HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids

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The potential role of ascorbic acid (AA) in biological systems has stimulated wide, multidisciplinary interest in this compound. Its determination is of interest in many fields (e.g., food, clinical, plant or pharmaceutical analysis).

In the past few years, many methods, based on different analytical techniques, have been developed for the determination of AA and its oxidation product, dehydroascorbic acid (DHA). This review aims to make clear the differences, the progress, and the advantages and the disadvantages of individual approaches using high-performance liquid chromatography (HPLC). We discuss HPLC methods for the determination of AA and DHA from the points of view of separation mechanism and detection technique. Stability of these analytes is a key issue in obtaining reliable method validation, so we also included this aspect in the discussion.

Keywords: Ascorbic acid; Dehydroascorbic acid; Detection; Determination; High-performance liquid chromatography; HPLC; Method validation; Separation; Stability

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1. Introduction

The biologically-active isomer of ascorbic acid (vitamin C, AA) is L-ascorbic acid, but there is still some discussion about the activity of L-dehydroascorbic acid [1,2]. D-ascorbic acid (isoascorbic acid, erythorbic acid, iso-AA) is legally used as an antioxidant food additive, but it has only 5% of the antiscorbutic effect of AA [3]. AA is widely distributed in plant material and, as the human body cannot synthesize it, fruit and vegetables are the major sources for the human diet. AA is rapidly oxidized to dehydroascorbic acid (DHA) due to the presence of two hydroxyl groups in its structure. Oxidation reactions can be induced by exposure to increased temperatures, high pH, light, presence of oxygen or metals and enzymatic action. This reaction is reversible and a principal step in the antioxidant activity of AA. Further oxidation generates diketogluconic acid (DKG), which has no biological function and the reaction is no longer reversible [2,4,5].

The potential role of AA in biological systems has stimulated wide, multidisciplinary interest in this compound. The role of AA in metabolism is complex. Its action in protecting against the oxidizing effect of free radicals is of crucial importance. Its presence is necessary for the activity of dopamine β-hydroxylase, as well as for the synthesis of collagen and the prevention of coronary heart diseases [6] and cancer [7].

Its concentration, or more accurately, the AA/DHA ratio can be an indicator of the redox state of an organism. That is why, sometimes, AA, together with DHA, has been determined in all kinds of biological materials using different analytical methods.

The content of vitamin C in fruits (the sum of the contents of AA plus DHA) is used as an index of the health-related quality of fruits, so interest in the simultaneous analysis of AA and DHA has also increased greatly in food analysis [8]. There are many problems to be overcome during simultaneous analysis of AA and DHA, and, even if there seem to be the following common method-development problems, these are somewhat highlighted during analysis of AA and DHA:

1) selectivity and sensitivity of the method;
2) the choice of internal standard (IS) for quantitation purposes;

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3) retention of AA and DHA in the high-performance liquid chromatography (HPLC) system;
4) the choice of appropriate detection; and
5) stability of AA and DHA in solutions.

A lot of methods based on the reversible redox reaction of AA oxidation/DHA reduction were developed in the past. Unfortunately, they often lacked true specificity, and were therefore prone to interferences by other reducing agents [9]. For example, spectrophotometric or enzymatic methods could interfere with analytes {e.g., ferrous or cuprous ions (spectrophotometry), sugars or glucuronic acid (spectrophotometry), or citrate and hydrogen peroxide (enzymatic methods) [10]}. The lack of sufficient sensitivity in analysis of biological materials has been especially evident (e.g., tissue samples).

In general, the most preferred analysis methods have utilized HPLC, because they provide higher selectivity than spectrophotometric, titration or enzymatic methods and they do not usually need derivatization. Depending on the type of detection technique employed, HPLC methods are generally more sensitive. Ultraviolet (UV) or electrochemical detection (ED) was used most commonly. More rarely, fluorescence detection (FD) (which needs derivatization of DHA and additional oxidation of AA to DHA) or mass-spectrometry (MS) detection provide the highest sensitivity and selectivity. However, MS is quite expensive and it has high running costs, which make it inaccessible for many laboratories.

A small number of methods, for the determination of AA and DHA, have utilized ISs. In spite of this, the IS approach is much more convenient for the determination of analytes in complex matrices, including food and biological fluids, in which these analytes are often determined. Such samples usually involve a complicated sample-preparation step, so an IS should be required.

Hydrochinon [11] or an isomer of AA (iso-AA) [12] could be used as ISs because they are of similar structure and will therefore have similar chromatographic and extraction behavior. Unfortunately, iso-AA could already be present in real samples of food material or biological fluids. Moreover, both compounds may be oxidized during storage and sample preparation. With the use of reduction agent (in some electrochemical methods), quantitation will be problematic. The subtraction method (to be discussed later), which was often utilized for simultaneous determination of AA and DHA, could not allow the use of these ISs [13]. Hippuric acid [14], nicotinic acid [15], uric acid [16], chlorogenic acid [17] or 4-hydroxyacetanilide [18] have been used as ISs in the HPLC assay for AA/DHA.

