Table III. Retention Times of Pyrazines on a C-18 Reversed-Phase Column[a]

pyrazine	ret time,[b] min	pyrazine	ret time,[b] min
tetramethyl	7.3	2-methoxy-3-isobutyl	13.3
2-methoxy-3-isopropyl	10.7	2-methoxy-6-isobutyl	15.5

[a] Mobile phase: 72% aqueous methanol buffered with 0.01 M $(NH_4)H_2PO_4$ and adjusted to pH 2.6 with concentrated phosphoric acid. [b] Flow rate 0.8 mL/min.

oxypyrazines standards (Figure 4b; Table III). In the wine spiked with methoxypyrazines, several peaks were observed near the retention time of TMP; some of them may have been TMP and the TMP breakdown unknowns (Figure 4c), as TMP has been previously reported in wines by Kosuge et al. (1971). Recovery of the 3IBP ranged between 43 and 62%. Assuming 50% recovery, the minimum detection level by this technique is 1.2 μg/L. In contrast, the recovery of IPP was extremely inefficient, in all cases being less than 19%.

ABBREVIATIONS USED

SDE	simultaneous distillation–extraction
3IBP	2-methoxy-3-isobutylpyrazine
TMP	tetramethylpyrazine
IPP	2-methoxy-3-isopropylpyrazine
6IBP	2-methoxy-6-isobutylpyrazine
DDW	double-distilled water

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Simultaneous Analysis of Ascorbic and Dehydroascorbic Acid by High-Performance Liquid Chromatography with Postcolumn Derivatization and UV Absorbance

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A high-performance liquid chromatography (HPLC) procedure has been developed for the rapid and simultaneous estimation of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in fresh fruits and vegetables. Isocratic separation of these components was accomplished by anion-exchange chromatography using acetonitrile–0.05 M KH_2PO_4 (75:25, v/v) as eluant. AA was determined by monitoring its absorbance at 254 nm, while DHAA detection was achieved by fluorescence as a result of postcolumn derivatization involving the condensation of DHAA with o-phenylenediamine (OPDA), to form a highly fluorescent quinoxaline derivative. The procedure allows detection for both forms of vitamin C at levels well below those usually found in orange juice.

INTRODUCTION

Fruits and vegetables constitute the major sources of vitamin C for human diets. The total vitamin C consists of the sum of ascorbic acid and its oxidized form, dehydroascorbic acid. Both forms have equal antiscorbutic activity (Tannenbaum, 1974).

Numerous methods for the analysis of vitamin C activity have been described. The most commonly used are the 2,6-dichlorophenolindophenol visual titration (AOAC, 1975), the spectrophotometric method with dinitrophenylhydrazine derivitization of DHAA (Roe et al., 1948), and the microfluormetric method by condensation of

DHAA with OPDA (AOAC, 1975). However, these methods are not specific and are often limited by the number of interferring substances present in foods. In addition, it is difficult to visually determine the end point when these methods are used with colored solutions. Pelletier and Brassard (1977) described an improved photometric method based on 2,4-dinitrophenylhydrazine for the AA and DHAA determination in foods. Though their method eliminated interference from other compounds, it is time consuming and requires special sample preparation.

Recently, due to the development of commercial HPLC systems, quantitative measurement of AA and DHAA in various substances has been reported by many investigators. Procedures vary in the type of column, elution conditions, detection systems, and the extraction technique

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used to stabilize AA and DHAA. AA can be determined easily by HPLC, with UV detection, but the determination of DHAA is complicated by its extremely low UV absorptivity. A procedure using two reversed-phase HPLC columns in series for the separation of AA and DHAA was reported by Finley and Duang (1981). They used water with a counterion reagent (tri-n-butylamine) as a mobile phase. AA and DHAA were detected at 254 and 210 nm, respectively. Rose and Nahrwold (1981) and Wimalasiri and Wills (1983) used a similar detection system with a single ion-exchange column and a mobile phase of acetonitrile–water containing 2.5 mM potassium dihydrogen phosphate. Doner and Hicks (1981) reported a separation of AA and DHAA by HPLC on a Zorbax-NH_2 column. The AA was monitored at 268 nm, while refractive index (RI) detection allowed the detection of DHAA. However, neither RI nor low wavelength (210 nm) can detect small amounts of DHAA such as that present in foodstuffs. Also, the low wavelength used introduces problems of solvent purity.

