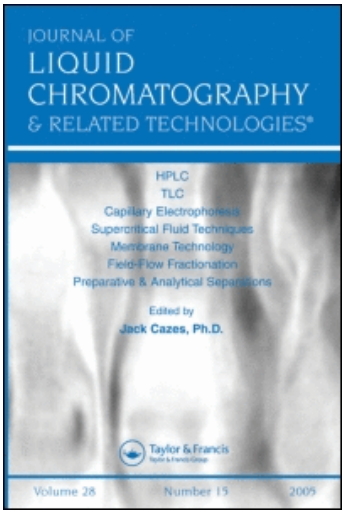

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Quantitative Analysis of Ascorbic Acid and Isoascorbic Acid in Foods by High-Performance Liquid Chromatography with Electrochemical Detection

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QUANTITATIVE ANALYSIS OF ASCORBIC ACID AND ISOASCORBIC ACID IN FOODS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

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ABSTRACT

A procedure is presented for the direct and simultaneous determination of ascorbic acid (AA) and isoascorbic acid (IAA) in food products by paired-ion reverse-phase high-performance liquid chromatography. Three Supelcosil C18 columns were used with a pH 5.4 mobile phase containing 0.04 M sodium acetate, 5 mM tetrabutylammonium hydrogen sulfate and 0.015% metaphosphoric acid. Food samples, preserved with metaphosphoric acid and diluted with mobil phase, were injected (20 μl) using an autosampler. Detection of AA and IAA was by amperometry using a glassy carbon electrode and Ag/AgCl reference electrode. The applied potential was +0.6 volt and the sensitivity was 20 nA. As little as 0.5 ng of each component could be detected under these conditions. When the same samples were incubated with homocysteine to reduce dehydroascorbic acid (DHAA) and dehydroisoascorbic acid (DHIAA) to AA and IAA respectively and reinjected into the system the values for total AA and IAA were obtained. The concentration of the oxidized forms of DHAA and DHIAA could then be calculated by substraction.

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INTRODUCTION

D-Isoascorbic acid (IAA), also known as erythorbic acid or D-araboascorbic acid, is a C-5 epimer of L-ascorbic acid (AA) and has 5% of the vitamin activity of AA (1,2). In spite of this, for economic reasons, IAA is frequently used as an antioxidant in foods (3,4).

There are several highly sensitive high-performance liquid chromatography (HPLC) methods for the direct measurement of AA in foods and biological fluids (5). Some of these methods are also able to measure dehydroascorbic acid (DHAA) (6-8) and/or IAA (8-12). The method of Kutnink et al (10) has a high sensitivity for the measurement of AA, IAA and uric acid in human plasma; however, it does not measure DHAA. On the other hand the method of Vanderslice and Higgs (8,9) separates and quantifies AA and IAA and their corresponding oxidised forms, but is not as sensitive as the aforementioned method because it requires a post-column derivatizaion. The method of Shüep and Keck (12) has been used to measure AA and IAA in processed meat products, but it has a low sensitivity which might be improved by the use of an amperometric detector.

Our purpose is to describe an extraction and HPLC procedure that separates and quantifies simultaneously the amounts of AA and IAA in food samples. The method can be easily adapted for the measurement of the oxidized forms: dehydroascorbic acid and dehydroisoascorbic acid (DHIAA), after convertion to the reduced state by homocysteine (7,13). The analysis detects total AA (+DHAA) and total IAA (+DHIAA). The concentrations of the oxidized forms can then be calculated by subtraction.

MATERIALS AND METHODS

Samples. Processed meats and other foods were obtained from local supermarkets. Ground beef, suspected of being adulterated by the addition of IAA, was obtained by

inspectors of Consumer and Corporate Affairs Canada from meat retailers.

