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Assay for both ascorbic and dehydroascorbic acid in dairy foods by high-performance liquid chromatography using precolumn derivatization with methoxy- and ethoxy-1,2-phenylenediamine

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ABSTRACT

   A procedure for the simultaneous determination of both ascorbic and dehydroascorbic acid in dairy foods by high-performance liquid chromatography using precolumn derivatization with 4-methoxy- and 4-ethoxy-1,2-phenylenediamine is presented. The derivatives are isolated by solid-phase extraction and analysed by fluorescence detection on a resin-type reversed-phase column at pH 9. Retention times are 2 and 3.2 min for the derivatives of ascorbic and dehydroascorbic acid, respectively. Relative standard deviations of the within- and between-assay tests are 7.1 and 5.5%, respectively, for ascorbic and 11 and 9%, respectively, dehydroascorbic acid. The limits of detection are 50 and 70 fmol per 5-μl injection for ascorbic and dehydroascorbic acid, respectively.

INTRODUCTION

   Milk and dairy products are a valuable source of vitamins for human nutrition. These foods may be heated by a range of processes and stored for various periods of time. Vitamin C, consisting of both ascorbic acid (AA) and dehydroascorbic acid (DHAA), is known to be labile during both processing and storage. It seems important to measure the effects on vitamin C and to ensure that losses are at a minimum, if possible. Also, it is of interest for the consumer to know the final level of vitamin C.
   A variety of methods for the assay of vitamin C in foods and biological samples are available. They include biological, chemical, polarographic, enzymatic and chromatographic procedures. The literature in this area has been reviewed by Jaffe [1] and by Bui-Nguyén [2]. Chemical techniques combined with spectrophotometric or spectrofluorimetric detection are still in use. Oxidation–reduction titration of AA with dichlorophenolindophenol is still very popular, although unspecific [3]. Another class of colorimetric reactions involves the reaction of AA with metal ions such as Fe^{3+} [4,5]. Enzymatic assays for AA utilize the ability of ascorbic acid oxidase to deplete ascorbic acid. The depletion may be followed by spectrophotometric [6] or polarographic methods [7]. Total vitamin C (AA + DHAA) may be measured by ox-

idation of AA to DHAA, followed by derivatization of the DHAA with either 2,4-dinitrophenylhydrazine [8] or 1,2-phenylenediamine [9] to give a coloured or a fluorescent derivative.

New developments in this area include high-performance liquid chromatography (HPLC) on anion-exchange [10–13], reversed-phase [14–16] and bonded-phase NH_2 [17] columns. Either ultraviolet [13,17] or electrochemical [14–16] detection is applied. However, these techniques are sensitive to AA only.

A precolumn derivatization procedure includes the reaction of DHAA with 1,2-phenylenediamine, followed by HPLC [18,19]. With prior oxidation of AA to DHAA, total vitamin C may be measured in the second run.

Using a post-column derivatization technique, both AA and DHAA may be measured separately in one run [20]. In this instance, the post-column reagent solution contains both 1,2-phenylenediamine and an oxidizing agent. After separation by HPLC, DHAA is derivatized and AA is oxidized and derivatized, to give the fluorescent quinoxaline. However, disadvantages include both the use of 20-m heated (65–70°C) and cooled coils and retention times of up to 20 min.

In this work, 4-methoxy- and 4-ethoxy-1,2-phenylenediamine were applied to the sequential precolumn derivatization of both AA and DHAA. The derivatives formed exhibit at least a 10-fold increase in fluorescence, compared with the quinoxaline derived from 1,2-phenylenediamine. They can be selectively isolated by solid-phase extraction and analysed by HPLC at pH 9 in a single run. The retention times are 2 and 3.2 min.

EXPERIMENTAL

Apparatus

The HPLC system consisted of Beckman (Berkeley, CA, U.S.A.) Model 100A precision-flow metering pumps, a Hamilton (Reno, NV, U.S.A.) PRP-1 column (150 × 4.1 mm I.D.) with a 5-μm polystyrene–divinylbenzene copolymer packing, a 5-μl loop, a Shimadzu (Kyoto, Japan) C-R1A data processor and a Merck–Hitachi (Darmstadt, Germany) Model F1000 spectrofluorimeter for HPLC applications, equipped with both a 150-W high-pressure xenon lamp and a 12-μl flow cell. The excitation and emission wavelength were set at 375 and 475 nm, respectively. The mobile phase was delivered at 1 ml/min. A Perkin-Elmer (Beaconsfield, U.K.) LS-5 luminescence spectrometer was used to scan the excitation and emission wavelengths of the methoxy- and ethoxyquinoxaline derivatives of DHAA.

