Simple In-Line Postcolumn Oxidation and Derivatization for the Simultaneous Analysis of Ascorbic and Dehydroascorbic Acids in Foods

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Abstract

A new analytical procedure for the simultaneous determination of L-ascorbic acid (AA), isoascorbic acid (IAA), L-dehydroascorbic acid (DHAA), and isodehydroascorbic acid (IDHAA) in food by high-performance liquid chromatography (HPLC) is developed. After separation on an HPLC column, an in-line oxidation of AA and IAA to DHAA and IDHAA, respectively, is performed on a short column of activated charcoal. The dehydroascorbic acids are derivatized with a 1,2-phenylenediamine solution in a heated capillary Tefzel reactor into fluorescent quinoxaline compounds and monitored fluorometrically. The chromatographic method provides good separation of LAA, LDHAA, and their diastereoisomers in a relatively short time (~10 min). After optimization of postcolumn derivatization conditions, calibration runs and recovery tests are performed. The fluorescent response in terms of peak area is highly proportional to the concentration of all derivatives examined over a range of 0.1 to 100 µg/mL solution for LAA, LDHAA, IAA, and IDHAA. Recoveries were in the range of 97 to 103%. The detection limit is 0.1 mg of each ascorbic acid derivative per 100 g food. A wide variety of foods (fruits, fruit juices, vegetables, vegetable products, milk, liver, and sausage) are analyzed by the developed procedure. The Vitamin C (LAA and LDHA) contents determined according to the present analytical method are in the same order of magnitude as the result of precolumn derivatization and the fluorometric methods. The described method is a highly specific procedure for determining Vitamin C in food. It is simple to handle, only slightly susceptible to disturbance, perfectly suitable for serial determinations, and yields reproducible results.

Introduction

The levels of Vitamin C, the essentially needed antioxidant vitamin, are determined by its dietary supply (mainly from fruits and vegetables). The total Vitamin C consists of the sum of ascorbic acid (AA) and its oxidized form, dehydroascorbic acid (DHAA), that has, at most, 10% of the vitamin activity of AA (1). However, in some literature, both forms have been reported to show the same antiscorbutic activity (2). Because AA performs its antioxidant function via its oxidation to DHAA, the ratio of AA/DHAA can show redox status in biological systems. All of the aforementioned facts make it increasingly important to develop analytical methods that ensure simple, rapid, reliable, sensitive, and simultaneous determination of AA and DHAA in foodstuffs.

Because of its many advantages, high-performance liquid chromatography (HPLC) became superior to other analytical methods. Qualitative determination of AA and DHAA has been a task in many laboratories. Most of the nonsimultaneous HPLC procedures based on either the reduction of DHAA to AA by dithiothreitol (3–5) or oxidation of AA to DHAA and condensation of the total DHAA with 1,2-phenelenediamine (OPDA) produce fluorometrically measurable 3-(1,2-dihydroxyethyl) furo(3,4-6)quinoxaline-1-one (DFQ). The oxidation has come out by using ascorbic oxidase (6) and, lately, activated charcoal (7).

Simultaneous HPLC determination of AA and DHAA has been achieved by several methods that can be classified into three groups; (*a*) those based on dual wavelength monitoring at 210–214 nm (for DHAA) and 245–265 nm (for AA) after separation on amino-bonded columns under weak ion-exchange conditions (8,9), two reversed-phase columns under ion-pair chromatographic conditions (10), reversed-phase column (11), or coupled with organic acid columns (12) using phosphate buffer as the mobile phase; (*b*) methods based on precolumn derivatization in which DHAA has been derivatized by reacting with OPDA to produce DFQ, which has a greater ultraviolet (UV) absorptivity than DHAA (13,14); and (*c*) methods based on postcolumn derivatization, such as that of Vanderslice and Higgs (15) who separated AA from DHAA on an ion-exchange

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column and oxidized it to DHAA by a postcolumn oxidizing reagent ($HgCl_2$). The original and derived DHAA has reacted with OPDA to form fluorometrically active DFQ that could be monitored by a fluorescence detector. The same authors (16) modified the procedure by using three altex ODS-C_{18} columns to separate AA, DHAA, IAA, and DHIAA in approximately 50 min using ion-paired chromatography. Kacem et al. (17) replaced postcolumn oxidation with UV detection of AA at 254 nm followed by postcolumn derivatization of DHAA to DFQ and fluorescence detection.