We discuss individually retention in the HPLC system, choice of appropriate detection, and stability of AA and DHA in solutions. There have been several review articles dealing with determination of AA in different matrices, using a vast number of various methods. Two recently published review articles described flow-injection methods [19] and non-spectrometric methods for the determination of AA (titrimetric methods, electrochemical methods, chemiluminescence, fluorescence, and chromatographic methods) [20]. One review article in 2001 generally focused on the determination of dietary anticancer compounds in biological fluids, but also included a summary concerning AA and DHA [21]. Since 1973, when use of HPLC for AA determination was first attempted, previous review articles are now quite old (e.g., a review of AA methodology for the determination of AA in biological samples, food products and pharmaceuticals from 1985 [22] or a review of currently new methods for determination of vitamin C from 1988 [23]. A review concerning bio-analytical methods for AA and DHA was published in 1992 [24], as was an analytically focused review about vitamins [25]). Methods concerning properties of DHA and its determination were reviewed by Deutsch in 2000 [2].

The aim of this review is to give a clear overview of HPLC analytical techniques and problems concerning simultaneous analysis of AA and DHA. The main focus is on the mechanism of chromatographic separation, the approach to detection and aspects of stability. Table 1 presents an overview of commonly-used HPLC methods for the determination of AA and DHA used in the period 1999–2008 in various fields of applications. We present the methods in chronological order.

2. HPLC methods

AA and DHA belong to the group of very small polar molecules that are difficult to retain in conventional reversed-phase (RP) chromatographic systems and separate from the dead volume. That is important, especially in bio-analytical assay, in which ballast compounds from biological matrices are eluted together with dead volume or at the beginning of the chromatogram.

The principal approaches to the determination of AA and DHA by LC are:
- RP;
- ion exchange;
- ion pair; and,
- ion exclusion.

The mobile phases are often very complex, with more than two components containing various modifiers or reagents. The approach of hydrophilic interaction liquid chromatography (HILIC), which is much simpler and more elegant, has become popular recently.

2.1. Reversed-phase chromatography

Conventional ODS stationary phases are widely used for the determination of AA and DHA (Table 1). However, RP methods often suffer from poor resolution of AA and

Table 1. Overview of chromatographic methods for the determination of ascorbic and dehydroascorbic acid in chronological order

Determined substances	Matrix	Stationary phase analytical column	Mobile phase	Detection	AA t_R [min]	Validation data	Ref., Year
AA, iso-AA DHA, iso-DHA – post-column derivatization o-phenyldiamine	Food multivitamins plasma	Ion-pair Jupiter C_{18} (250 × 4.6 mm, 5 μm)	2.3 mM DTMACl 2.5 mM Na_2EDTA 66 mM phosphate buffer 20 mM acetate buffer pH 4.5	UV 254 nm FD 350 nm 430 nm	7.7	r^2 = 0.9999 rec = 97.5–101.3% AA RSD = 2.4–3.7% AA RSD = 4.3–5.8% DHA	[39] 1999
AA, pyruvate, lactate	Microdialysis of striatum	Ion-exclusion Polypore H (100 × 4.6 mm, 10 μm)	4 mM sulfonic acid	UV 214 nm	1.6	r^2 = 1.000 $RSD_{inter} < 1.27\%$ $RSD_{inter} < 6.05\%$ LOD = 0.5 μM	[51] 1999
AA, DHA – reduction by 2-mercaptoethanol glutathione	Ocular fluids	Waters RP-C18 (10 μm)	0.2% M KH_2PO_4 pH 3.0	ED	7.1	not given	[74] 1999
AA, DHA – precolumn reduction DTT	Solid AA kinetic study of degradation induced by moisture	Lichrospher 100 RP C18 e (250 × 4.6 mm, 5 μm)	0.1% TFA pH 2.7	DAD 245 nm	2.1	RSD = 1.05–2.21%	[28] 1999
AA	Food	Inertsil ODS-3 (150 × 4.6 mm, 5 μm)	20 mM monosodium L-glutamate pH 2.1 – phosphoric acid	ED Ag/AgCl 400 mV	4.6	rec ⩾ 90% AA RSD = 2.5% LOD = 0.1 ng/5 μl	[29] 2000
AA	Food	Inertsil ODS-3 (150 × 4.6 mm, 5 μm)	20 mM GMP pH 2.1 phosphoric acid	ED Ag/AgCl 400 mV	4	accuracy ⩾ 90% AA RSD = 2.7% LOD = 0.1 ng/5 μl	[30] 2000
AA, DHA – reduction TCEP iso-AA, UA	Biological samples	Ion-pair Luna C_{18} (150 × 4.6 mm, 3 μm)	100 mM sodium dihydrogenphosphate 100 mM sodium acetate 1.0 mM EDTA 0.189 mM n-DTMACl 36.6 μM tetraoctylammonium bromide MeOH pH 5.4 o-phosphoric acid	ED Ag/AgCl 300 mV	10	$r^2 > 0.99$ rec = 103.5% RSD_{intra} = 1.5% RSD_{inter} = 3.5%	[40] 2000
AA, DHA – reduction DTT	Fruit vegetables	HiChrom C_{18} (250 × 4.0 mm, 5 μm)	0.2 M KH_2PO_4 pH 2.4 (adj. by phosphoric acid)	UV 254 nm 210 nm	9	r^2 = 0.9992 accuracy = 81.7–105.9% RSD < 1%	[61] 2000

