High-Pressure Liquid Chromatographic Determination of Ascorbic Acid in Cooked Sausages

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ABSTRACT

The purpose of this paper was to study and optimize both extraction and high-pressure liquid chromatography (HPLC)–UV detection procedures to develop a proper method for the determination of ascorbic acid content in cooked sausages. A simple and sensitive reversed-phase HPLC method for the NH2-bonded phase has been described for the determination of ascorbic acid content in cooked sausages. Various extracting agents were tested to solubilize the vitamin, with 5% (wt/vol) metaphosphoric acid giving the best results. Samples were chromatographed with UV detection at 248 nm on a 25-cm Spherisorb NH2 cartridge with a 0.4-cm inside diameter with a mixture of 0.02 M potassium phosphate buffer solution (pH 3.6) and acetonitrile (40:60, vol/vol) for the mobile phase. The method's precision within a day was 1.2%, and its precision between days was 3.8%. The detection limit was 1.6 mg/100 g. Recovery ranged from 91.4 to 96.2% for ascorbic acid added to meat samples. Twenty samples of six different products were analyzed in duplicate. For the samples analyzed, the mean value for ascorbic acid ranged between 21.555 and 24.899 mg/100 g of fresh weight.

Vitamin C comprises essentially two compounds, L-ascorbic acid, a strong reducing agent, and its oxidized derivative L-dehydroascorbic acid. Although most vitamin C in body fluids and tissues is in its reduced form, both ascorbic acid and dehydroascorbic acid have biological activity and are interconvertible by an oxidation-reduction reaction. Vitamin C is found almost exclusively in foods of plant origin. Aside from the kidney, no animal food is considered a significant source of this vitamin. Vitamin C is readily lost in cooking because of its water solubility and is generally more abundant in raw plant foods (2).

Numerous high-pressure liquid chromatography (HPLC) methods for the determination of vitamin C content have been developed over the years. There is interest in the possibility of determining both ascorbic acid and dehydroascorbic acid contents for an estimation of total vitamin C activity and in separating ascorbic acid from other compounds that have not shown vitamin C activity. Several extraction techniques have been developed for the reduction of dehydroascorbic acid to ascorbic acid by homocysteine (3, 7), including extraction with a mixture of metaphosphoric acid and ethanol (6, 7), extraction by enzymatic oxidation of ascorbic acid to dehydroascorbic acid followed by o-phenylenediamine derivative fluorimetric detection (8, 9), and oxidation of ascorbic acid and derivatization with 2,4-dinitrophenylhydrazine (5). For the analysis vitamin C content, methods involving HPLC have been used because of their high selectivity and accuracy. These assays have used various bonded stationary-phase material and solvent compositions, together with either UV or electrochemical detection.

Even though meat and other major ingredients used in the preparation of cooked sausages are poor sources of vitamin C, L-ascorbic acid is added to these products as a preservative because of its antioxidant properties. In addition, vitamin C is sometimes added to meat products to increase their vitamin C content.

The purpose of this paper was to study and optimize both extraction and HPLC-UV detection procedures to develop a proper method for the determination of ascorbic acid in cooked sausages.

MATERIALS AND METHODS

Apparatus and liquid chromatography conditions. A model HP1090 high-performance liquid chromatograph (Hewlett-Packard, Madrid, Spain) equipped with a Rheodyne 7010 autosampler and an HP 3390A integrator (Hewlett-Packard) was used. The chromatographic column was a stainless steel cartridge (25 cm with an inside diameter of 0.4 cm; Teknokrom, Sant Cugat del Vallés, Barcelona, Spain) packed with Spherisorb NH2 (5 μm). A 10-μl volume of eluate was chromatographed, with a mixture of 40% potassium phosphate buffer solution (0.02 M, pH 3.6) and 60% acetonitrile isocratically pumped at a flow rate of 1.000 ml/min for the mobile phase. The oven temperature was 35°C. Absorbances were monitored at 248 nm with a variable-wavelength HP 8452A detector (Hewlett-Packard).

Reagents. The reference standard of L-ascorbic acid was obtained from Merck (Darmstadt, Germany). Metaphosphoric acid was obtained from Sigma Chemical Co. (St. Louis, Mo.), as was EDTA. All reagents for chromatographic conditions were of...
HPLC grade, and all other chemical reagents used were of analytical grade. Mobile phase solution was filtered through a 0.45-
μm Millipore membrane filter and degassed prior to use.

**Samples.** Six commercially purchased cooked sausages (‘lunch,’ ‘chopped pork,’ ‘chopped beef,’ ‘chopped turkey,’
‘vitaminized chopped,’ and ‘Sicilian mortadella’ sausages) were analyzed. All of the sausages analyzed were composed of meats, fat, water, sugars, salt, different spices, and some additives, such as preservatives, including vitamin C. These types of sausage differed mainly in meat composition (quantities of pork, beef, and turkey meats, depending on the product) and grinding degree.

**Sample preparation.** Five grams of finely ground samples were transferred into a 50-ml beaker in duplicate. Twenty milliliters of freshly prepared metaphosphoric acid solution (5%, wt/vol) and 1 ml of EDTA solution (0.1 mg/ml) were added, and the mixtures were then stirred well at room temperature for 2 min. The contents of the beaker were quantitatively transferred to a 50-
ml amber volumetric flask by washing the beaker at least twice with water and bringing the flask to volume with water. The contents of the flask were then centrifuged for 5 min at 3,000 rpm, and the upper solution was filtered through Albet no. 1305 filter paper and diluted (1:2) with ultrapure HPLC water. Finally, samples were refiltered through Millipore filters (0.45 μm) into amber vials for liquid chromatographic analysis.