The objective of the present work was to develop in-line postcolumn oxidation of AA to DHAA by using short columns packed with activated charcoal after the weak ion-exchange chromatographic separation of the vitamers. Postcolumn derivatization conditions were optimized. The method was compared to other methods and applied to the analysis of selected fruits and vegetables.

Experimental

Reagents

AA, OPDA, ammonium dihydrogen phosphate, methaphosphoric acid, acetic acid, and acetonitrile (Merck, Darmstadt, Germay); activated charcoal (Sigma, St. Louis, MO); and isoascorbic acid (Fluka, Buchs, Switzerland) were obtained and used as received. Bidistilled water was used to prepare the solutions. Fruits and vegetables were purchased from a local market.

Standard solutions

A stock solution of ascorbic and isoascorbic acid was prepared by dissolving 100 mg of each acid in 100 mL of 3% metaphosphoric acid solution containing 8% acetic acid. The stock solution was diluted to different concentrations with the same solvent for calibrations and recoveries.

To prepare standard DHAA and DHIAA, 50 mL of diluted stock solution (50 µg/mL) was oxidized by shaking thoroughly with 1 g activated charcoal for 15 min, in accordance with ISO-1986 (7). The mixture was then filtered through filter paper (S&S 595 1/2, Schleicher & Schuell, Germany). The first 5-mL aliquot was discarded, and the remaining filtrate was taken for analysis.

Sample extraction

Fruit and vegetable samples (25–100 g) were homogenized by a warring blender in the presence of an extraction solvent (acetic acid containing metaphosphoric acid) for 2 min. The final volume was brought about with the same extracting solvent. The mixture was filtered and, if necessary, centrifuged before filtration. In order to fall within a convenient calibration range, the amount from each sample was taken according to the accepted vitamin level.

HPLC conditions

HPLC was performed with a system consisting of a Shimadzu (Tokyo, Japan) model LC-10AT pump, a Perkin-Elmer (Norwalk, CT) model ISS-101 autoinjector fitted with a 20-µL loop, a Shimadzu model RF-551 fluorometric detector set at an excitation of 350 nm and an emission of 420 nm, and a Merck L-5025 column thermostat. The detector signals were recorded and manipulated by a Getaway 2000 computer system installed with Shimadzu Class LC-10 chromatographic software.

The separation of vitamers was performed on a Grom-Sil-NH_2 (250 mm × 4.6-mm i.d., 5 µm) column under weak ion-exchange conditions. The mobile phase was 70% acetonitrile in 0.05M ammonium dihydrogen phosphate (pH 5). The eluent was flushed with helium gas and pumped into the system with a flow rate of 1.0 mL/min.