Sample Preparation. Samples (3-5 g) were homogenized in a polytron homogenizer in enough cold 17% metaphosphoric acid (J.T. Baker Inc. Phillipsburg, NJ) to give a final concentration of 0.85% (w/v) as described by Pelletier and Brassard (1). Homogenates were centrifuged at 30,000 x g in a refrigerated centrifuge for 30 min. The supernatant was filtered through a Millex-GS, 022 μm filter unit (Millipore, Bedford, MA). Usually 500 μl of clear supernatant was mixed with 115 μl 45% K_2HPO_4 buffer pH 9.8, to give a final pH of 7.1. After maintaining the mixture at 25°C for 30 min, 0.85% metaphosphoric acid was added to bring the final volume to 2 ml. A 100 μl aliquot of the treated supernatant was diluted to 10 ml with mobile phase buffer. A 20 μl aliquot of this was injected into the system. This procedure allowed for the estimation of AA and IAA. For the determination of DHAA and DHIAA, a second aliquot of clear supernatant was mixed with 45% K_2HPO_4 buffer, pH 9.8, containing 1% homocysteine (Sigma Chemical Co., St. Louis, MO) and kept at 25°C for 30 min. The rest of the procedure was identical to that used for AA and IAA. A second 20 μl injection in the HPLC system resulted in values for AA + DHAA and IAA + DHIAA. Therefore the concentrations of DHAA and DHIAA could be calculated by subtraction.

Preparation of Standards. AA (BDH Chemicals Ltd. Poole, England) and IAA (Sigma Chemical Co, St. Louis, MO) stock standards were prepared at a concentration of 2.5 mg/ml with 0.85% metaphosphoric acid. Intermediate standards (50 μg/ml) were prepared by diluting the stock standards 1:50 with metaphosphoric acid. The intermediate standards were used to prepare a calibration curve. Usually, 500 μl aliquots of several diluted intermediate standards were treated with 45% K_2HPO_4 buffer, pH 9.8, containing 1% homocysteine and kept at 25°C for 30 min. After diluting the standard in the same form as the sample, 20 μl aliquots containing 0.5 to 2.5 ng of AA and IAA, were injected into the HPLC system.

HPLC Analysis. A Spectra-Physics (San Jose, CA) system consisting of a SP 8800 pump, SP 8760 autosampler cooler, SP 8780 autosampler and a ChromJet integrator was used. A SSI pulse dampener (Supelco, Oakville, ONT.). Three 25 cm x 4.6 mm, 5 μm Supelcosil LC-18-DB columns at room temperature were used to separate AA and IAA. A 3 cm x 4.6 mm Brownee (San Jose. CA) Spheri-5-RP-18 guard column was also used. The amperometric detection system (BAS, West Lafayette, IN) included an LC48 controller and an electrode flow cell consisting of a glassy carbon electrode, a stainless steel electrode top, and an Ag/AgCl reference electrode. The applied potential was + 0.6 volts (oxidative) and the sensitivity range was 20 nA.

The mobile phase was 0.08 M sodium acetate buffer, pH 5.4 containing 5 mM tetrabutylammonium hydrogen sulfate (Sigma) and 0.015% metaphosphoric acid. Before the solution was brought to a final volume the pH was adjusted to 5.4 with a few drops of glacial acetic acid. The mobile phase, was filtered through a 0.22 μm filter (Millipore Corp, Bedford, MA) followed by degassing for one hour with stirring while under vacuum. During the chromatography, helium gas was bubbled continuously into the mobile phase. The mobile phase was run isocratically at room temperature at a 0.4 ml/min flow rate. Samples and standards in a volume of 20 μl were injected into the chromatograph by using the autosampler, maintained at 4-5°C with the cooling system. A calibration curve of at least 5 standards was run daily. Peak heights for these standards were stored and the integrator automatically calculated a quadratic fit through the levels. Sample peaks were automatically compared with the calibration curve in order to obtain the concentration.

