# Determination of a Surfactant (Sodium 6-*O*-Palmitoyl-L-Ascorbate) in Bread by High Performance Liquid Chromatography[1, 2]

D. J. MAURO, D. L. WETZEL, P. A. SEIB, and R. C. HOSENEY

ABSTRACT

Bread samples enzymatically digested for 1 hr were analyzed by high performance liquid chromatography in 10 min for sodium 6-*O*-palmitoyl-L-ascorbate, a dough conditioner at 0.5% of the flour weight with 2.3% relative precision for different loaves. A microBondapak C-18 reverse phase column was used with methanol-water (75/25) by volume to give efficient separation. Sample preparation, digestion conditions, and the chromatographic system were optimized for maximum analyte recovery. At least 81.7% of the added dough conditioner survived bread making. With the use of a suitable detector, the procedure may be generally applicable to surfactants in bread.

Cereal Chem. 56(3):152-155

Salts of 6-acyl esters of L-ascorbic acid serve as dough conditioners and as antistaling agents in bread making (Ofelt et al 1958, Hoseney et al 1977). We were interested in determining the fate of those salts in bread because L-ascorbic acid is, for the most part, destroyed during bread making (Quadri et al 1975) and because the 6-acyl esters of L-ascorbyl 6-laurate, 6-palmitate, or 6-stearate contain vitamin C potency (Inagaki et al 1968). We chose sodium 6-*O*-palmitoyl-L-ascorbate (SPA) to represent the general class of 6-acyl esters.

An analytical technique was needed to more accurately establish the survival rate of the 6-acyl ester of L-ascorbate during the bread making process. A procedure suitable for routine analysis was also desired. This article concerns a method developed to determine SPA in bread.

[1] Contribution 939-J, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Manhattan, KS 66506.
[2] Presented at the AACC 60th Annual Meeting, Kansas City, MO, October 1975.

0009-0352/79/000019$3.00/0


## MATERIALS AND METHODS

SPA was prepared as described previously (Hoseney et al 1977). All solvents used were spectrograde. The water was distilled, deionized, and redistilled from alkaline potassium permanganate. The methanol (Mallinckrodt anhydrous analytical reagent grade) was refluxed and redistilled from magnesium and recrystallized iodine according to Vogel's procedure (1956). The amylases were type II-A bacterial from *Bacillus subtilus* (Sigma Chemical Co.), malt amylase PF (Wallerstein), and amyloglucosidase grade III from *Aspergillus* (Sigma Chemical Co.). All other chemicals were reagent grade. A Waters Associates model 202 high-pressure liquid chromatograph equipped with a differential ultraviolet (254 nm) detector, a second pump, and a programmer were used in the isocratic mode. A nonrotating stem, high-pressure stainless-steel

valve, and a Varian stop flow septumless injector in series, respectively, were installed between the pump and the column of the chromatograph with the column connected directly to the injector. A Hamilton syringe and Houston Omnigraphic recorder were used with the system. A Waters Associates (Milford, MA) reverse phase microBondapak C-18 (10$\mu$ particle size) stainless-steel column (30 cm × 4.6 mm i.d.) was used for the chromatograms shown. The eluting solvent mixture selected was methanol/water (75:25) by volume.

Bread loaves with 0.5 g of SPA per 100 g of flour were baked with a straight-dough pup-loaf (100 g flour) procedure (Finney and Barmore 1945) and stored at 0°C until analyses. The samples were freeze-dried and then ground in a Tekmar A-10 water-cooled analytical mill (Tekmar Co., Cincinnati, OH) long enough to allow at least 50% by weight of the ground material to pass through a 13XX wire (openings of 104 $\mu$). The 2.0-g ground sample and 250 mg of sodium dodecyl sulfate were added to 50 ml of $\alpha$-amylase solution (5,000 Sigma units), prepared by adding 60 mg of $\alpha$-amylase to 500 ml of pH 6.95–7.00 0.1*M* potassium dihydrogen phosphate buffer. (One Sigma unit releases 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C.) This mixture was then digested with occasional stirring at 48°C for 1 hr. After digestion the samples were rapidly filtered with suction through a glass fiber pad (984H Ultra, Whatman, Inc.). Cellulose filters were unsuitable because they retain significant quantities of the analyte to lower the recovery. A 25-$\mu$l aliquot of the filtrate was injected directly into the chromatograph. Control loaves were treated identically and spiking of the control bread was done to individual ground samples before digestion.

The SPA chromatographic peak from the bread digestate was identified by retention time of pure analyte and by spiking digested bread with the known substance. By the width at half height method, peak areas for known quantities were obtained and a calibration curve was prepared.

