DETERMINATION OF L-ASCORBYL 6-PALMITATE IN BREADMAKING USING REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) WITH ELECTROCHEMICAL (EC) DETECTION

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1986

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LIST OF ABBREVIATIONS

AA	L-Ascorbic acid
AP	L-Ascorbyl 6-lpalmitate
DMSO	Dimethylsulfoxide
EC	Electrochemical
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
RDA	Recommended daily allowances
UV	Ultraviolet

INTRODUCTION

L-Ascorbyl 6-palmitate (AP) is a GRAS food additive used to preserve oils and to emulsify carotene-based food colors (CFR, 1983). AP has useful surfactant and antioxident properties in breadmaking, as well (Hoseney et al 1977). AP strengthens bread dough, increases loaf-volume, improves crumb grain, and replaces shortening. Furthermore, AP (0.38%, based on flour), like sodium stearoyl 2-lactylate (SSL), softens bread crumb more effectively than a mixture of distilled monoglycerides (0.5%) and shortening (3%) (Koch, 1981).

From a nutritional standpoint, L-ascorbyl 6-palmitate has vitamin C potency equivalent to L-ascorbic acid (AA) in guinea pigs (Inagaki et al 1968) and in humans (De Ritter et al 1951). The Food and Drug Administration (FDA, 1979) proposed AP as a form of vitamin C in over-the-counter drugs; however, to the author's knowledge no final action by the commissioner of FDA has been published. Besides providing vitamin C activity, AP also may enhance iron absorption by tissues from bread. Morck et al (1980) used the dual iron isotopic assay in humans to show that, compared to control bread, approximately a 25% increase in iron absorption occurred from white bread that had been baked with 0.4% AP (flour-weight basis). The increase was just below a significance level of $p = 0.001$ (Morck 1985).

Incorporating L-ascorbyl 6-palmitate in bread.

L-Ascorbyl 6-palmitate can be incorporated in bread dough by addition of premixes of AP with other lipids (Koch 1981). The

premixes were prepared by one of four ways: (1) dispersing 8 - 13 parts of AP in 100 parts of vegetable oil or plastic shortening, (2) premelting AP (17 parts) in shortening (100 parts) at 120°C and allowing the mixture to solidify, (3) mixing at high speed 90 parts of AP with 10 parts of mono-diglyceride or SSL, and (4) mixing AP into flour at 20,000 rpm. Furthermore, one experiment indicated a water-dispersion of AP can be added directly to a bread formula, if the emulsified particles of AP are less than 240 µm. On the other hand, Koch (1981) found commercial samples of AP suspended in water were not fully effective in dough. Dark specks on the crust of bread occurred that were probably due to large particles of AP not incorporated into the dough (Koch 1981). Cantrell et al (1980) reported use of a water-dispersion of AP in commercially produced hamburger buns.

Koch (1981) demonstrated the shortening-sparing action of AP in white bread. A preblend of 0.25 g of AP in 1.0g of shortening/ 100 g of flour or 0.38 g in 1.0 g of soy oil was found to be as effective as 3 g of a non-emulsified shortening. A premix of 0.38 g AP with 3.0 g of soy, peanut, cottonseed, or corn oil gave excellent bread. Furthermore, it was possible to replace all shortening or oil in bread using a 9:1 premix of AP with a hydrophilic surfactant. At the optimum level of 0.38% for the 9:1 premix of AP and monoglyceride, grain and volume of bread were equal to that of bread made with 3% shortening.

Assay for L-ascorbyl 6-palmitate in bread.

Mauro et al (1979) devised a 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. The extraction medium which they used consisted of 50 ml 0.1 M aqueous phosphate buffer (pH 7.0), 250 mg sodium dodecyl sulfate and 6 mg α-amylase. The aqueous phosphate buffer at pH 7.0 favored the migration of 6-acyl group to form L-ascorbyl 5-palmitate.

We report here an improved assay for AP in bread using high-performance liquid chromatography with electrochemical detection (HPLC-EC). The new procedure has several advantages over the prior one; (1) no α-amylase digestion of bread crumb was needed; (2) the EC detector was about 5X more sensitive than the UV detector; (3) AP was more stable in the new extraction medium (DMSO-acid) than in the phosphate buffer (pH 7.0) used in the past, and (4) in the new extraction medium the ester group on the 6-hydroxyl of AP did not migrate or exchange with other hydroxyls on the ascorbate molecule as they apparently did in the phosphate buffer. We have used the new procedure to compare the stability of AP to that of L-ascorbic acid (AA) in freshly made and aged bread.

EXPERIMENTAL

Materials.

L-ascorbyl 6-palmitate (AP, m.p. 116-117^{o}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) cocktail 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).

High performance liquid chromatography (HPLC).

The HPLC system consisted of a 6000A solvent delivery system (Waters Associates, Inc., Milford, Mass), 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 (20 µl, 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 x 4.6 mm, stainless steel, Sulpelcosil LC-18, particle size 5 µm; Supelco, Inc., Bellefonte, PA). AA was determined also on a reverse-phase column (Alltech C-18, 250 x 4.6 mm, particle size 5 µm). The analytical columns were protected by a precolumn with a 40 x 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 5.0). The mobile phase for AA assay contained

80 mM acetate buffer (pH 4.2), 0.1 mM disodium ethylenediaminetetraacetate and 1.0 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 to integrated areas of known amounts of the standard compound, while those of AA were done by comparing peak height values.

Stability of the sodium salt of AP in phosphate buffer (pH 7.0) at 48°C.

Sodium L-ascorbyl 6-palmitate (SAP) was prepared as reported 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 0.1 M phosphate buffer (pH 7.0) containing sodium dodecyl sulfate (250 mg) and 6 mg of α-amylase (Baccilus substilus, Sigma Chemical Co., St. Louis, MO). The mixture was shaken gently in a water bath at 48°C, and 20 µl of the mixture was injected directly into the chromatograph at various period of times.

Breadmaking.

Pup-loaves were made according to the procedure of Finney and Barmore (1943). The full-formula dough contained the following ingredients : flour, 100 g (14% m.b.); water, 66 g; sucrose, 6 g; non-fat dry milk, 4 g; soy oil, 2 g; instant dry

yeast, 0.72 g; sodium chloride, 1.5 g; enzyme-active malt (240°L), 0.5 g; and potassium bromate, 1 mg. Immediately prior to mixing, AP (150 mg per dough) was preblended with soy oil (2 g), and the mixture added to 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 (5.0 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, while all others were baked for 24 min at 218°C. After baking, loaf volume and weight were recorded. Loaves were cooled 1 hour under ambient room conditions and stored 0, 1, 3, 5, and 10 days at 25° in a polyethylene bag (0.038 mm thickness). Immediately after storage the bread was freeze-dried, ground in a Waring blender, and then stored at -20°C until assay. The moisture of all pup-loaves in this study was 31 - 33%, while freeze-dried bread was 1.5 - 4.0% (AACC 1983). Prior to assay, the bread was ground with a mortor and pestle to pass a U.S. No. 60 wire-mesh screen.

Determination of L-ascorbyl 6-palmitate.

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, 45.0 ml) and freshly prepared 30% aqueous metaphosphoric acid (5.0 ml) containing L-ascorbic acid (AA, 600 mg). A 2 ml aliquot of an AP stock solution was diluted to 50 ml with 90% aqueous DMSO, and the mixture stirred 10 min at 25°C. A 2 ml aliquot of the 90%

DMSO solution was pipetted into a 10 ml volumetric flask, and methanol was added to volume. After mixing, a small amount of white precipitate was removed by centifugation (15,000 rpm, Eppendorf Model 5412 Microcentrifuge, Brinkmann Instruments Inc., Westbury, NY), and 20 µl of the clear supernatant was injected into the chromatograph. The white precipitate was found to contain no AP by mixing with a small amount of methanol followed by UV spectroscopy and thin-layer chromatography of the methanol extract on silica gel. The thin-layer plate was developed with a mixture of $CHCl_3$ and CH_3OH (80:20; V/V) and visualized by spraying with 50% aqueous sulfuric acid and charing 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. To the freeze-dried bread (1 g) was added a 2 ml aliquot of an AP stock solution prepared as described in the previous paragraph. Then, 48 ml of 90% DMSO in water was added, and the mixture stirred on a magnetic stir plate at 25° for 0.5 - 5.0 h. After different extraction periods, an aliquot (2 ml) was pipetted into a volumetric flask (10 ml) and made to volume with methanol. The mixture was centrifuged and 20 µl of the clear supernatant, which contained 48, 72, 96, 192, or 384 ng of AP, was injected into the chromatograph using the loop injector.