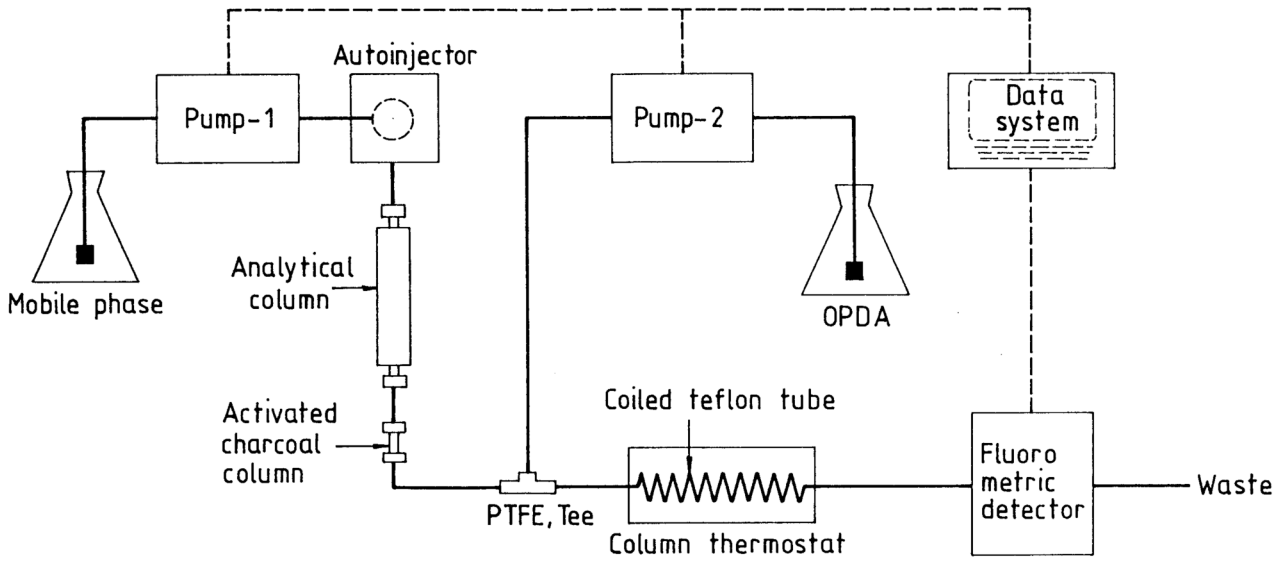

Figure 1. Block diagram of the HPLC system with in-line postcolumn oxidation and derivatization.

In-line postcolumn oxidation

To achieve rapid and effective in-line postcolumn oxidation of AA to DHAA, the flowing stream from the analytical column was passed through a slurry-packed activated charcoal column (2 cm × 4.6-mm i.d.) (Figure 1). The lab-made column was packed by pressing the slurry of charcoal–eluent in the short column using a column-packing appliance (Knaur) and isocratic pump. One stainless steel and two glass fiber (5 µm) sieves (Grom) were fitted on the two sides of the column before and after packing, which was performed at no higher than 50 bar.

In-line postcolumn derivatization

The derivatization reagent was OPDA dissolved in 24 mL of 8% acetic acid–3% metaphosphoric acid and 10 mL of 2.5M sodium acetate buffer (pH 5.2). The volume was adjusted to 500 mL with double-distilled water. After degassing, the reagent was pumped to meet the second stream from the column in a poly(tetrafluoroethylene) "Tee" (Sykam GmbH, Germany). The mixture passed the column heater through a 20-m Teflon tube before entering the fluorometric detector. The Teflon tubing coming from the thermostat was 0.5 m with no cooling applied between reactor and detector.

Calibration and recovery

Known amounts of standard AA and DHAA were injected as solutions in acetic acid containing 3% metaphosphoric acid to the analytical column. The fluorescent response, in terms of peak area, was plotted versus the concentration of all the examined acids in a range of 0–250 µg/mL for AA and DHAA and 0–100 µg/mL for IAA and IDHAA.

For the recovery test, proper amounts of AA and DHAA standards were added as solutions in the extracting solvent to the various fruits and vegetables during extraction. The peak area of AA and DHAA in spiked and unspiked samples, as well as percent recovery, was calculated. Triplicate runs were performed for each spiked or unspiked sample.

Results and Discussion

Figures 2 and 3 are the HPLC chromatograms of four Vitamin C derivatives in a standard solution and apple extract (spiked and unspiked prior to extraction), respectively. Although separation was performed under chromatographic conditions similar to those used by Kacem et al. (17), resolution of AA, DHAA, and their isoderivatives was much better with our slightly modified elution mode. The elution order of the isoderivatives was opposite to that obtained by Vanderslice and Higgs (16), who used three altex ODS-C_{18} columns under ion-pair conditions that made the last vitamer (IAA) elute in approximately 50 min. With the weak ion-exchange mode applied in the present work, all vitamers eluted in 10 min with no marked shift in elution times after several hundreds of injections.

It is noteworthy that the incorporation of a charcoal column in the analytical system as an in-line postcolumn oxidizing agent caused a slight increase in retention time (≈50 s) of each peak on chromatogram. According to our experiments, 2 cm was the optimal column length for obtaining symmetrical peaks and 100% oxidation of AA and IAA to DHAA and IDHAA,

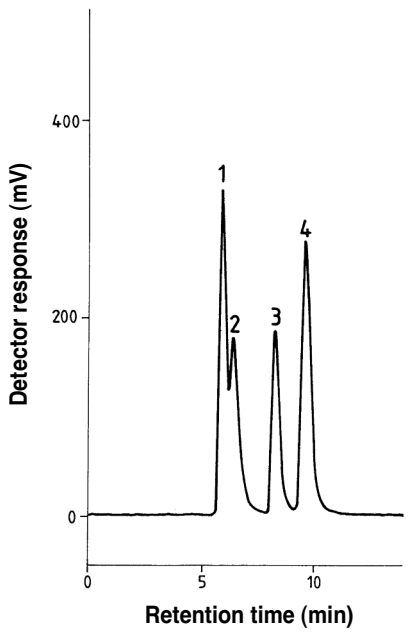

Figure 2. HPLC profile of four standard ascorbic acid derivatives as separated by weak ion-exchange mode and fluorometrically monitored after postcolumn oxidation and derivatization: 1, DHAA; 2, IDHAA; 3, IAA; 4, AA.

