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Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples

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Abstract

The total vitamin C amount in different food and plasma samples was determined by a dual detection system, after HPLC separation, with direct detection of ascorbic acid and indirect fluorimetric detection of dehydroascorbic acid after a post-column *O*-phenyldiamine derivatisation. The two active forms of vitamin C and their D-isomers were separated within 10 min. The repeatability was determined by measurement of several fruits and vegetables and ranged from 0.3 to 1.9% (relative standard deviation) for vitamin C. The reproducibility, based on double determinations, ranged from 1.9 to 3.6% for vitamin C, depending on the matrix. The reproducibility, based on several determinations of reference materials, ranged from 2.4 to 3.7% for ascorbic acid and from 4.3 to 5.8% for dehydroascorbic acid, again depending on the matrix.

Keywords: Vitamins; Ascorbic acid; Dehydroascorbic acid; Isoascorbic acid; Dehydroisoascorbic acid

1. Introduction

In the field of nutritional research and control, vitamin C analyses involve determination and quantification of L-ascorbic acid (AA) and L-dehydroascorbic acid (DHAA) in foodstuffs and in biological samples e.g., plasma samples. The D-isomer of ascorbic acid, isoascorbic acid (IAA) is not found in natural products but may be present in so-called vitamin C-enriched food products or in products where IAA is added as an antioxidant. Therefore, a vitamin C method should be able to distinguish between the two ascorbic acid isomers and their primary oxidation products.

On the one hand, metaphosphoric acid may provide the most efficient ascorbic acid extraction, by preventing oxidation compared to citric acid, acetic acid, perchloric acid and orthophosphoric acid. On the other hand, metaphosphoric acid may cause serious analytical interactions with silica-based column materials, e.g., C_{18} or NH_2 bonded-phases. These interactions may result in drifts in the baseline and retention time. However, attempts to solve this problem often result in incomplete separation of the four compounds. Also, the presence of minerals e.g., Fe^{3+} and Cu^{2+} in samples may require use of a

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chelating agent, such as ethylenediaminetetraacetic acid (EDTA). However, this may result in time-consuming analyses due to the long retention time of EDTA.

In 1988 Lloyd et al. [1] introduced a method based on a polystyrene–divinylbenzene polymer column (PLRP-S) later modified by Wanderslice and Higgs [2]. The PLRP-S column is compatible with injections of high concentrations of metaphosphoric acid and provides a very stable system without drift of baseline and retention time.

Nutritional research and control analyses of vitamin C require the capability to analyse a broad variety of matrices e.g., vegetables, fruit, processed foods, milk products, whole diets, supplemented special diets, blood plasma, multivitamin tablets etc., all containing a high amount of hydrophilic potentially interfering compounds. However, in our experience the PLRP-S column system did not fulfil these requirements.

In contrast to ascorbic acid, dehydroascorbic acid has a weak UV absorption and no response to electrochemical detection (ED). In order to increase the sensitivity for dehydroascorbic acid, derivatisation prior to or after the chromatographic separation is necessary. Prior to high-performance liquid chromatography (HPLC), dehydroascorbic acid may be reduced to ascorbic acid by L-cysteine [3] or dithiothreitol [4] or derivatised with *O*-phenyldiamine [5,6] to form the fluorophore 3(1,2-dihydroxyethyl)furo[3,4-*b*]quinooxaline-1-one (DFQ) [7]. Dehydroascorbic acid and ascorbic acid can be detected simultaneously by means of a post-column on-line derivatisation. Karp et al. [8] used a post-analytical column, solid-state reduction of DHAA to AA. A more frequently used method is a post-column reaction [9,10] by means of an on-line post-column oxidation of AA to DHAA with Cu^{2+} [10] or Hg^{2+} [2] followed by a derivatisation with *O*-phenyldiamine. In this way ascorbic acid and dehydroascorbic acid were detected simultaneously and fluorometrically as DFQ.

In the present study, we chose a method providing simultaneous data on AA and DHAA, i.e., a post-column derivatisation method.

This method gives an optimal separation of AA and IAA, DHIAA and DHAA in addition to a minor variation in retention time during an analytical sequence. We suggest a combination of 1% metaphosphoric acid and 0.5% oxalic acid, pH 2, as extraction buffer. This buffer provides a high stabilisation of AA and DHAA and leads to minimal interactions with the chromatographic system. AA and IAA were detected directly by UV or ED and DHIAA and DHAA were detected fluorometrically as DFQ after a post-column derivatisation with *O*-phenyldiamine.

The method was validated in agreement with the NMKL procedure NR4 1996 (Nordic Committee on Food Analysis) [11] and tested on a large number of samples and matrixes.

2. Experimental

2.1. Chemicals

All reagents were of highest commercial available purity. All water used was Millipore water.