Therefore, most of the HPLC analytical procedures used are based on either the reduction of DHAA to AA and detection of the total ascorbic acid (TAA) by UV or oxidation of AA to DHAA, and the TAA is determined by fluorometry after condensation of DHAA with OPDA. Dennison et al. (1981) described an HPLC method for the analysis of total vitamin C in beverages by UV measurement of AA after reduction of DHAA with homocysteine. Keating and Haddad (1982) reported the simultaneous determination of AA and DHAA using precolumn derivatization. DHAA was converted into a fluorophore with OPDA. The detection was made at 290 nm for AA and 348 nm for the fluorophore. Speek et al. (1984) developed an HPLC method for the simultaneous determination of total vitamin C based on precolumn enzymatic oxidation of AA to DHAA. The latter is condensed with OPDA and detected fluorometrically. DHAA can be determined with omission of the oxidation step.

While these methods give increased sensitivity for the estimation of DHAA, the addition of the derivatization step increases the complexity and adds another variable to the analysis. Also, problems were encountered with the stability of the derivative.

Recently, Vanderslice and Higgs (1984) proposed an HPLC method with fluorometric detection and postcolumn derivatization involving oxidation of AA to DHAA followed by reaction with OPDA to form a fluorescent product.

In this study we have examined the system proposed by Vanderslice and Higgs (1984) and modified it to obtain an estimation that includes AA and DHAA as a separate value, using a single injection and omitting the oxidation step of AA.

MATERIALS AND METHODS

Reagents. Ascorbic acid (Aldrich), dehydroascorbic acid (Aldrich), o-phenylenediamine (Kodak), metaphosphoric acid (Fisher), potassium phosphate monobasic (Fisher), and HPLC-grade acetonitrile (Fisher) were obtained and used as received. Double distilled deionized water was used to prepare solutions.

Apparatus. High-performance liquid chromatography was performed with a system incorporating a Waters Associates Model 6000A pump, a Waters Model U6K injector, a Spectra Physics Model 8440 variable-wavelength ultraviolet detector (Spectra-Physics) set at 254 nm, and a Fisher Recordall Series 5000 recorder. Separation of AA from DHAA was achieved by use of an Alltech NH_2 column in the weak-anion-exchange mode. The mobile phase
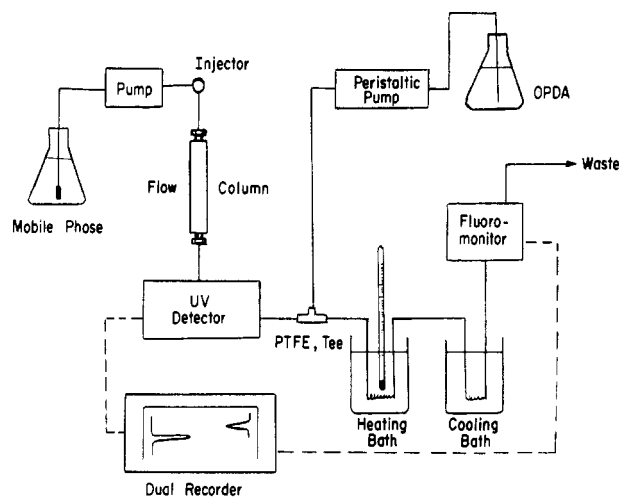

Figure 1. HPLC system with postcolumn derivatization and tandem ultraviolet and fluorometric detection.

was 75% acetonitrile in 0.05 M monobasic potassium phosphate (pH 5.9). The eluant was filtered through a 0.45-μm Millipore filter (Gelman Sciences, Inc. Ann Arbor, MI) and subsequently degassed by applying a vacuum. The flow rate was 1.5 mL/min.