substances		analytical column					
AA, pyruvate, lactate	Dialysates	Ion-exclusion Polypore H (220 × 4.6 mm, 10 μm)	4 mM sulphuric acid	UV 214 nm	3.5	$r^2 \geqslant 0.999$ $RSD_{intra} < 3.0\%$ $RSD_{inter} < 7.3\%$ LOD = 0.5 μM	[52] 2000
AA (9 water-soluble vitamins)	Multivitamin tablets	μ-Bondapack C_{18} (300 × 3.9 mm, 10 μm)	A: MeOH B: 0.1 M KH_2PO_4 pH 7.0 gradient elution	DAD 265 nm	2.0	$r^2 = 0.9995$ rec = 95% LOD = 0.1 μg/ml	[68] 2001
AA	Multivitamin-mineral tablets	Ion-pair Hypersil BDS-C_{18} (250 × 4.0 mm, 5 μm)	Ion-pair solution-ACN (98:2) 10 mM hexanesulfonic acid sodium salt, acetic acid, TEA	DAD 275 nm	2.2	rec = 89–115% rec = 95–112% $r^2 = 0.999$ LOQ = 10 μg/ml	[41] 2001
AA, DHA – reduction DTT	Parenteral nutrition mixtures	Ion-pair Luna C_{18} (150 × 4.0 mm, 5 μm)	MeOH 0.067 M phosphate buffer pH 7.8 (40:60) + cetrimide	UV 278 nm	4.20	$r^2 = 0.9999$ AA RSD < 1% AA $r^2 = 0.9936$ DHA RSD < 2.2% DHA	[42] 2001
AA, β-carotene	13 beverages	Ion-exchange Kromasil NH_2 5 μm-100Å (250 × 4.6 mm, 5 μm)	Acetic acid in water 0.1 M	UV 250 nm	9.3	$r^2 = 0.9997$ RSD = 2.1 ± 1.5% LOD = 1.2 mg/l LOQ = 4.0 mg/l	[50] 2002
AA	Food	Inertsil ODS-3 (150 × 3.0 mm, 5 μm)	pH 2.1 – phosphoric acid 0.2%	ED Ag/AgCl 400 mV	4	rec $\geqslant$ 90% AA $RSD_{intra} = 2.8\%$ $RSD_{inter} = 3.7\%$ LOD = 0.1 ng/5 μl	[31] 2003
AA, GA, ART, MAP	Cosmetic products	Ion-pair Mightysil RP-18 GP (250 × 4.6 mm, 5 μm)	0.005 M potassium dihydrophosphate buffer TBAH MeOH, phosphoric acid	UV 240 nm 220 nm	4	r = 0.9996 RSD = 3.5% rec = 94.8–95.6%	[43] 2003
AA, DHA – derivatization with o-phenylene diamine	Arthritic rats' serum	Nucleosil C18	ACN:60 mM phosphoric acid pH 2.0 (80:20)	UV 254 nm FD 350 nm 425 nm	1.7	$r^2 = 0.9996$ $RSD_{intra} < 1.2\%$ $RSD_{inter} < 2.9\%$	[32] 2003
AA, epicatechin gallic acid	Star fruit *(Averrhoa carambola L.)*	Shim-Pack VP-ODS (250 × 4.6 mm, 5 μm)	A: 0.1% formic acid in water B: MeOH gradient elution	DAD 280 nm ESI-MS-MS	14.3	not given	[69] 2004
AA (other water-soluble and fat-soluble vitamins)	Pharmaceutical preparations food	MetaChem Polaris C18-A (150 × 4.6 mm, 3 μm)	0.010% TFA pH 3.9 MeOH	DAD 280 nm ESI-MS full scan	2.8	$r^2 = 0.9986$ RSD = 2.1% LOD = 25.55 ng/ml LOQ = 85.16 ng/ml	[70] 2004

Table 1 (*continued*)