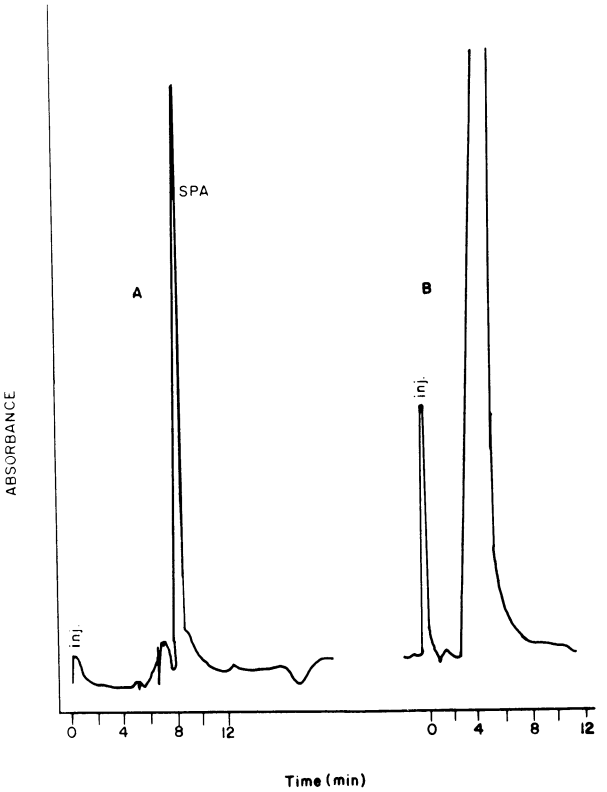


Fig. 1. Effect of enzyme on SPA chromatogram A is SPA digested in the presence of bacterial $\alpha$-amylase; chromatogram B is SPA digested in the presence of amyloglucosidase.

## RESULTS AND DISCUSSION

An average of 81.7% of the original compound was accounted for in the bread (Table I) with the above method, but omitting the filtration step, on different samples of bread containing 0.5% 6-*O*-palmitoyl-L-ascorbate.

A column and solvent system was readily selected and optimized that effectively separated the analyte from the other ultraviolet-absorbing compounds in the bread digestate. The major problem was devising sample preparation before chromatographic injection to give reproducible, high recovery of analyte from the complex matrix of the sample. In preliminary experiments no SPA was detected in simple ether extracts of bread. Recoveries of SPA added to ground control loaves before digestion were 50% or less. Furthermore, if the same amount of SPA (0.5% based on flour) were added before baking, only 12–25% of the compound could be recovered from $\alpha$-amylase digestate of the bread. The low recoveries were considered to result from one or more of the following: esterase activity in the $\alpha$-amylase, incomplete breakdown of a possible starch SPA complex, and formation of a protein SPA complex.

Esterase activity in the bacterial $\alpha$-amylase was not the problem. Under the digestion conditions used on bread, only 11% of SPA recovered after 1 hr was lost through digestion for an additional hour. Surfactants used as dough conditioners have been reported to complex with starch, and digestion of starch with $\alpha$-amylase has been used to free the surfactants (DeStefanis and Ponte 1972). In this work the remaining particulate material in the digested bread crumb always gave some blue color with iodine after as long as 5 hr

**TABLE I**
**Recovery of Sodium 6-*O*-Palmitoyl-L-Ascorbate (SPA) Added to Control Loaves and Loaves Baked with SPA at a Level of 0.5 g/100 g of Flour**

| | Maximum Recovery from Control Bread[a] (%) | Recovery Values for a Single Loaf[b] (%) | Recovery Values from Four Loaves[c] (%) | Survival Rate from Four Loaves[d] (%) |
|---|---|---|---|---|
| | 96.6 | 77.4 | 77.4 | 81.1 |
| | 95.4 | 77.0 | 80.0 | 83.5 |
| | 94.5 | 77.5 | 77.8 | 81.5 |
| | | 76.0 | 77.4 | 81.1 |
| | | 76.0 | | |
| Mean value | 95.5 ± 1.0 | 76.8 ± 1.1 | 78.1 ± 1.8 | 81.7 |
| Rel. std. dev. | 1.0 | 1.4 | 2.3 | |

[a] Maximum recovery was determined by adding 6.3 mg of SPA to 2.0 g of dried ground bread containing no SPA.
[b] Successive injections of the digestate from one loaf.
[c] Mean values based on replicate recovery determinations.
[d] Survival = actual recovery/average maximum recovery.