Postcolumn Derivatization. The system used for the postcolumn derivatization was similar to that described by Vanderslice and Higgs (1984). After separation of AA and DHAA on the analytical column, the flowing stream from the UV detector was mixed with a second stream containing the derivatization reagent (OPDA) in a mixing PTFE Tee (Rainin Catalog No. 45-1003). The final eluant was passed through a heating coil and then into a cooling coil, before entering a fluorometric detector (Aminco Fluoromonitor) equipped with an ultraviolet mercury light source (Type GE No. F4T4/BL, 4 W), a Corning 7-51 exitation filter, a Wratten 2A emission filter, and a 70-μL flow cell whose output is sent to the recorder (Figure 1). All postcolumn tubing was 0.40 mm i.d. Teflon. The reaction path length was 20 m and was maintained at constant temperature 63 °C, while the cooling coil (22 °C) was 2 m. The fluorogenic reagent consisted of 0.05% (w/v) OPDA in distilled water and was pumped using a Gilson Minipuls 2 peristaltic pump at a flow rate of 0.5 mL/min.

Sample Preparation. Fresh fruits and vegetables were purchased from a local market and homogenized in a domestic blender. A sample (20 g) was then blended with 3% (w/v) metaphosphoric acid solution (50 mL) for 2 min and diluted to volume (100 or 200 mL) with extracting solution. The resulting solution was filtered through paper (Whatman 541), and a portion of the filtrate was purified by percolation through a C_{18} Sep-Pak (Waters Associates, Milford, MA), a short plastic column containing μ-Bondapak C_{18} as described by Wimalasiri and Wills (1983). The C_{18} Sep-Pak was placed on the Luer tip of the syringe barrel and the column preconditioned with 4 mL of methanol followed by 10 mL of water. The sample (4 mL) was then passed through the Sep-Pak. The first 3 mL was discarded, and the remaining 1 mL was collected for analysis. The Sep-Pak C_{18} can be reused up to eight times provided it is washed with methanol and water between samples. Frozen orange juice was first diluted according to package directions. The resulting solution was filtered and purified as above. The injection volume was 20 μL.

Recovery Study. Proper amounts of ascorbic acid and dehydroascorbic acid standards were added as solutions in metaphosphoric acid to the various fruits and vegetables during extraction so that the AA and DHAA content of
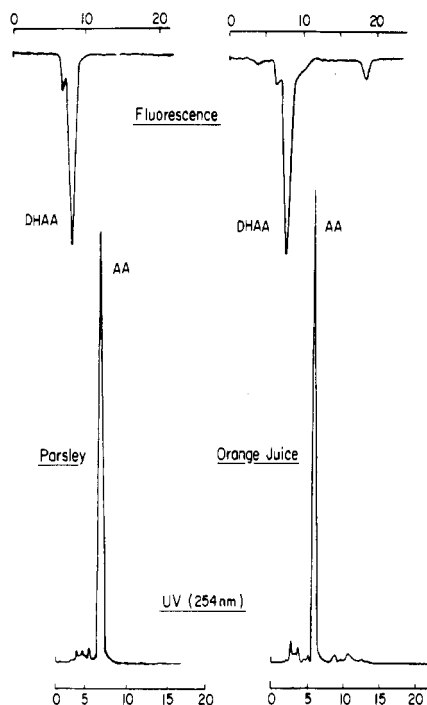
Figure 2. Typical HPLC chromatograms of orange juice and parsley monitored by tandem ultraviolet (UV, 254 nm) and fluorometric detection.

Table I. Ascorbic Acid and Dehydroascorbic Acid Content of Various Foods

sample[a]	concentration, mg/100 g		
	AA	DHAA	TAA
broccoli, fresh	81.5 ± 2.5	6.2 ± 0.9	87.7 ± 1.6
orange juice	42.7 ± 1.1	2.9 ± 0.2	45.6 ± 1.0
fruit punch	51.0 ± 1.9	3.9 ± 0.2	54.9 ± 2.1
orange drink	45.7 ± 1.6	1.0 ± 0.1	46.7 ± 1.5
parsley	148.7 ± 7.4	9.7 ± 1.0	158.5 ± 7.0
tomato fresh	9.1 ± 0.4	1.1 ± 0.2	10.2 ± 0.5
strawberry	51.0 ± 1.9	6.1 ± 0.2	57.1 ± 1.8
banana	7.7 ± 0.6	3.0 ± 0.1	10.7 ± 0.6

[a] Number of samples = 4. Mean ± SD.

the spiked samples approximately doubled that of the unspiked. The AA and DHAA of spiked samples were then determined as described previously, and percent recovery was calculated.

