethylene bags at 25°C, the bread was freeze-dried and assayed for AP. In one experiment, one freshly baked loaf was placed in a desiccator, and the desiccator was evacuated with a water aspirator for 40 min and refilled with nitrogen. The bread in the desiccator was stored 5 days in the dark at 25°C, freeze-dried and assayed for AP.

To assay bread baked with AP, the ground bread (1g) was added to 2 mL of a solution of AA (600 mg) dissolved in a mixture of DMSO (45 mL) and 30% metaphosphoric acid (5 mL). Then, 48 mL of 90% aqueous DMSO was added, and the mixture stirred on a magnetic stir plate at 25°C for 0.5-5 hr. At different extraction times, an aliquot (2 mL) was removed by pipette, diluted to 10 mL with methanol, and the mixture analyzed by HPLC as previously described.

#### Determination of AA

AA was determined by the HPLC method described by Pachla and Kissinger (1979), except the mobile phase was 80 mM acetate buffer (pH 4.2) containing 0.1 mM EDTA and 1 mM Q8. A standard curve was derived as follows. Stock solutions of L-ascorbic acid (0.1-1.2 mg) were prepared in 3% aqueous metaphosphoric acid (10 mL) that had been freshly made and degassed. Immediately prior to injection, an aliquot (1 mL) of a stock solution was diluted 25-fold with cold

degassed 0.05 M perchloric acid, and 0.02 mL of the mixture containing 8 - 96 ng of AA was injected.

Blank bread was spiked with AA, and the recovery of AA was determined. To an Erlenmeyer flask (25 mL) was added freeze-dried bread (1 g) and an aliquot (0.5-5 mL) of a standard solution containing 0.1-1.2 mg of AA in freshly prepared 3% aqueous metaphosphoric acid. The volume of the liquid phase in all mixtures was adjusted to a total of 10 mL by adding 3% metaphosphoric acid. The mixtures were stirred at 25°C for 30 min, centrifuged, and an aliquot (1 mL) of the clear supernatant was pipetted into a 25 mL volumetric flask. Cold perchloric acid (0.05M) was added to volume, and a 0.02 mL aliquot was immediately injected into the chromatograph. The blank bread sample gave a small peak at R<sub>T</sub> 5.8 min, which was the same R<sub>T</sub> as that of added AA. The intensity of the peak at R<sub>T</sub> 5.8 min for bread spiked with AA was corrected for that peak in the blank sample.

Bread was baked from dough containing 63.8 mg of AA added per 100g flour. The bread was assayed for AA after storing 0, 1, 3, 5, and 10 days in polyethylene bags at 25°C. To assay bread for AA, freeze-dried bread was ground, and the ground bread (1 g) extracted with 10 mL of 3% aqueous metaphosphoric acid for 30 min at 25°C. After centrifugation, an aliquot (1 mL) of the clear supernatant was diluted to 25 mL volume with cold 0.05 M perchloric acid, and 0.02 mL of the resulting solution was injected into the chromatograph. The percentage of AA recovered was corrected for the amount of AA in the blank bread sample.

## RESULTS & DISCUSSION

### Loss of AP in phosphate buffer at pH 7

When AP (6.3 mg) was stirred in a mixture of 50 mL 0.1M phosphate buffer at pH 7 containing 0.5% sodium dodecyl sulfate and 6 mg bacterial  $\alpha$ -amylase (Mauro et al., 1979), HPLC-EC revealed that AP was unstable. This solvent is the same as the extraction/digestion medium recommended by Mauro et al. (1979), who extracted bread for 1 hr at 48°C with no filtration of the extract. Approximately 35% destruction of AP occurred under those conditions (Fig. 1). After 3 hr stirring, the loss of AP increased to approximately 80%. An exponential

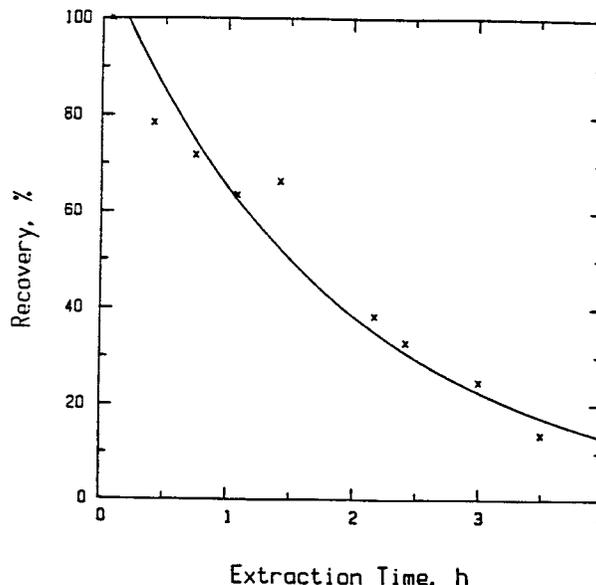


Fig. 1—Recovery (%) of L-ascorbyl 6-palmitate (AP) from a pH 7 phosphate buffer. AP (6.3 mg) was stirred in 50 mL of 0.1M aqueous phosphate buffer (pH 7) containing 250 mg sodium dodecyl sulfate and 6 mg  $\alpha$ -amylase at 48°C (Mauro et al., 1979), centrifuged, and an aliquot (0.02 mL) injected into the chromatograph. HPLC was done at 25°C using EC detection (+ 0.72 V) on a reverse-phase column with mobile phase a mixture (77/23; v/v) of methanol and 80 mM acetate buffer (pH 5) at 2 mL/min.

