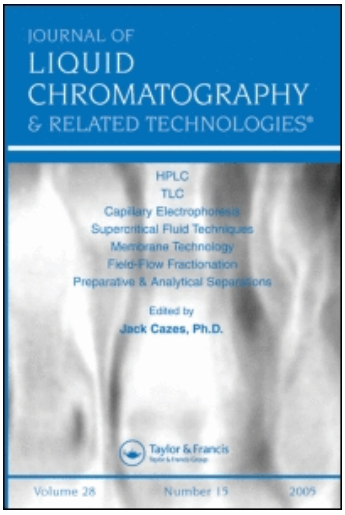

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Procedure for the Separation and Quantitative Analysis of Ascorbic Acid, Dehydroascorbic Acid, Isoascorbic Acid, and Dehydroisoascorbic Acid in Food and Animal Tissue

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To cite this Article Behrens, Willy A. and Madere, Rene(1994) 'A Procedure for the Separation and Quantitative Analysis of Ascorbic Acid, Dehydroascorbic Acid, Isoascorbic Acid, and Dehydroisoascorbic Acid in Food and Animal Tissue', Journal of Liquid Chromatography & Related Technologies, 17: 11, 2445 — 2455
To link to this Article: DOI: 10.1080/10826079408013492
URL: http://dx.doi.org/10.1080/10826079408013492

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(11), 2445-2455 (1994)

A PROCEDURE FOR THE SEPARATION AND QUANTITATIVE ANALYSIS OF ASCORBIC ACID, DEHYDROASCORBIC ACID, ISOASCORBIC ACID, AND DEHYDROISOASCORBIC ACID IN FOOD AND ANIMAL TISSUE*

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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 and animal tissues by reverse phase high-performance liquid chromatography. Two PLRP-S columns in series were used with a pH 2.2 mobile phase containing 20 mM phosphate buffer and 0.17% metaphosphoric acid. An amperometric detector set at 0.7 volt and 20 mA was used. As little as 0.5 ng of each compound could be detected. 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, DHAA and DHIAA, could then be calculated by substraction.

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

*Bureau of Nutritional Sciences Publication #430

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(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 derivatization.

We reported a procedure for the direct and simultaneous determination of AA and IAA in food products by paired-ion reverse-phase high performance liquid chromatography with electrochemical detection (13). When the same samples were incubated with homocysteine to reduce 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 could then be calculated by subtraction. The procedure is highly sensitive but was subject to interferences when it was applied to biological fluids and tissues. Modifications to this procedure are reported here and include the use of polystyrene divinyl benzene polymer (PLRP-S) columns as proposed by Vanderslice and Higgs (9) but conserving our approach of treating the sample with homocysteine (7,14) in order to estimate AA, IAA, DHAA and DHIAA by a double injection into the HPLC system.

MATERIALS AND METHODS

HPLC System

A chromatographic system (Spectra-Physics, San Jose, CA) consisting of a SP8800 pump, SP8760 autosampler cooler, SP8780 autosampler and a Chromjet

integrator was used. A pulse dampener (SSI, Supelco, Oakville, ONT) was incorporated into the system. The amperometric detection system (BAS, West Lafayette, IN) included an LC-4B 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 +0.7 volts (oxidative) and the sensitivity range was 20 mA. Two 25 cm x 4.6 mm, 5 μm particle size, PLRP-S columns (Polymer Laboratories Inc., Amherst, MA) connected in series were used to separate AA and IAA. For food analysis, the columns were kept at 22°C and for biological fluids and tissue analysis they were cooled to 5°C. The mobile phase was 20 mM sodium phosphate monobasic, monohydrate containing 0.17% metaphosphoric acid at a final pH 2.2. The mobile phase was filtered through a 0.22 μm GS filter (Millipore Corp., Bedford, MA), degassed for half hour using a water aspirator and followed by bubbling helium continuously. For food analysis the flow was 0.7 ml/min at 22°C, but for tissues the flow was set at 0.6 ml/min and the columns cooled at 5°C. Samples and standards in a volume of 20 μl were injected into the chromatograph using the autosampler, maintained at 4-5°C. A calibration curve of at least 5 standards was run daily. Peak area 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 calculate sample concentration.