Reagents and chemicals

Unless stated otherwise, analytical-reagent grade chemicals were used. Ascorbic acid, oxalic acid, 70% perchloric acid, 85% orthophosphoric acid, 25% ammonia solution, methanol, ethanol, bromine, 1,2-phenylenediamine and palladium phthalocyanine and HPLC-grade acetonitrile, dipotassium hydrogenphosphate and 1-propanesulphonate were obtained from E. Merck (Darmstadt, Germany). Column chromatographic grade C_{18} silica gel LiChroprep (particle size 25–40 μm) and Florisil (100–200 mesh) were from Merck. 4-Methoxy-1,2-phenylenediamine dihydrochloride (98%) and 4-ethoxy-2-nitroaniline (97%) were from Aldrich (Steinheim, Germany) and Aminex 50W-X2 (Na^+) (200–400 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Stock solutions were 0.5% oxalic acid, 150 mmol/l H_3PO_4, 1 mol/l KH_2PO_4, 1 mol/l K_2HPO_4 for HPLC stored in a refrigerator, C_{18} silica gel LiChroprep in ethanol (10 g in 95 ml), saturated aqueous bromine, phosphoric acid buffer (150 mmol/l H_3PO_4 adjusted to pH 2 with 1 mol/l KH_2PO_4) and a 30% (v/v) slurry of Aminex 50W-X2 (H^+) in water.

Solutions prepared on the day of use were mobile phase for HPLC, consisting of 16% acetonitrile in both 50 mmol/l K_2HPO_4 and 5 mmol/l 1-propanesulphonate, adjusted to pH 9 with 150 mmol/l H_3PO_4, 10 mmol/l ascorbic acid made by dissolving 1 mmol of ascorbic acid in 100 ml of 0.5% oxalic acid and 1 μmol/l dehydroascorbic acid as a standard solution. The last solution was prepared by transferring 100 μl of 10 mmol/l ascorbic acid into a 10-ml screw-capped volumetric flask (Pierce Europe, Oud-Beijerland, The Netherlands), half-filled with 0.5% oxalic acid, followed by addition of aqueous bromine as an oxidizing agent. The excess of bromine was removed by streaming nitrogen over the fluid surface. The flask was diluted to the mark with water, sealed and shaken. The resulting solution contained 100 μmol/l DHAA. A 100-μl volume of this solution was diluted in 10 ml of phosphoric acid buffer.

Solutions prepared just prior to use were 100 mg per 100 ml 4-ethoxy-1,2-phenylenediamine ditrifluoroacetic acid in phosphoric acid buffer (pH 2) and 100 mg per 100 ml 4-methoxy-1,2-phenylenediamine ditrifluoroacetic acid in phosphoric acid buffer (pH 2).

Solid-phase extraction columns were made on the day of use by filling 1-ml filtration tubes (containing frits only) with 1 ml of a suspension of C_{18} silica gel in ethanol. The filled tubes were allowed to drain. The packed adsorbent was rinsed twice with 1 ml of water.

Cation-exchange columns were made on the day of use by filling filtration tubes with 1 ml of a suspension of 30% Aminex 50W-X2.

Derivatization reagents

4-Methoxy-1,2-phenylenediamine dihydrochloride (3 g) was dissolved in 100 ml of water, decolorized with Florisil and filtered through a 0.45-μm pore membrane filter. The solution was loaded onto a 25 × 5 cm I.D. Prepbar column (E. Merck) filled with reversed-phase C_{18} silica, particle size 7 μm. Gradient elution with ethanol was performed at 10 ml/min (gradient from 0 to 60% in 60 min) in the presence of 0.5% trifluoroacetic acid. The 4-methoxy-1,2-phenylenediamine peak at 280 nm was identified by reacting one drop of effluent with 5 ml of 100 mmol/l disodium α-ketoglutarate in 1 mol/l KH_2PO_4 and observing blue-green fluorescence under UV light. The fraction was evaporated to dryness and the residue was dissolved in 100 ml of water and freeze-dried. The substance was stored in a 250-ml glass bottle under nitrogen at room temperature.

4-Ethoxy-1,2-phenylenediamine was prepared from 4-ethoxy-2-nitroaniline. 4-Ethoxy-2-nitroaniline (3 g) was dissolved in 200 ml of methanol and hydrogenated with hydrogen on 250 mg of palladium phthalocyanine as catalyst. The fluid was passed through a 0.45-μm PTFE membrane to remove palladium, decolorized with Florisil, evaporated to dryness, the residue dissolved in 100 ml of water, purified by HPLC using gradient elution with ethanol as described above (gradient from 0 to 80% in 60 min), evaporated to dryness, the residue dissolved in water, freeze-dried and stored under nitrogen.