## DETERMINATION OF L-ASCORBYL 6-PALMITATE . . .

decay curve was fitted to the data giving % recovery =  $111.7e^{-0.54t}$ , where  $t$  = extraction time in hours. The half-life of AP was approximately 1.5 hr. Meanwhile, an unidentified peak began to appear as a shoulder on the AP peak after 30 min incubation (Fig. 2). The unknown peak ( $R_T$  6.3 min) intensified with extraction time up to 3.5 hr, while the AP peak ( $R_T$  7 min) decreased. Our chromatographic column contained 5  $\mu$ m particles and apparently provided improved resolution over the analytical system used by Mauro et al. (1979) who detected only a single peak with  $R_T$  the same as AP on a 10  $\mu$ m particle column (reverse phase).

The unknown peak in the chromatogram ( $R_T$  6.3 min) was probably L-ascorbyl 5-palmitate due to 6-5 *O*-acyl migration. Both peaks at  $R_T$  6.3 and 7 min in the 3.5-hr extract disappeared when the extract was stirred with charcoal (Fig. 2D). Charcoal treatment oxidizes the 2,3-ene diol in ascorbic acid and its 5- or 6-derivatives to the dehydro form of the compounds (Tolbert and Ward, 1982). The dehydro form of AA and its 5- or 6-palmitate ester would not be detected by HPLC-EC at + 0.72V. Mauro et al. (1979) reported a 95.5% recovery of AP in bread that had been spiked with the sodium salt of AP. However, their analytical system probably did not resolve the 5- and 6-acyl esters of AA. The instability of AP is not conducive for quantitative analysis of that derivative in the phosphate buffer extraction medium used by Mauro et al. (1979).

### New solvent system to extract AP from dough and bread

A new medium, in which AP is stable for at least 5 hr, was devised to extract AP from bread. The extracting solvent was 90% dimethylsulfoxide containing 0.12% metaphosphoric acid and 0.05% AA. Ninety-percent DMSO is a powerful solvent for starch, either in gelatinized or granular form (Killion and Foster, 1960). Furthermore, DMSO forms strong complexes with helical amylose (Simpson et al., 1972). Should AP complex with amylose in bread, as suggested by Maura et al. (1979), the large excess of DMSO in the extraction medium would dissociate the complex and release AP. Thus, enzymatic

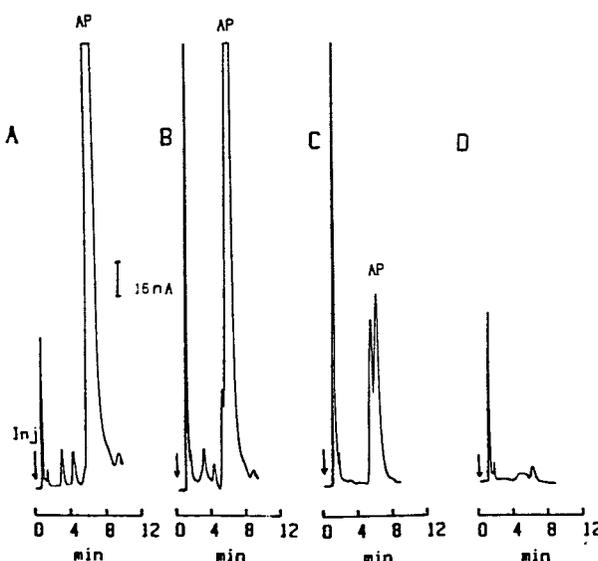


Fig. 2—Chromatograms of L-ascorbyl 6-palmitate (AP) stirred in a pH 7 phosphate buffer. A solution of AP (6.3 mg) in 50 mL of 0.1M phosphate buffer (pH 7), containing 250 mg sodium dodecyl sulfate and 6 mg  $\alpha$ -amylase was stirred at 48°C (Mauro et al., 1979). A, 5 min stirring; B, 1-hr stirring; C, 3.5-hr stirring; and D, extract C after charcoal treatment (150 mg charcoal per 10 ml extract and stirred 10 min at 25°C). After centrifugation, HPLC-EC assay was done as described in caption to Fig. 1.

digestion of the starch is not needed to dissociate complexed AP. It was further speculated that DMSO would dissociate AP should it complex with protein. Ascorbic acid and metaphosphoric acid were included in the new solvent to maintain AP in its reduced form.