Determined substances	Matrix	Stationary phase analytical column	Mobile phase	Detection	AA t_R [min]	Validation data	Ref., Year
L-AA, iso-AA L-DHA – reduction TCEP (subtraction)	Blood	Ion-pair Chromolith PerformanceC_{18} (100 × 4.6 mm)	2.5 mM NaH_2PO_4 2.5 mM DTMACl 1.25 mM Na_2EDTA 2% ACN	UV 264 nm	0.8	r^2 = 0.999 RSD = 4.2–4.5% RSD = 4.3–4.6% LOD = 1.5 μmol/l LOQ = 4.95 μmol/l	[44] 2005
AA	Human lymphocytes	CSC-Kromasil C18 (250 × 4.6 mm, 5 μm)	0.2 M KH_2PO_4/H_3PO_4 pH 3.0 2 mM EDTA	UV 245 nm	–	r^2 = 0.989 RSD_{inter} = 0.25–9.98% RSD_{intra} = 1.2–12.49%	[62] 2005
AA (B_1, PP, B_6, B_5, B_9, B_{12}, B_2, B_8)	Polyvitaminated premixes	YMC-Pack Pro C_{18} (250 × 4.6 mm, 5 μm)	A: 0.025% TFA pH 2.6 B: ACN gradient elution	UV 210 nm 275 nm	6.4	r^2 = 0.9956 RSD_{intra} = 2.1% RSD_{inter} = 5.0 and 5.9%	[63] 2005
AA, GSH, GSSG, UA	Urinary bladder	Hypersil C18 (250 × 4.0 mm, 5 μm)	50 mM potassium phosphate pH 3.0 adjusted by o-phosphoric acid	ED Coularray 300 mV	4	r^2 > 0.996 RSD_{intra} < 4.4% RSD_{inter} < 9.4% rec = 98.9–112.6% LOQ = 500 nmol/l	[75] 2005
AA carotenoids	Tomato kiwi mango	Coupled Symmetry C18 (75 × 4.6 mm, 3.5 μm) Atlantis C18 (150 × 2.0 mm, 5 μm)	70% MeOH 30% acetic acid 0.05%	MS-SIM ESI^- $[M–H]^-$ = 175	3.99	rec = 85% LOD = 10 μl/l LOQ = 50 μl/l	[77] 2005
AA, DHA – reduction DTT	Human milk	Spherisorb ODS2 C_{18} (250 × 4.6 mm, 5 μm)	0.1% acetic acid : MeOH (95:5)	UV 254 nm	3.7	rec = 95.55 ± 1.18% RSD^{intra} = 3.09% RSD_{inter} = 4.03%	[71] 2006
AA (taurine and other water soluble vitamins) IS = hippuric acid	Multivitamin tablets	Johnson Spherigel C_{18} (250 × 4.6 mm, 5 μm)	B: MeOH A: 5 mM heptafluorobutyric acid gradient elution	ESI-MS ESI^- $[M–H]^-$ = 175.4	3.6	rec = 98.3% RSD_{intra} = 0.5% RSD_{inter} = 1.1% LOD = 12 μl/l LOQ = 20 μl/l	[14] 2006
AA	Wines	PLRP-S 100A (150 × 4.6 mm, 5 μm)	A: water-trifluoroacetic acid (99:1) B : ACN-A (80:20)	UV 243 nm	2.3	r^2 = 0.999 rec > 95% RSD = 0.8–2.2% LOD = 1 mg/l LOQ = 5 mg/l	[38] 2006

substances		analytical column					
AA, AA-2G and other derivatives of AA	Enzymatic hydrolysates	Interstil CN-3 (250 × 4.6 mm, 5 μm)	MeOH 28.6 mM H_3PO_4–NaH_2PO_4 pH 2.1 (65:35) + 20 mg/l dithiothreitol	UV 240 nm	4.5	r^2 = 1.000 rec = 97.3–106.3% RSD = 0.5–2.9%	[64] 2006
AA, DHA – reduction DTT	Banana, papaya, mango pineapple	Ion-exclusion Shodex RSpak KC-811 (250 × 4.6 mm, 5 μm)	0.2% o-phosphoric acid	UV 245 nm	6	rec = 99 ± 6% LOD = 0.1 mg/l	[54] 2006
AA, iso-AA	Fortified food products	Ion-pair LiChrospher RP-18 (250 × 4.6 mm, 5 μm)	ACN sodium acetate pH 5.4 decylamine TCEP	UV 265 nm	12.8	r^2 = 0.9999 rec = 93–105% LOD = 0.1 μg/100 g	[3] 2006
AA, sugars, organic acids	Blackberry	Ultrasphere ODS (250 × 4.6 mm, 5 μm)	0.5% m-phosphoric acid	DAD 200-360 nm	–	r > 0.99	[33] 2006
AA, acetaminophen DHA – derivatization OPDA	Pharmaceuticals	Ion-pair Phenomenex Synergie 4u hydro RP (150 × 4.6 mm, 4 μm)	5 mM CTMAB 0.04% sodium phosphate buffer pH 3.5 ACN (90:10)	UV 245 nm 360 nm	4	R. S. D. ⩽ 1.40 % rec = 99.7–101.8% LOQ = 50 pmol	[45] 2007
AA, DHA – reduction DTT or BAL	Strawberries tomatoes, apples	1. C18 Spherisorb ODS2 (250 × 4.6 mm, 5 μm) 2. NH_2-Spherisorb S5 (250 × 4.6 mm, 5 μm)	1. 0.01% sulphuric acid pH 2.6 2. 10 mM potassium dihydrogen phosphate buffer pH 3.5 ACN (60:40)	UV 245 nm	–	rec = 93.6–104.4% RSD = 0.6–3.9% LOQ < 0.61 mg/100 g LOD < 0.18 mg/100 g	[8] 2007
AA, iso-AA AA-2G, AA-2βG	Food beverages	HILIC Interstil Diol (250 × 4.6 mm, 5 μm)	ACN:66.7 mM ammonium acetate (85:15)	UV 260 nm	8	r = 0.9996 RSD_{intra} = 0.5–2.8% RSD_{inter} = 0.5–2.1% $accuracy_{inter}$ = 98.7–105.8% $accuracy_{intra}$ = 99.1–104.1% LOD = 0.3 μg/ml	[59] 2007
L-AA, L-DHA – derivatization OPDA	Foods	NovaPak C18 (150 × 3.9 mm, 4 μm)	80 mM phosphate buffer pH 7.8 MeOH (84–16%)	FD 355 nm 425 nm	1.89	r = 0.9997 LOQ = 0.82 μg/ml LOD = 0.27 μg/ml	[73] 2007
AA, oxalic acid citric acid, malic acid, cis-aconitic acid, quinic acid, fumaric acid	*Castanea sativa*	Ion-exclusion Nucleogel Ion 300 OA (300 × 7.7 mm, 5 μm)	sulphuric acid 0.01 M	UV 214 nm	30	not given	[53] 2007