Sample Preparation

Samples (0.5-3.0 g) were homogenized with 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 (15). Homogenates were centrifuged at 30,000 x g in a refrigerated centrifuge for 30 min. The supernatant was filtered through a Millex-GS 0.22 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 determination 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.

Samples

Processed meats and other foods were obtained from local supermarkets. Plasma and tissues were obtained from five male Wistar rats (Charles River, Canada, St. Constant, Quebec) that were fed an AIN-76 diet (16) for three months. After this period, they were fed for 5 days the same diet but containing 100 g IAA/kg diet.

Tissues were collected from animals anesthetized with halothane (2% in oxygen) (Fluothane, Ayerst Laboratory, Montreal). Plasma and samples of liver, adrenal gland and spleen were stored at -75°C until analysis.

RESULTS AND DISCUSSION

A chromatogram of standards is shown in figure 1. The AA and IAA peaks, 1 ng each, were well resolved on a stable baseline. Usual retention times were 15.6 min for AA and 18.3 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 1.7 to 1.8 min apart. In our previous method (13), the retention times were 27.3 for AA and 28.6 min for IAA, therefore two PLRP-S columns in this case performed much better than three C18 reverse-phase columns even in more adverse conditions. The columns were run at 0.6 ml/min because they were cooled down to 5°C to allow for a better separation of AA and IAA. As will be shown later, when the columns are used for food analysis the flow was 0.7 ml/min and the temperature was kept at 22°C and the retention times were shorter.

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 an almost linear relationship. Figure 2 shows a calibration graph for AA and IAA. 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.

In our previous method (13) the detector response for AA and IAA was almost equal, however with the present system the response for IAA was smaller. As recoveries for both were the same (97-102%) this has to be attributed to a different mobile phase or to an unknown factor.

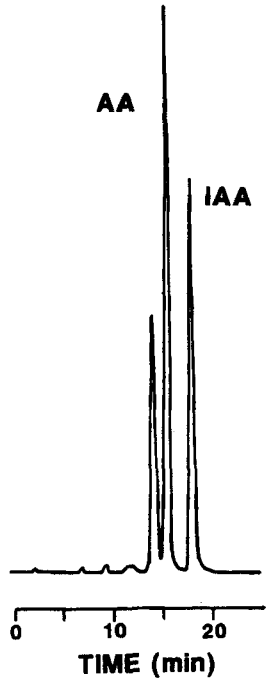

Figure 1. Chromatogram of standards. AA: ascorbic acid and IAA: isoascorbic acid (1 ng each). The retention times were 15.6 min for AA and 18.3 min for IAA. The peak at 14.0 min corresponds to homocysteine.

Feeding rats a diet containing 10% IAA for five days produced high levels of this compound in plasma and the selected tissues, liver, spleen and adrenal gland (Figure 3). As can be seen in the chromatograms, the peaks of AA and IAA were well separated on a stable baseline and no interfering peaks were observed, except in the case of adrenal gland where an unidentified peak with a retention time of 17.5 min was close to IAA (18.3 min), however this was not a problem for the quantification of IAA. This interfering peak required the cooling of columns at 5°C. The values for AA, IAA, DHAA and DHIAA obtained after feeding rats a diet containing 10% IAA for 5 days, are presented in Table 1. As expected, the adrenal gland accumulated the highest

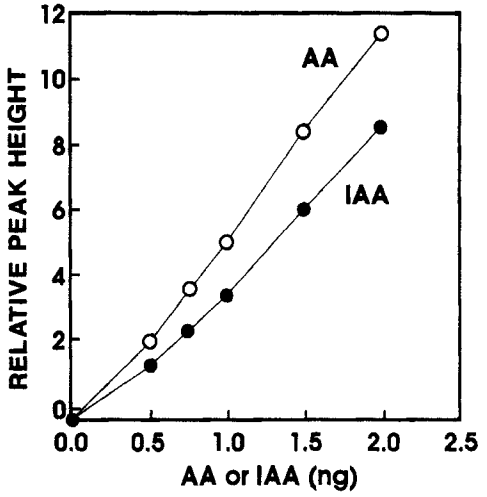

Figure 2. Calibration curve for standard solutions of ascorbic acid (AA) and isoascorbic acid (IAA) in the range of 0.5 to 2.0 ng.