Metaphosphoric acid is known to chelate metal ions that accelerate  $O_2$ -oxidation of AA (Bradley et al., 1973). Upon chelation, the metals are much less active in promoting oxidation of AA, and presumably AP. The level of AA in 50 mL of the extraction medium used in this work provided a 12 to 120 molar excess of AA over AP when 1g dry bread contained 0.5 mg to 5 mg of AP. The excess AA ensured that AP remained in its reduced form during assay. AA did not interfere with the resolution of AP during chromatography, since AA was eluted in the void volume of the reverse-phase column. Leung and Loewus (1985) have used dithiothreitol (0.1%) to prevent oxidation of AA to its dehydro form during HPLC-EC determination.

Fig. 3A shows the stability of AP when stirred in the DMSO-acid medium with and without AA (0.05%). Without AA, the intensity of the AP response was 4 to 9% lower than with AA, depending on extraction time. Furthermore, no apparent migration of the 6-acyl group on AP occurred in the DMSO-acid medium; only one peak at  $R_T$  7 min was observed over the entire 5 hr extraction period. Similar results were obtained when AP and bread were stirred together in the extraction medium with and without AA (Fig. 3B).

### Recovery of AP from bread baked with AP and extracted for different periods of time

Bread was baked from dough containing 0 and 0.15% AP, stored in polyethylene bags for 0 and 10 days and freeze-dried. The bread was extracted with the DMSO-acid medium with and without added AA, and the extraction period was varied from 0 to 5 hr. The concentration of AP in the extract rose during the first hour of extraction, then reached a plateau value (Fig. 4). Once again, slightly lower amounts of AP were recovered when the 1-hr extraction medium contained no AA. Chromatograms of the bread extracts are shown in Fig. 5. The blank bread gave no signal for AP (Fig. 5A), whereas AP was detected at  $R_T$  = 7 min in the extracts of blank bread spiked with AP (Fig. 5B) and from bread baked with AP (Fig. 5C). The AP peak was base-line resolved and had a detection limit of 2 ng AP in 0.02 mL of extract. From these results, an extraction period of 1-hr was used routinely for the determination of AP in bread. AA (0.05%) was used in our extraction medium because it improved recovery of AP by about 4% (Fig. 4).

### Recovery of AA from bread spiked with AA

Chromatograms typical of those used to determine AA in bread extracts are shown in Fig. 6. Extracts from blank bread when spiked with AA (0.4 mg/g of dry bread) showed a major peak eluting at 5.8 min with base-line resolution (Fig. 6C). AA could be detected as low as 1 ng in the 0.02 mL extract. The recovery of AA from spiked bread was quantitative when compared to a standard solution of AA in 3% metaphosphoric acid. When the extract of bread (C) was stirred with charcoal, the peak at  $R_T$  5.8 min disappeared (Fig. 6D). Surprisingly, blank bread (A) contained a small amount of a compound with  $R_T$  5.8 min (Fig. 6A), which disappeared upon treatment with charcoal (Fig. 6B). Spiking of the blank bread extract with AA increased the intensity of the peak at  $R_T$  5.8 min (Fig. 6C). If the unknown peak in blank bread was AA, the quantity would be equivalent to 40 ppm AA based on the original amount of formula flour or 0.04 mg/g flour. The 40 ppm does not take into account the loss of AA incurred during baking. Thus, the level of the unknown compound at  $R_T$  5.8 min in the dough might exceed 40 ppm.

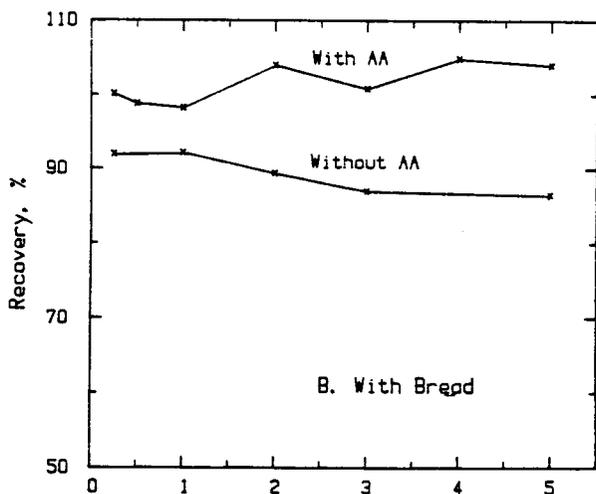
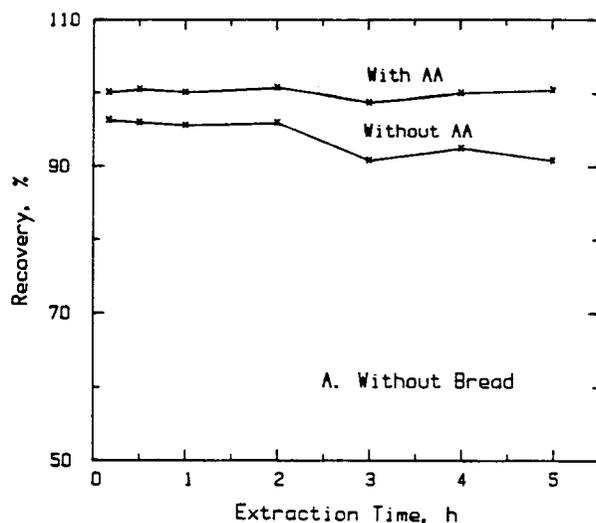