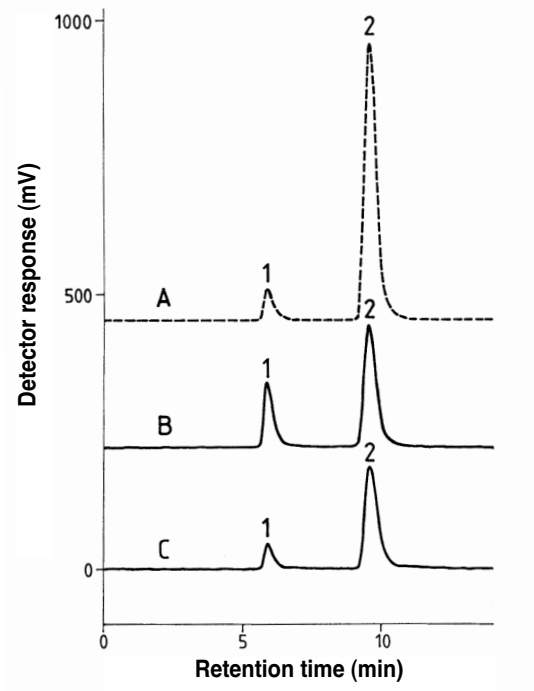

Figure 3. Chromatograms of Vitamin C derivatives from apple spiked with AA (A), spiked with DHAA (B), and unspiked (C).

with no degraded products observed. Also, this column was not harmful to DHAA from standard solutions or food extracts. Moreover, the oxidizing column has a long life and is capable of oxidizing more than 2000 injected samples. An index of the decreased capacity of the oxidizing column is the marked peak broadening and decrease in percent oxidation, which can be controlled by monitoring the remains of unoxidized AA using a UV detector.

Optimization of postcolumn derivatization

The in-line postcolumn derivatization of dehydro derivatives to the correspondent DFQs was carried out by pumping the fluorogenic reagent and HPLC eluent at a flow rate of 0.3 and 0.8 mL/min, respectively. The use of acetic and metaphosphoric acid solutions to prepare the OPDA reagent resulted in much higher detector response and reagent stability than those obtained when water was the solvent for the fluorogenic reagent (data not shown). The pH of the mixed streams was close to the optimum value (≈3) recommended for the condensation of DHAA and OPDA to form DFQ (14). Most important for the condensation of DHAA to DFQ is the concentration of OPDA. As the concentration was increased, the rate of derivatization was accelerated. The maximum OPDA concentration was found to be 0.4%, over which neither a significant increase in DFQ intensity nor a negative effect on its stability was observed (Figure 4). The negative effect of the increased OPDA concentration in the reagent solution was reported by Zapata et al. (14) in their study on conventional precolumn derivatization of DHAA.

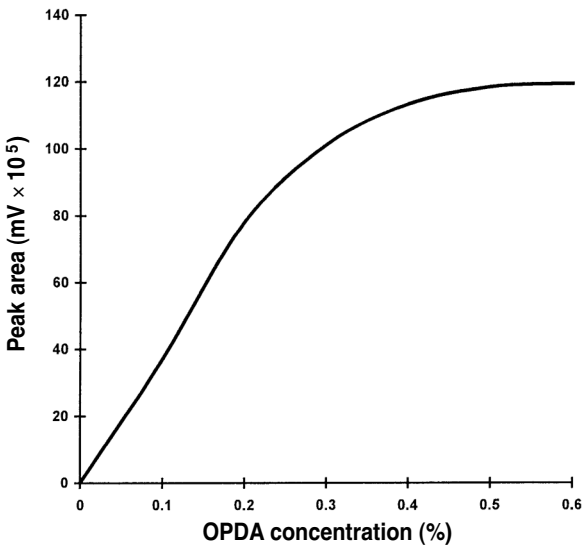

Figure 4. Effect of OPDA concentration in the fluorogenic reagent on formation of the fluorescent compound (DFQ) of dehydro derivatives.