Metaphosphoric acid (MPA) [40–44% $(HPO_3)_n$, 56–60% $(NaPO_3)_n$] (Aldrich), oxalic acid (Sigma), disodium oxalate (Sigma), sodiumthiosulphate-5-hydrate (Merck), iodine sublimated (I_2) (Merck), potassium iodide (KI) (Sigma), dodecyltrimethylammonium chloride (Fluka), sodium acetate (Merck), trisodiumcitrate-2-hydrate (Merck), acetic acid, 100% (Merck), phosphoric acid, 85% (Merck), KH_2PO_4 (Merck), Na_2EDTA (Merck), *O*-phenyldiamine (OPD) (Sigma), L-ascorbic acid (Sigma), D-isoascorbic acid, (erythorbic acid), (Sigma).

2.2. Chromatographic conditions

The liquid chromatographic system consisted of a Waters 6000 A eluent pump (Waters, Milford, MA, USA) coupled to a Waters Wisp 710 B refrigerated autosampler set at 4°C. The column temperature was kept at 20°C during analyses by a Croco-Sil column cooler (Cluzeau Info Lab., Sainte-Foy-La-Grande, France). Ascorbic acid was detected on a Jasco 870-UV (Jasco Spectroscopic, Tokyo, Japan) detector set at 247 nm or an ESA Coulochem II coulometric electrochemical detector (ESA, Bedford, MA, USA) equipped with a Model 5020 guard cell operating at

+200 mV and a Model 5010 dual analytical cell operating at −200 mV and +150 mV[1].

The post-column reagent was delivered by a Dionex RP-1 pump (Dionex, USA) operated at 0.3 ml/min and mixed with the HPLC mobile phase in a 20 m×0.3 mm I.D. reaction coil, kept at 55°C. Dehydroascorbic acid was detected as DFQ on a Jasco 820-FP fluorescence detector set at excitation wavelength 350 nm and emission wavelength 430 nm. The analytical HPLC column was a Jupiter C_{18} (5 μ, 300 Å, 250×4.6 mm I.D., Phenomenex, Torrance, CA, USA) equipped with a Phenomenex security guard system and operated at 1.2 ml/min. The mobile phase was 2.3 m*M* dodecyltrimethylammonium chloride and 2.5 m*M* Na_2EDTA in a 66 m*M* phosphate–20 m*M* acetate buffer adjusted to pH 4.50. The post-column reagent was 28 m*M* *O*-phenyldiamine in a 12 m*M* trisodium citrate–55 m*M* Na_2EDTA buffer adjusted to pH 3.70. A 20-μl aliquot of standards and samples was injected.

To obtain stable retention times, the column was flushed with water and methanol for every 100–150 injections. After the wash the column was equilibrated with eluent and saturated with metaphosphoric acid by injection of 20 μl standard 10 to 12 times prior to analysis.

All samples were injected in doublets and data were collected on a Turbochrom Navigator and Turbochrom Result Manager software from Perkin-Elmer (Norwalk, CT, USA). The sample amounts were computed with overlapping brackets sequence, based on one external standard per four samples.

2.3. Sample preparation

The extraction procedure for foodstuff samples in this study was based on a well-proven accredited laboratory HPLC method for AA and IAA analysis (AB1132), see below.

Precautions. All extracts were prepared immediately before analyses and kept cold and protected against daylight during extraction and analysis.

[1]The potential referees to the ESA reference electrode, a pH sensitive electrode. At pH 3, the ESA reference potential is 300 mV lower than a Ag/AgCl electrode. The ESA reference will change +60 mV per pH unit increasing.

2.3.1. Extraction buffers

All foodstuff samples were extracted in 1% (w/v) metaphosphoric acid with 0.5% (w/v) oxalic acid adjusted to pH 2. However, samples with high amounts of starch were extracted in 2% (w/v) metaphosphoric acid and 1% (w/v) oxalic acid–ethanol (50:50) and afterwards diluted a minimum of two times with 1% (w/v) metaphosphoric acid.

2.3.2. Foodstuffs

A 25–30-g sample was weighed into 80–100 ml extraction buffer and transferred to a Waring blender together with another 50–70 ml of extraction buffer. Oxygen was evacuated by leading a stream of carbon dioxide through the suspension for 3 min. The sample was blended for exactly 5 min. The resulting solution was quantitatively filled up to 250 ml with the extraction buffer. An aliquot was centrifuged for 10 min at 9000 *g* and 4°C and filtered on a 0.45-μm filter prior to analysis.

2.3.3. Multivitamin tablets

Five tablets were crushed and extracted in 250 ml 5% (w/v) metaphosphoric acid and 1% (w/v) oxalic acid. The extracts were diluted 20–50 times with 1% (w/v) metaphosphoric acid and 0.5% oxalic acid, pH 2. Aliquots were centrifuged for 10 min at 9000 *g* and 4°C and filtered on a 0.45-μm filter before analyses.

