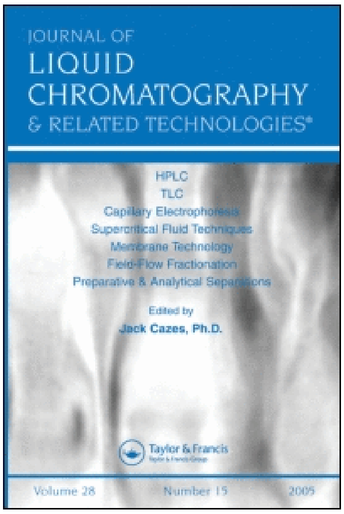

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# Comparison of Two Methods of Ascorbic Acid Determination in Vegetables

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# COMPARISON OF TWO METHODS OF ASCORBIC ACID DETERMINATION IN VEGETABLES

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## ABSTRACT

Vegetables (broccoli, Brussels sprouts, cauliflower, green beans, spinach, and turnip) were analyzed for ascorbic acid using a modified AOAC method and compared to a high performance liquid chromatography (HPLC) method. The HPLC method employed a weak ion exchange μBondapak $NH_2$ column and detection at 254 nm. The two methods did not differ in ascorbic acid values for fresh and three-week stored broccoli, cauliflower, green beans, turnip and three-week stored Brussels sprouts and spinach. A higher ascorbic acid content was found for fresh Brussels sprouts and spinach when measured by the HPLC method.

## INTRODUCTION

Measurement of ascorbic acid by direct titration with 2,6-dichloroindophenol dye has been the most used method because it is simple and rapid. The method is applicable to citrus fruit juices and vitamin tablets which do not contain interfering substances. The major problem with this method has been color interferences which make the titration endpoint difficult to determine. The dye is also reported to react with numerous substances but an acceptable alternative method has not been found which is as easy and avoids these problems (1).

Chemical substances which are capable of reducing the dye in the AOAC procedure are copper and iron ions, sulfite, thiosulfate, D-isoascorbic acid, tannins, betanin, and sulfhydryl compounds such as cysteine and glutathione (1). These compounds may be either naturally present in foods or added during the processing of foods. Several vegetables reported high in ascorbic acid (2) also contain various sulfur-containing compounds. Ascorbic acid values for these vegetables may in part be due to sulfur-containing compounds which reduce the dye.

Due to the unstable nature of ascorbic acid, prior to and during chromatographic analysis, numerous HPLC methods are reported in the literature (3,4,5,6,7,8,9,10,11). The advantages of an HPLC method are chromatographic separation of potentially interfering compounds and elimination of the reaction with the dye which can take place with compounds other than ascorbic acid. Therefore, the objective of this study was to compare the 2,6 dichloroindophenol modified AOAC ascorbic acid method with an HPLC method using vegetables of high sulfhydryl content. Green beans were also included which contain minimal sulfur-containing compounds.

## MATERIALS AND METHODS

### Vegetable Preparation

Fresh vegetables were analyzed for ascorbic acid, total sulfur, non-protein sulfhydryl and moisture contents as soon as possible after harvest. All vegetables were also analyzed after a three-week storage period. Storage conditions consisted of a walk-in cooler maintained at 2°C and 95-100% relative humidity. Weight loss during storage was used to adjust raw values. Vegetables were prepared for analysis as follows. Each broccoli head was trimmed to three inches, florets broken apart and a random sample was taken from each head. Two Brussels sprouts were cut in half, one half of each sprout was used for each ascorbic acid method. Each cauliflower head was trimmed of leaves, florets were broken apart, and a random sample was taken from each head. The outer leaves of each spinach plant were used. A wedge was cut from each turnip. Ten green beans were cut in half; 5 stem ends and 5 blossom ends were analyzed for each ascorbic acid method. Seven replicate samples were analyzed for each ascorbic acid method, moisture and total sulfur. Six replicate samples were used for non-protein sulfhydryl analysis.