Sample preparation, derivatization and extraction

A 1-g cheese sample or 1 ml of milk was homogenized in or mixed with, respectively, 18 ml of 0.5% oxalic acid, 1 ml of 70% perchloric acid was added and the precipitate was removed by centrifugation at 1600 g.

The supernatant (0.5 ml) was transferred into a test-tube, followed by addition of 0.8 ml of 1 mol/l KH_2PO_4 for buffering at pH 2 and 0.5 ml of 100 mg per 100 ml 4-ethoxy-1,2-phenylenediamine for derivatization. The reaction time was 45 min in the dark at room temperature. The solution was passed through a solid-phase extraction column and a cation-exchange column, attached to one another by means of an adapter, and collected in a test-tube. Two 1-ml volumes of water were passed through the columns for rinsing and collected in the same test-tube. Saturated aqueous bromine (50 μl) was added to oxidize AA. The excess of bromine was removed by bubbling with nitrogen for 10 min. [A manifold for delivering nitrogen to ten test-tubes held in a rack was constructed from a length of polyethylene tubing (30 cm × 1.2 cm I.D. × 1.4 cm O.D.) and ten pieces of PTFE capillary tubing (100 mm × 1.8 mm I.D. × 2 mm O.D.). The polyethylene tubing was sealed at one end with a stopper and punctured a long its length in ten places, corresponding to the positions of the ten test-tube holes in the rack. The PTFE capillaries were then forced into the puncture holes. The unit was attached at one end to the nitrogen source and fixed in position, allowing immersion of capillary tubing tips into the test-tube fluid for nitrogen bubbling.]

A 0.5-ml volume of 100 mg per 100 ml 4-methoxy-1,2-phenylenediamine was added and allowed to react for 45 min in the dark. The solution was passed through the solid-phase extraction column used previously. The column was rinsed with 1 ml of water. The derivatives of interest were recovered from the adsorbent by elution with 1 ml of 15% acetonitrile and 0.5% of trifluoroacetic acid in water, and immediately neutralized by adding 5 μl of 25% ammonia solution. (For this purpose, a 25% ammonia solution was stored in a 30-ml serum-type reaction vial sealed with a 13.5 mm O.D. polyethylene stopper. When in use, the stopper was replaced with one having a 1 mm diameter hole, through which the ammonia solution was sampled with a 5-μl syringe-pipette with a disposable PTFE-tip.) HPLC analysis was performed within a few hours.

RESULTS AND DISCUSSION

1,2-Phenylenediamine reacts with α-keto acids and with DHAA to yield fluorescent quinoxaline derivatives under mild conditions [21]. The present derivatization reagents also undergo this type of reaction to generate presumably methoxy- and ethoxyquinoxalines. However, these derivatives exhibit at least a 10–13-fold higher fluorescence than the quinoxaline of DHAA. Excitation and fluorescence maxima are observed at 375 and 475 nm, respectively.

Two-step sequential derivatization is applied to the simultaneous assay of both AA and DHAA by HPLC. First, the sample is derivatized with 4-ethoxy-1,2-phenylenediamine, followed by isolation of both products of derivatization and removal of excess of reagent. The latter is removed by adsorption on a cation-exchange resin. The derivatives are retained by the reversed-phase adsorbent. The AA in the sample is oxidized to DHAA and then derivatized with 4-methoxy-1,2-phenylenediamine.

Each derivatization reaction could be followed by measuring the increase in fluorescence. Stable plateau values of fluorescence were attained within 45 min at pH 2, 3, 4 and 5 at room temperature.

The derivatives were recovered from the reversed-phase adsorbent by elution with 15% acetonitrile and 0.5% trifluoroacetic acid in water. Under these conditions, the derivatives of keto acids and orange-coloured products are largely retained by the adsorbent. Usually colourless eluates were obtained. These were immediately neutralized with a 25% ammonia solution, with which pH 8–8.5 was easily obtained. When neutralized, the derivatives were found to be very stable at room temperature, and could be analysed by HPLC within 24 h without noticeable changes in the fluorescence.

A typical chromatogram representing 5 pmol each of AA and DHAA per 5-μl injection is shown in Fig. 1. Separation at pH values between 3 and 5.5 on both resin- and silica-based reversed-phase columns was unsatisfactory, as the elution profile of each derivative gave a major peak with a shoulder on both sides. This drawback could not be corrected by ion-pair reagents in the mobile phase.

