ELSEVIER

Journal of Chromatography A, 881 (2000) 309–316

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Enzymatically validated liquid chromatographic method for the determination of ascorbic and dehydroascorbic acids in fruit and vegetables

Vural Gökmen*, Nermin Kahraman, Nilay Demir, Jale Acar

Food Engineering Department, Hacettepe University, 06532 Beytepe, Ankara, Turkey

Abstract

A liquid chromatographic method has been described for the determination of total vitamin C, ascorbic acid (AA) and dehydroascorbic acid (DHAA) in fruits and vegetables. The complete separation of AA and DHAA could be achieved on a C_{18} column using 0.2 *M* KH_2PO_4 (pH adjusted to 2.4 with H_3PO_4) as the mobile phase at a flow-rate of 0.5 ml/min. Since the detection sensitivity was poor for DHAA even at 210 nm, it was estimated as the difference between the total AA after DHAA reduction and AA content of the original sample, using dithiothreitol (DTT) as the precolumn reductant. The reaction times for the complete conversion of DHAA to AA at room temperatures were 150, 120, 90 and 75 min for 1, 2, 4 and 8 mmol DTT per mmol of DHAA, respectively. The percentage recovery ranged from 81.7 to 105.9. AA contents of some selected fruits and vegetables were analyzed comparatively by liquid chromatography and enzymatic assay to validate the method. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Food analysis; Fruit; Vegetables; Ascorbic acid; Dehydroascorbic acid; Vitamins; Dithiothreitol

1. Introduction

An accurate and specific determination of the nutrient contents of foods is becoming extremely important as researchers learn more about the relationship of dietary intake and human health. One such nutrient is vitamin C, which is widely distributed in plant materials, with fruit and vegetables being the major source in most human diets. The biologically active forms, ascorbic acid (AA) and dehydroascorbic acid (DHAA), have equal antiscorbutic activity in men.

Various methods have been reported for the determination of vitamin C in foods or biological fluids including titration, spectrophotometry, fluorometry, voltammetry, electrophoresis and high-performance liquid chromatography (HPLC) in the last decades [1–12]. Most of these methods, other than HPLC, are time-consuming and may give overestimates due to the presence of oxidizable species other than AA, and/or do not measure DHAA. Although HPLC has brought some advantages, compared to direct measurement techniques, due to elimination of interferences, numerous efforts have been directed to increase sensitivity using specific detection systems such as electrochemical [13] and fluorometric [14] detectors. However, sensitivity is still an important problem for the direct measurement of DHAA by HPLC using common detection systems like a UV–Vis detector. Usually, DHAA is determined as the difference between the total AA after DHAA reduction and AA content of the original sample. Various reducing agents, such as homocysteine [15], dithio-

*Corresponding author.

0021-9673/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(00)00080-7

threitol [16] and L-cysteine [10] have been previously studied for the reduction of DHAA to AA.

This paper reports an HPLC procedure for the rapid estimation of AA and DHAA in fruits and vegetables. The chromatographic conditions applied allowed to separate almost all constituents of the original sample. DTT was used as the precolumn reductant to convert DHAA to AA. The reduction conditions were optimized in terms of DTT concentration, reaction time, and stability. Various fruits and vegetables were analyzed to determine their AA and DHAA contents and the results were confirmed by an enzymatic assay (EA).

2. Experimental

2.1. High-performance liquid chromatograph

A Varian model 9010 liquid chromatograph was used. It was equipped with a Rheodyne model 7125 injector, 10 μl sample loop and a Hewlett-Packard Series 1100 diode array detector set at 210 and 254 nm. A stainless steel, 250×4 mm I.D., C_{18} (5 μm) column (HiChrom) operated at ambient temperature was used. It was protected by a C_{18} guard column at dimensions of 10×4 mm I.D. A 0.2 *M* KH_2PO_4 (Merck) in deionized water solution was used as the mobile phase with a flow-rate of 0.5 ml/min. The pH of the mobile phase was adjusted to 2.4 by H_3PO_4 (Merck).

2.2. Ascorbic and dehydroascorbic acid

A stock solution of AA (Merck) was prepared daily by dissolving 10 mg of AA in 100 ml of deionized water (100 μg/ml). It was diluted with deionized water to obtain the final concentrations of 10, 20, 30, 40 and 50 μg/ml. DHAA was prepared through the oxidation of ascorbic acid stock solution by activated charcoal.