2.3.4. Plasma samples

A 500-ml volume of plasma was stabilised immediately after preparation by addition of 500 ml 10% metaphosphoric acid and frozen at −80°C until analysis. A 500–1000-ml volume of stabilised plasma was centrifuged at 14 000 *g* and 4°C for 10 min. One volume of the supernatant was added to two volumes of 0.4 *M* acetate buffer, pH 3.9 resulting in a metaphosphoric acid concentration of approximately 1.7% and pH 2 of the extract. A 500-ml volume of the extract was transferred to a Whatmann vectaspin micro (Whatman International, Maidston, UK) centrifuge tube, equipped with a 0.45-μm polypropylene filter and centrifuged at 10 000 *g* at 4°C for 5–10 min.

2.4. Standards

Ascorbic and dehydroascorbic acid were quantified based on external standards. In foodstuffs analyses, samples were quantified within the concentration range of 10–150 μg/ml for AA and 1–50 μg/ml for DHAA. External standards were 50 μg/ml and 5 μg/ml for AA and DHAA, respectively. In plasma and foods with very low vitamin C contents, AA was quantified in the concentration range of 0.1–10 μg/ml and DHAA in the range of 0.05–5 μg/ml. External standards 1 μg/ml and 0.5 μg/ml for AA and DHAA, respectively. All standards were prepared in the extraction buffer used during the analysis. A DHAA stock solution, of 500 μg/ml, was prepared from AA standards by adding small amounts of a 0.1 *M* iodine solution (I_3^-) stepwise to 50 ml ascorbic acid (1 mg/ml) until the solution had a constant light yellow colour. To reduce the surplus iodine two crystals of sodium thiosulphate were added and the standard was filled up to 100 ml with extraction buffer. AA and DHAA standards were mixed and diluted to the concentrations required.

3. Results

3.1. Chromatography

Figs. 1 and 2 represents typical chromatograms of standards, frozen green beans and human blood plasma. Fig. 1A–C shows chromatograms of ascorbic acid and isoascorbic acid and Fig. 2A–C shows chromatograms of dehydroascorbic acid and dehydroisoascorbic acid. In this study, ascorbic acid was measured in food samples by UV detection and by ED in blood plasma samples. The analytical run-time was set at 14 min for food analyses in order to flush oxalic acid and metaphosphoric acid from the column between injections. When analysing plasma a run-time of 10 min was found adequate. The relative standard deviation (RSD) of the retention time during a 23 h run of food extracts was 2.38% ($n=80$) for AA and 0.46% ($n=80$) for DHAA. When analysing plasma samples under the conditions described here, the RSD of the retention time was 4.90% ($n=130$) for AA and 6.30% ($n=130$) for DHAA. The resolution between AA and IAA was 2.3 for 50 μg/ml standards and 1.03 for DHAA and DHIAA in a 5 μg/ml standard.

3.2. Post-column reaction

The post-column reaction between DHAA and *O*-phenyldiamine was optimised with regard to the reaction time, reaction temperature, pH and OPD concentration. A citric acid buffer was preferred to a phosphate buffer in the OPD reagent, due to higher stabilisation of OPD. Furthermore, precipitation occurred when the OPD reagent was based on a phosphate buffer. During the optimisation the DHAA concentration was 50 μg/ml, and this concentration was chosen as the highest expected DHAA concentration in any food extracts. The length of the reaction coil was set at 20 m in order to limit peak broadening. The net flow-rate was 1.5 ml/min, resulting in a reaction time of approximately 1 min, however, no significant increase in the fluorescence response was observed at reaction times up to 4 min.

Fig. 3 shows that the fluorescence response reaches a plateau at approximately 25 m*M* OPD under the conditions described in Section 2.2. From an economic consideration 28 m*M* was chosen. Fig. 4 shows that the fluorescence response reaches a plateau between 55–60°C and tends to decrease slightly at higher temperatures under conditions described in Section 2.2. Fig. 5 shows that the fluorescence response reaches a local maximum at pH 3.70 corresponding to a pH of approximately 4.10 in the reaction coil. However, by reducing the length of the reaction coil to 10 m, we found linearity up to a concentration of 20 μg/ml DHAA, $r^2=0.9999$

3.3. Linearity

Quantification of AA and DHAA was based on bracket calibrations of external standards. For average foodstuff analyses the linearity on a four-point calibration curve was checked ranging from 10 to 150 μg/ml for AA and 1 to 50 μg/ml for DHAA ($r^2>0.9999$). For plasma analyses and foodstuffs with low vitamin C contents a four-point calibration curve was used, ranging from 0.1 to 10 μg/ml AA