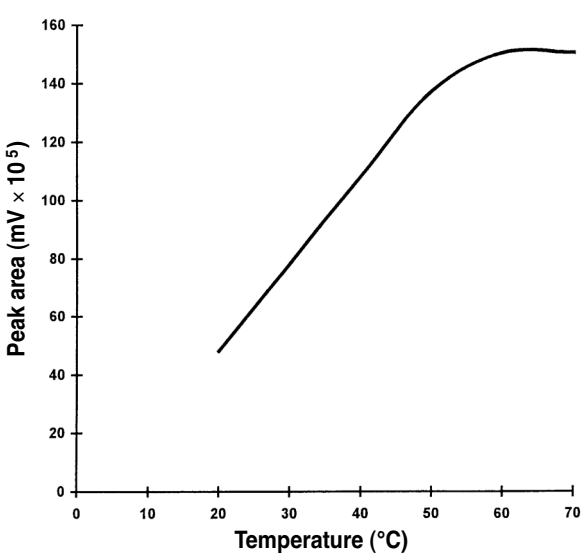

Figure 5. Effect of temperature of postcolumn derivatization on condensation of OPDA with DHAA to form DFQ.

Another factor affecting the derivatization rate is the reactor temperature. At temperatures below 25°C, the condensation reaction was very slow. Raising the reactor temperature to 50°C caused an almost linear increase in DFQ response (Figure 5). The maximum response was approached at 60°C, over which no more DFQ was formed as a function of elevated temperature. The maximum temperature was found similar to the 63°C reported by Kacem et al. (17), who applied a similar postcolumn derivatization procedure to determine dehydro derivatives of AA.

By using the optimized HPLC procedure, linear calibration curves were obtained for all Vitamin C derivatives examined in this work in the range 0–80 µg/mL (Figure 6). The correlation coefficient of the linear regression equation ranged between 0.999 and 1.0, denoting that the method is very adequate for sensitive and accurate quantitative analysis of Vitamin C. The limit of detection was 10–20 ng, with AA and DHAA being of higher response than their isoderivatives.

Stability of standard solutions and food extracts

To achieve precise and reliable quantitation, Vitamin C derivatives should be stable during preinjection preparation. As shown in Table I, two solvent systems were compared for

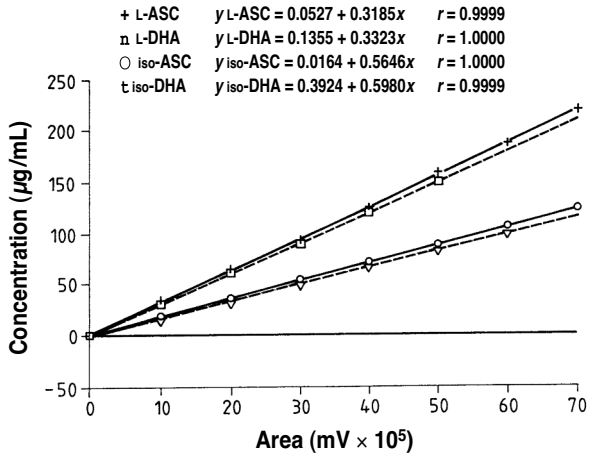

Figure 6. Calibration curves of AA, DHAA, IAA, and IDHAA as determined by HPLC with in-line postcolumn oxidation and derivatization.

their effect on the stability of Vitamin C derivatives in standard solutions and spiked food extracts. In an acetic-acid-containing metaphosphoric acid solution, DHAA was so unstable that 64 and 40% of the initial concentration could be retained by the standard solution after 8 and 24 h of ambient storage, respectively. In food extracts prepared by acidic solution only, DHAA lost 36–54% of the spiked quantity after 2 h of storage time. The decrease in DHAA as a function of storage time was accompanied by remarkable increases in AA concentration (29–53%) most probably due to the presence of reducing agents that catalyze the conversion of DHAA to AA in the extract of fruits and vegetables. It is of interest that after a 24-h storage, AA was highly stable in all of the standard solutions and food extracts tested in this work.