2.3. EA kit for ascorbic acid

A commercially available assay kit supplied by Boehringer Mannheim was used. Measurements were performed following the manufacturer's recommended procedure [17]. Absorbances were measured using a Schimadzu model 2101 UV–Vis scanning spectrophotometer.

2.4. Sample preparation

Fruit and vegetable samples were portioned into small pieces. Four parts of deionized water were added into one part of portioned fruit, or vegetable (dilution factor, $F=5$). The mixture was homogenized using a Virtis homogenizer at medium speed for 2 min. The spiking with ascorbic acid for recovery trials were performed in this step. The homogenized sample was filtrated through a S&S No.589[3] black band filter paper (Schleicher and Schuell), then through a 0.45 μm Millipore disposable filter, keeping the sample away from direct sunlight. The clarified sample was divided into two parts. One part was synchronously analyzed for the AA content (C_{f_1}) of the sample by HPLC and EA for an accurate comparison of both methods. Carrez clarification was avoided to prevent poor recovery for EA as recommended by the manufacturer.

DTT was added into the other part at a ratio of 1 mg/ml (or less for the samples having relatively lower amounts of DHAA), and it was kept in the dark for 90–120 min to convert any DHAA to AA. After complete conversion of DHAA was achieved, the sample was analyzed for its total AA content (C_{f_2}) by HPLC.

2.5. Calculation of the results

The DHAA content of the sample was calculated by subtracting the initial AA content from the total AA after conversion. Concentration of AA in the final test solution (C_{f_1} and C_{f_2}) was determined using the calibration graph based on concentration (μg/ml) vs. peak area (mAUs), prepared daily running fresh standard solutions:

$$\text{AA in the sample (mg/100 g)} = \frac{C_{f_1}F}{10}$$

$$\text{DHAA in the sample (mg/100 g)} = \frac{(C_{f_2} - C_{f_1})F}{10}$$

3. Results and discussion

3.1. Chromatographic efficiency

A typical chromatogram depicting the separation of two forms of vitamin C, AA and DHAA, is shown in Fig. 1. AA elutes slightly after 9 min while DHAA comes off just after AA in the C_{18} column using 0.2 *M* KH_2PO_4 (pH 2.4) as the mobile phase at a flow-rate of 0.5 ml/min. The amounts of AA and DHAA were not influenced by subsequent chromatographic analysis. Addition of 1 mg DTT per ml of mobile phase had no remarkable change in the peaks of AA and DHAA.

The specific detection wavelength of AA was 254 nm, while that of DHAA was 210 nm. Even though DHAA can be resolved from AA, the sensitivity is insufficient owing to lack of chromophores in its structure, even when monitoring at 210 nm, the maximum for DHAA. Relative sensitivity of DHAA was 1.14×10^{-2} at 254 nm and 3.05×10^{-2} at 210 nm, when that of AA is assumed to be 1. UV response (y) of AA over a concentration (x) range of 1 to 100 μg/ml was linear ($y=10.471x+4.1138$) with a regression coefficient (r^2) of 0.9992. The reproducibility was also good in terms of retention time. Day-to-day variations in retention time was found to be less than 1% over a period of 3 months, even ±5°C fluctuations in the ambient temperature was recorded.

As low as 10 μg of AA injected into the column, which corresponds to an approximate concentration of 0.5 mg AA/100 g sample, could be detected sensitively. However, the poor UV response of DHAA did not allow to measure DHAA naturally present in fruits and vegetables, even at relatively high amounts.

The detection wavelength alone brought up to a specificity to the method. Some of the acidic constituents of the samples which appeared as interferences at 210 nm could be eliminated at 254 nm (Fig. 2). However, the chromatographic conditions defined here may also be used to determine the organic acid composition of fruits and vegetables with little modification in the sample preparation. The organic acids commonly found in fruits and vegetables could be resolved from each other as shown in Fig. 3.

3.2. Reduction reaction efficiency

The reduction of DHAA to AA was catalyzed using DTT as the precolumn reductant (Fig. 4).