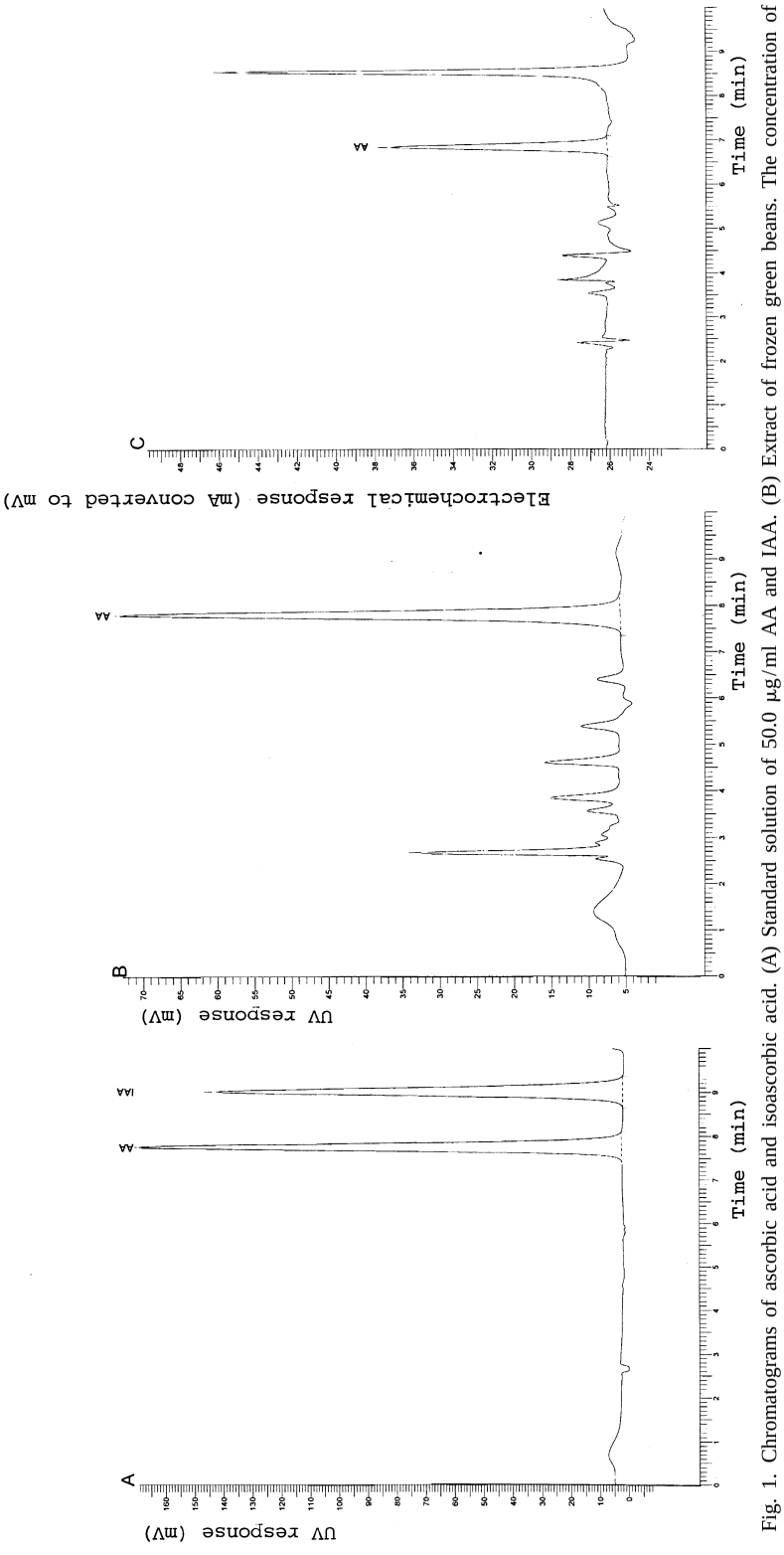

Fig. 1. Chromatograms of ascorbic acid and isoascorbic acid. (A) Standard solution of 50.0 μg/ml AA and IAA. (B) Extract of frozen green beans. The concentration of ascorbic acid was 21.1 μg/ml. (C) Human blood plasma extract. The concentration of ascorbic acid was 0.6 μg/ml in the extract.