RESULTS AND DISCUSSION

A chromatogram of standards is shown in figure 1. The AA and IAA peaks are well resolved on a stable baseline. Each chromatogram was allowed to run for

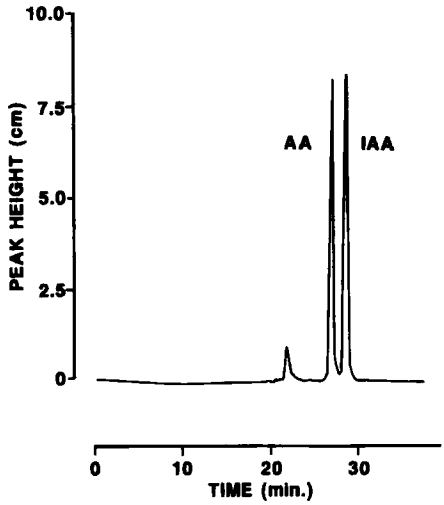

Figure 1. Chromatogram of a standard containing ascorbic acid (AA) and isoascorbic acid (IAA).

40 min. Usual retention times were 27.3 min for AA and 28.6 min for IAA. These retention times were reproducible and changed slightly with different batches of mobile phase. Nevertheless, the separation of the AA and IAA peaks remained 1.3 to 1.5 min. apart. The lowest detection level for AA or IAA was set at 0.5 ng; at this concentration the chromatograph characteristics were still far from the generally accepted definition of chromatography detection limit, namely the amount of analyte which produces a peak height more than two times the noise level.

The observed detector response of the integrator for standard solutions of AA and IAA, in the range of 0.5 to 2.5 ng, revealed a non-linear dependence. Figure 2 shows a calibration graph for IAA. An almost identical graph (not shown) was obtained for AA. To test reproducibility a standard solution composed of 1.5 ng each of AA and IAA was injected into the system ten times. Following calibration, values

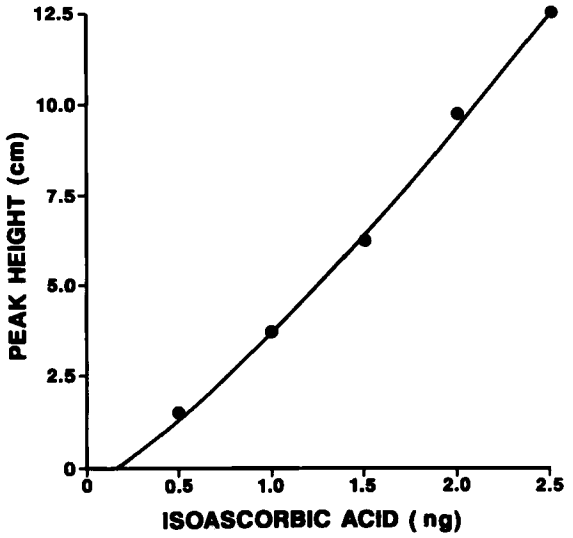

Figure 2. Isoascorbic acid standard curve. An almost identical curve was obtained for ascorbic acid.

were for AA, 1.55 ± 0.02 ng and a retention time of 27.34 ± 0.040 min., and for IAA, 1.54 ± 0.03 ng and a retention time of 28.60 ± 0.04 min. The test was carried out in a time span of almost 7 hrs. As we intended to use an autosampler overnight it was necessary to test the stability of AA and IAA for a longer period of time. Consequently, two solutions containing 1.0 and 2.0 ng of AA and IAA were automatically and alternately injected into the system twelve times for a total time of 18 hrs. Retention times and concentrations varied very little. For AA (1.0 ng), observed values were 1.03 ± 0.02 ng and a retention time of 27.32 ± 0.01 min, while for IAA (1.0 ng), 1.00 ± 0.02 ng and a retention time of 28.59 ± 0.01 min. When the standard was 2.0 ng, AA values were 2.01 ± 0.03 ng and 27.32 ± 0.01 min, and for IAA 2.00 ± 0.04 ng and 28.58 ± 0.01 min.

TABLE 1.

Ascorbic and Isoascorbic acid content of selected processed meats and recovery of added standards.