Bread was baked from dough containing 150 mg of AP per 100 g 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 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, freeze-dried bread (1 g) was ground by mortor and pestle to pass a 60-mesh screen. To the ground bread was added 2 ml of a solution of AA (600 mg) dissolved in a mixture of DMSO (45.0 ml) and 30% metaphosphoric acid (5.0 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 h. At different extraction times, an aliquot (2 ml) was removed by pipette, diluted with four volumes of methanol and the mixture treated as previously described.

Determination of L-ascorbic acid.

AA was determined by the HPLC method described by Pachla and Kissinger (1979), except using a modified mobile phase consisting of 80 mM acetate buffer (pH 4.2), 0.1 mM EDTA and 1.0 mM octyltriethylammonium phosphate. 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 prepared 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 20 µl of the mixture containing 8 - 96 ng of AA was injected.

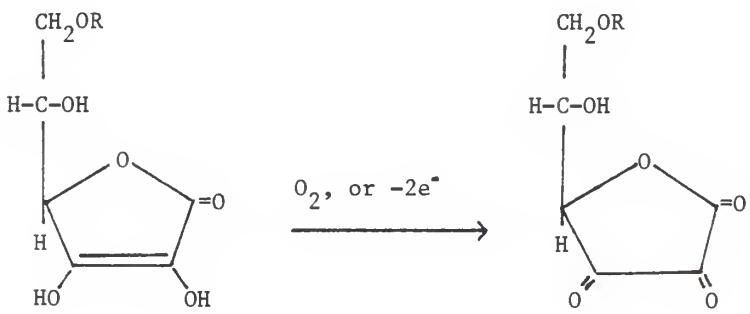
Blank bread was spiked with AA, and the recovery of AA was determined. To an erlenmeyer flask (25 ml) was added freeze-

dried bread (1.0 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 room temperature for 30 min, centrifuged, and an aliquot (1 ml) of the clear supernatant was pipetted into a 25 ml volumetric flask. Cold perchloric acid (0.05 M) was added to volume, and a 20 µl aliquot was immediately injected into the chromatograph. The blank bread sample gave a peak at the retention time (R_T) = 5.8 min, which was the same as that of added AA. The intensity of the peak at R_T = 5.8 min for bread spiked with AA was corrected for the AA in the blank sample.

Bread was baked from dough containing 63.8 mg of AA added per 100 g 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 20 µl 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 AND DISCUSSION

Electrochemical (EC) detection of L-ascorbic acid (AA) and its derivatives is known to be approximately 5X more sensitive than UV detection (Brunt and Bruins 1979; Grün and Loewus 1983). In crude extracts, amperometric (electrochemical) analysis gives fewer signals from interfering substances than UV detection. For those reasons, we decided to use HPLC-EC to follow the survival of L-ascorbyl 6-palmitate (AP) and L-ascorbic acid (AA) in bread. The oxidation of AA and AP by the EC detector occurred as shown below.



R = H; L-ascorbic acid
R = $CO(CH_2)_{14}CH_3$; L-ascorbyl 6-palmitate

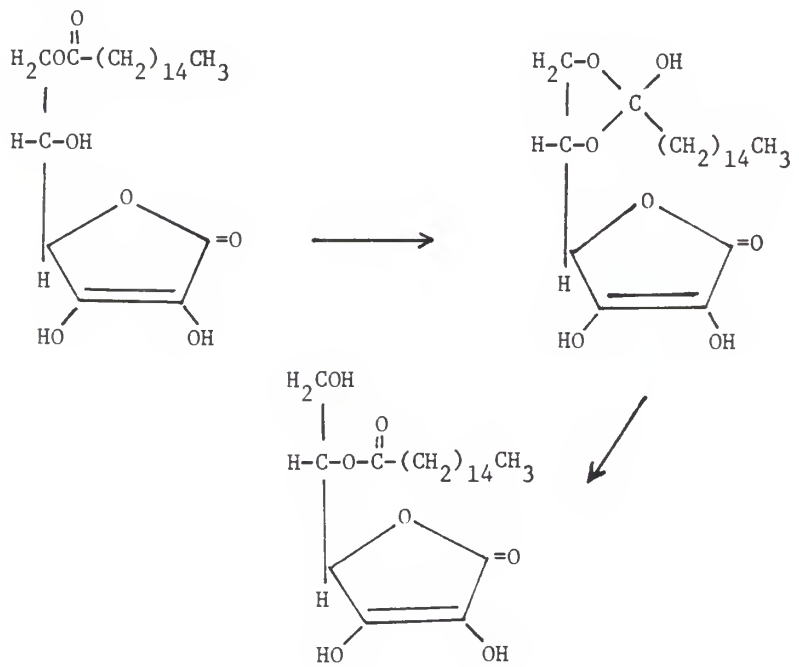
R = H; dehydro ascorbic acid
R = $CO(CH_2)_{14}CH_3$; dehydro ascorbyl 6-palmitate

Loss of AP in phosphate buffer at pH 7.0.

When we stirred AP (6.3 mg) in the extraction medium containing 50 ml of 0.1 M phosphate buffer (pH 7.0), 0.5% sodium dodecyl sulfate and 6 mg bacterial ∝-amylase (Mauro et al 1979), HPLC-EC showed AP was unstable in the extraction medium. Using the extraction/digestion conditions recommended by Mauro et al

(1979) of 1 h at 48° with no filtration of the extraction mixture, we found approximately 35% destruction of AP after one hour stirring (Fig. 1). After 3 h stirring, the loss of AP increaseed to approximately 80%. Meanwhile, an unidentified peak began to appear as a shoulder on the AP peak after 30 min extraction (Fig. 2). The unknown peak (R_T 6.3 min) intensified with extraction time up to 3.5 h, while the AP peak (R_T 7.0 min) decreased. Mauro et al (1979) found only one peak for AP after similar treatment using a reverse-phase column (10 µm particle size) developed with a mixture of methanol and water (75:25; V/V). Our separating system which utilized 5 µm particles provided better resolution.

It appears that the unknown peak is due to acyl migration to form L-ascorbyl 5-palmitate. A mechanism for 6-O to 5-O acyl migration is shown below.



Both peaks at retention time (R_T) 6.3 and 7.0 min in the 3.5-h extract disappeared when the extract was stirred with charcoal (Figs. 2C and 2D). Charcoal treatment is known to oxidize 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 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-acylated derivatives. The instability of AP in the phosphate buffer extraction medium may lead to errors without careful timing of the assay.

New solvent system to extract AP from dough and bread.

We devised a new medium to extract AP from bread in which AP is more stable. The new medium is a 498/2 (V/V) mixture of 90% aqueous dimethylsulfoxide (DMSO) and 30% aqueous metaphosphoric acid containing 12% L-ascorbic acid (AA), or in other words, 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 hypothesized by Mauro et al (1979), the large excess of DMSO in the extraction medium would dissociate the complex and release AP into the medium. Thus, no enzymatic digestion of the starch is needed to dissociate complexed AP. Meanwhile, DMSO has lower dielectric constant than water. Ascorbic acid and metaphosphoric

acid were included in the new solvent medium to maintain AP in its reduced form.

After extraction of bread or dough, the DMSO-acid extract was mixed with four volumes of methanol to precipitate starch and other polymers. Centrifugation gave a clear alcoholic supernatant in which AP was soluble (Cort 1974). The clarified supernatant was then chromatographed on the HPLC-EC system.

Metaphosphoric acid is known to chelate metal ions that accelerate O_2-oxidation of AA (Bradley et al 1973). After chelation, the metals are much less active in promoting oxidation of AA, and presumably AP. The level of AA in the extraction medium used in this work provided a 12 to 120 molar excess of AA over AP when 1 g of dry bread containing 5 mg to 0.5 mg of AP was extracted with 50 ml of medium. The excess AA insured 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 approximately 4 percentage points 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 7.0 min was observed over the entire 5 h extraction period. Similar results

were observed 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 breads were extracted with the DMSO-acid medium with and without added AA, and the extraction period was varied from 0 to 5 h. The concentration of AP in the extract rose during the first hour of extraction, then reached a plateau (Fig. 4). Once again, slightly lower concentrations of AP were detected when the 1-h extraction medium contained no AA. Chromatograms of the bread extracts are shown in Fig. 5. The blank bread gave no signal at R_T 7.0 min, where AP was eluted in the extracts of blank bread spiked with AP and from bread baked with AP. The AP peak was base-line resolved and had a detection limit of 2 ng AP in the 20 µl of extract. From these results, we set an extraction period of 1-h for the determination of AP in bread. AA (0.05%) was used in our extraction medium because it improved recovery of AP by 3 - 4 percentage points (Fig. 4). In fresh bread, the maximum recovery was 50%; in 10 day old bread 30%.