To improve the stability of DHAA, acetonitrile was added to the standard solutions or food extract immediately after preparation or extraction with acidic solution. As a result, the stability of DHAA was increased to the maximum, so that 100% and 98% recoveries could be attained after storage of 8 and 24 h, respectively. As for AA and IAA, about 3% of the initial amount was converted to DHAA after the 24-h storage of acetonitrile-containing preparations.

Vitamin C in selected fruits and vegetables

Table II summarizes the data of the Vitamin C content of some fruits and vegetables as determined by different methods, including ISO, precolumn, and postcolumn oxidation. The postcolumn oxidation method developed in the present work was more advantageous than the others due to its ability to separate and detect DHAA from AA, even when they existed at very low concentrations in food extracts such as that of champignons. For the fresh vegetables and fruits examined, AA ranged from 0.1 to 212 mg/100 g, and DHAA ranged from trace to 6.8 mg/100 g. As for other foods tested, AA ranged from 0.1 mg/100 g in milk to 71.3 mg/100 g in sausage. These values are within the range given by Daood et al. (18), Romero Rodriguez et al. (19), Vanderslice and Higgs (15), Mannino and Pagliarini (20), and Thompson and Trenerry (21).

Conclusion

In general, relative recovery of Vitamin C in food samples spiked with AA was higher than in samples spiked with DHAA, particularly when ISO and precolumn oxidation methods were applied. It seems that stability of added DHAA during extraction is influenced by the chemical composition of the food extract. For instance, in tomato, champignons, and black currant extracts, the lowest percent recovery was obtained (86–87%), whereas in the other food extracts, it was almost stable, and high percent recovery could be attained. With our inline post-column oxidation method using acetonitrile in preinjection preparation of the samples, such instability problems were not encountered. The percent recovery of DHAA and Vitamin C was in the range of 99 and 104%. It should be mentioned that none of the examined samples contained isoderivatives of ascorbic acid.

Table I. Stability of AA, DHA, IAA, and IDHA in Different Solvents at Room Temperature

Sample	Storage time (h)	Recovery (%)*					
		Solved in acid			Solved in acid–acetonitrile		
		AA	DHA	Total§	AA	DHA	Total§
L-AA	1	100	0	100	100	0	100
	8	98	1	99	99	1	100
	24	97	2	98	97	3	100
L-DHA	1	0	100	100	0	100	100
	8	0	64	64	0	100	100
	24	0	40	40	0	98	98
IAA	24	–‖	–	–	97	3	100
IDHA	24	–	–	–	0	98	98
Apple + L-AA	2	100	0	100	100	0	100
	24	–	–	–	97	3	100
Apple + L-DHA	2	44	52	96	0	100	100
	24	–	–	–	0	99	99
Orange juice + L-AA	2	99	1	100	100	0	100
	8	–	–	–	100	0	100
Orange juice + L-DHA	2	29	64	92	0	100	100
	8	–	–	–	0	100	100
Black currant nectar + L-AA	2	99	0	99	100	0	100
Black currant nectar + L-DHA	2	33	50	83	0	99	99
Tomatoes + L-AA	2	99	0	99	101	0	101
Tomatoes + L-DHA	2	34	53	87	1	99	100
Red pepper + L-AA	2	99	0	99	101	0	101
Red pepper + L-DHA	2	53	46	99	0	100	100

* Mean values of 2–3 analyses, variation ± 2%.
† *m*-Phosphoric acid–acetic acid (3%:8%).
‡ *m*-Phosphoric acid–acetic acid (3%:8%) with acetonitrile (1:2).
§ Sum of AA and DHA.
‖ Not analyzed.