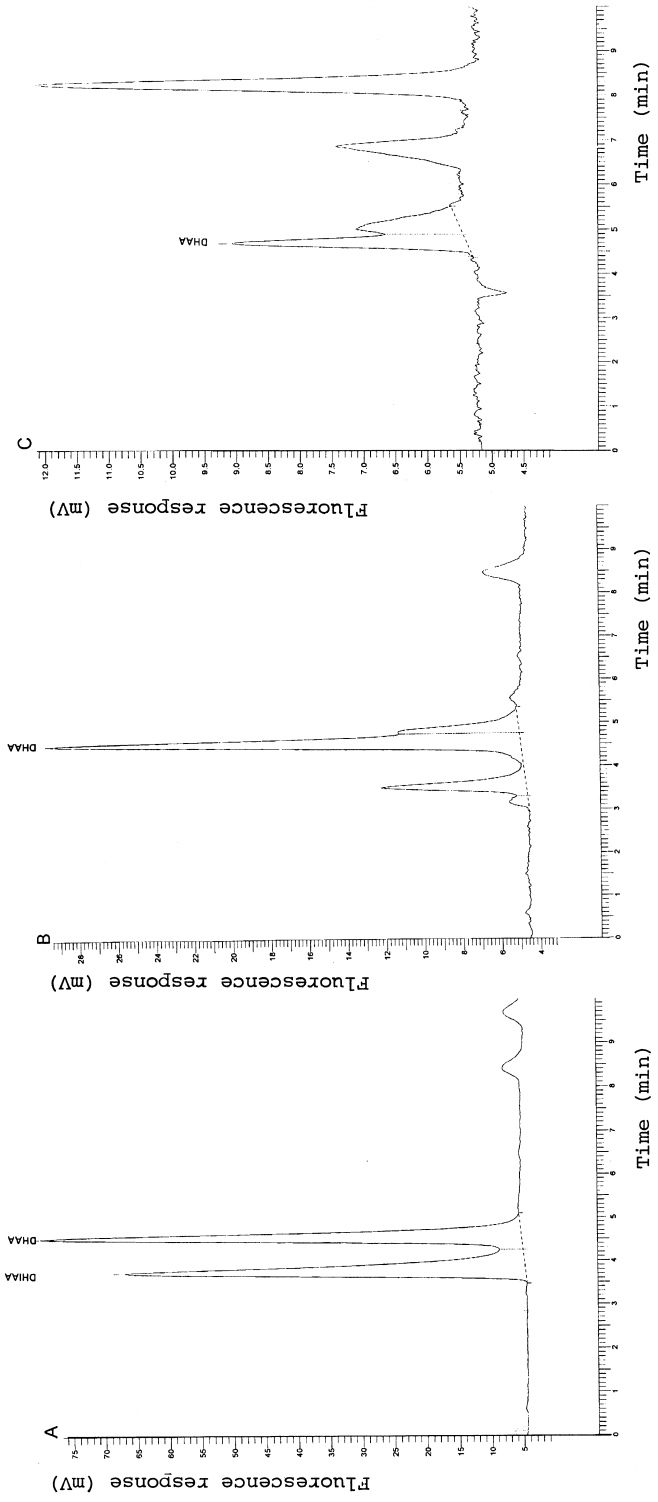

Fig. 2. Chromatograms dehydroascorbic acid and dehydroisoascorbic acid. (A) Standard solution of 5.0 μg/ml DHAA and DHIAA. (B) Extract of frozen green beans. The concentration of dehydroascorbic acid was 1.8 μg/ml. (C) Human blood plasma extract. The concentration of dehydroascorbic acid was 0.2 μg/ml in the extract.

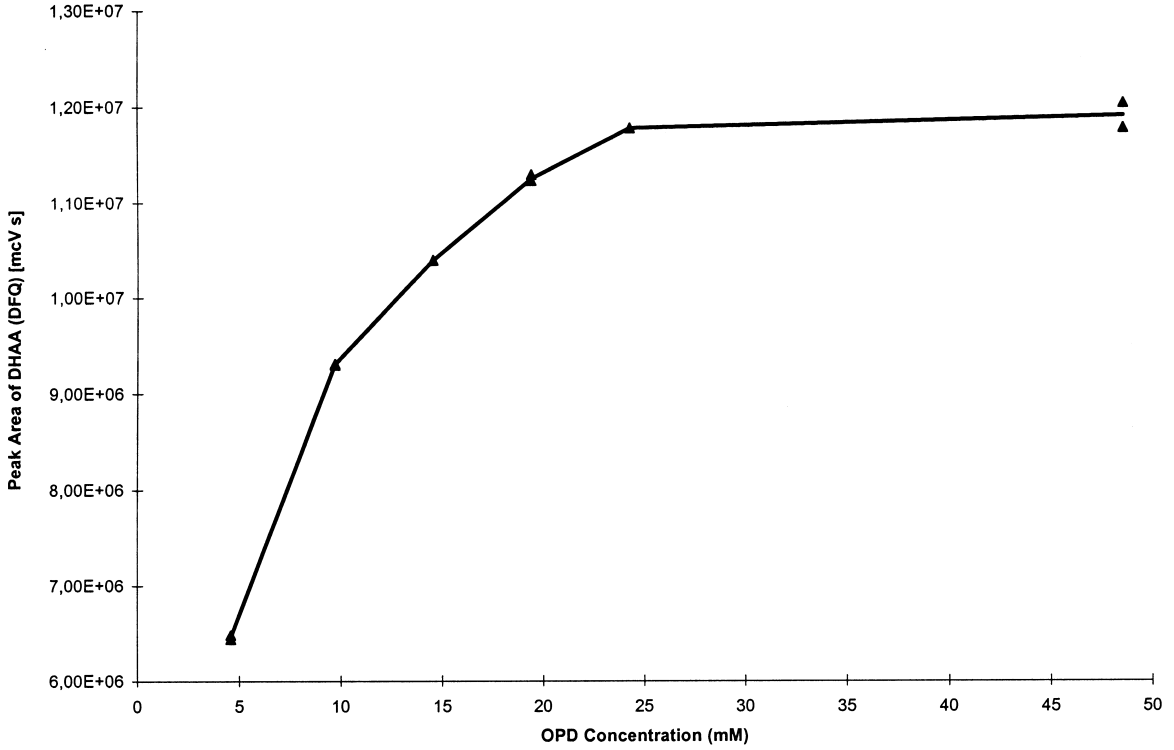

Fig. 3. Optimisation of the OPD concentration. The DHAA standard was 50 μg/ml and the reaction temperature was 55°C. The OPD buffers were adjusted to pH 3.70.