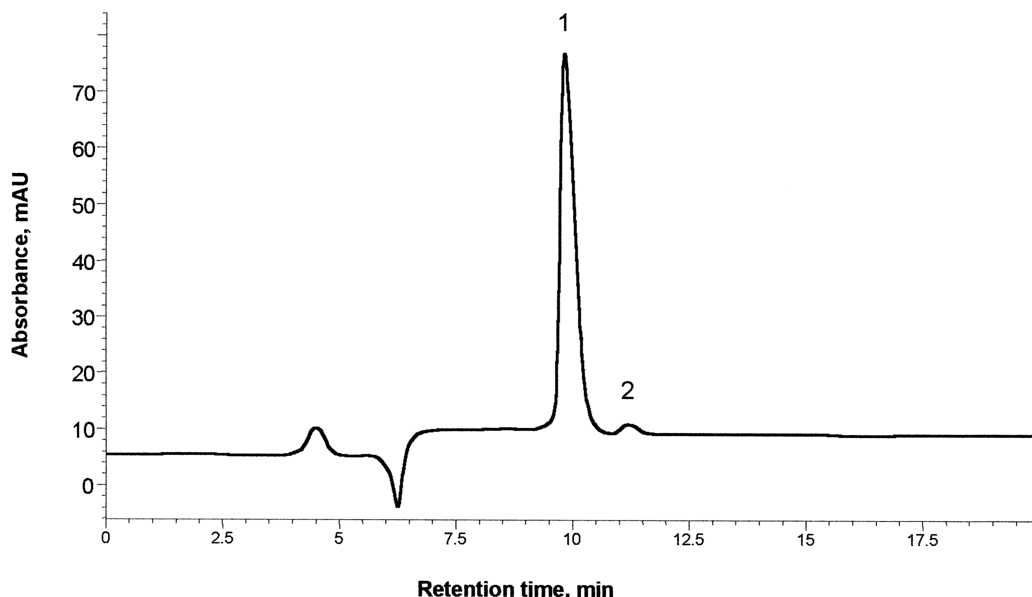

Fig. 1. Separation of AA and DHAA (each 50 mg/l). Peaks: 1: AA, 2: DHAA.

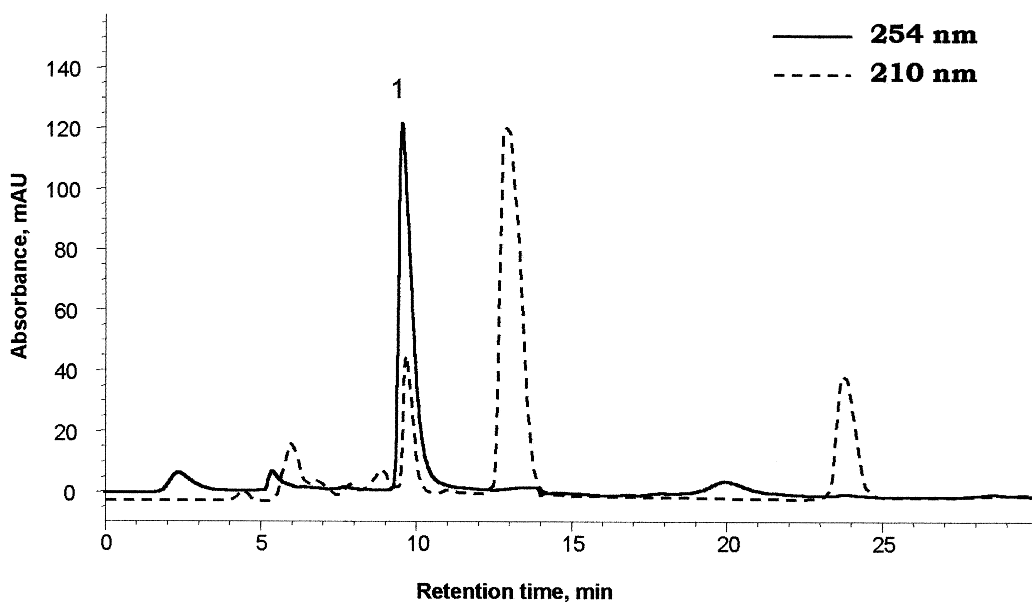

Fig. 2. Chromatograms of fresh lemon juice monitored at 254 and 210 nm, Peak: 1: AA.