Table 1 (*continued*)

Determined substances	Matrix	Stationary phase analytical column	Mobile phase	Detection	AA t_R [min]	Validation data	Ref., Year
AA, IS = nicotinic acid	Semi-solid pharmaceutical/ cosmetic preparations	LiChrospher 100-RP 18 (250 × 4.6 mm, 5 μm)	0.2% m-phosphoric acid/ MeOH/ ACN (90:8:2)	UV 254 nm	3.5	rec = 95.46–101.54% RSD_{intra} = 0.38 % RSD_{inter} = 1.22% LOD = 0.05 μg/ml LOQ = 0.17 μg/ml	[15] 2007
AA, UA iso-AA = IS	Plasma	Ion-pair Supelco LC 18DB (250 × 4.6 mm, 5 μm)	40 mM sodium acetate 1.3 mM Na_2EDTA 1.5 mM DTEAP 7.5% MeOH pH 4.65 by acetic acid	ED 280 mV	3.2	results given for stability	[46] 2007
AA, DHA – reduction DTT vitamin E fatty acids	Human milk	Spherisorb ODS2 C_{18} (250 × 4.6 mm, 5 μm)	MeOH acetic acid 0.1% (95:5)	UV 254 nm	–	rec = 95.06 ± 1.12% RSD = 2.44% RSD = 3.63%	[65] 2008 [66] 2008
AA, vitamins E and A iron, selenium	Infant milk-based powdered formula	Spherisorb ODS2 C_{18} (250 × 4.6 mm, 5 μm)	MeOH acetic acid 0.1% (95:5)	UV 254 nm	–	rec = 95.06 ± 1.12% RSD = 2.44% RSD = 3.63%	[67] 2008
AA, IS = chlorogenic acid	Tablets	ZIC-HILIC (150 x 2.1 mm, 3.5 μm)	ACN 50 mM ammnonium acetate buffer pH 6.8 (78:22)	UV 268 nm	4.6	rec = 98.81–104.45% RSD = 3.04–3.72% R = 0.9995	[17] 2008

AA, Ascorbic acid; AA-2G, 2-O-α-D-glucopyranosyl-L-ascorbic acid; AA-2βG, 2-O-β-D-glucopyranosyl-L-ascorbic acid; ACN, Acetonitrile; ART, Arbutin; BAL, 2,3-dimerccapto-1-propanol; CLD, Chemiluminiscence detection; DAD, Diode-array detection; DHA, Dehydroascorbic acid; DKG, Diketogluconic acid; DTT, Dithiotreitol; ED, Electrochemical detection; EDTA, Ethylene diamine tetraacetic acid; FD, Fluorescence detection; GMP, Disodium guanosine-5'-monophosphate; ESI, Electrospray ionization; GA, Glycolic acid; IS, Internal standard; LOQ, Limit of quantification; LOD, Limit of detection; MAP, Mg ascrorbyl phosphate; MeOH, methanol; MS, Mass spectrometry; ODS, Octadecylsilica; OPDA, o-pheylenediamine; rec, Recovery; RSD, Relative standard deviation, which describes method precision; TCEP, [2-caboxyethyl]phosphine; TEA, Triethylamine; TFA, Trifluoroacetic acid; UA, Uric acid; UV, Ultraviolet.
Ion-pairing agents: CTMAB, Cetyltrimethylammonium bromide; DTEAP, Dodecyltriethylammonium phosphate; DTMABr, Dodecyltrimethylammonium bromide; DTMACl, Dodecyltrimethylammonium chloride; HTMAB, Hexadecyltrimethylammonium bromide; TAF, Tridecylammonium phosphate; TAHS, Tetrahexylammonium hydrogen sulphate; TBAP, Tetrabutylammonium phosphate; TBAH, Tetrabutylammonium hydroxide.

the dead retention volume. To get sufficient retention, a very high percentage of water, usually in inorganic/organic acid or inorganic buffer (sometimes even 100%), must be applied in combination with low pH. Neutral forms of acids, which arise in acidic pH, are better retained on the ODS stationary phase.

A mobile phase below the pK_a of AA (4.17) is necessary for ion-suppression RP separation. Perchloric acid has frequently been employed as an ion suppressant for the determination of organic acids by RP HPLC due to its strong acidity and powerful suppressive action at low application concentration (mM level). This kind of chromatography is sometimes called ''ion-suppression reversed-phase chromatography'' [26,27]. In analysis of AA and DHA, trifluoroacetic acid (TFA), [28] sulphuric acid [8] or phosphoric acid [29–33] at very low pH values around 2 were applied as ion-suppression RP mobile phases. One of two main drawbacks of such an approach is the 100% concentration of the water fraction in mobile phase. It is very well known that water mobile phases not containing an organic modifier can negatively influence separation efficiency on C18 stationary phase or they can even cause so-called ''hydrophobic collapse of stationary phase'' in long-term use [34].

The second drawback – low pH – also accelerates degradation of silica-based analytical columns due to dissolution of the base silica material. This problem could be very well solved by using a pre-saturation column or changing the silica support for a hybrid [35], zirconia [36] or polymer support [37] that possesses higher chemical stability. The problem of the stability of silica-based columns at low pH was taken into account in only one case, in which a polymer-based stationary phase was used [38].