Product	AA mg/100 g	IAA mg/100 g	% Recovered AA	% Recovered IAA
Polish Sausage A.	58.5*	15.4	94	94
Polish Sausage B.	tr	66.1	107	110
Pepperoni A.	18.8	26.4	94	94
Pepperoni B.	tr	39.1	101	102
Salami	tr	28.2	104	107
Beef Wiener	tr	23.8	99	100
Chicken Wiener	0	19.4	102	106
Bologna	tr	29.9	105	108
Summer Sausage	tr	39.4	105	108
Kolhassa Sausage	tr	34.1	99	97
Frankfurters	0	35.5	100	104
Mock Chicken	tr	18.5	97	96

* The values represent the average of duplicate determinations. Individual values did not differ by more than 3% from the average value.

tr: traces

Recovery studies were performed by determining AA and IAA levels in several samples of foods with and without the addition of 0.2 ng of AA and IAA standard. Table 1 shows that, with the exception of two products that also contained AA, the presence of IAA was confirmed in all the processed meats analyzed. Recoveries of AA added were in the range of 94 to 107% and for IAA from 94 to 110%. Figure 3 shows several chromatograms from processed meat containing AA and/or IAA. Fig. 3A shows an analysis of a sample of pepperoni prepared with AA and IAA. Fig. 3B is a sample of wiener containing only IAA, while Fig. 3C shows a sample of salami also containing only IAA. In Fig. 3D the same sample of salami was spiked

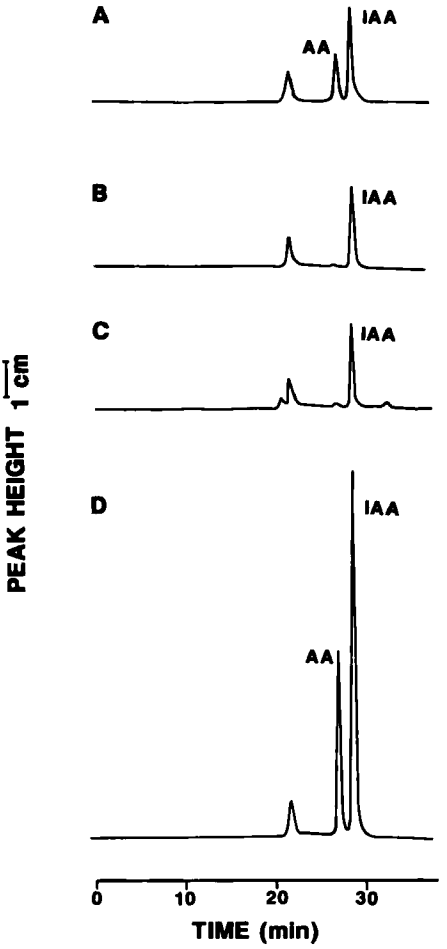

Figure 3. Chromatograms of selected processed meats. A) Pepperoni prepared with AA and IAA. B) Wiener prepared with IAA. C) Salami prepared with IAA. D) Same sample of salami but spiked with a standard containing AA and IAA.

TABLE 2

Ascorbic acid and isoascorbic acid content of ground beef samples suspected of adulteration

Sample #	AA mg/100 g	IAA mg/100 g
1	0	60.5*
2	0	12.9
3	81.7	0
4	37.1	0
5	0	0
6	0	0
7	10.6	0
8	0	32.9
9	20.8	0
10	0	9.6
11	0	0
12	0	0

* The values represent the average of duplicate determinations, individual values did not differ by more than 3% from the average value.

with a standard of 1 ng each of AA and IAA. As indicated above, the recovery in this case was 104 and 107% respectively.

Analysis of twelve samples of ground beef suspected of containing IAA revealed that eight of them contained either AA or IAA (Table 2). Several authors have reported that vitamin C improves pigment and lipid stability in ground beef (15,16). Apparently it is being used by some retailers to maintain the fresh appearance of the ground beef.