In the extract, AA should remain constant during derivatization of DHAA. This requirement was fulfilled by performing the derivatization at pH 2, as shown in recovery experiments (Table I). Different amounts of both AA and DHAA were added to oxalic acid–perchloric acid extracts of milk, buffered with 1 mol/l KH_2PO_4 at pH 2, and with 1 mol/l potassium oxalate (pH 5) at pH 3 and 4, followed by two-step derivatization, extraction and HPLC. The recoveries of AA ranged between
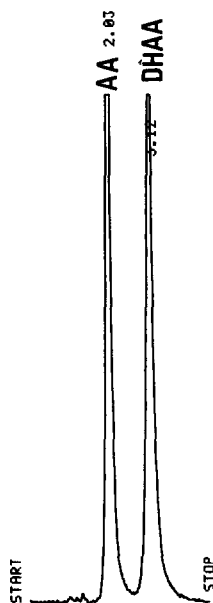

Fig. 1. Separation of methoxy- and ethoxyquinoxaline of DHAA, with retention times of 2.03 and 3.12 min as indicated. Peaks represent 5 pmol each of AA and DHAA. Elution at 1 ml/min; injection volume, 5 μl; recording attenuation, 8 mV full-scale. For other details, see text.

TABLE I

RECOVERIES OF AA AND DHAA ADDED TO 0.4-ml VOLUMES OF OXALIC ACID–PER-CHLORIC ACID EXTRACTS OF MILK

Procedure	Amount added (pmol)		Amount measured (pmol) (mean ± S.D.)		Recovery (%)	
	AA	DHAA	AA	DHAA	AA	DHAA
Derivatization at	0	0	612 ± 68	167 ± 29		
pH 2 (n = 5 at each level)	100	100	715 ± 52	265 ± 24	103	98
	200	200	850 ± 67	345 ± 43	119	89
	400	400	1050 ± 78	532 ± 51	108	91
	800	800	1432 ± 45	913 ± 36	103	93
Derivatization at	0	0	276 ± 13	167 ± 7		
pH 3 (n = 5 at each level)	100	100	339 ± 26	301 ± 13	63	134
	200	200	450 ± 14	395 ± 19	87	114
	400	400	641 ± 39	589 ± 26	91	106
	800	800	908 ± 49	1064 ± 27	79	112
Derivatization at	0	0	244 ± 19	200 ± 7		
pH 4 (n = 5 at each level)	100	100	235 ± 7	411 ± 19	0	211
	200	200	250 ± 26	635 ± 33	0	217
	400	400	204 ± 11	1050 ± 30	0	213
	800	800	20 ± 3	2281 ± 99	0	260

103 and 119% at pH 2 and 63 and 91% at pH 3. No recovery of AA was observed at pH 4. The AA losses were recovered as DHAA.

Dairy foods such as raw and pasteurized milk, yoghurt, UHT-processed milk and cheese were analysed by derivatization at pH 2. Results with details of the origin of the foods and type of heat treatment are given in Table II.

TABLE II

AA AND DHAA CONTENTS OF DAIRY FOODS EXPRESSED IN mg PER 1000 ml OR 1000 g, RESPECTIVELY

Results are means ± S.D. (n = 6).

Food	AA	DHAA
Raw cow's milk[a]	5.9 ± 0.3	1.52 ± 0.2
Pasteurized milk[b,c]	4.0 ± 0.25	0.39 ± 0.03
Yoghurt[b]	0.27 ± 0.3	1.52 ± 0.2
UHT milk by indirect heating[b,d,e]	1.1 ± 0.05	0.21 ± 0.03
UHT milk by indirect heating[b,d,f]	0.14 ± 0.03	0.07 ± 0.007
UHT milk by direct heating[b,d,g]	4.15 ± 0.21	0.28 ± 0.014
UHT milk by direct heating[b,d,h]	3.90 ± 0.13	0.28 ± 0.02
Pasteurized milk[b,h]	5.0 ± 0.3	0.46 ± 0.03
Tilsiter cheese[b]	1.76 ± 0.3	0.32 ± 0.04