Recovery of AA from bread spiked with AA.

Chromatograms typical of those used to determine AA in bread extracts (3% aqueous metaphosphoric acid for 0.5 h) are shown in Fig. 6. Blank-bread extracts 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

20 µl of 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 a 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 is AA, the quantity is equivalent to 40 ppm AA (4 mg/100 g flour) based on the original amount of formula flour. The 40 ppm does not take into account the loss of AA incurred during baking. Thus, the level of the unknown material at R_T 5.8 min in the dough may exceed 40 ppm.

Standard curves for determination of AP or AA in bread.

Bread containing no AP was spiked with three levels of AP, and the spiked bread extracted at 25°C in 90% DMSO containing 0.12% metaphosphoric acid and 0.05% AA. After 1 hour stirring, the mixture was diluted with 4 volumes of methanol, and the clarified mixture injected into the chromatograph. Fig. 7 shows that the 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 showed a coefficient of variation of ± 3% with 5 separate injections of the final methanolic solution containing 120 ng AP/20 µl, which

is equivalent to 0.15% AP in the flour if all AP survived breadbaking. A small amount of precipitation occurred when the DMSO-acid extraction medium was diluted 5-fold with methanol. The precipitate was shown to be free of AP by UV and by thin-layer chromatography. Presumably some inorganic phosphate salts precipitated in the methanolic solution.

Blank bread was also spiked with AA and its recovery was quantitative when compared to a standard curve. Fig. 8 shows the linear response of the HPLC-EC determination when bread was spiked with 0.01 - 0.12% (based on flour). The coefficient of variation of 5 injections of AA (96 ng/20 µl, or 0.12% of AA in bread based on flour) was $\pm$ 0.8%.

Fig. 9 shows a flow chart of the analytical procedures for the determination of AP and AA in bread.

Bread made from dough containing AP or AA.

Bread was made containing 150 mg AP or an equivalent amount of AA (63.8 mg) per pup-loaf (100 g flour). Dough containing AP that had been fermented 3 h, moulded, and proofed was found to have retained 72.9% of AP, while the fresh bread retained 50.7% of the AP added to the flour. The loss of AA in the dough was not determined; 58.1% of 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 condition than used in the laboratory (218^{o}C for 24 min). Furthermore, the surface area to

crumb volume is higher in pup-loaves than in commercial-sized loaves. Since the loss of AP or AA would be high in crust, one might expect higher survival of AP and AA in large vs small loaves.

A previous report (Mauro et al 1979) gave 78% recovery of AP in bread when 0.48% AP was added to the bread formula. When we increased AP in our formula 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), which were equal to 45 mg and 180 mg, respectively, of AP in 100 g of fresh bread. It seems likely that the recoveries reported in this work are more accurate than those previously reported, since AP is more stable in the DMSO-acid extraction medium and rearranged products might not have resolved in the earlier study.

AP recovered from bread is predominantly in its reduced form, and not its dehydro-form. When 10-day old bread baked with 0.15% AP was stirred 1 h 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, then the large excess of AA in the extraction medium would have increased the recovery of AP by more than the 3 - 4 percentage points observed.

It is surprising that AA survived better in fresh bread than

did AP. Fig. 10 shows 58.1% retention of AA vs 50.7% for AP in baked bread that had been cooled one hour. The AA remaining in bread did not include the unknown in the blank bread which eluted on the chromatogram with the same R_T as AA (Fig. 6A). The intensity of the peak for AA in bread baked with added AA was corrected for the intensity of the peak in the blank bread.

The recovery of AA from bread was 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 survival of over one-half of the added AA in freshly baked bread can be explained by the anaerobic conditions in the dough due to O_2-uptake by yeast (Seib 1985). ElKassabany et al (1979) used a fluorescence spectroscopic assay for AA and reported that less than 5% retention of AA (initially 100 ppm based on flour) remained in a yeasted dough after mixing and 60 min fermentation. Quadri et al (1975) used the fluorometric assay procedure and found approximately a 10% retention of AA in bread (initial level of 77 mg in pup-loaf). Apparently, the survival of AA during bread making 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 method of drying the bread, the length of bread storage (as discussed below), and the method of extracting AA from the matrix material.

Loss of AP and AA in aged bread.

Fig. 10 shows the storage losses of AP and AA when the

vitamin C breads were held in polyethylene bags at 25°. The AA curve showed a rapid loss of AA after 1 day, with no AA detected after 10 days. AP disappeared at approximately one-third the rate of AA, and gave approximately 30% retention after 10 days. AP most likely complexed with amylose during baking, and 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 = % retention and x = days stored at 25°. AP was predicted to disappear from bread after 23 days at room temperature and AA after 9.4 days.

Fig. 10 also shows that baking with 0.15% AP in the formula gave approximately 9% of the adult RDA of vitamin C in one slice of bread stored for 3 days, and approximately 6% RDA in bread stored 10 days. In 1985, the cost of adding 0.15% AP ($ 30 per Kg AP) to a one-pound 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 (Table 1). Storage under N_2 practically eliminated the loss of AP.

ACKNOWLEDGEMENTS

The author wishes to express her great appreciation to Dr. Paul A. Seib, major professor, for his guidence, encouragement and advice in the completion of this work. Appreciation is extended to Dr. Karl J. Kramer of U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture for providing invaluable help and facilities for the project, and for being on the author's graduate committee. The author also extends her sincere appreciation to Dr. Keith C. Behnke for being on the graduate committee and for reviewing this thesis.

Dr. Charles W. Deyoe, Head, Department of Grain Science and Industry, is also gratefully acknowledged for providing funds needed to carry out this work. Dr. Doreen Liang, Mr. YangSheng Wu, and Mrs. Debi Rogers are thanked for their assistance on many occasions.

In addition, the author wishes to express her deep thanks to her parents, and her husband for their boundless suport, encouragement, and understanding through these years of study.

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Table 1. Retention (%) of L-ascorbyl 6-palmitate (AP) and L-ascorbic acid (AA) in dough, fresh bread and stored bread[a].

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

[a]Bread held in polyethylene bags at ambient conditions.

[b]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.

[c]Baked with 150 mg AP/pup-loaf, and stored 5 days under N_2 in a desiccator.

Fig. 1. Recovery (%) of AP in the extraction medium used by Mauro et al (1979). AP (6.3mg) was stirred in 50 ml of 0.1 M aqueous phosphate buffer (pH 7.0) containing 250 mg sodium dodecyl sulfate and 6 mg α-amylase at 48^{o}C, centrifuged, and aliquot (0.02 ml) injected into the chromatograph. HPLC was done at 25^{o} 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 5.0) at 2 ml/min.