### HPLC Method

The HPLC method of Bradbury and Singh (12) was modified. Approximately 25 g of each vegetable was accurately weighed and homogenized with 100 mL aqueous 5% metaphosphoric acid for 2 min in an Osterizer. The slurry was centrifuged at 1100 x g for 10 min. The supernatant was filtered through Whatman no. 4 filter paper. An aliquot was diluted to 10 mL to obtain ascorbic acid amounts of 0.25 to 1.0 $\mu$g. This aliquot was filtered through a C18 SEP-PAK cartridge (Waters Associates, Milford, MA) for color removal and then passed through a 0.45 micron membrane. 20 $\mu$L were loaded on a weak ion exchange Waters $\mu$Bondapak $NH_2$ column (3.9 mm x 30 cm). The mobile phase was aqueous 0.005 M $KH_2PO_2$ adjusted to pH 4.6 and acetonitrile 30:70 (v/v) which was continuously sparged with helium during operation. A flow rate of 1 mL/min was maintained on a Beckman model 110A pump. Ascorbic acid eluted at 4 min and monitored at 254 nm on a variable wavelength Hitachi model 100-40 Spectrophotometer.

### Dichloroindophenol Method

The AOAC method (13) was modified as follows. Approximately 25 g of each vegetable was weighed and homogenized for 2 min in 100 mL of aqueous 3% metaphosphoric acid. The slurry was centrifuged for 10 min at 1100 x g and the supernatant filtered through Whatman no. 4 filter paper. An aliquot was diluted to obtain 0.01 to 0.03 g ascorbic acid and adjusted to pH 3.6 with citrate buffer (0.5 M citric acid in 1 N NaOH). This solution was filtered through a C18 SEP-PAK cartridge prior to reaction with 2,6-dichloroindophenol. Absorbance was determined at 520 nm on a Bausch and Lomb Spectronic 20.

### Sulfur, Sulfhydryl, and Moisture Methods

Total sulfur was measured by combusting a 0.2 g sample at 1400°C in a Leco Sulfur Analyzer (Leco Corporation, St. Joseph, MI). Non-protein sulfhydryl content was analyzed using a modified Ellman's method (14). Vegetables were dried at 110°C for ca. 24 hours for moisture determination.

### Statistical Analysis

SAS was used to statistically analyze the data (15).

## RESULTS AND DISCUSSION

The ascorbic acid content of the six vegetables for the two analytical periods and two methods are illustrated in FIGURE 1. No significant differences occurred between the two methods when analyzing fresh and stored broccoli, cauliflower, green beans, and turnip, and stored Brussels sprouts and spinach. While changes in ascorbic acid are likely with