Although DTT has been reported before as an effective reductant, some parameters, particularly DTT amount and reaction time, require optimization. It was clearly seen that DTT amount directly affected the rate of reduction (Fig. 5) when increasing amounts of DTT (1, 2, 4 and 8 mmol) were added into 1 mmol of DHAA solution in water. The reaction was carried out at room temperature in a

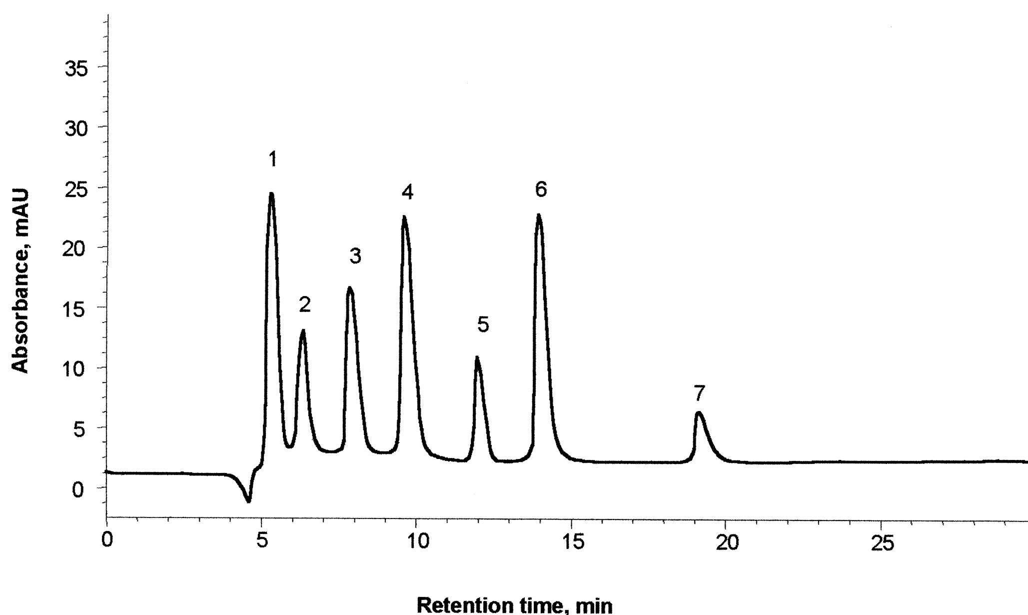

Fig. 3. Separation of organic acids. Peaks: 1: oxalic acid, 2: quinic acid, 3: L-malic acid, 4: AA, 5: citric acid, 6: fumaric acid, 7: succinic acid.

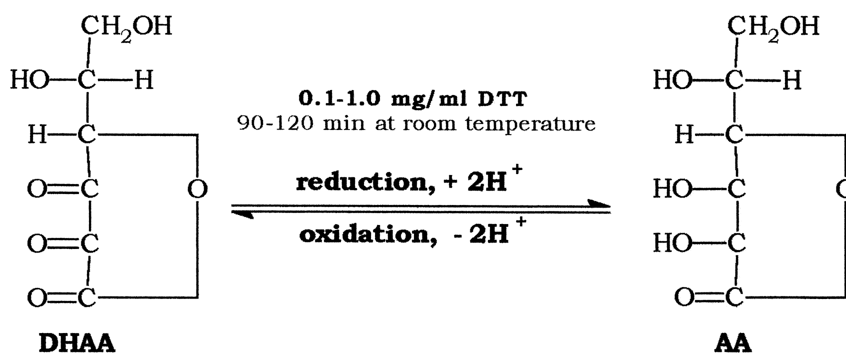

Fig. 4. Reduction scheme of DHAA to AA using DTT.

dark place. The complete conversion could be achieved within 150, 120, 90 and 75 min for 1, 2, 4 and 8 mmol DTT, respectively. The reduction followed a zeroth order kinetics pattern. The data for the first hour were used to obtain the zeroth order rate constants, which were estimated to be 0.9533, 1.1722, 1.4411 and 1.8378 mol/min for 1, 2, 4 and 8 mmol DTT, respectively.