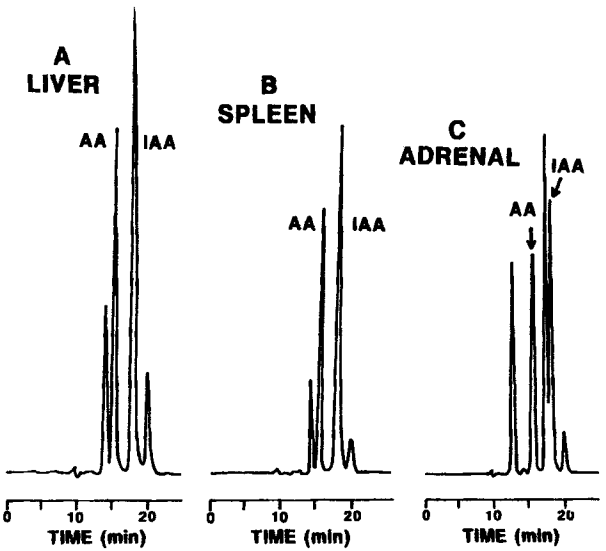

Figure 3. Chromatograms of tissues from rats fed a diet a diet containing 10% of isoascorbic acid (IAA). Retention times were almost identical to those in Fig. 1. In panel A is liver, in panel B is spleen and in panel C is adrenal. In the latter tissue there was an unidentified peak with a retention time of 17.5 min.

TABLE 1

Ascorbic Acid, Dehydroascorbic Acid, Isoascorbic Acid and Dehydroisoascorbic Acid Content of Plasma and Selected Tissues of Rats Fed a Diet Containing 10% of Isoascorbic Acid.

Tissue	Total AA	AA	DHAA	Total IAA	IAA	DHIAA
Plasma	9.09* ±1.65	6.48 ±1.27	2.61 ±0.52	53.52 ±14.69	40.38 ±12.11	13.14 ±6.89
Spleen	376.6 ±56.8	257.8 ±32.0	118.6 ±31.1	456.6 ±116.4	299.4 ±89.9	157.2 ±30.7
Liver	114.8 ±33.8	101.6 ±38.0	13.2 ±7.7	123.20 ±42.8	102.2 ±31.7	21.2 ±15.2
Adrenal	1794.4 ±418.9	1505.2 ±530.0	289.0 ±156.6	1983.6 ±433.0	1465.4 ±558.8	518.2 ±512.9

* The values are mean ± SD, and are expressed in μg/g of tissue, or ml of plasma. There were 5 rats in the group.

levels of AA. Interestingly, the levels of IAA are equal to those of AA under our dietary conditions. Total AA and Total IAA were present mainly in the reduced forms (AA and IAA) with the exception of spleen in which the oxidized forms (DHAA and DHIAA) represented approximately 50% of the total vitamin. A complete study of the effect of dietary IAA (and DHIAA) on the tissue levels of AA (and DHAA) will be presented later.

As indicated above, food analysis was performed with the two PLRP-S columns at 22°C, because no interfering peaks were observed in any of the samples selected. Chromatograms (not shown) showed AA and IAA well resolved on a stable baseline. Because of the higher temperature and a higher flow (0.7 ml/min), retention times were 11.9 min. for AA and 13.4 min. for IAA. The calibration curve (not shown) was similar to that in the tissue analysis. Recoveries studies were performed with all food samples and the values were in the range 94-104%.

Table 2 shows a sample of selected processed foods, in which meat products, without exception, presented variable levels of IAA and DIAA. In some products, such

TABLE 2

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
skimmed 1% milk	nd	—	—	nd	—	—
Condensed milk	1.47*	0.79	0.68	nd	—	—
Evaporated milk (2%)	19.60	16.80	2.80	nd	—	—
Cherry cheesecake	10.55	4.40	6.15	nd	—	—
Diet product, chocolate	22.75	10.34	12.41	nd	—	—
Iced Tea	nd	—	—	10.43	7.34	3.09
Cooked Ham	tr	—	—	72.16	68.99	3.17
Sausage	1.44	0.63	0.81	12.81	7.63	5.18
Chicken spread	5.13	4.24	0.89	42.13	29.46	12.67
Fruit Preservative	8433.0	7767.0	667.0	nd	—	—

* Values are expressed as mg/100 g or ml and represent the average of duplicate determinations, individual values did not differ by more than 3% from the average value.
nd: not detected, tr: traces.

as condensed milk, cheesecake and a diet product, DHAA was as high or higher than AA. On the other hand, products that contained IAA, the oxidized form was in small proportion. A fruit preservative, a product to be used in home fruit canning, contained only AA and DHAA at very high levels. These values represent a selected sample of a more widespread survey that is underway in our laboratory.