of digestion. However, the soluble material gave no blue color with iodine after 20 min of digestion. To maximize starch digestion, the effect of the particle size of the bread, temperature of digestion, and use of other amylolytic enzymes were examined. Particle size was reduced using a high-speed (20,000 rpm) hammer mill to increase the surface area for enzyme attack. That increased recoveries only slightly. Increasing the temperature of the digestion bath from 30 to 48°C decreased the time of maximum recovery from 5 to 1 hr, but the percentage of SPA recovered did not increase. To determine if the source of enzyme would affect analyte recovery, we compared three enzyme sources: bacterial $\alpha$-amylase, malt $\alpha$-amylase, and a crude glucoamylase. For the two $\alpha$-amylase sources, no cleavage of SPA was apparent. A chromatogram of the sample-glucoamylase digestate showed no SPA peak. The increase in the amount of L-ascorbic acid appearing with the solvent peak (Fig. 1) indicated that SPA was cleaved to L-ascorbate and palmitate.

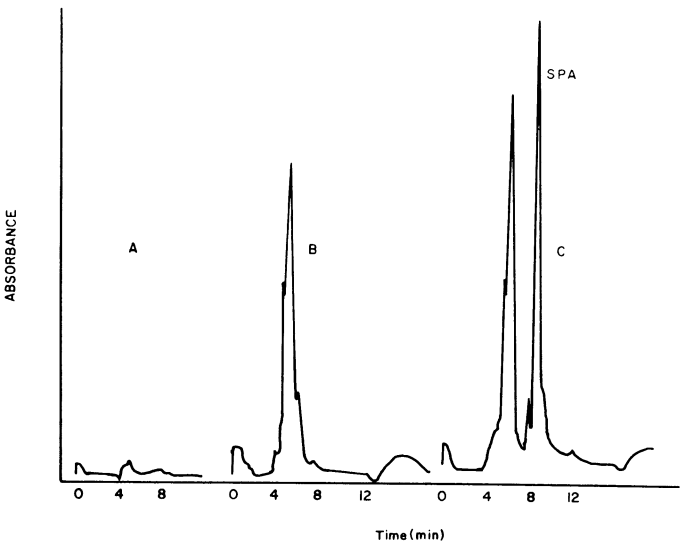


Fig. 2. Effects of sodium dodecyl sulfate on recovery of SPA. Chromatogram A is sodium dodecyl sulfate in 50 ml of enzyme solution, chromatogram B is control bread plus 250 mg of sodium dodecyl sulfate, chromatogram C is bread with SPA baked in at 0.5% level plus 250 mg of sodium dodecyl sulfate in 50 ml of enzyme solution.

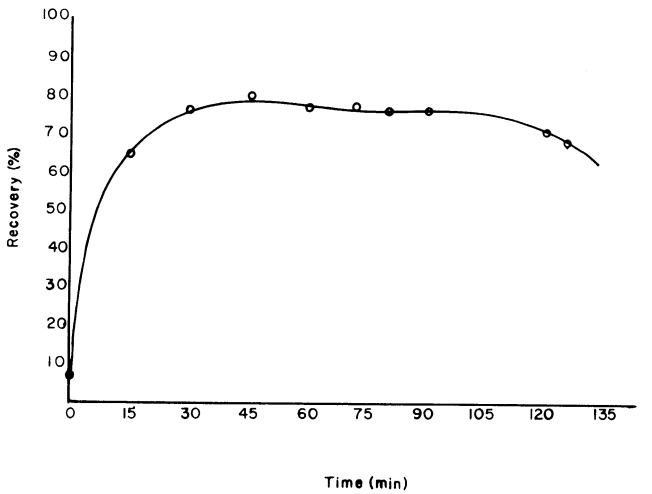


Fig. 3. Recovery of SPA as a function of length of enzyme digestion at 48°C.

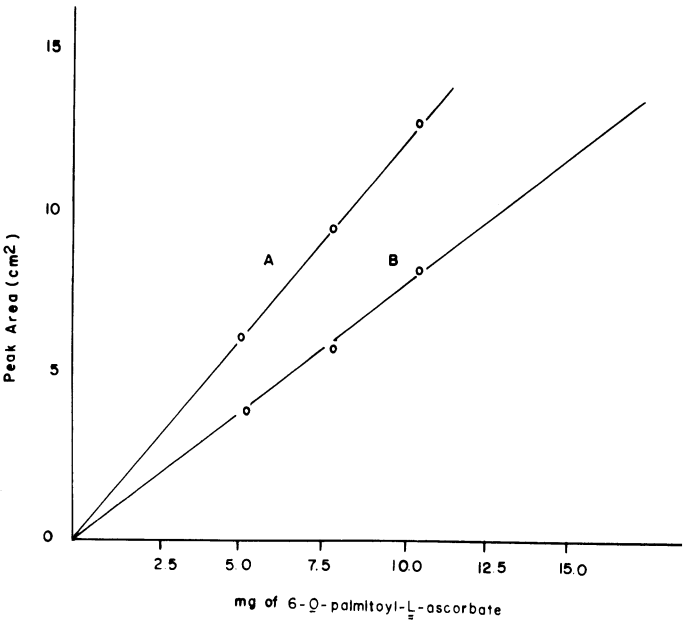


Fig. 4. Recovery of SPA from unfiltered (A) and filtered (B) $\alpha$-amylase digestate with added sodium dodecyl sulfate.