Calibration Curves. Samples of reagent-grade AA and DHAA in the mobile phase were combined to contain 2.0 AA + 0.5 DHAA, 4.0 AA + 1.0 DHAA, 6.0 AA + 2.0 DHAA, 8.0 AA + 3.0 DHAA, and 10.0 AA + 4.0 DHAA mg/100 mL. The standard mixtures had to be prepared daily. Aliquots (20 μL) of the combined solutions were injected into the chromatographic system, and the resulting peak heights were plotted against concentrations for the calibration curve.

RESULTS AND DISCUSSION

By using the HPLC procedure described, linear calibration curves were obtained for DHAA in the range 0–4 mg/100 mL and for AA in the range 0–10 mg/100 mL. Correlation coefficients of the linear regression equation were 0.9994 for AA and 0.9999 for DHAA, and the limits of detection were 0.05 μg for AA and 0.01 μg for DHAA.

Typical chromatograms of orange juice, parsley, tomato, and strawberry are shown in Figures 2 and 3. These illustrate the ability of tandem ultraviolet–fluorometry detection to determine simultaneously AA and DHAA. In all samples, the AA and DHAA peaks were well resolved with no interference; the DHAA peak has a shoulder in
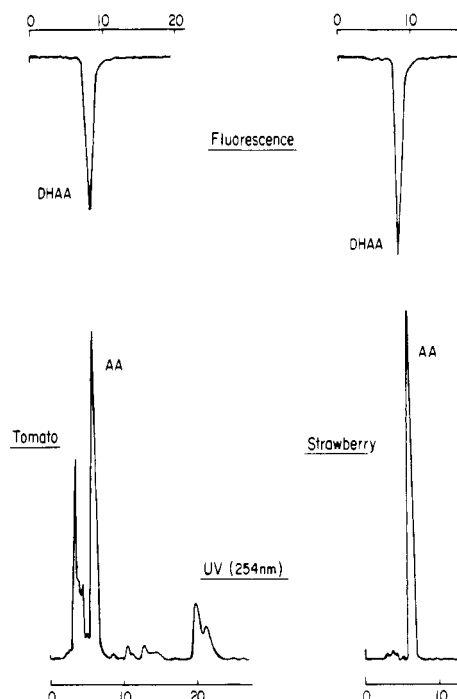

Figure 3. Typical HPLC chromatograms of tomato and strawberry, monitored by tandem ultraviolet (UV, 254 nm) and fluorometric detection.

Table II. Recovery of Ascorbic Acid and Dehydroascorbic Acid from Spiked Samples

sample[a]	recoveries, %		
	AA	DHAA	TAA
broccoli	97.7 ± 2.9	110.7 ± 4.6	104.2
orange juice	99.5 ± 2.7	101.7 ± 0.8	100.6
fruit punch	97.0 ± 0.7	102.5 ± 1.1	99.8
orange drink	96.7 ± 1.5	101.7 ± 2.2	99.2
parsley	91.0 ± 1.9	111.7 ± 2.5	101.4
tomato	96.0 ± 1.2	104.7 ± 1.5	100.4
strawberry	96.5 ± 1.1	105.7 ± 1.5	101.0
banana	91.2 ± 1.9	102.5 ± 1.1	96.9

[a] Number of samples = 4. Mean ± SD.

some of the samples. The procedure was successfully applied to the analysis of vitamin C in different food products. The results are presented in Table I. Although direct comparison was not made with other methods of analysis such as the dye titration or the fluorometric method, the levels of AA and DHAA were found to be in the same order of magnitude as those reported in the literature (Ashoor et al., 1984; Wills et al., 1983; Wimalasiri and Wills, 1983). The data in Table II show that both AA and DHAA are completely recovered from the samples examined. The recoveries ranged from 91 to 99.5% for AA and from 101 to 112% for DHAA. The slightly higher recoveries for DHAA could be due to the oxidation of some of the AA to DHAA during extraction and sample preparation. The recoveries for the total vitamin C (AA + DHAA) for all samples studied are more than 99%. The lowest value, 97, was noted in the case of banana. As both forms of the vitamin are biologically active, the procedure provide a valuable tool for the measurement of AA and DHAA in food products specially for those where both compounds are already in solution and extraction is not necessary. With other food products, where homogenization and extraction are required, some loss of AA may occur during or following the extraction. Therefore, conducting the extraction at 3 °C might be helpful in preventing potential

conversion of AA to DHAA.