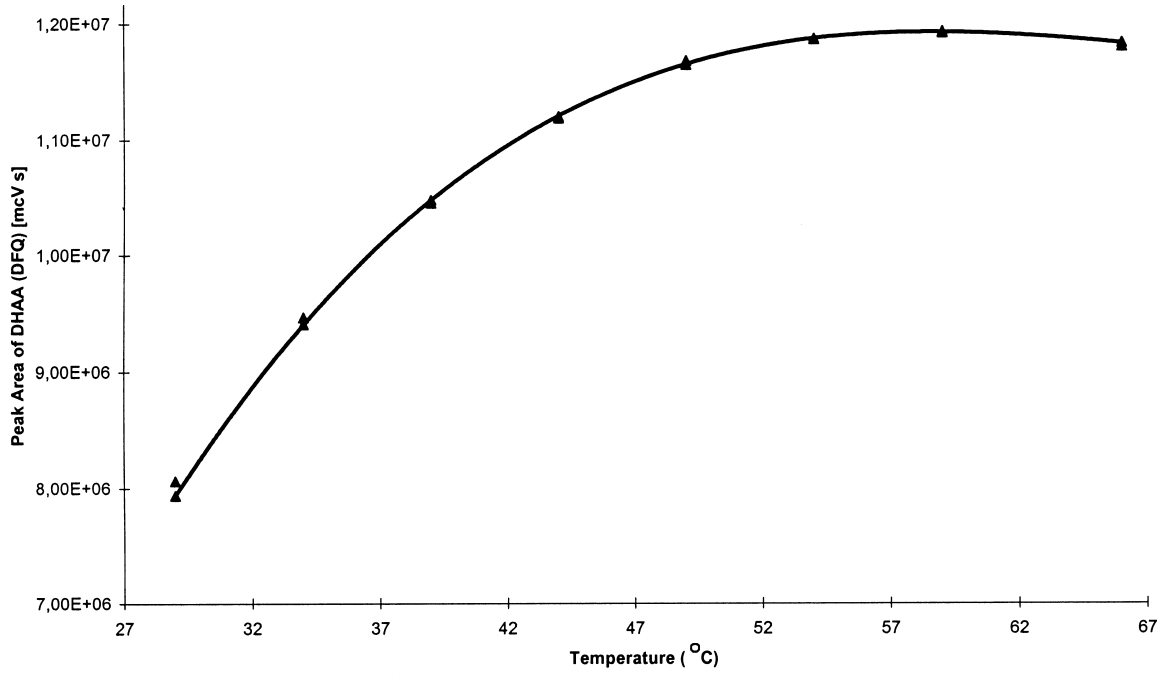

Fig. 4. Optimisation of the post-column reaction temperature. DHAA standards were 50 μg/ml and the OPD concentration was 28 m*M*, pH 3.70.