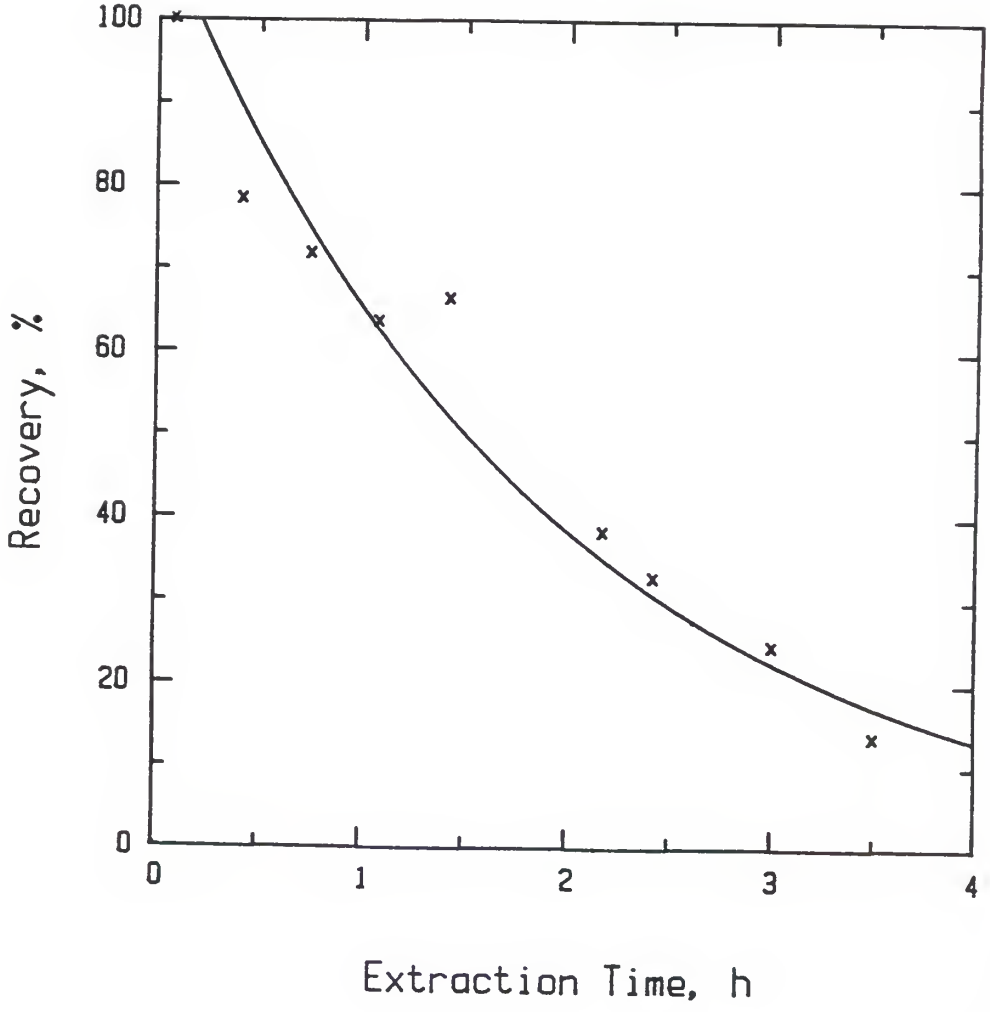

100
80
60
40
20
0
Recovery, %
0
1
2
3
4
Extraction Time, h

Fig. 2. HPLC-EC assay of AP (6.3 mg) stirred in the extraction medium of Mauro et al (1979) containing 50 ml of 0.1 M phosphate buffer (pH 7.0), 250 mg sodium dodecyl sulfate and 6 mg α-amylase at 48°C. (A). 5 min stirring; (B). 1-h stirring; (C). 3.5-h stirring; and (D). extract (C) after charcoal treatment (150 mg charcoal per 10 ml extract and stir 10 min at 25°C). After centrifugation, HPLC-EC assay was done as described in caption to Fig. 1.

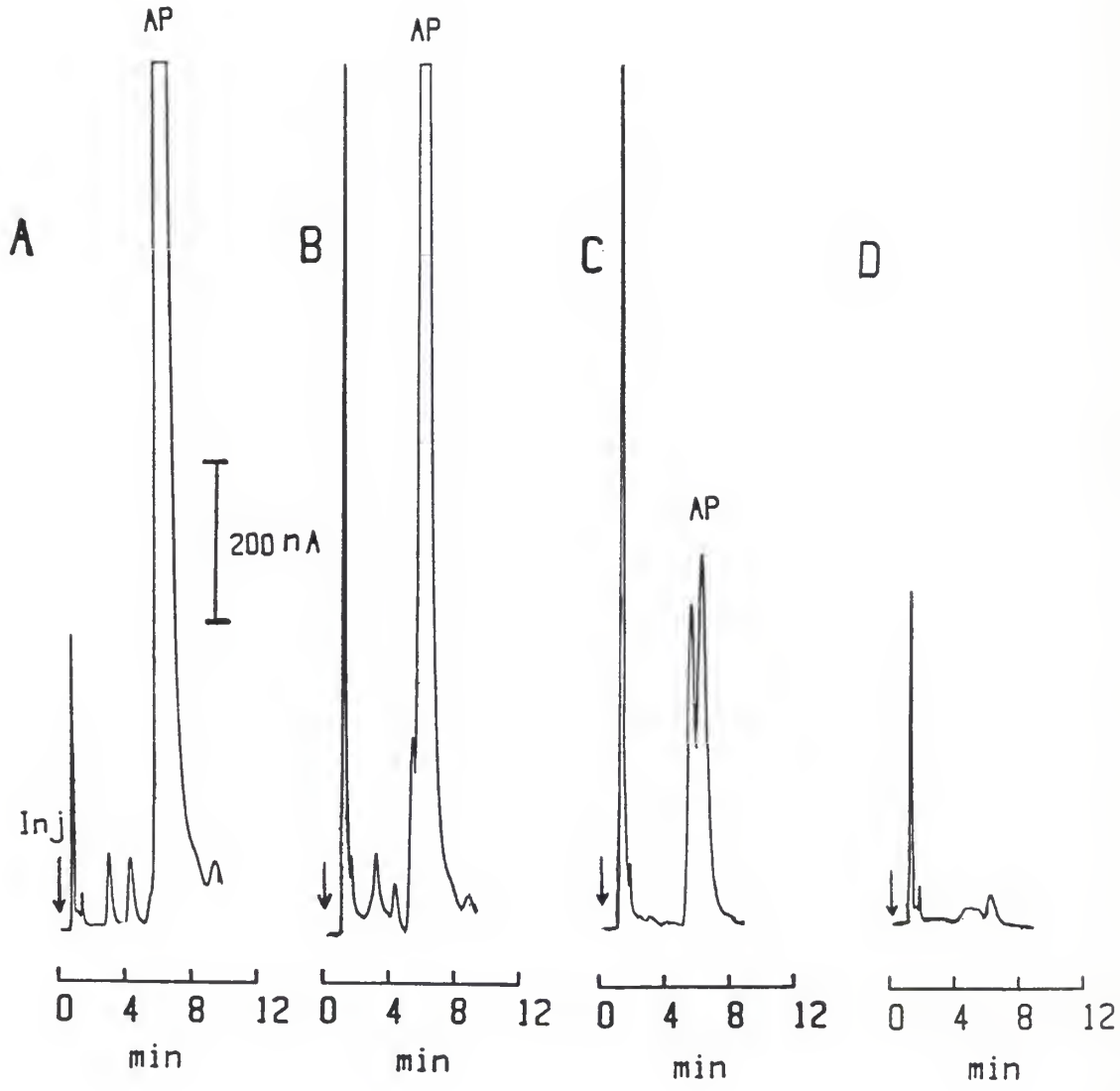
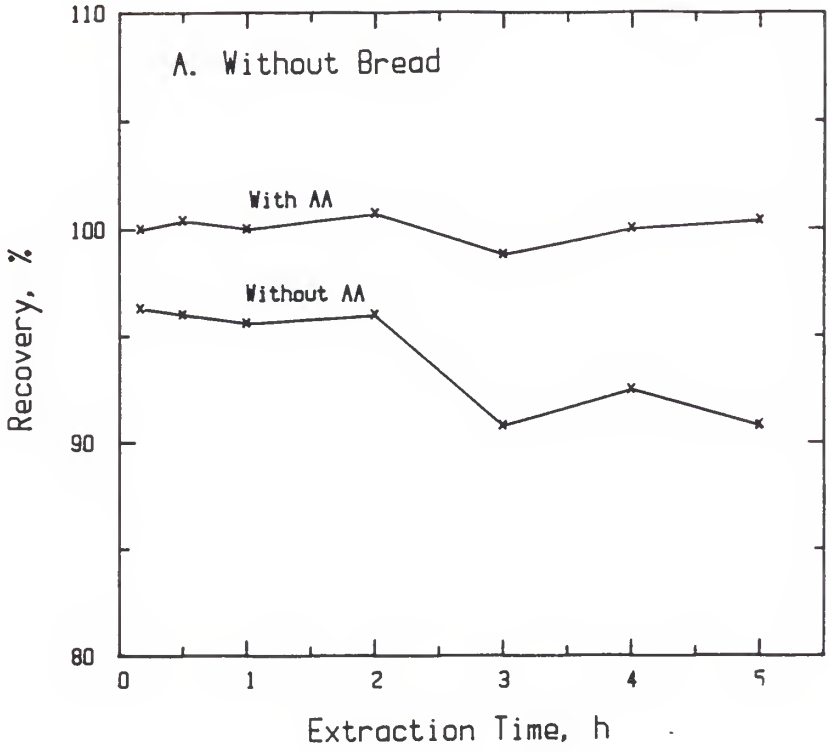

AP
AP
AP
A
B
C
D
200 nA
Inj
0 4 8 12
min
0 4 8 12
min
0 4 8 12
min
0 4 8 12
min

Fig. 3. (A). Recovery of AP from the DMSO-acid extraction medium (90% DMSO containing 0.12% metaphosphoric acid) at 25° with and without 0.05% AA in the medium. 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 ± 0.6%.