**Procedure for standard solutions.** A stock solution of 1,000
μg of L-ascorbic acid per ml in 2% (wt/vol) metaphosphoric acid solution mixed with 2 ml of EDTA (0.1 mg/ml) was prepared daily and stored in darkness in a refrigerator. Working standard solutions (200 and 250
μg/ml) were prepared immediately before use, since even under these acid conditions degradation of ascorbic acid was observed as a function of the number of hours of storage. Aliquots of these solutions were treated as samples. The resulting peak areas were plotted against concentration (from 50 to 200 μg) for the calibration curves. The vitamin content of the sample extracts were obtained by interpolation on the standard curve.

**RESULTS AND DISCUSSION**

Ascorbic acid is sensitive to heat and light, which rapidly convert it to dehydroascorbic acid. The decomposition is catalyzed by the presence of metals, such as iron and copper, the temperature, the pH, and the oxygen concentra-
tion. The choice of an adequate extraction method is critical for obtaining the most stable conditions for ascorbic acid. In addition, because most cooked sausages are poor sources of vitamin C, experimental conditions during the analysis must be carefully studied and controlled. We assayed the vitamin C extraction with metaphosphoric acid and trichloroacetic acid at a 5% (wt/vol) concentration in two different meat products (lunch and vitaminized chopped sausages) and a mixture of standard solutions. The addition of a very small quantity of disodium EDTA, a very good chelant agent for divalent cations (1), is recommended. The results presented in Table 1 show that both agents allow similar extraction of vitamin C. Nevertheless, we observed that extraction with metaphosphoric acid produced chromatograms with fewer impurities, so we decided to use this extraction agent. Another advantage associated with the use of metaphosphoric acid is an increase in the mean life of the chromatographic column.

Because of the polar nature of ascorbic acid, we carried out reversed-phase HPLC on the NH2-bonded phase, which separates the vitamin without the use of an ion-pair reagent, necessary for the improvement of retention for other columns. Good separation was achieved with a Spherisorb NH2 (5 μm) stationary-phase column by using a mixture (60/40) of acetonitrile and 0.02 M phosphate buffer adjusted to pH 3.6 with orthophosphoric acid to avoid ascorbic acid oxidation. The amount of phosphate buffer was regulated to achieve an adequate retention time. The vitamin was eluted

**TABLE 1. Effect of extracting agent on L-ascorbic acid determinationa**

<table>
<thead>
<tr>
<th>Extracting agent</th>
<th>Value for sample (mg/100 g)</th>
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</thead>
<tbody>
<tr>
<td>Metaphosphoric acid</td>
<td>22.643 ± 1.093</td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>21.503 ± 1.050</td>
</tr>
</tbody>
</table>

a Data are expressed as means ± standard deviation; n = 6 in duplicate.
in about 4.0 min at a flow rate of 1.000 ml/min. The influence of temperature (35, 40, and 60°C) on the retention time was studied, because retention is strongly influenced by temperature (4) when separation is carried out on an NH₂ column. In our study, the retention times for the vitamin C peak varied from 2.76 min at 60°C to 3.43 min at 35°C. Even though the retention time decreased with increasing temperature, the best separation was achieved at 35°C. Typical chromatograms for the elution of a standard solution of ascorbic acid and a representative cooked sausage (chopped pork) are shown in Figures 1 and 2.

With this chromatographic system, the calibration curve was prepared for different days (12 times over 3 months) to verify the applicability of this method to the quantification of vitamin C in cooked sausages. Linearity was observed from 50 to 200 μg, which is more than adequate for the detection of vitamin C in the range of 16.0 to 67.0 mg/100 g in the samples studied. The linear regression coefficient was 0.9988. The detection limit for L-ascorbic acid was found to be 1.600 mg/100 g, with a signal-to-noise ratio of 3. Table 2 shows the calibration data for L-ascorbic acid.

To determine the precision of the method, six aliquots of one sample were tested in parallel, with the coefficient of variation within a day being 1.2%. The variation among days was determined by analyzing in duplicate 10 independent replicate samples of the same material over 3 months. The coefficient of variation between days was 3.8% on average. Reproducibility was tested for a representative sample by adding two standard concentrations of L-ascorbic acid (Table 3) prior to extraction, and the mean percentage of recovery ranged from 91.4 to 96.2%.

In the present study, ascorbic acid content was determined according to the HPLC method above, developed for six commercially cooked sausages (lunch, chopped pork, chopped beef, chopped turkey, vitamin-chopped, and Sicilian mortadella sausages) collected weekly from a food factory over a 4-month period. Table 4 gives the results for the vitamin contents of the meat samples assayed. Each value represents an average of 20 samples analyzed in duplicate. In all samples except those of the vitamin-chopped sausage (with vitamins added), values ranged from 22.456 to 25.868 mg/100 g for ascorbic acid.

In conclusion, vitamin C can be properly determined in cooked sausages by a simple, rapid, and low-cost HPLC method. Sample preparation includes only one extraction step with the stabilizing metaphosphoric acid, followed by filtration for the HPLC injection. The HPLC method studied and proposed for the quantification of ascorbic acid produces an ascorbic acid peak that is very well resolved on a stable baseline. For the reasons mentioned above, this technique is an efficient and favorable alternative to ion-pair reversed-phase liquid chromatography, and its application results in a precise, accurate, and sensitive procedure that can be used for routine assessment of vitamin C in cooked sausages.

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**REFERENCES**