2.2. Ion-pair chromatography

Ion-pair chromatography is also used very widely in analyzing AA and DHA [3,39–46]. Ion-pairing reagents, together with inorganic buffers, were often used as additives in analyzing AA and DHA. However, use of inorganic buffers causes many problems. Inorganic salts can build up in the flow-line elements, such as check-in and check-out valves. This may result in malfunction of these valves. Switching from aqueous buffer to an eluent with high content of organic solvent must be done carefully and gradually (after washing the system with water) in order to prevent buffer precipitation. Moreover, inorganic buffers are not compatible with MS detection because they are not volatile so they should be avoided in LC-MS applications.

The mobile phase required for ion pairing is often very complex, with five or even more components being necessary [39,40,46]. The reproducibility, and the selectivity, of such a method may be poor. Retention greatly depends on the type and the concentration of the ion-pairing agent and the analytical column. Ion-pairing reagents are not at all recommended for LC-MS applications, because they often remain inside the ion source and give false positive signals, even a long time after their use. Some of them are also incompatible because of their lack of volatility.

In conventional chromatography, ion-pairing reagents usually decrease column life-time, so optimization must be done carefully and only low concentrations can be used. They also tend to cause instability in the chromatographic separation or gradually increase pressure with each subsequent injection of sample. There is also a danger of precipitation with other components of the mobile phase [47]. To conclude, the ion-pairing chromatographic approach is not ideal for the modern analytical laboratory.

2.3. Ion-exchange chromatography

The ion-exchange chromatographic approach was often used in the first chromatographic methods for AA determination [48,49]. As AA is a weak organic acid, it can be well retained on anion-exchange stationary-phase type SAX (strong anion exchanger). Recently, the ion-exchange approach was sometimes applied in a somewhat modified manner, using an amino-modified stationary phase, where amino group figured as WAX. Inorganic buffer or acid at low pH [50] was typically used as the mobile phase. This approach did not become very popular in analysis of AA and DHA.

2.4. Ion-exclusion chromatography

Few of the recent methods for the determination of AA and DHA are based on an ion-exclusion approach [51–54]. This approach was common in the 1990s [55]. The stationary phase in ion-exclusion chromatography is often based on sulphonated spherical PS/DVB resins in different ionic forms (e.g., Polypore H or Nucleogel Ion 300). The mobile phase is typically an inorganic acid (e.g., sulphuric, phosphoric or sulphonic) without any organic modifier. Retention on ion-exclusion columns is controlled by electrostatic repulsion forces, hydrophobic interactions and the size-exclusion effect. The ionic functional group (SO_3^-) of the ion exchanger repels ions of the same charge by electrostatic force, preventing them from entering the PS-PVD pore system. Strongly ionized solutes are excluded from the pore system. Neutral solutes not affected by electrostatic forces enter the pore system and partition due to hydrophobic interactions. Weakly ionized solutes (e.g., organic acids, including AA and DHA) elute somewhere in between, due to electrostatic forces and partial penetration into the pores where hydrophobic interactions occur. Size exclusion also affects retention as larger molecules are excluded from the pore system [56].

Ion-exclusion chromatography is a much more elegant approach compared to RP or ion-exchange chro-

matography. Stationary-phase stability at very low pH is assured by the polymeric base of stationary phase.

2.5. HILIC (Hydrophilic interaction liquid chromatography)

HILIC is an alternative to conventional RP-HPLC or NP-HPLC (normal-phase HPLC) and it is very convenient for the analysis of small polar molecules that are weakly retained or eluted with dead volume in conventional RP-HPLC systems. NP-HPLC was often replaced by HILIC because of bad reproducibility, low solubility of polar compounds in NP mobile phases and great difficulties when connection with MS detection was required.

Retention and separation of polar compounds on polar stationary phases with partly aqueous eluents is not a new separation mode in HPLC. The first applications were published more than 30 years ago. After these attempts, for a long time, HILIC was mostly confined to carbohydrate analysis. In the early 1990s, new polar stationary phases started to emerge, and the practice was given the name HILIC. Recently, this approach has gained great attention because of the increased need to analyze polar compounds in complicated mixtures. Another reason for its popularity is the widespread use of MS coupled to HPLC [57].

In HILIC, analyte retention is believed to be by partitioning of the analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, with the main components usually being 5–40% water in ACN. Conceptually, it is the most rational way to address very hydrophilic and uncharged compounds. The mobile phase comprises a high percentage of an organic solvent (typically acetonitrile), which is complemented by small percentage of the water/volatile buffer part. The water-enriched liquid layer is established within the stationary phase, thus partitioning solutes from the mobile phase into the hydrophilic layer. The primary mechanism of separation is partitioning based on hydrogen bonding, and the secondary mechanism, which could influence selectivity, is electrostatic interaction with charged stationary phases. Elution is enabled by increasing the polarity of mobile phase, thus the content of water component [57,58].

The applications now encompass most categories of polar compounds, charged as well as uncharged, although HILIC is particularly well suited for solutes lacking charge where coulombic interactions cannot be used to mediate retention. Under the HILIC conditions, stationary phase is of polar character, usually containing hydroxyl-ethyl, diole or amino groups, or it could be a special kind of ''zwitterionic'' stationary phase or some others. The advantage of HILIC conditions is the utilization of a high percentage of organic solvent, which enables the possibility of combining LC with MS detection together with high sensitivities [57,58].