Fig. 3—Recovery of L-ascorbyl 6-palmitate (AP) from the DMSO-acid extraction medium with and without AA in the medium (A). The extraction medium was 90% DMSO containing 0.12% metaphosphoric acid with and without 0.05% L-ascorbic acid (AA). The initial concentration of AP in the medium was 1.5 mg/50 mL, and an aliquot of the DMSO-acid extract was diluted 5-fold with methanol prior to injection (0.02 ml) into the chromatograph. The standard error of the determination with 0.05% AA was  $\pm 0.6\%$ . Stirring time vs recovery of AP from spiked bread (B). A mixture of dry ground bread (1 g) and AP (1.5 mg) was stirred at 25°C in 50 mL of the extraction medium with and without 0.05% AA. The extracts were diluted 5-fold with methanol, and 0.02 mL of the clear supernatant was injected. HPLC-EC was done as described in the caption to Fig. 1. The standard error of the determination with 0.05% AA was  $\pm 2.7\%$ .

#### Standard curves for determination of AP or AA in bread

Figure 7 is an outline of the analytical procedures used to determine AP and AA in bread. The AP assay method gave a linear response when 0.06–0.48% AP (based on flour) was added to blank bread. The recovery of AP in the spiked bread was 99% compared to a standard curve with no bread. The recovery of AP in the HPLC-EC method exhibited a  $\pm 3\%$  coefficient of variation with five separate injections of the final methanolic solution containing 120 ng AP/0.02 mL, which is the theoretical concentration of AP expected in extracts of bread baked with 0.15% AP in the flour, provided that all AP survives breadbaking. A small amount of precipitation occurred when the

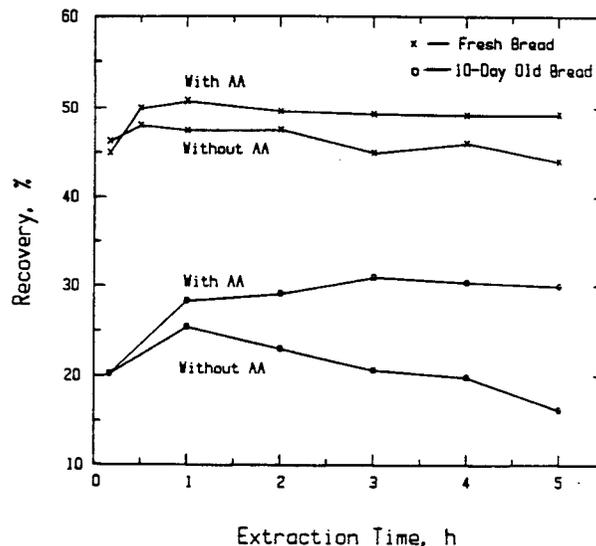


Fig. 4—Recovery of L-ascorbyl 6-palmitate (AP) from bread baked with AP in the dough. Bread was baked containing 63.8 mg AP/100 g flour. The bread was stored in polyethylene bags 0 and 10 days, then freeze-dried and ground. Ground bread was stirred 5 hr in the DMSO-acid medium with and without 0.05% AA, and the extracts diluted 5-fold with methanol prior to injection of the clear supernatant (0.02 mL). HPLC-EC was done as described in the caption to Fig. 1. The standard error in the AP assays for 1–5 hr with 0.05% AA was  $\pm 1.3\%$  and  $\pm 3.4\%$ , for 0 day and 10 day bread, respectively.