This HPLC procedure provides a relatively fast and sensitive technique for the simultaneous determination of AA and DHAA in foodstuffs and beverages. The method is simple and requires a minimum of sample preparation during the simultaneous determination of AA and DHAA. Further, this HPLC method measured AA and DHAA directly, which eliminates the need for the oxidation of AA to DHAA or the reduction of DHAA to AA prior to the analysis. The procedure was also found to be very useful for measurement of AA and DHAA in browned samples of orange juice where many interferring compounds limited the use of the dye titration and the microfluorometric methods.

Registry No. AA, 50-81-7; DHAA, 490-83-5.

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Qualitative and Quantitative Analysis of Monensin A Sodium Salt in the Low-Nanogram Range by Thin-Layer Chromatography and Fast Atom Bombardment Mass Spectrometry

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Fast atom bombardment mass spectrometry (FAB-MS) was used for quantitation of monensin A sodium salt in samples of chicken fat after separation using thin-layer chromatography. The method used offers the possibility to measure amounts of monensin A sodium salt at levels below 10 ng, corresponding to concentrations below 10 ng/g in chicken fat. The low values (10% or lower) obtained for the relative standard deviation using FAB-MS give a clear advantage over the conventional thin-layer bioautographic method.

INTRODUCTION

Monensin A, the major component of four polyether antibiotics produced by *Streptomyces cinnamonensis*, is frequently used as a feed supplement to control coccidiosis in chickens (Haney and Hoehn, 1968; Shumard and Callender, 1968). Methods have been developed for monensin determination at the milligram/kilogram (ppm) level in feeds based on microbiological (Kline et al., 1970; Kavanagh and Willis, 1972; Martinez and Shimoda, 1983), colorimetric (Golab et al., 1973), thin-layer chromatographic (TLC) (Asukabe et al., 1984), and high-performance liquid chromatographic (Macy and Loh, 1983) procedures. A semiquantitative thin-layer bioautographic assay procedure has been reported for the determination of monensin A residues at the microgram/kilogram (ppb) level in chicken tissues (Donoho and Kline, 1968). However, the identity of monensin is normally not confirmed.

The molecular formula of monensin A sodium salt (in the rest of this paper referred to as monensin A) has been determined by high-resolution mass spectrometry (Chamberlin and Agtarap, 1970). In a recent report (Chang et al., 1984), the mass spectra of three coccidiostats, including monensin, obtained by fast atom bombardment mass spectrometry (FAB-MS) are given. A method for quantitative analysis of corticosterone with progesterone as internal standard using FAB-MS has been described (Tanaka, 1983). In this paper we have used TLC and FAB-MS for quantitative determination of monensin A in the low nanogram/gram (ppb) range in chicken fat samples with monensin B sodium salt (in the rest of this paper referred to as monensin B) as internal standard.

MATERIAL AND METHODS

Chemicals. Monensin A and B sodium salts were gifts from Dr. M. Beran, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. Monensin A sodium salt standard was also supplied by Lilly Research Laboratories (Indianapolis, IN). All other chemicals were of analytical reagent grade.

Extraction. A 20-g chicken fat sample was extracted by methods similar to those reported earlier (Martinez and Shimoda, 1983; Donoho and Kline, 1968).

Thin-Layer Chromatography. The samples, dissolved in methanol, were applied to precoated silica gel TLC plates (20 × 20 cm; E. Merck, Darmstadt, FRG). The TLC plates were developed in a solvent system of carbon tetrachloride–benzene–methyl cellosolve (80:10:10). The TLC plates were air-dried, and a small band with the same R_f

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