(B). Stirring time *vs* recovery of AP from spiked bread. A mixture of dry ground bread (1g) and AP (1.5 mg) were 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 ± 2.7%.


A. Without Bread
With AA
Without AA
Recovery, %
110
100
90
80
0
1
2
3
4
5
Extraction Time, h

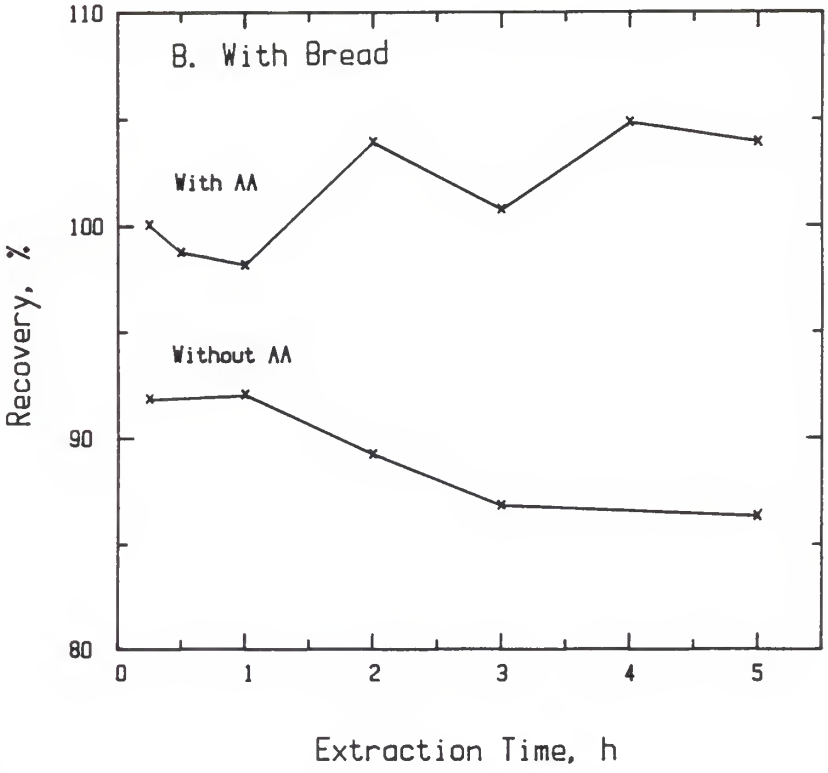

B. With Bread
With AA
Without AA
Recovery, %
110
100
90
80
0
1
2
3
4
5
Extraction Time, h

Fig. 4. Recovery of AP from bread baked with AP in the dough (63.8 mg per 100 g flour). Bread was stored in polyethylene bags o and 10 days then freeze-dried and ground. Ground bread was stirred 5 h 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). The standard error in the AP assays for 1-5 h with 0.05% AA was $\pm$ 1.3% and $\pm$ 3.4%, for 0 day and 10 day bread, respectively.

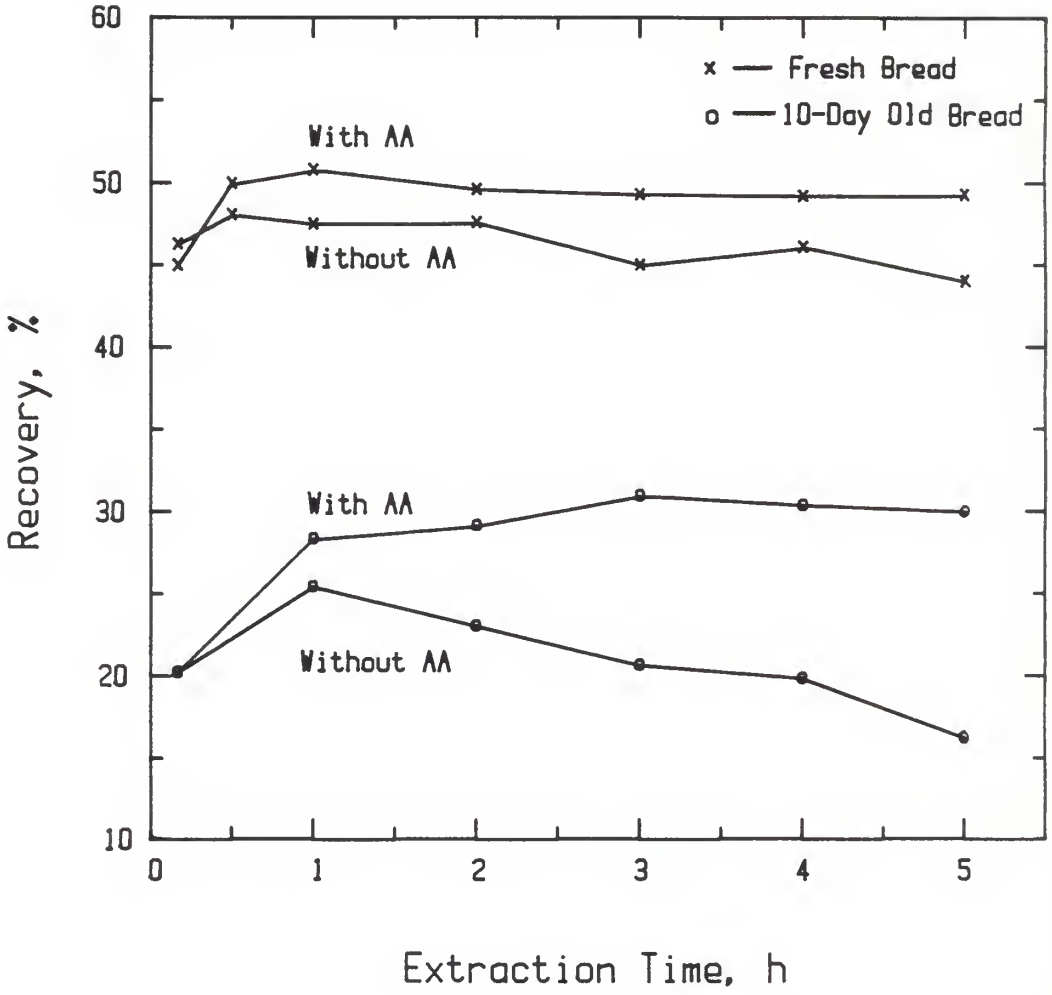

60
50
40
30
20
10
0
1
2
3
4
5
Recovery, %
Extraction Time, h
× — Fresh Bread
o — 10-Day Old Bread
With AA
Without AA
With AA
Without AA

Fig. 5. HPLC-EC chromatograms of (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 (1 g) was extracted 1 h 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% AA. 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.