The method described here allows for the quantification of AA, IAA, DHAA and DHIAA in food samples and in tissues of animals, and is highly sensitive. The amperometric detection permits quantification at levels of 0.5 ng/20 μl injection, but

requires a second injection in the chromatograph, after treatment with homocysteine, to determine the oxidized forms. On the other hand, the method of Vanderslice and Higgs (8,9) only requires one injection; however, after separation on the column, the compounds are converted into fluorescence derivatives, which require a more complex HPLC system and makes the method more difficult and less sensitive.

REFERENCES

1. J. Fabianek, and H. Hepr. Antiscorbutic activity of d-araboascorbic acid, Proc. Soc. Exp. Biol. Med., 125: 462-465 (1967).

2. O. Pelletier and C. Godin. Vitamin C Activity of D-isoascorbic acid for the guinea-pig, Can. J. Physiol. Pharmacol., 47: 985-991 (1969).

3. J.C. Bauernfeind and D.M. Pinkert. Food processing with added ascorbic acid, Adv. Food Res., 18: 220-315 (1970).

4. B. Borenstein. The role of ascorbic acid in foods, Food Technol., 41: 98-99 (1987).

5. L.A. Pachla, D.L. Reynolds, and P.T. Kissinger. Analytical methods for determining ascorbic acid in biological samples, food products and pharmaceuticals, J. Assoc. Off. Anal. Chem., 68: 1-12 (1985).

6. R.C. Rose and D.L. Nahrwold. Quantitative analysis of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography, Anal. Biochem., 114: 140-145 (1981).

7. W.A. Behrens and R. Madère. A highly sensitive high-performance liquid chromatography method for the estimation of ascorbic and dehydroascorbic acid in tissues, biological fluids, and foods, Anal. Biochem., 165: 102-107 (1987).

8. J.T. Vanderslice and D.J. Higgs. Chromatographic separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroisoascorbic acid and their quantitation in food products, J. Micronutr. Anal., 4: 109-118 (1988).

9. J.T. Vanderslice and D.T. Higgs. Separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroascorbic acid in food and animal tissue, J. Micronutr. Anal., 7: 67-70 (1990).

10. M.A. Kutnink, J.H. Skala, H.E. Sauberlich and S.T. Omaye. Simultaneous determination of ascorbic acid, isoascorbic acid 9erythorbic acid) and uric acid in human plasma by high-performance liquid chromatography with amperometric detection, J. Liquid Chromatog., 8: 31-46 (1985).

11. L.L. Lloyd, F.P. Warner, J.F. Kennedy and C.A. White. Quantitative analysis of vitamin C (L-ascorbic acid) by ion suppression reversed phase chromatography, Food Chem., 28: 257-268 (1988).

12. W. Schüep and E. Keck. Measurement of ascorbic acid and erythorbic acid in processed meat by HPLC, Z. Lebensm Unters. Forsch., 191: 290-292 (1990).

13. W.A. Behrens and R. Madère. Quantitative analysis of ascorbic acid and isoascorbic acid in foods by high-performance liquid chromatography with electrochemical detection, J. Liquid Chromatog., 15: 753-765 (1992).

14. R.E. Hughes. The use of homocysteine in the estimation of dehydroascorbic acid, Biochem. J., 64: 203-208 (1956).

15. O. Pelletier and R. Brassard. Determination of vitamin C (L-ascorbic acid and dehydroascorbic acid) in food by manual and automated photometric methods, J. Food Sci., 42: 1471-1477 (1977).

16. American Institute of Nutrition. Report of the American Institute of Nutrition Ad Hoc Committee on standards for nutritional studies, J. Nutr., 107: 11340-1348 (1977).

Received: September 29, 1993
Accepted: January 14, 1994