The amount of DTT required depends on how much DHAA is present. This is simply estimated from the approximate expected DHAA concentration of the final test solution. According to the sample preparation procedure proposed here, the DHAA concentration varies between 0 to 100 μg/ml in the samples when dilutions were taken into account. Thus, as low as 0.1 mg of DTT per ml of test solution is enough to reduce all DHAA to AA, but higher amounts would be expected to shorten the reaction time and ensure the prevention of oxidation. It is important to reduce the consumption of expensive chemicals for routine analytical methods. The results reported here now simply recommend to use relatively lower amounts of DTT reported before to achieve an adequate conversion in an acceptable

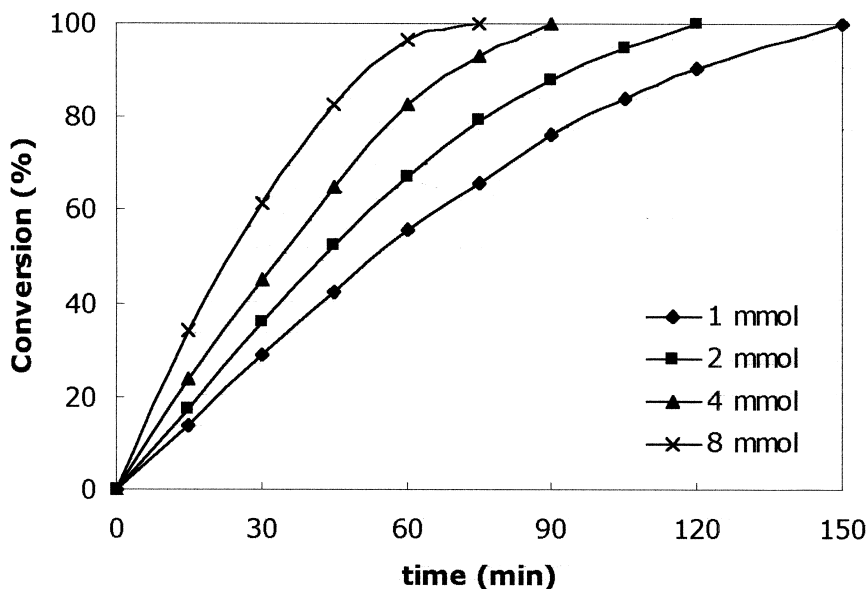

Fig. 5. Effect of the DTT amount on the reduction of DHAA to AA.

duration [16]. Although other precolumn reductants have been reported for the conversion of DHAA to AA, the amounts required were as high as 30 to 40 mol per mol of DHAA [1,15].

The effect of DTT on the analysis of total vitamin C in fruits and vegetables is also illustrated in the chromatograms shown in Fig. 6. The signal of total AA after the conversion of DHAA using DTT almost doubled at 254 nm for the tomato. DTT remaining in the test solution after reduction did not cause any interference.

3.3. Sample preparation efficiency

Most of the methods propose to use acid solutions as the extracting solvent in order to stabilize vitamin C [2,6]. In this study, the effect of buffer at pH 2.4 (mobile phase) and distilled water as the homogenizing/extracting solvent was comparatively determined. The extraction yield of distilled water was found out to be ca. 5% higher than that of the buffer. The percentage recovery of vitamin C as AA ranged from 81.7 to 105.9 using the samples spiked with 5 and 10 mg AA per 100 g of sample.

The stability of total AA formed after the conversion of DHAA was examined in fresh orange juice (pH<4.0) in which AA is naturally present, and in cauliflower extract (pH>5.5) in which DHAA is naturally present. Results have shown that AA formed by DTT was stable for 1 h in the dark in both matrices, but started to decrease after 1 h at a slow rate up to 3 h, and increasing rate afterwards (Fig. 7). It is recommended to measure the total AA just after completion of the reaction (90–120 min), because the stability of AA might be lower in foods in which oxidation potential is higher.

3.4. Applicability

Applicability of the method was verified analyzing some selected fruits and vegetables and comparing the results with those obtained by a well established EA (Table 1). The HPLC and EA results were in good agreement with each other for AA contents of various fruits and vegetables. The deviations of HPLC results from those obtained by EA were found out to be less than ±10%, even for relatively low and high AA contents. This deviation is acceptable from the viewpoint of a confirmatory test. DHAA contents of the samples were determined by only HPLC since the EA kit was for the measurement of AA alone.