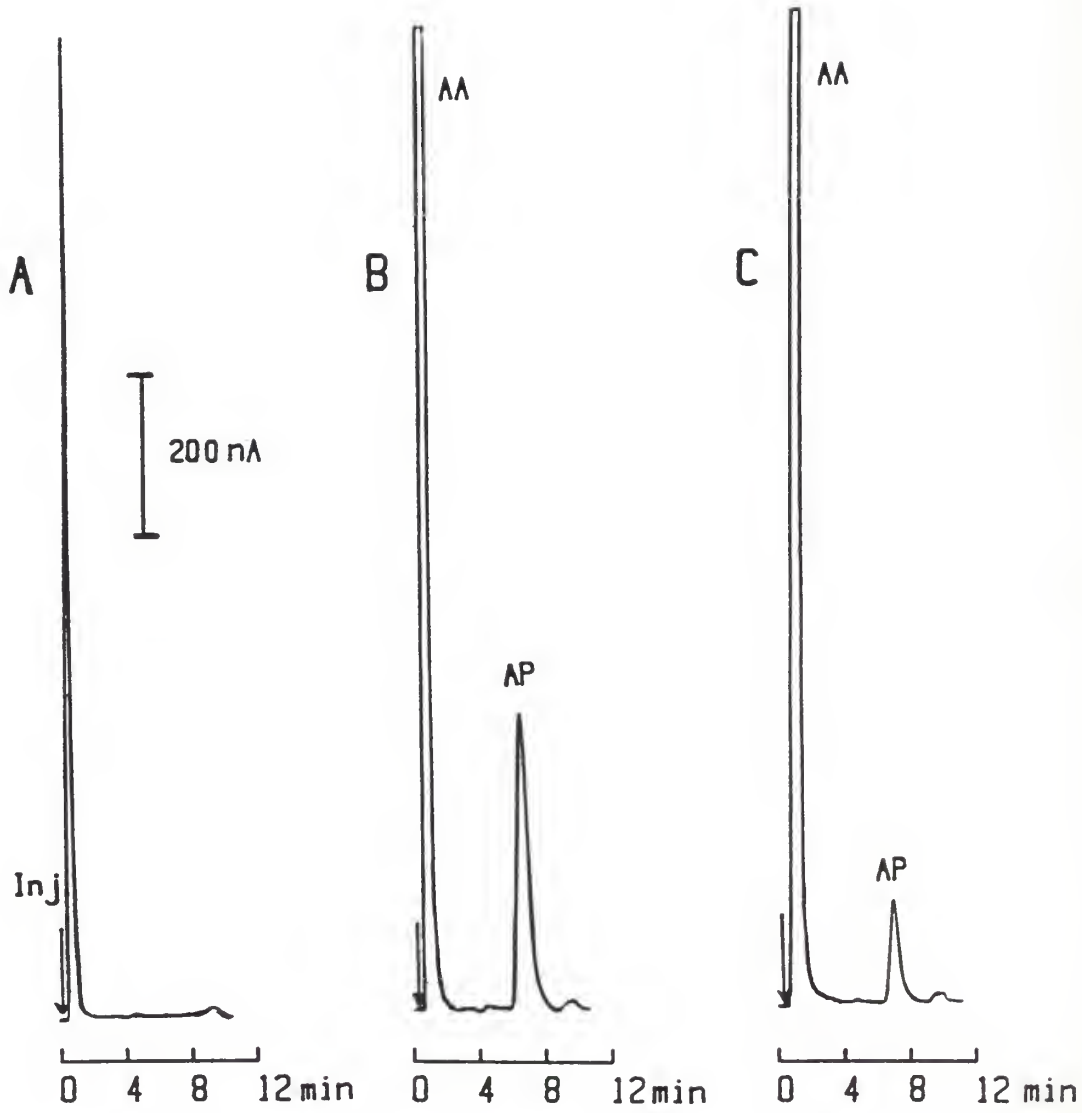

A
200 nA
Inj
0 4 8 12 min
B
AA
AP
0 4 8 12 min
C
AA
AP
0 4 8 12 min

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); (C). extract of bread (A) spiked with AA (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). Freeze-dried bread (1g) was extracted 30 min at 25°C with 10 ml of 3% aqueous metaphosphoric acid. After centrifuging, an 1 ml aliquot of the supernatant was diluted to 25 ml with cold 0.05 M 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 Alltech C-18 reverse phase column with mobile phase (1 ml/min) of 80 mM acetate buffer (pH 4.2) containing 1.0 mM of octyltriethylammonium phosphate (Q8) and 0.1 mM EDTA.

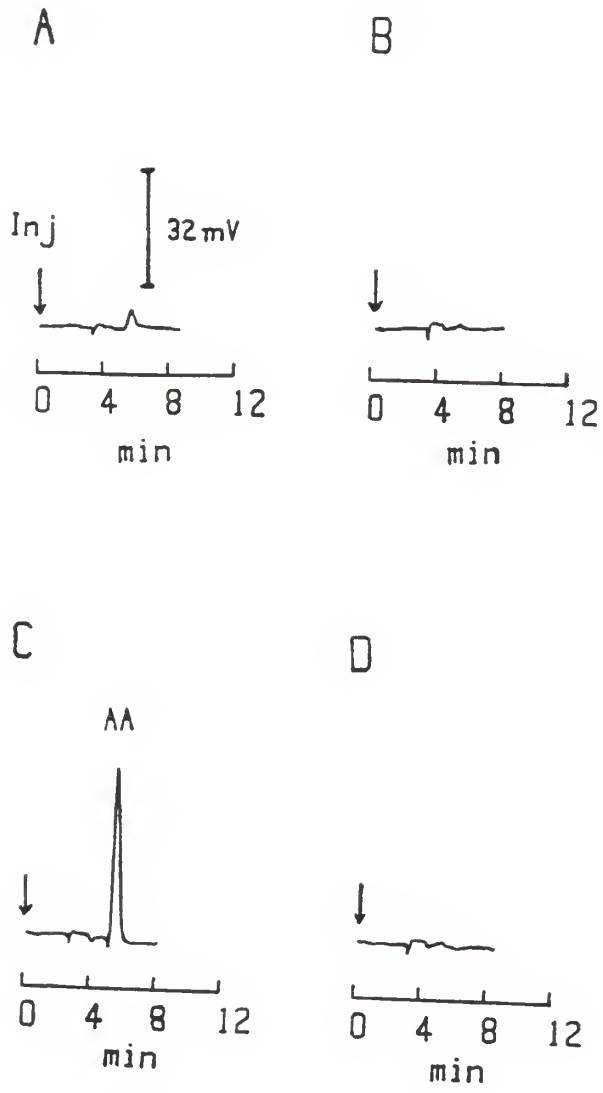

A
B
Inj
32 mV
0 4 8 12
min
0 4 8 12
min
C
D
AA
0 4 8 12
min
0 4 8 12
min

Fig. 7. Standard curve to determine L-ascorbyl 6-palmitate (AP) in bread. Freeze-dried blank bread (1 g) was spiked with AP (0.6 - 4.8 mg/g of bread) and extracted 1 h at 25°C with 50 ml of 90% DMSO containing 0.12% metaphosphoric acid and 0.05% AA. The extract was diluted with 4 volumes of methanol, centrifuged, and an aliquot (0.02 ml) injected into the chromatograph. The HPLC-EC conditions are given in the caption to Fig. 1.