Figure 4 shows three chromatograms of ground beef samples. Fig. 4A shows results from a sample of ground beef adulterated with IAA and 4B results from a sample adulterated with AA. Fig. 4C shows a chromatogram of a sample of normal

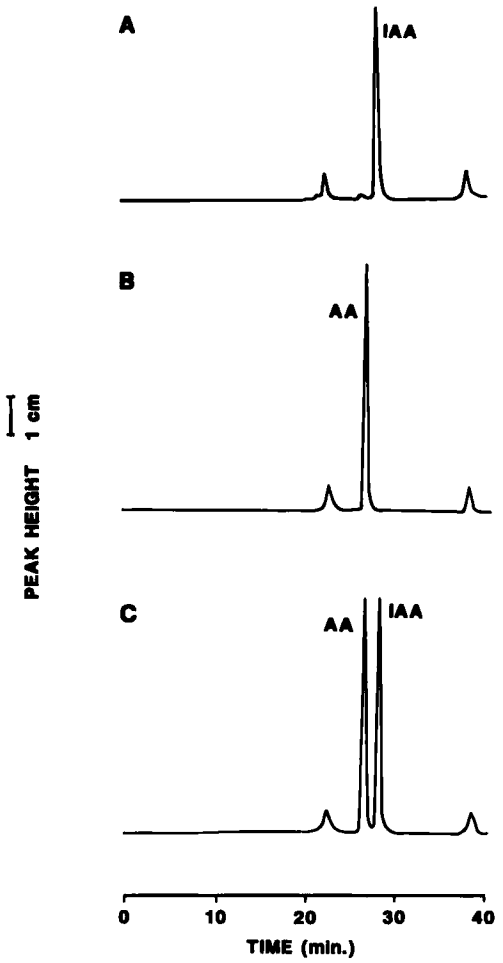

Figure 4. Chromatograms of selected ground beef samples. A) Ground beef adultered by the addition of IAA. B) Ground beef adulterated by the addition of AA. C) Normal ground beef spiked with a standard containing AA and IAA.

TABLE 3.

The content of Ascorbic acid and Isoascorbic acid and their corresponding oxidized forms in selected foods.

Food	Total AA	AA	DHAA	Total IAA	IAA	DHIAA
Applesauce	77.6*	73.3	4.3	10.7	9.1	1.6
Baby food-peaches	33.5	31.6	1.9	7.1	6.2	0.9
Baby food-rice cereal[1]	37.7	28.9	8.8	nd		
marmalade-peaches	20.1	18.7	1.4	nd		

* Values are expressed as mg/100 g of food and represent the average of duplicate determination, individual values did not differ by more than 3% from the average value.

nd: not detected.

[1] The sample was composed of 35% infant formula and Rice cereal with bananas fortified with iron.

ground beef spiked with an AA and IAA standard. Recoveries of AA and IAA, were in the range of 95 to 105%.

Both, the processed meats and ground beef samples were prepared in the presence of homocysteine, therefore the indicated values for AA and IAA are total values, which also included DHAA and DHIAA. Following a similar procedure described earlier (7), in which samples were incubated with or without homocysteine, it is possible with the present method to estimate DHAA and DHIAA. Table 3 shows a few examples of selected foods in which DHAA is a minor component of the total AA in the food with the exception of baby food-cereal in which 23% of the total was composed of DHAA. It is likely that iron, which is added to these cereals, catalyses the oxidation of AA and IAA, thus accounting for these high levels. However, the values are not far from those described in powdered infant formula (17) or for some canned food (18), using the same procedure. A broader survey of food products is under way in our laboratory to estimate the amount of AA and/or IAA added by manufacturers.

In summary, the extraction and HPLC procedures reported here allow for the quantitation of AA, IAA, DHAA and DHIAA in food samples. The amperometric detection permits quantitation at levels of 0.5 ng. This high sensitivity makes this method useful in analysing biological fluids and tissues.

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