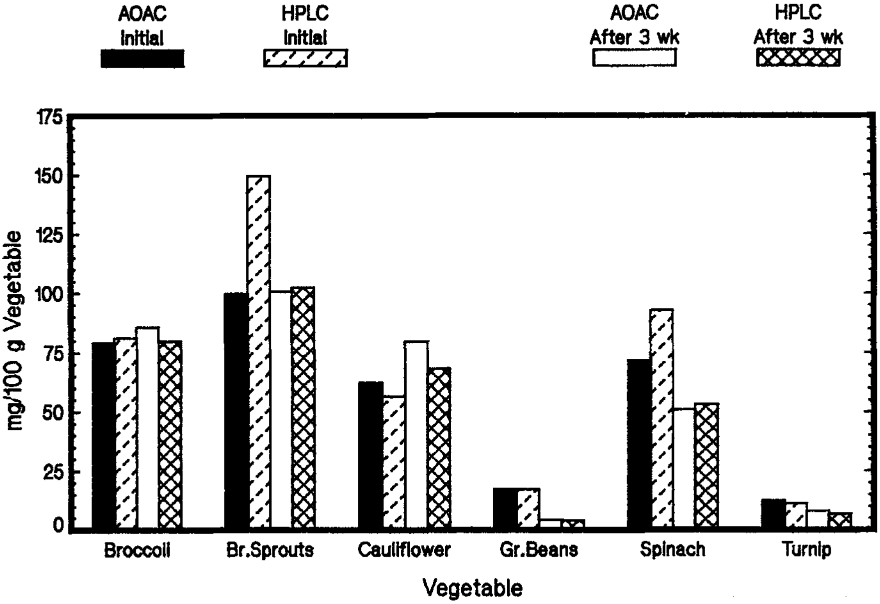

FIGURE 1. Ascorbic acid content of six vegetables analyzed by a modified AOAC method and an HPLC method. Each vegetable was analyzed initially after harvest and after 3 weeks storage at 2°C and 95-100% relative humidity.

certain vegetables, the methods measured the changes in a similar manner. Significant differences resulted between the two methods for fresh Brussels sprouts and spinach at $p \leq 0.05$. These higher ascorbic acid contents for fresh Brussels sprouts and spinach using the HPLC method are difficult to explain. The variability in leaves within a fresh spinach plant may have been demonstrated, although this variability was not present after three-weeks storage.

The AOAC ascorbic acid contents (mg/100 g) for the vegetables in this study were: broccoli, 79.0; Brussels sprouts, 99.7; cauliflower, 62.3; green beans, 17.4; spinach, 71.8; and turnip, 12.6. Haytowitz and Matthews (2) list the ascorbic acid values (mg/100 g) for these same vegetables, as: broccoli, 93.2; Brussels sprouts, 85.0; cauliflower, 71.5; green beans, 16.3; spinach, 28.1; and turnip, 21.0. Ascorbic acid contents were higher in this study than that listed by Haytowitz and Matthews (2) for Brussels sprouts and spinach; comparable for green beans; and lower for broccoli and

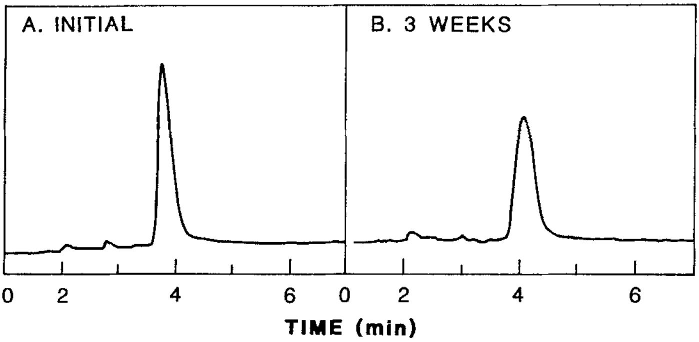

FIGURE 2. Chromatograms of ascorbic acid in fresh and stored broccoli. The ascorbic acid peak eluted at 4 min.

turnip. These differences may be due to different growing conditions such as season, location, cultivar, fertilization, irrigation, amount of sunlight and heat stress.

Chromatograms for broccoli are illustrated in FIGURE 2. The only compound known to co-elute under the ascorbic acid peak is erythorbic acid. Erythorbate is not known to exist naturally in plants and animals (16). The HPLC method could measure compounds in fresh Brussels sprouts and spinach other than ascorbic acid which may elute with ascorbic acid. Cysteine, dehydroascorbic acid and citric acid were not detected at 254 nm under the HPLC method conditions. Therefore, these compounds did not produce the higher ascorbic acid values with the HPLC method for fresh Brussels sprouts and spinach.

Total sulfur and non-protein sulfhydryl contents of the freshly harvested vegetables are listed in TABLE 1.
Total sulfur content of Brussels sprouts was greater than that found in the other vegetable and non-protein -SH content of spinach was significantly higher than the other vegetables ($P \leq 0.05$). These higher levels of total sulfur or non-protein -SH may be responsible for the differences in the AOAC and HPLC results. Changes in the amount or form of sulfur compounds during storage may result in more or less interference in one or both of the methods.