The HILIC approach has so far been applied in two methods determining AA, unfortunately without taking into account DHA. AA was analyzed together with its more stable derivatives in food and beverages [59] and in pharmaceutical preparations using chlorogenic acid as IS [17].

3. Detection techniques

Simultaneous direct detection of AA and DHA is quite a complicated analytical problem. There are many detection techniques available in HPLC. However AA and DHA demonstrate very different properties in UV absorption, fluorescence and ED. For example, AA, by contrast with DHA, has a strong response in UV and also electrochemically. However, FD is only possible, when DHA is subjected to derivatization. Generally, there is a need to transform one form into the other in order to be able to determine the amount of both compounds using one detection technique or it is necessary to connect two detection techniques in parallel.

3.1. Ultraviolet detection

The most commonly used HPLC detection technique in AA assay is definitely UV spectrophotometry. UV detection [15,17,60–67] or DAD [33,41,68–70] has been used in most analytical methods for the determination of AA. AA has its absorption maximum in the range 244–265 nm, depending on the composition of mobile phase, especially in presence of buffers of various pH values. The wavelength applied most often has been 254 nm [10], then 245 nm or 265 nm (see Table 1). Lower UV-detection wavelengths are sometimes used when the method has been utilized for detecting more compounds (e.g., pyruvate and lactate [51,52] or AA and DHA together [61]).

DHA in solution absorbs UV light well at 185 nm, but it has a little absorbance above 220 nm [2]. In order to perform simultaneous HPLC analysis of AA and DHA with simple UV detection, it is necessary to derivatize DHA using 4,5-dimethyl-1,2-phenylenediamine [45], which is a fluorogenic agent highly specific for DHA, then record the chromatogram at 360 nm (which is an excitation wavelength often used in FD).

Most methods using UV detection determine AA without DHA, because of the poor UV-absorption properties of DHA. Some methods employ a parallel detection technique – FD – in order to be able to get sensitive, selective quantitation of DHA [32,39].

For the simultaneous determination of AA and DHA using UV detection, the reduction of DHA must be integrated into the method. First, the content of AA is determined in the original sample to get the initial concentration of AA. Subsequently, the reduction of DHA in the sample is performed, whereby any DHA

should be converted to AA. After the conversion, the sample is analyzed for total AA, so the same sample is injected in duplicate:
1) original sample for initial AA concentration; and,
2) converted sample for total AA concentration.

The content of DHA is calculated by subtracting the initial AA content from the total AA content after the conversion [8,28,40,42,44,54,61,65,66,71]. The method is conventionally called the subtraction approach. DTT (dithiothreitol) [8,28,42,54,61,65,66,71] is the most common reducing agent. TCEP (tris[2carboxylethyl]phosphine hydrochloride) [40,44] could be an alternative, which assures conversion of DHA to AA. L-cysteine has also been reported as a reducing agent prior to HPLC analysis [72].

From the point of view of increasing sensitivity and selectivity for DHA, the subtraction approach is better than UV detection at low wavelengths (below 220 nm). Generally, UV detection can lack sufficient selectivity in complex matrices (e.g., biological material, food or plant samples) for both AA and DHA. This is because UV absorbance as a physico-chemical property is additive and it is not specific enough, because many organic molecules with UV absorbance similar to that of AA or DHA could be present in matrices, where AA and DHA are being analyzed.

3.2. Fluorescence detection

As parent chemical structures, AA and DHA have no florescence properties. FD enables determination of AA after its oxidation to DHA and subsequent derivatization, which is quite a complicated, multi-step and time-consuming procedure. That is probably why FD is not so widely used in practical analysis of AA and DHA [32,39,73]. Derivatization of DHA either pre-column or post-column is often used to enhance the sensitivity of the detection and to eliminate interferences from complicated matrices.

Simultaneous determination of AA and DHA can be accomplished by post-column, on-line derivatization. O-phenyldiamine was employed as a post-column derivatization agent to form quinoxaline fluorescent derivative 3-(1,2-dihydroxyethyl)furol[3,4-b]quinoxaline-1-one [32,39]. Detection wavelengths were set at 355 nm for excitation and 425 nm for emission. A dual-detection system was often employed, where AA was detected by UV detection and DHA by FD [32,39].

If a dual-detection system is not available, oxidation of AA to DHA is necessary, and the subtraction principle described in Section 3.1. above must be applied. In order to get the amount of total AA, it has to be oxidized to DHA. Recently, there was a report of using an in situ peroxide radical generated by thermal decomposition of an azo-compound – 2,2'-azobis(2-amidinopropane) dihydrochloride [73]. Older methods utilized oxidation of AA by iodine solution.

3.3. Electrochemical detection

AA is relatively reactive and easy to detect in coulometric and amperometric systems. DHA must be determined using the subtraction approach (AA amount before reduction and after reduction, see Section 3.1. above), because it is electrochemically inactive.

HPLC methods for the determination of AA using ED provide the highest sensitivity, specificity, and also reduce substance interference. Specificity and elimination of substance interference are achieved by setting an optimal voltage for both electrodes, which is obtained by reviewing the hydrodynamic voltammetry (HDV) plot (graph of response or current versus potential or voltage). This varies for each electro-chemically active substance [74]. ED, either amperometric or coulometric, has often been employed in analysis of AA and DHA [29–31,40,46,74–76].