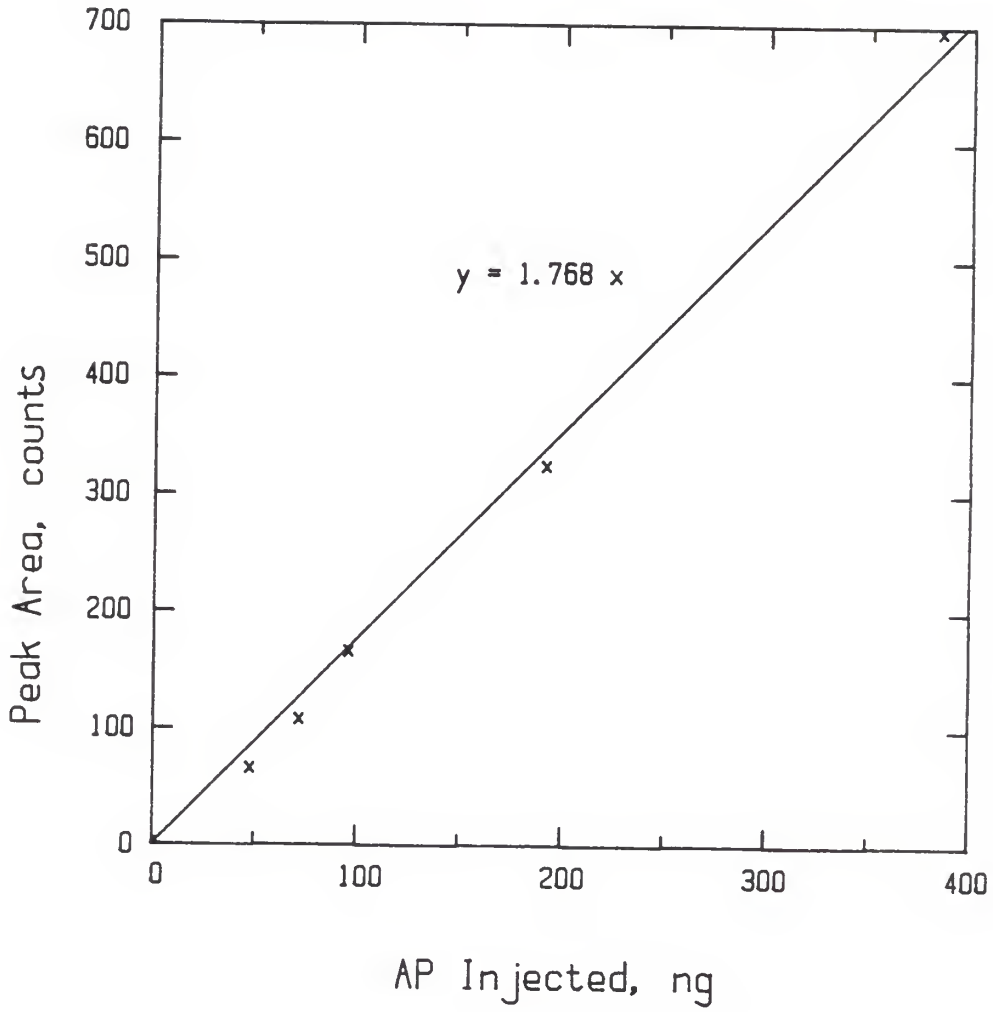

700
600
500
400
300
200
100
0
0
100
200
300
400
y = 1.768 x
Peak Area, counts
AP Injected, ng

Fig. 8. Standard curve to determine L-ascorbic acid (AA) in bread. Freeze-dried blank bread (1 g) was spiked with AA (0.1 - 1.2 mg) and the mixture extracted with 10 ml of 3% metaphosphoric acid. After centrifugation an aliquot of the clear supernatant was diluted 25-fold with cold 0.05 M perchloric acid and immediately assayed using the HPLC-EC system as described in the caption to Fig. 6.

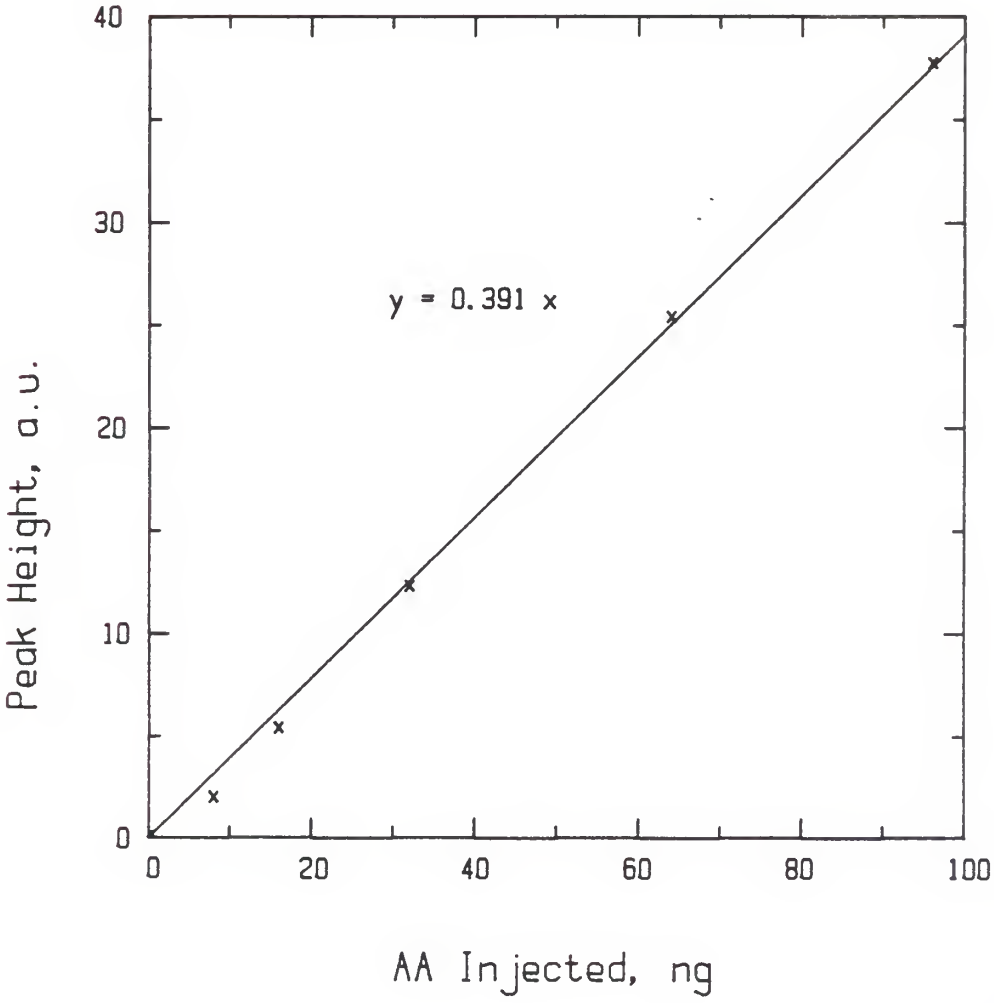

40
30
20
10
0
0
20
40
60
80
100
y = 0.391 x
Peak Height, a.u.
AA Injected, ng

Fig. 9. Flow chart for the analytical determination of AP and AA in dough and bread.