A second surfactant was added in excess to the digestion mixture to compete with the analyte for possible binding sites on polymeric surfaces such as those of protein or undigested starch. This also allowed us to test the theory that such binding caused the initial poor recoveries. Three digestion solutions were prepared with 50 ml of enzyme containing 250 mg of sodium dodecyl sulfate, which had been reported (Weber and Osborn 1969) to bind with protein. Digest 1 contained only these substances, digest 2 also contained 2.0 g of control (blank) bread, and digest 3 also contained 2.0 g of control (blank) bread and pure SPA. The chromatograms in Fig. 2 show that sodium dodecyl sulfate introduced no interfering ultraviolet-absorbing components. Furthermore, the average recovery of SPA from digest 3 was 95.5%. These results suggest that SPA binds to the surfaces in the digestion mixture. When bread baked from dough containing SPA was analyzed, 78.1% of the SPA was recovered from the whole loaf. Increasing the level of sodium dodecyl sulfate in the digestion was tried but recovery of SPA did not increase. The optimum digestion time of the ground bread was determined by chromatographing the digesting material every 15 min for 120 min (Fig. 3). A slight decline in recovery was noted upon overdigestion.

Actual recovery of SPA from bread digested 1 hr was repeatedly 78.1%. Considering the average maximum recovery of SPA added to the control bread after drying and grinding of 95.5%, the survival of SPA during bread making was then 81.7%. Having established survival, the optimized procedure used for maximum recovery studies was adapted for routine analysis of SPA in bread. A filtering step using glass fiber filter discs was added to the analytical scheme since repeated injection of unfiltered solutions on the column soon clogged the 2-$\mu$ stainless-steel frit at the head of the column and greatly reduced the life of the column. Some SPA (present as suspended micelles) was inevitably lost by all filtering procedures attempted, but the loss was reproducible over a range (Fig. 4) using the recommended procedure described. With 2.0-g bread samples, 63% (average of seven determinations) of the total added SPA could be recovered from the filtered solution with a relative standard deviation of 1.09. Thus, multiplication of the quantity found in the range by 1.58 resulted in the corrected value for SPA in bread.

The survival value includes both crumb and crust. Because the crust is subjected to more severe conditions during baking, survival from the crumb alone may be higher. Thus, the survival value of

81.7% should be considered a minimum. A routine procedure has been developed for SPA that may be generally applicable to surfactants in bread, providing that they are ultraviolet-absorbing or that detection by another means and subsequent quantitation is possible.

## LITERATURE CITED


DeSTEFANIS, V. A., and PONTE, J. G., Jr. 1972. Determination of crumb softeners and dough strengtheners in baked foods. (Abstr. 88). Cereal Sci. Today 17(9):276.

FINNEY, K. F., and BARMORE, M. A. 1945. Varietal responses to certain baking ingredients essential in evaluating the protein quality of hard winter wheats. Cereal Chem. 22:225.

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.

INAGAKI, C., ARAKAWA, N., SUZURI, N., SAGO, Y., and KOGAMI, K. 1968. Metabolism of fatty acid esters of L-ascorbic acid. III. Biochemical activity of various L-ascorbic acid derivatives. Vitamins 37:152 (Japan) [Chem. Abstr. 68:66890a].

OFELT, C. W., MEHLTRETTER, C. L., MacMASTERS, M. M., OTEY, F. H., and SENTI, F. R. 1958. Effect on crumb firmness. II. Action of additives in relation to their chemical structure. Cereal Chem. 35:142.

QUADRI, S. F., LIANG, Y. T., SEIB, P. A., DEYOE, C. W., and HOSENEY, R. C. 1975. Stability of L-ascorbate 2-sulfate and L-ascorbic acid in wheat foods and milk. J. Food Sci. 40:837.

VOGEL, A. I. 1956. A Textbook of Practical Organic Chemistry (3rd ed.). 169 pp. Longmans, Green and Co.: London.

WEBER, K., and OSBORN, M. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406.


[Received December 12, 1977. Accepted August 16, 1978]