Metaphosphoric acid is used to extract ascorbic acid due to its ability to stabilize ascorbic acid to air oxidation (1). It is also Total sulfur content of Brussels sprouts was greater than that found in the other

TABLE 1.

Total Sulfur and Non-Protein Sulfhydryl Contents of Selected Vegetables.

| Vegetable | Total Sulfur[1] mg/100 g | Non-Protein -SH[2] mmoles/g |
|---|---|---|
| Broccoli | 121.32 ± 13.01 | $0.934^a$ ± 0.175 |
| Brussels Sprouts | 172.53 ± 17.27 | $1.004^a$ ± 0.238 |
| Cauliflower | 72.24 ± 8.98 | $0.997^a$ ± 0.174 |
| Green Beans | 13.85 ± 1.18 | $0.883^a$ ± 0.117 |
| Spinach | 44.31 ± 5.00 | $1.833^b$ ± 0.581 |
| Turnip | 21.31 ± 2.46 | $0.908^a$ ± 0.191 |

[1] Results are means and standard deviations of 7 replicate samples.

[2] Results are means and standard deviations of 6 replicate samples.

[a,b] Mean values in the same column followed by the same letter are not significantly different as determined by Duncan's Multiple range at the $p \leq .05$ level of significance.

vegetable and non-protein -SH content of spinach was significantly higher than the other vegetables ($P \leq 0.05$). These higher levels of total sulfur or non-protein -SH may be responsible for the differences in the AOAC and HPLC results. Changes in the amount or form of sulfur compounds during storage may result in more or less interference in one or both of the methods.

Metaphosphoric acid is used to extract ascorbic acid due to its ability to stabilize ascorbic acid to air oxidation (1). It is also an excellent protein precipitant (16). Proteins containing -SH groups would therefore not be in the vegetable extract. However, free amino acids such as cysteine and glutathione (a sulfhydryl- containing tripeptide) are found in vegetables. These non-protein containing -SH compounds could remain in the vegetable extract and cause false high ascorbic acid values.

Preliminary work was done in which cysteine (a sulfhydryl-containing amino acid) was added to the ascorbic acid standard used for the dichloroindophenol spectrophotometric method. This resulted in an increase of 10 - 35% ascorbic acid when compared to a standard without added cysteine.

Further experimentation included analysis of broccoli and its ascorbic acid extract for total sulfur. Approximately 50% sulfur remained in the broccoli extract which was assumed to be non-protein -SH compounds or free sulfur. These sulfur compounds were thought to interfere with the dichloroindophenol dye as did cysteine. Analysis of the ascorbic acid extracts of the vegetables for various sulfur compounds may give insights to the higher ascorbic acid values found in Brussels sprouts and spinach for the HPLC method.

The ascorbic acid content for the vegetables measured by the dichloroindophenol method was expected to be higher than ascorbic acid contents of vegetables analyzed by HPLC because of the potential for measuring compounds other than ascorbic acid. These data do not explain the higher ascorbic acid content of fresh Brussels sprouts and spinach when analyzed by the HPLC method. In this study, naturally occurring sulfur-containing compounds in foods did not appear to interfere with the dichloroindophenol dye used in the AOAC method. Different unknown compounds, specific to fresh Brussels sprouts and spinach could elute with ascorbic acid. With storage these compounds may be oxidized and therefore do not result in higher ascorbic acid contents when measured by HPLC versus the dichloroindophenol method.

HPLC and AOAC gave comparable results in fresh and three- week stored broccoli, cauliflower, green beans, turnip and three-week stored Brussels sprouts and spinach. Ascorbic acid contents when measured by the HPLC method were higher for fresh Brussels sprouts and spinach. The advantages of the HPLC method are the avoidance of color and chemical interferences with the dichloroindophenol dye. The AOAC method remains a rapid, easy and less expensive method for routine analysis.

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