Table II. Determination of Vitamin C in Foods by Different Analytical Methods

Food	Analytical method*	Content (mg/100 g) AA Mean value	AA Variation (±)	DHA Mean value	DHA Variation (±)	Vitamin C Mean value	Vitamin C Variation (±)	Recovery (%) AA	Vitamin C	DHA/ Vitamin C
Cauliflower	ISO	–‖	–	–	–	36.1	0.1	–	108	– 108
	HPLC–pre	36.2	0.2	3.2	0.1	39.4	0.2	–	102	– 96
	HPLC–post	35.4	0.4	2.6	0.2	39.1	0.3	100	101	102 102
Red pepper (Spanish)	ISO	–	–	–	–	215.3	3.0	–	97	– 94
	HPLC–pre	–	–	–	–	215.3	2.5	–	98	– 94
	HPLC–post	212.0	0.5	3.1	0.2	215.1	0.6	101	101	100 100
Potato	ISO	–	–	–	–	39.0	0.7	–	93	– 93
	HPLC–pre	31.4	0.5	1.7	0.2	33.1	0.5	100	100	95 92
	HPLC–post	32.0	0.3	1.8	0.2	33.8	0.3	101	100	101 101
Spinach (deep frozen)	ISO	–	–	–	–	31.5	0.2	–	105	– 103
	HPLC–pre	28.4	0.3	2.5	0.1	30.9	0.4	99	99	93 97
	HPLC–post	39.6	0.2	2.3	0.1	31.9	0.2	99	101	102 99
Tomatoes	ISO	–	–	–	–	14.3	0.2	–	99	– –
	HPLC–pre	–	–	–	–	14.8	0.2	–	98	– 87
	HPLC–post	14.2	0.2	0.4	0.1	14.6	0.2	100	101	99 99
Apple	ISO	–	–	–	–	23.1	0.5	–	98	– –
	HPLC–pre	19.4	0.3	3.3	0.2	22.7	0.3	–	102	– 95
	HPLC–post	19.8	0.2	2.5	0.2	23.3	0.3	100	101	99 100
Strawberry (deep frozen)	ISO	–	–	–	–	34.2	0.3	–	102	– 98
	HPLC–pre	30.0	–	5.9	0.2	35.9	0.3	–	97	86 98
	HPLC–post	30.0	–	5.2	0.2	35.2	0.2	98	99	103 103
Plums	ISO	–	–	–	–	4.2	0.2	–	98	– 93
	HPLC–pre	–	–	–	–	4.5	0.3	–	110	– 97
	HPLC–post	n.d.#	–	5.5	–	5.5	0.2	100	100	100 100
Mushroom (*Agaricus bisporus*)	ISO	–	–	–	–	0.2	0.2	–	86	– 86
	HPLC–pre	–	–	–	–	0.2	0.1	–	104	– 100
	HPLC–post	0.1	–	n.d.	–	0.1	0.1	98	98	100 99
Orange juice	ISO	–	–	–	–	42.4	0.2	–	94	– –
	HPLC–pre	–	–			43.6	0.2	–	100	– 90
	HPLC–post	36.9	0.2	6.8	0.4	43.7	0.3	99	100	100 100
Black currant nectar	ISO	–	–	–	–	37.6	0.3	–	93	– –
	HPLC–pre	–	–	–	–	36.9	0.3	–	95	– 82
	HPLC–post	33.0	0.2	4.7	0.1	37.7	0.2	99	99	100 100
Multivitamin fruit juice	HPLC–post	49.6	0.3	0.6	0.1	50.2	0.3	100	100	100 100
	declared	–	–	–	–	37.5	–	–	–	– –
Pig liver	ISO	–	–	–	–	14.9	0.2	–	105	– 107
	HPLC–pre	12.8	0.2	1.7	0.1	14.5	0.2	97	99	103 97
	HPLC–post	13.4	0.2	1.3	0.1	14.7	0.2	98	100	105 99
Sausage "Tee" (Becel)	ISO	–	–	–	–	72.2	0.1	–	107	– 106
	HPLC–pre	68.4	0.4	4.5	0.3	72.9	0.7	98	102	105 97
	HPLC–post	71.3	0.7	2.8	0.1	74.1	0.7	101	99	104 100
Milk, 3.5% fat pasteurized	ISO	–	–	–	–	0.7	0.02	–	99	– –
	HPLC–pre	0.1	0.01	0.6	0.03	0.7	0.01	90	102	– 99
	HPLC–post	0.1	0.01	0.6	0.04	0.7	0.01	90	102	100 100

* ISO is fluorometric method ISO 6557/1-1996 (7); HPLC-pre is reversed-phase HPLC, separation as quinoxaline derivate, fluorescence detection, Bognár (22); HPLC-post is amino-phase HPLC, postcolumn oxidation of L-AA to L-DHA, and derivatization to quinoxaline derivative (new method). Mean value and variation of 3 analyses.
† Vitamin C is L-ascorbic acid and L-dehydroascorbic acid.
‡ Added as L-ascorbic acid.
§ Added as L-dehydroascorbic acid.
‖ –, not analyzed.
n.d., not detected.

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