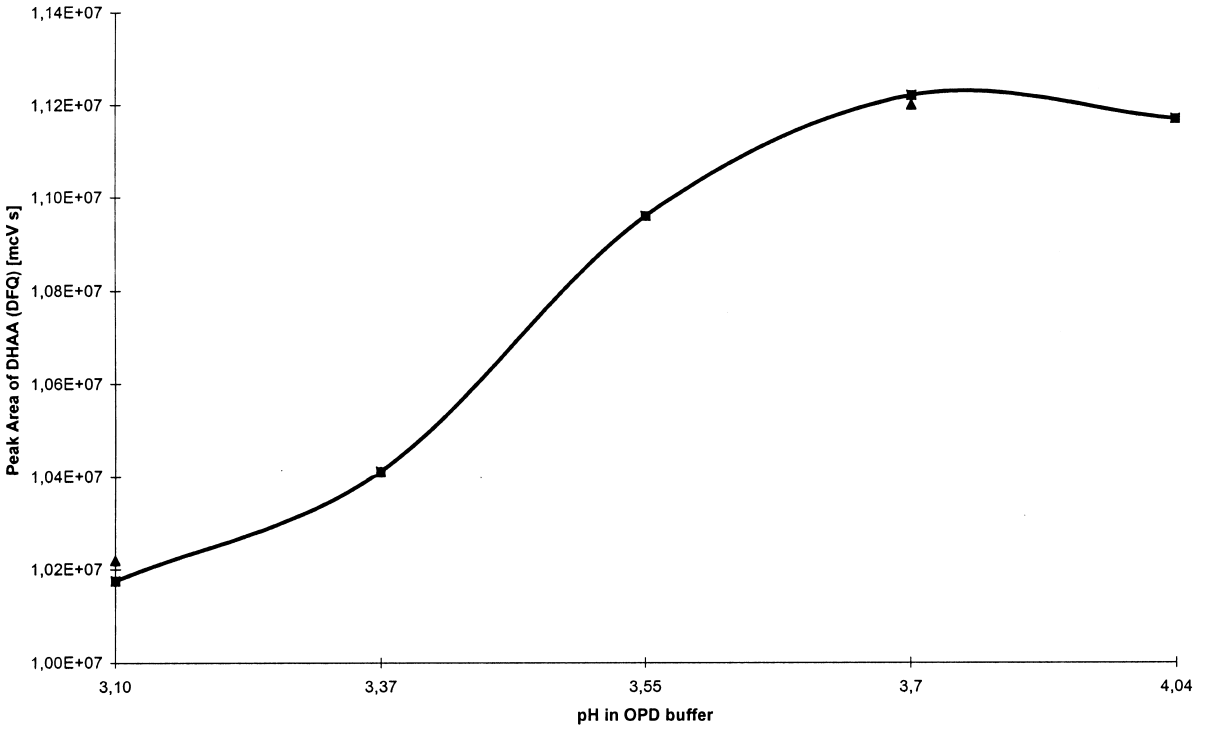

Fig. 5. Optimisation the OPD buffer pH. The DHAA standard was 50 μg/ml, OPD concentration was 28 m*M* and the reaction temperature was 55°C.

and 0.05 to 5 μg/ml DHAA (r^2>0.999). The calibration curves were forced through the origin.

3.4. Repeatability and reproducibility

The method was validated with respect to the within-day precision and the day-to-day precision. The precision (RSD) of injections of AA and DHAA standard, calculated on 20 repeated injections during 24 h, were 1.16% and 1.10%, respectively. The repeatability was checked during a 24-h run, where four foodstuff extracts were injected repeatedly. Table 1 shows the precision during analyses of different foodstuff extracts. The table also shows that

Table 1
Repeatability: within-day precision[a]

	AA			DHAA			Vitamin C[b]		
	Mean (μg/ml)	SD	RSD (%)	Mean (μg/ml)	SD	RSD (%)	Mean (μg/ml)	SD	RSD (%)
Broccoli (n=8)[c]	136.9	0.84	0.61	7.8	0.40	5.13	144.7	0.40	0.28
Tomato (n=8)	32.8	0.43	1.31	0.8	0.13	17.22	33.6	0.27	0.81
Plum (n=8)	3.1	0.11	3.44	2.7	0.03	1.29	5.8	0.11	1.87
Cabbage (n=8) (freeze–dried)	37.1	0.30	0.80	15.0	0.26	1.77	52.3	0.27	0.52

[a] Variation in AA and DHAA concentrations in some foodstuff extracts, repeatedly determined during 23 h. Samples were extracted in 1% MPA+0.5% oxalic acid, pH 2, and kept at 4°C and protected against daylight during the analysis.
[b] Sum of AA and DHAA.
[c] Number of determinations of the same extract based on double injections.

AA and DHAA are stabilised in the extracts during the 24-h analytical run. However, in the tomato extract and to some extent in the broccoli extract the DHAA concentration increased during the 24-h run. This may be due to the high AA/DHAA ratio in the extract; e.g., a slight AA oxidation will result in a large increase in DHAA concentration. The vitamin C concentration, i.e., AA+DHAA, was constant during the run.

The reproducibility was determined as the mean RSD of a number of double determinations of samples in the same matrix and in comparable matrices (R-charts) Table 2. Furthermore, the reproducibility was determined as the RSD of a number of single determinations of in house control materials (X-charts) Table 2.

Table 2
Reproducibility: day-to-day accuracy

	RSD (%)		
	AA	DHAA	Vitamin C
Samples[a]			
Green bean ($n=18$)[e]	2.2	2.0	1.8
Cauliflower ($n=18$)[f]	1.5	3.3	2.5
Plasma ($n=105$)	3.5[b]	18.3[c]	3.0
Cabbage ($n=4$)[d]	1.3	2.3	3.6
Vegetables[g]	3.1	5.1	1.9
Fruits[h]	2.8	8.5	3.4
Control material[i]			
Cabbage ($n=8$)[j]	3.66	5.75	
Nidina ($n=8$)[k]	2.37	4.25	

[a] The reproducibility is calculated as the mean RSD of the number (n) of individual double determinations.
[b] Mean 1.37 μg/ml (0.44–3.22 μg/ml).
[c] Mean 0.09 μg/ml (0.02–0.4 μg/ml).
[d] Cabbage extract diluted and treated as plasma.
[e] Raw and processed beans from the same batch with decreasing AA and DHAA extract amounts ranging from 17.6 to 3.6 μg/ml and 2.6 to 0.9 μg/ml, respectively.
[f] Raw and processed cauliflower from the same batch with decreasing AA and DHAA amounts, ranging from 85.5 to 32.4 μg/ml and 6.1 to 0.8 μg/ml, respectively.
[g] Broccoli, parsley, cucumber, green pepper, pea.
[h] Strawberry, plum, apple, peach.
[i] The reproducibility is calculated as the RSD of number (n) of single determinations of a homogeneous control material.
[j] Freeze–dried.
[k] Cow's milk protein breast milk substitute.