Table 1 clearly indicates that fruits and vegetables exhibit different AA and DHAA profile. In other

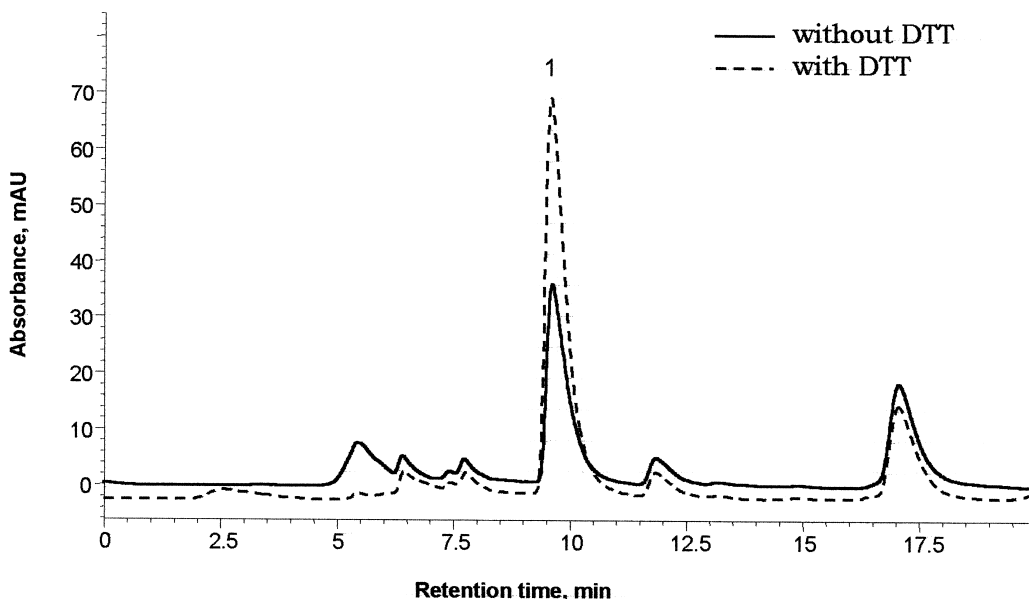

Fig. 6. Chromatograms of tomato monitored at 254 nm with and without DTT. Peak: 1: AA.

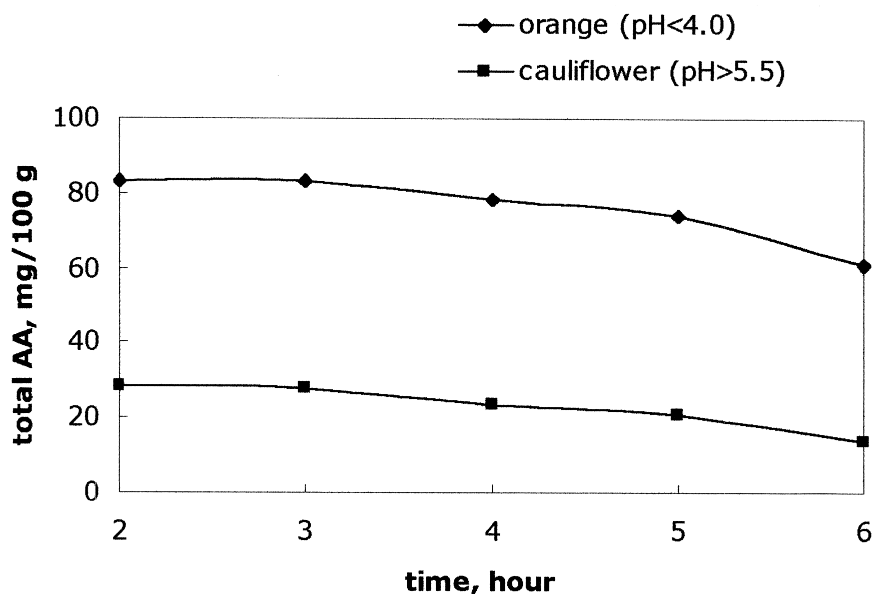

Fig. 7. The stability of AA formed after the reduction of DHAA.

words, AA is dominantly present in fruits, while DHAA in vegetables. It is very interesting to note the relation between AA/DHAA contents and pH of fruits (pH<4.0) and vegetables (pH>5.0).