[a] Obtained from the herd at our institute.
[b] On retail sale.
[c,e,f,g,h] Local dairy plants.
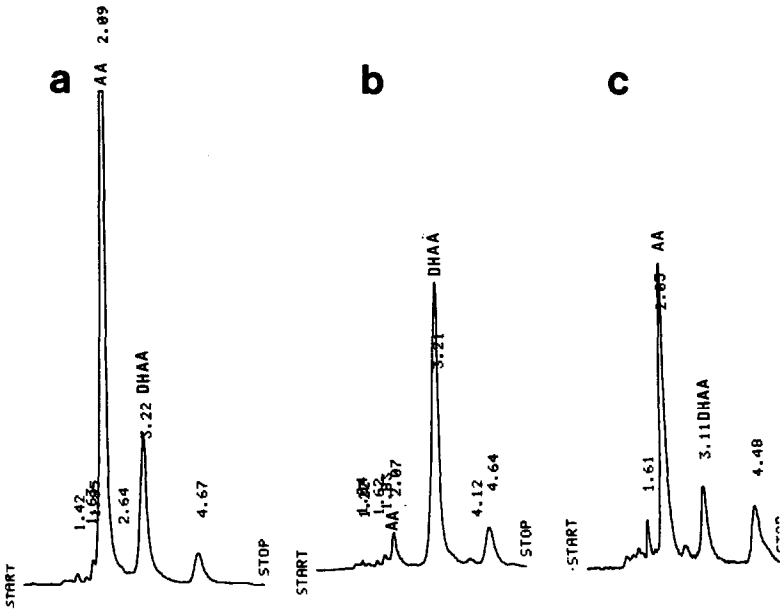[d] Ultra-high-temperature-processed milk (135–150°C).

Fig. 2. Elution profiles for the analysis of (a) raw milk, (b) yoghurt and (c) Tilsiter cheese. Peaks for AA and DHAA are indicated. Numbers at peaks indicate retention times in min.
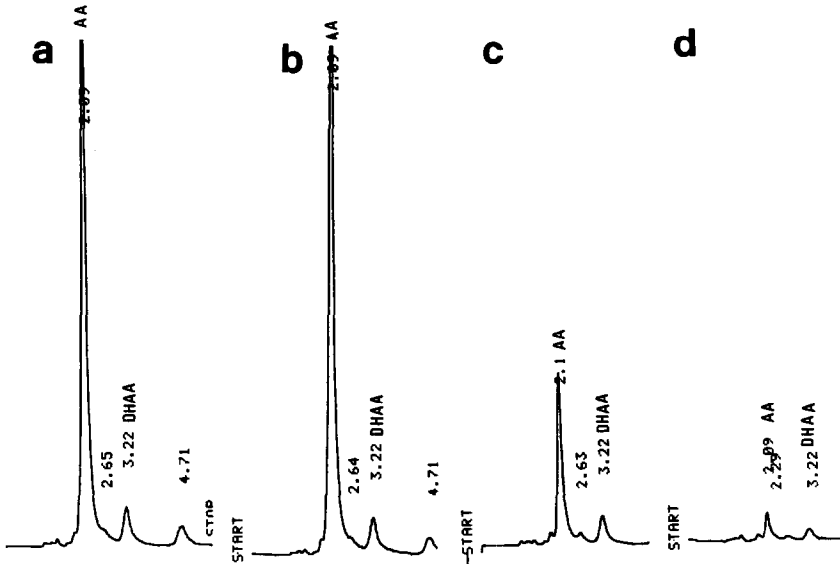
Fig. 3. Elution profiles for the analysis of UHT milk by (a,b) direct and (c,d) indirect heating at dairy plants given in Table II. Numbers at peaks indicate retention times in min.

TABLE III

PRECISION OF THE ASSAY

Parameter	Amount in 1-ml aliquot of Tilsiter cheese (pmol) (mean ± S.D.)	
	AA	DHAA
Within-assay ($n=9$)	500 ± 36	90 ± 6
Relative standard deviation	7.1%	5.5%
Between-assay ($n=6$)	479 ± 45	93 ± 8
Relative standard deviation	11%	9%

Typical chromatograms for the analysis of dairy foods given in Table II are shown in Figs. 2 and 3. As expected, the highest values were found in both raw and pasteurized milk, with as much as 80% in the form of AA. The yoghurt contained a quarter of the amount found in pasteurized milk, mostly in the form of DHAA. UHT-processing by indirect heating with heat exchangers destroyed as much as 80–90% of the vitamin in raw milk, in contrast to processing by direct heating with steam. In contrast to Emmental and Gruyère cheese, which contained less then 0.2 mg/kg, Tilsiter cheese showed concentrations of about 2 mg/kg, with 80% in the form of AA.

The reproducibility of the procedure was tested on a Tilsiter cheese, which was stored in a refrigerator for 10 days. The results are given in Table III. The within- and between-assay relative standard deviations were 7.1 and 5.5%, respectively for AA and 11 and 9%, respectively, for DHAA. These values are within acceptable limits.

At a signal-to-noise ratio of 3, the detection limits were calculated to be about 50 and 70 fmol of AA and DHAA, respectively, per 5-μl injection.

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