Table 3
Accuracy – recovery (%) in two matrices

	AA	DHAA
Plasma ($n=5$)	97.5	95.5
Cabbage ($n=5$)	101.3	98.7

3.5. Accuracy

To determine the accuracy, recovery was measured in several matrices. Table 3 shows some recovery results. Furthermore, the analysis was evaluated by participation in the BIPEA proficiency-testing program (Table 4) [12] and by analyses of certified reference materials, Table 5.

4. Discussion

The combination of metaphosphoric acid as extraction buffer and a silica-based, reversed-phase column often results in pronounced variations in retention time and in baseline drift in addition to an inadequate separation of AA, IAA, DHAA and DHIAA. Use of a PLRP-S column may to some extent solve this problem. However, it requires a complicated post-column on-line oxidation and derivatisation system to compensate for inadequate resolution.

The present method was developed in order overcome several problems that may occur during vitamin C analysis, i.e., a method optimised in regard to stabilisation and separation of AA/IAA and DHAA/DHIAA and elimination of baseline- and retention time drift.

Ascorbic acid is easily oxidised to DHAA in blood samples [10] and should be deproteinised and stabilised immediately after collection. Lykkesfeldt et al. [4] showed that a concentration of 5% metaphosphoric acid stabilises AA and DHAA during a two-month storage at −20°C. Extraction of foodstuff samples have been performed in different buffers, i.e., 0.85% MPA [13,14], 0.1 *M* citric acid+5 m*M* EDTA [15,16], 3% MPA+8% acetic acid [2,17]. On the other hand, we found that 1% (w/v) metaphosphoric acid+0.5% (w/w) oxalic acid adjusted to pH 2 provides an excellent extraction and stabilisation of ascorbic acid and dehydroascorbic acid in food

Table 4
Accuracy – proficiency testing (BIPEA)

Matrix	Results			Reference vitamin C (mg/100 g)	Tolerance vitamin C (mg/100 g)
	AA (mg/100 g)	DHAA (mg/100 g)	Vitamin C (mg/100 g)		
24 h diet (n=21)[a]	n.d.[b]	14.15	14.15	14.89	4.47
Baby food (n=23)	79.52	2.84	82.36	82.59	24.78
Breast milk substitute (n=21)	78.00	3.80	81.80	82.99	24.90
Milk powder (n=24)	35.43	7.30	42.73	44.94	13.48

[a] Number of participating laboratories.
[b] Not detected.

Table 5
Accuracy – certified reference material (BCR)

Matrix	Results			Reference vitamin C (mg/100 g)	Uncertainty[a] vitamin C (mg/100 g)
	AA (mg/100 g)	DHAA (mg/100 g)	Vitamin C (mg/100 g)		
CRM421 (milk powder)	47.2	26.8	74.0	76.9	3

[a] The uncertainty is defined as the half-width of the 95% confidence interval of the mean of the data set averages.

extraction. Further, by using this buffer no interference between the metaphosphoric acid extract and the reversed-phase ion-pair HPLC system was observed.

Dehydroascorbic acid standards were prepared by oxidation of AA standards with an iodine solution and the surplus iodine reduced by sodium thiosulphate. AA and DHAA standards were then mixed and no increase in DHAA or decrease in AA was observed during the next 24 h. Hydrolysis of DHAA to 2,3-diketogulonic acid in a solution at pH 2 was investigated by means of C^{13} nuclear magnetic resonance (NMR) [18]. The reduction of DHAA to AA under effect of surplus thiosulphate was investigated, yet, no formation of either 2,3-diketogulonic acid or ascorbic acid was observed.

For starchy foodstuffs such as potatoes, Wanderslice et al. [16] proposed addition of one volume of *n*-butanol after homogenisation of the sample to precipitate starch from the aqueous solution. This method implied centrifugation at 48 400 *g* which is, however, incompatible with the performance of routine analyses. Homogenisation of samples in 2% MPA–ethanol (1:1) results in precipitation of starch and stabilisation of AA and DHAA. Injection of 50% ethanol will affect the column equilibration and result in a splitting of the DHAA peak, however, by reduction of the ethanol concentration to a maximum of 25% in the extract, no effect was seen on the DHAA peak shape (data not shown).

The method based on a PLRP-S column described by Vanderslice and Higgs [2] was compatible with injections of high MPA concentrations, without retention or baseline drift and with separation of AA, IAA, DHAA and DHIAA. However, when detecting AA and IAA directly with UV detection, the insufficient separation of hydrophilic impurities or EDTA may cause inaccurate integration.

In this method we chose to detect AA and IAA directly by UV or ED. To achieve optimal separation, we based the chromatographic system on a silica-based ODS column material with an enlarged range of pH stability compared to traditional ODS column material. By combining a phosphate buffer with an acetic acid buffer and EDTA in the mobile phase, we achieved a system with high capacity and relatively short run-time. Further, the combination of the Jupiter column and the *n*-dodecyltrimethylammonium chloride detergent resulted in a system with high stability to injection of 1–2% MPA extracts.

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