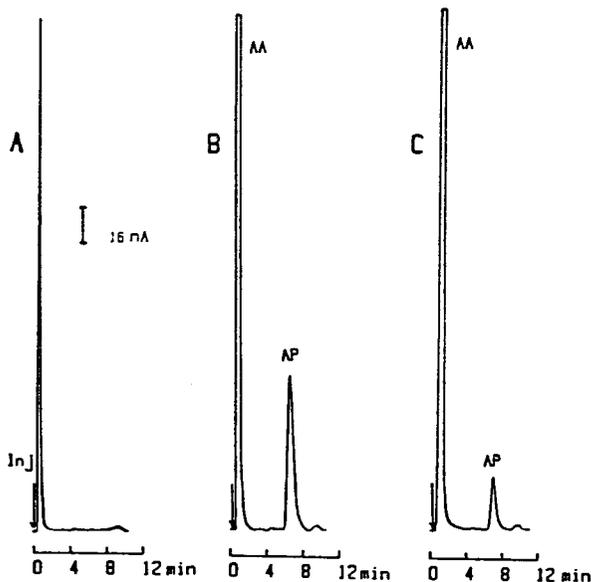


Fig. 5—HPLC-EC chromatograms of DMSO-acid extracts of bread made with and without L-ascorbyl 6-palmitate (AP). A, bread with no AP in the dough; B, bread A spiked with AP (2.4 mg/g freeze-dried bread); and C, bread baked with AP in the dough (initially 1.5 mg/g flour). Freeze-dried bread A (1g) was extracted 1 hr at 25°C with 50 ml of 90% DMSO containing 0.12% metaphosphoric acid, while breads B and C were extracted with the same medium plus 0.05% L-ascorbic acid. The extracts were diluted with 4 volumes of methanol, centrifuged, and an aliquot (0.02 mL) of the clear supernatant injected into the chromatograph. HPLC was done as described in the caption to Fig. 1.

DETERMINATION OF L-ASCORBYL 6-PALMITATE . . .

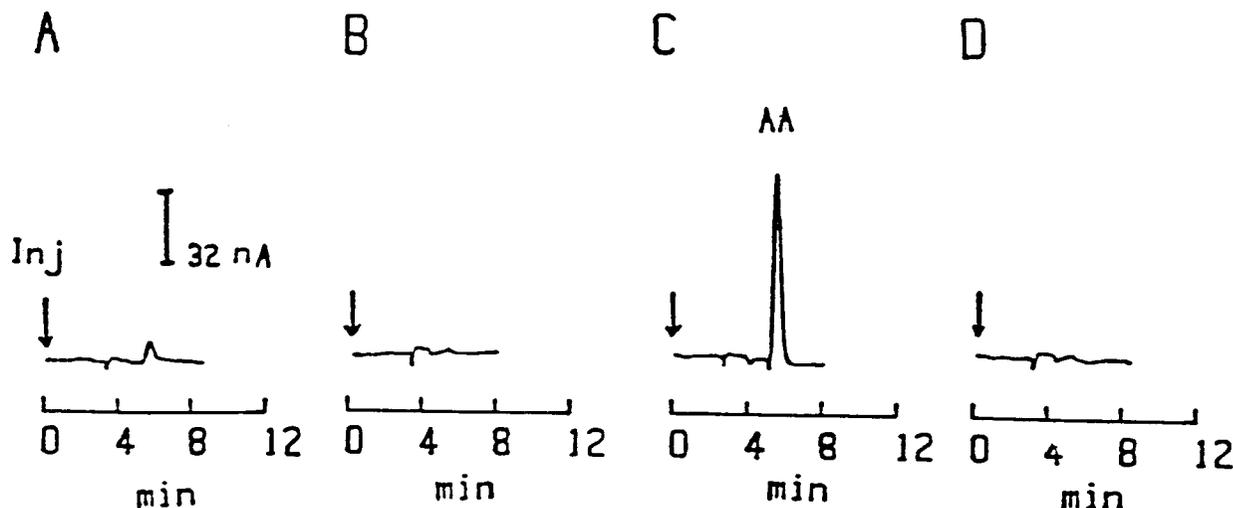


Fig. 6—Reducing substance(s) detected by HPLC-EC in bread extracts. A, Extract of straight-dough bread (0-day old); B, extract A stirred 10 min with charcoal (150 mg charcoal per 10 ml extract), then diluted with perchloric acid; C, extract of bread A spiked with L-ascorbic acid (0.4 mg/g of dry bread); and D, extract from bread C treated with charcoal prior to injection (150 mg charcoal per 10 ml extract for 10 min), then diluted with perchloric acid. Freeze-dried bread (1 g) was extracted 30 min at 25°C with 10 mL of 3% aqueous metaphosphoric acid. After centrifuging, a 1 ml aliquot of the supernatant was diluted to 25 mL with cold 0.05M perchloric acid, and an aliquot (0.02 mL) of the diluted mixture was injected immediately into the chromatograph. HPLC was done at 25°C using an EC detector (+ 0.72V) and an Alltech C-18 reverse-phase column with mobile phase (1 mL/min) of 80 mM acetate buffer (pH 4.2) containing 1 mM of octyltriethyl-ammonium phosphate (Q8) and 0.1 mM EDTA.