The amounts of AA and DHAA naturally present in some fruits and vegetables have also been reported previously. Some reports agree with our results when the individual contributions of AA and DHAA to the total vitamin C contents are taken into account [14,18]. However, some results reported elsewhere are conflicting with respect to AA and DHAA contents, particularly for vegetables [2,6].

Table 1
AA and DHAA contents of some selected fresh fruits and vegetables

Sample	Vitamin C (mg/100 g)		
	AA		DHAA, HPLC
	HPLC	Enzymatic	
Lemon	53.8	51.3	0.9
Orange	43.5	42.7	3.5
Grapefruit	33.7	32.0	1.2
Kiwi	34.2	31.6	6.5
Strawberry	29.0	29.0	5.4
Tomato	7.9	8.6	5.4
Green pepper	1.6	1.8	22.5
Parsley	nd	nd	218.4
Mint	nd	nd	50.3
Cabbage	5.5	5.6	20.1
Onion, fresh	1.8	1.5	4.6
Green bean	nd	nd	7.3
Soybean	nd	nd	nd

4. Conclusion

A simple HPLC method for the determination of two forms of vitamin C in fruits and vegetables was described. The sample preparation was optimized in a logical manner to consume lesser amounts of reagents, but to obtain an acceptable result in terms of sensitivity, stability and reproducibility. Due to low UV sensitivity for DHAA, direct analysis of this molecule was impossible even at excess quantities, while an adequate determination of total vitamin C could be achieved by conversion of DHAA to AA with DTT and detection of the reduced form.

Fruits and vegetables significantly differed according to the natural occurrence of AA and DHAA. This should be taken into consideration when determining the vitamin C losses during food processing, because these two forms have different resistances against thermal degradation and oxidation.

Extension of the method to the determination of organic acids originated from fruits and vegetables appears as an advantage. The organic acids commonly present in fruits and vegetables are resolved under the chromatographic conditions described here.

References

[1] D.B. Dennison, T.G. Brawley, G.L.K. Hunter, J. Agric. Food Chem. 29 (1981) 927.
[2] P. Wimalasiri, R.B.H. Wills, J. Chromatogr. 256 (1983) 368.
[3] B. Luckas, Fresenius Z. Anal. Chem. 323 (1986) 496.
[4] A. Hernanz, J. Clin. Chem. Clin. Biochem. 26 (1988) 459.
[5] Y. Maeda, S. Ochi, T. Masui, S. Matubara, J. Assoc. Off. Anal. Chem. 71 (1988) 502.
[6] R.J. Bushway, J.M. King, B. Perkins, M. Krishnan, J. Liq. Chromatogr. 11 (1989) 3415.
[7] Official Methods of Analysis, AOAC, Arlington, VA, 15th ed., 1990, Method 967.21
[8] Z.Q. Gao, A. Ivaska, T.X. Zha, G.Q. Wang, P.B. Li, Z.F. Zhao, Talanta 40 (1993) 399.
[9] M. Chiari, M. Nesi, G. Carrea, P.G. Righetti, J. Chromatogr. A 645 (1993) 197.
[10] H. Iwase, I. Ono, J. Chromatogr. A 654 (1993) 215.
[11] J.M. Irache, I. Ezpeleta, F.A. Vega, Chromatographia 35 (1993) 232.
[12] V. Gökmen, J. Acar, Fruit Process. 5 (1995) 198.
[13] L.S. Liau, B.L. Lee, A.L. New, C.N. Ong, J. Chromatogr. 612 (1993) 63.
[14] J.T. Vanderslice, D.J. Higgs, J. Chromatogr. Sci. 22 (1984) 485.
[15] R.C. Rose, D.L. Nahrwold, Anal. Biochem. 114 (1981) 140.
[16] L.W. Doner, K.B. Hicks, Anal. Biochem. 115 (1981) 225.
[17] Methods of Enzymatic Bioanalysis and Food Analysis, catalogue No. 409 677, Boehringer Mannheim GmbH, Germany
[18] J.W. Finley, E. Duang, J. Chromatogr. 207 (1981) 449.