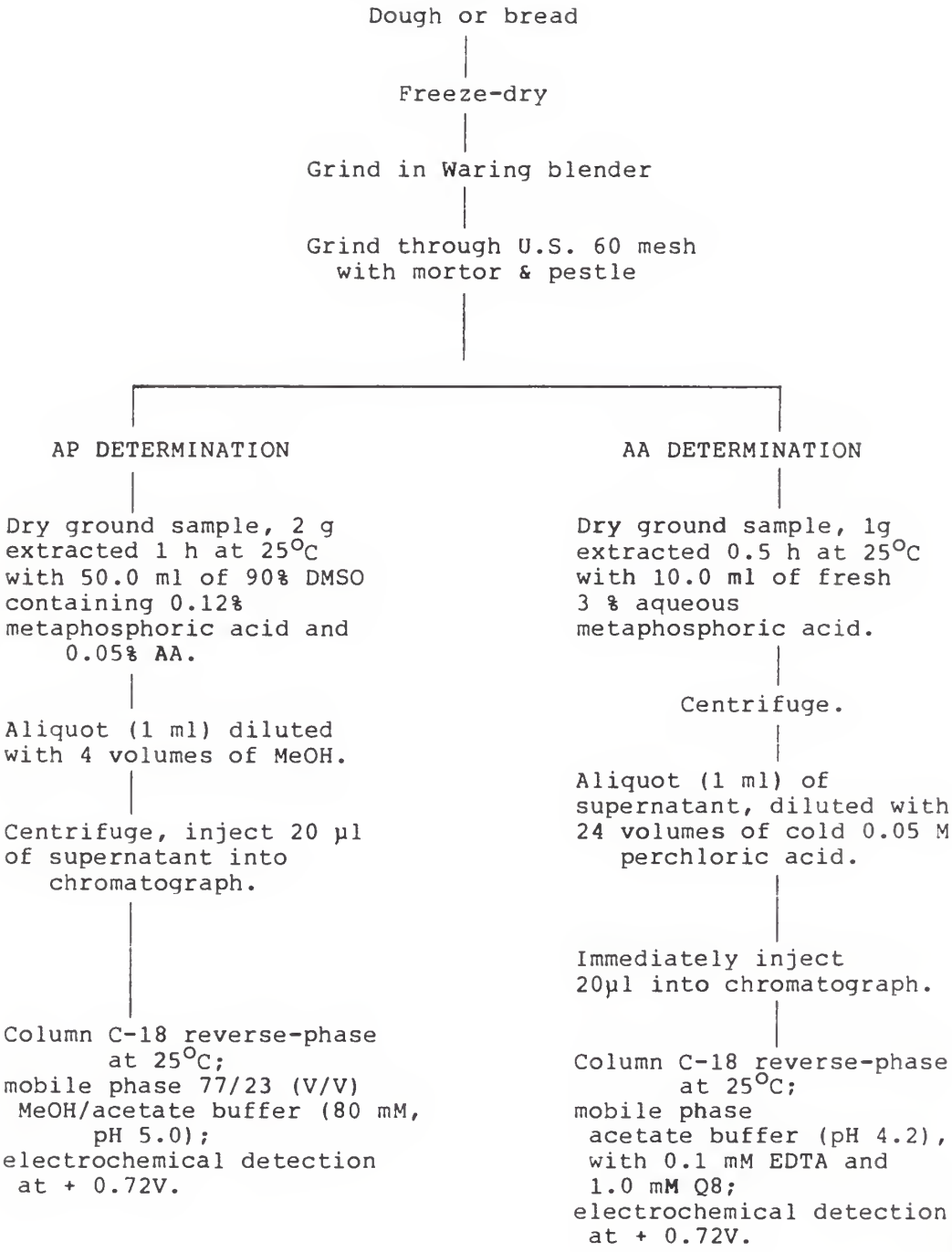
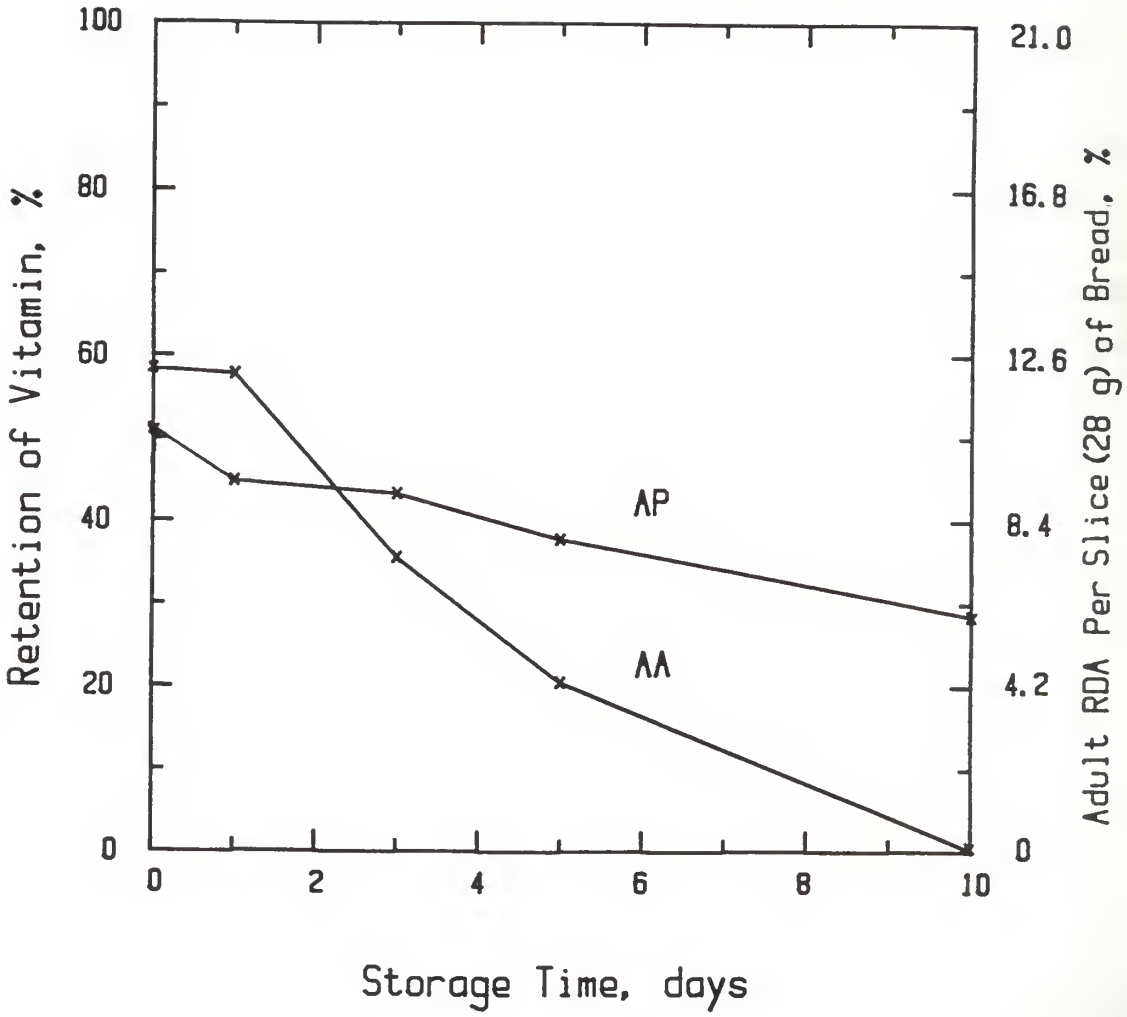

Dough or bread
Freeze-dry
Grind in Waring blender
Grind through U.S. 60 mesh with mortor & pestle
AP DETERMINATION
Dry ground sample, 2 g extracted 1 h at 25°C with 50.0 ml of 90% DMSO containing 0.12% metaphosphoric acid and 0.05% AA.
Aliquot (1 ml) diluted with 4 volumes of MeOH.
Centrifuge, inject 20 µl of supernatant into chromatograph.
Column C-18 reverse-phase at 25°C; mobile phase 77/23 (V/V) MeOH/acetate buffer (80 mM, pH 5.0); electrochemical detection at + 0.72V.
AA DETERMINATION
Dry ground sample, 1g extracted 0.5 h at 25°C with 10.0 ml of fresh 3 % aqueous metaphosphoric acid.
Centrifuge.
Aliquot (1 ml) of supernatant, diluted with 24 volumes of cold 0.05 M perchloric acid.
Immediately inject 20µl into chromatograph.
Column C-18 reverse-phase at 25°C; mobile phase acetate buffer (pH 4.2), with 0.1 mM EDTA and 1.0 mM Q8; electrochemical detection at + 0.72V.

Fig. 10. Retention of L-ascorbic acid (AA, initially 63.8 mg per pup-loaf) and L-ascorbyl 6-palmitate (AP, initially 150 mg per pup-loaf) in bread stored in polyethylene bags at 25°C. The RDA of vitamin C per slice was calculated based on bread containing 32% moisture, and an RDA of 60 mg L-ascorbic acid per day per person.


100
80
60
40
20
0
Retention of Vitamin, %
21.0
16.8
12.6
8.4
4.2
0
Adult RDA Per Slice (28 g) of Bread, %
AP
AA
0
2
4
6
8
10
Storage Time, days

DETERMINATION OF L-ASCORBYL 6-PALMITATE IN BREADMAKING USING REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) WITH ELECTROCHEMICAL (EC) DETECTION.

BY

TSUI-HWA TRACY HUNG

B. S., National Chung Hsing University, R.O.C., 1977

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1986

ABSTRACT

The survival of L-ascorbyl 6-palmitate (AP) and L-ascorbic acid (AA) in breadmaking was determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC). AP was found to be stable for up to 5 hours at 25°C in 90% dimethylsulfoxide containing 0.12% metaphosphoric acid and 0.05% AA. When the DMSO-acid medium was used to extract bread that had been spiked with 0.6 - 4.8 mg AP / g of dry bread, AP was recovered quantitatively in the extraction medium. The DMSO-acid extract was diluted with four volumes of methanol, centrifuged , the supernatant injected into a reverse phase column, and the column was developed with methanol/buffer (77/23 V/V) at pH 5.0. AP was base-line resolved with R_T approximately 7 min, and the system had a detection limit of 2 ng of AP. Duplicate analyses differed by only 2%.

Reverse phase HPLC-EC also was used to assay AA in bread. Bread was extracted using 3% aqueous metaphosphoric acid, the extract centrifuged, and the clear supernatant diluted with cold 0.05 M aqueous perchloric acid. AA was base-line resolved with R_T 5.8 min when the column was developed by an acetate buffer (pH 4.2) containing EDTA and ion-pairing agent.

Bread was baked containing 150 mg of AP or 63.8 mg of AA per 100 g flour. Fresh bread after cooling one hour retained 50.7% AP and 58.1% AA. During storage of bread in polyethylene bags at 25°C, AA was lost approximately three times faster than AP. AA could not be detected in bread after 10 days storage, whereas 30% AP still remained. Bread baked with 0.15% AP in the formula

contained 9% of the adult RDA for vitamin C after 1 - 3 days storage, and 6% RDA at 10 days. Storage of bread under N_2 practically eliminated the loss of AP.