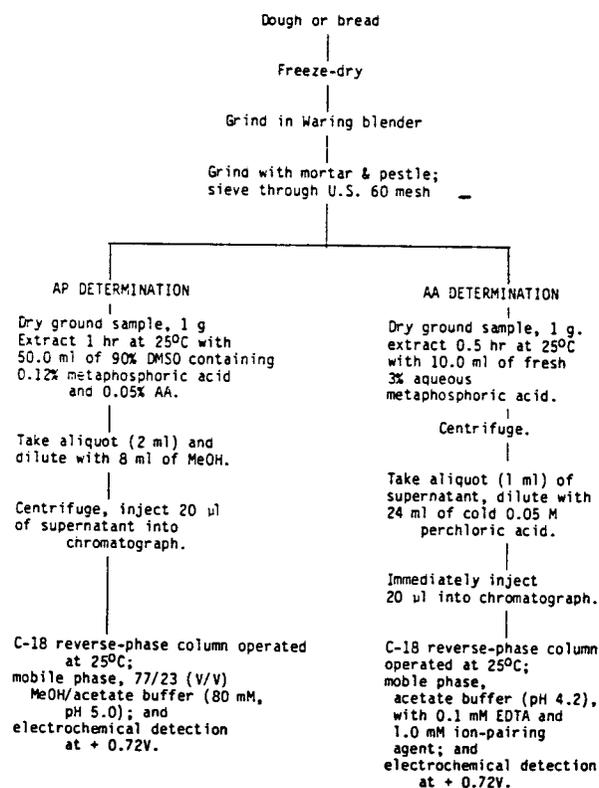


Fig. 7—Flow chart for the analytical determination of AP and AA in dough and bread.

DMSO-acid extraction medium was diluted five-fold with methanol. A methanol extract of the precipitate was shown to be free of AP by UV and by thin-layer chromatography. Presumably some inorganic phosphate salt precipitated in the methanolic solution.

Blank bread was also spiked with AA; its recovery was quantitative when compared to a standard curve. Once again, a linear response was observed when bread was spiked with 0.01–0.12% (based on flour) and assayed by HPLC-EC. The coefficient of variation of five injections of AA (96 ng/0.02 mL or 0.12% of AA in bread based on flour) was ± 0.8%.

Bread made from dough containing AP or AA

Bread was made containing 150 mg AP or an equimolar amount of AA (63.8 mg) per pup-loaf (100 g flour). Dough containing AP that had been fermented 3 hr, molded and proofed was found to have retained 72.9% AP, whereas the fresh bread retained 50.7% AP added to the flour. The loss of AA in the dough was not determined; 58.1% AA was retained immediately after baking. Thus, the loss of AP during mixing, fermenting, and proofing was 27% and the loss during baking was 22%. Perhaps, recovery of AP or AA in fresh bread would increase if the loaves were baked under less severe conditions than those used in the laboratory (218°C for 24 min). Furthermore, the ratio of surface area to crumb volume was higher in pup-loaves than in commercial-sized loaves. Since the loss of AP or AA would be high in crust, there might be higher survival of AP and AA in larger vs smaller loaves.

A previous report (Mauro et al., 1979) listed 78% recovery of AP in bread when 0.48% AP was added to the bread for-

Table 1—Retention (%) of L-ascorbyl 6-palmitate (AP) and L-ascorbic acid (AA) in dough, fresh bread and stored bread<sup>a</sup>

Sample	Age of bread, d	AP, initially 150 mg per pup loaf	AA, initially 63.8 mg per pup loaf
Dough	-	72.9	—
Bread	0	50.7 (58.7) <sup>b</sup>	58.1 (61.1) <sup>b</sup>
Bread	1	44.6	57.5
Bread	3	43.1 (51.6) <sup>b</sup>	35.3 (40.9) <sup>b</sup>
Bread	5	37.6 (45.2) <sup>c</sup>	20.2
Bread	10	28.3	0

<sup>a</sup> Bread held in polyethylene bags at 25°C.

<sup>b</sup> Values in parentheses are retention when AP was increased in a loaf from 150 mg to 480 mg, and AA was increased from 63.8 mg to 200 mg.

<sup>c</sup> Baked with 150 mg AP/pup-loaf, and stored 5 days under N<sub>2</sub> in a desiccator.

mula. When AP was increased from 0.15 to 0.48% in the present investigation, the recovery of AP in the fresh bread increased from 50.7% to 58.7% (Table 1). It seems likely that the recoveries reported in the present work were more accurate than those previously reported, since AP was stable during extraction from bread in the DMSO-acid medium and rearranged products might not have been resolved in the earlier study.

AP recovered from bread appeared to be predominantly in its reduced form, not its dehydro form. When 10-day old bread baked with 0.15% AP was stirred 1 hr in the new extraction medium with and without AA present, 28.3% and 25.4% of AP was recovered, respectively (Fig. 4, bottom two curves). Similar results were found for fresh bread (Fig. 4, top two curves), where 50.7% and 47.4% of AP was recovered, respectively, with and without AA present in the medium. If the bread contained substantial amounts of the dehydro form of AP, we assume the large excess of AA in the extraction medium would have increased the recovery of AP by more than 3-4%. The dehydro form of AA is not stable at pH 5-6 (Tolbert and Ward, 1982). White bread crumb has a pH ~ 5, which explains the low levels of the dehydro forms of AP and, presumably dehydro-AA, found in bread.

It is surprising that AA survived better in fresh bread than did AP. Figure 8 shows 58% retention of AA vs 51% for AP in 1 hr old bread. The remaining AA measured in bread was corrected for the unknown in the blank bread, which eluted at the same  $R_T$  as AA on the chromatogram (Fig. 6A).

The recovery of AA from bread increased from 58.1% to 61.1% when the level of AA in the formula was increased from 0.064% to 0.2% (640 to 2000 ppm, Table 1). The retention of over one-half of the added AA in freshly baked bread can be explained by the anaerobic conditions in the dough because of  $O_2$ -uptake by yeast (Seib, 1985). Elkassabany et al. (1979) used a fluorometric assay for AA and reported that less than 5% of AA (initially 100 mg per 100 g of flour) remained in a yeasted dough after mixing and 60 min fermentation. Quadri et al. (1975), using the fluorometric assay, found approximately a 10% retention of AA in bread (initial level of 77 mg per 100g flour). Apparently, the survival of AA during

breadmaking depends on the initial level of AA in the dough. In theory, the HPLC-EC method of determining AA in bread should give more accurate results than methods that do not separate and purify AA from the bread prior to quantitation. Recovery of AA from bread also depends on the length of bread storage (as discussed below), the method of drying the bread, and the method of extracting AA from the matrix material.

#### Loss of AP and AA in aged bread

Figure 8 shows the storage losses of AP and AA when the vitamin C-fortified breads were held in polyethylene bags at 25°C. The AA curve showed a rapid loss after 1 day, with no AA detected after 10 days. AP disappeared at approximately one-third the rate of AA, with approximately 30% retention after 10 days. AP most likely complexed with amylose during baking, so its accessibility to oxygen in the loaf was reduced. Linear regression equations were derived for the loss of AP and AA with storage time in bread. The equations were  $y = -2.1x + 48.8$  and  $y = -6.1x + 57.6$  for AP and AA, respectively, where  $y = \% \text{ retention}$  and  $x = \text{days stored at } 25^\circ\text{C}$ . AP was predicted to disappear from bread after 23 days at 25°C and AA after 9.4 days.

Figure 8 shows that baking with 0.15% AP in the formula gave approximately 9% of the daily adult RDA of vitamin C (ascorbic acid) in one slice of bread stored for 3 days, and approximately 6% RDA in bread stored for 10 days. In 1985, the cost of adding 0.15% AP (\$30 per kg AP) to a 1 pound (0.45 kg) loaf of white pan-bread was 1.4 cents. Recovery of AP from 5-day old bread increased from 37.6% to 45.2% when the bread was stored under nitrogen. Storage under  $N_2$  practically eliminated the loss of AP.

In conclusion, a new procedure for quantitative determination of AP in bread was developed that had several advantages over a prior method: (1) no  $\alpha$ -amylase digestion of bread crumb was needed; (2) the EC detector was about five times more sensitive than the UV detector; (3) AP was more stable in the new extraction medium (aqueous acid in 90% DMSO) than in the phosphate buffer (pH 7.0) used in the past; and (4) the ester group on the 6-hydroxyl of AP, in the new extraction medium, did not migrate or exchange with other hydroxyls on the ascorbate molecule as it apparently did in the phosphate buffer.

#### REFERENCES

- AACC. 1983. "Approved Methods of the AACC." Methods 44-19. American Association of Cereal Chemists. St. Paul, MN.
- Bradley, D.W., Emery G., and Maynard, J.E. 1973. Vitamin C in plasma: a comparative study of the vitamin stabilized with trichloroacetic acid or metaphosphoric acid and the effects of storage at  $-70^\circ$ ,  $-20^\circ$ ,  $4^\circ$  and  $25^\circ$  on the stabilized vitamin. *Clin. Chim. Acta* 44: 47.
- Brunt, K. and Bruins. C.H.P. 1979. Evaluation of the characteristics of the differential amperometric detector in combination with anion-exchange chromatography, using L-ascorbic acid as test compound. *J. Chromatogr.* 172: 37.
- Cort, W.M. 1974. Antioxidant activity of tocopherols, ascorbyl palmitate and ascorbic acid and their mode of action. *J. Am. Oil Chem. Soc.* 51: 321.
- Elkassabany, M., Hoseney, R.C., and Seib, P.A. 1979. Ascorbic acid as an oxidant in wheat flour dough. I. Conversion to dehydroascorbic acid. *Cereal Chem.* 57: 85.
- Finney, K.F. and Barmore, M.A. 1943. Yeast variability in wheat variety test baking. *Cereal Chem.* 20: 194.
- Grün, M. and Loewus, F.A. 1983. Determination of ascorbic acid in algae by high-performance liquid chromatography on strong cation-exchange resin with electrochemical detection. *Anal. Biochem.* 130: 191.
- Hoseney, R.C., Seib, P.A., and Deyoe, C.W. 1977. Use of salts of 6-acyl esters of L-ascorbic and D-isoascorbic acids in breadmaking. *Cereal Chem.* 54: 1062.
- Killion, P.J. and Foster, J.F. 1960. Isolation of high molecular weight amylose by dimethylsulfoxide dispersion. *J. Polymer Sci.* 46: 65.
- Koch, R.C., Seib, P.A., and Hoseney, R.C. 1987. Use of ascorbyl-6-palmitate in breadmaking. *J. Food. Sci.* In press.
- Leung, C.T. and Loewus, F.A. 1985. Concerning the presence and formation of ascorbic acid in yeast. *Plant Sci.* 38: 65.
- Mauro, D.J., Wetzell, D.L., Seib, P.A., and Hoseney, R.C. 1979. Determination of a surfactant (sodium 6-O-palmitoyl-L-ascorbate) in bread by high performance liquid chromatography. *Cereal Chem.* 56: 152.

—Continued on page 974

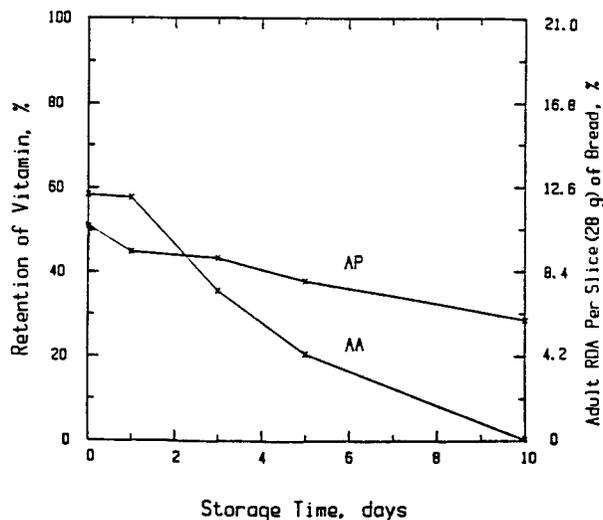


Fig. 8—Retention of L-ascorbic acid and L-ascorbyl 6-palmitate in bread stored in polyethylene bags at 25°C. The bread formulas, based on 100g of flour (14% moisture), contained 63.8 mg L-ascorbic acid or 150 mg L-ascorbyl 6-palmitate. The RDA of vitamin C (L-ascorbic acid) per slice was calculated based on bread containing 32% moisture, an RDA of 60 mg L-ascorbic acid per day, and assuming no dehydro form of the vitamin was present.

**DETERMINATION OF L-ASCORBYL 6-PALMITATE. . .From page 953**

---

Pachla, L.A. and Kissinger, P.T. 1979. Analysis of ascorbic acid by liquid chromatography with amperometric detection. *Methods Enzymol.* 62: 15.  
Quadri, S.F., Liang, Y.T., Seib, P.A., Deyoe, C.W., and Hosney, R.C. 1975. Stability of L-ascorbic acid in wheat foods and milk. *J. Food Sci.* 40: 837.  
Seib, P.A. 1985. Oxidation, monosubstitution and industrial synthesis of ascorbic acid. *Int. J. Vit. Nutr. Res. Supplement* 27: 259.  
Simpson, T.D., Dintzis, F.R., and Taylor, N.W. 1972. A V<sub>7</sub> conformation of dimethyl sulfoxide-amylose complex. *Biopolymers* 11: 2591.  
Tolbert, B.M. and Ward, J.B. 1982. Dehydroascorbic acid. Ch. 5, p. 101. In "Ascorbic Acid: Chemistry, Metabolism, and Uses." Seib, P.A. and Tolbert, B.M. (ed). American Chemical Society, Washington, DC.  
Ms received 3/14/86; revised 8/23/86; accepted 9/19/86.

---

Cooperative investigations between the Agricultural Research Service, U.S. Department of Agriculture, and the Kansas Agricultural Experiment Station, Manhattan, KS. Contribution No. 86-337-J. Departments of Grain Science and Industry and Biochemistry, Kansas Agricultural Experiment Station, Manhattan, KS 66506.

The authors thank Benjamin Borenstein and Howard Gordon of Hoffmann-La Roche, Inc. for providing L-ascorbyl 6-palmitate. We are also grateful to Yangsheng Wu for baking the bread, and to Robert W. Schrader and Ross Industries, Cargill, Inc., Wichita, KS for providing the flour.

Mention of a proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

---