ED is reliable, selective and highly sensitive, much more so than the UV detection method, and allows no interferences from non-related substances within the sample. However, it also has some disadvantages. Usually, a very long column-equilibration time is needed (17–48 h!). Certain conditions, namely ionic strength and pH of the buffers running through the electrochemical detector, must be kept constant and pressure changes must be minimized. Passivation (acid cleaning) is necessary on initial use of chromatography system and thereafter once every 6–12 months [74].

3.3.1. Amperometric detection. Amperometric detectors are ''flow-by'' detectors, in which the solution containing AA and the other components of the mobile phase flow around the detector. Only 1% of the mobile phase and analyte are oxidized by the selected voltage. The advantages are that the electrodes can be repolished and reused, and also that alterations to the mobile-phase-separation conditions may be well tolerated with respect to background current. A disadvantage of amperometric detection is that the sensitivity is decreased by contaminants on the electrode surface, which has relatively small area [76].

3.3.2. Coulometric detection. Coulometric detectors are ''flow-through'' detectors. The mobile phase and AA flow through the detector, which is porous. Although coulometric detectors should oxidize 100% of the mobile phase, in practice they oxidize approximately 70% of the mobile phase and the analyte. An advantage of coulometric detection is that it is very sensitive, minimizing the background current from mobile phase. A disadvantage is that slight impurities in the mobile phase could increase the background current markedly, and in this way decrease sensitivity. Coulometric detectors often require ultrapure water to minimize background. With well-optimized conditions, coulometric detectors may be

Table 2. An overview of approaches to stabilization

Determined substances	Matrix	Access of light	Temperature	Concentration	pH	Extraction agent/ treatment	Stabilization agent	Ref., year
AA, iso-AA DHA, iso-DHA	Food multivitamin tablets plasma	Protected from daylight	Cold, not specified centrifugation at 4°C	NA	pH 2.0	1% MPA + 0.5% OXA 1.7% MPA, 0.4 mM acetate buffer pH 3.9	MPA 5%	[39] 1999
AA, pyruvate, lactate	Microdialysis of striatum	NA	Laboratory	NA	Acidic	Direct injection of dialysates on LC		[51] 1999
AA, glutathione	Ocular fluids	NA	Extraction on ice	NA	Acidic	0.1% MPA + 0.1 M EDTA + 1 mM thiourea	MPA 0.3%	[74] 1999
AA	Urine plasma	Amber glass storage in darkness	Cold, not specified centrifugation at 4°C	Studied	Acidic	10% PCA 0.015% MPA	EDTA in tubes	[60] 1999
AA, DHA	Solid AA	NA	Laboratory	NA	Acidic	No extraction	MPA 1%	[28] 1999
AA	Food	Amber glass (effect of daylight and UV studied)	Studied column temperature 25°C	Studied	pH 2.1 studied	20 mM MSG pH 2.1 adjusted by phosphoric acid	MSG	[29] 2000
AA	Food	Amber glass	Laboratory	NA	pH 2.1	20 mM GMP pH 2.1 adjusted by phosphoric acid	MSA, IMP, GMP, DSA, trisodium citrate	[30] 2000
AA, DHA iso-AA, UA	Biological samples	NA	Cold extraction samples kept on ice	NA	Acidic	10% MPA 2 mM EDTA	10% MPA 2 mM EDTA	[40] 2000
AA, DHA	Fruit vegetables	Protected from daylight	Laboratory	NA	NA	Deionized water	–	[61] 2000
AA, pyruvate, lactate	Dialysates	NA	Laboratory	NA	Acidic	Direct injection of dialysates on LC		[52] 2000
AA (9 water-soluble vitamins)	Multivitamin tablets	NA	Laboratory	NA	Neutral	phosphate buffer pH 7.0	–	[68] 2001
AA	Multivitamin tablets	Amber glass	Autosampler 22°C	NA	Acidic	1% ammonia in DMSO + 2% acetic acid 0.01 M HCl	Pyrogallolol 0.1 M citric acid	[41] 2001

substances						treatment		
AA, DHA	Parenteral nutrition mixtures	Amber glass	Studied 5, 15, 25, 35°C	NA	NA	–	–	[42] 2001
AA, β-carotene	13 beverages	Amber glass	Laboratory	NA	NA	Direct analysis	–	[50] 2002
AA	Food	Amber glass	Sample preparation at 5°C	NA	pH 2.1	0.2% phosphoric acid pH 2.1 + 20 mM L-methionine	L-methionine	[31] 2003
AA, GA, ART, MAP	Cosmetic products	NA	Studied 10°C and 25°C Sample preparation at 25°C	NA	Studied pH 2 and 6.9	Distilled water deoxygenation by N_2	–	[43] 2003
AA, DHA	Arthritic rats' serum	NA	Laboratory	NA	pH 2.0	ACN:60 mM phosphoric acid pH 2.0	Phosphoric acid	[32] 2003
AA, epicatechin gallic acid	Star fruit *(Averrhoa carambola L.)*	NA	Laboratory sample preparation at 90°C	NA	Neutral	50% aqueous acetone	–	[69] 2004
AA (other water-soluble and fat-soluble vitamins)	Pharmaceutical preparations food	NA	Laboratory	NA	Acidic	TFA:MeOH (50:50)	TFA	[70] 2004
L-AA, iso-AA L-DHA	Blood	NA	Laboratory centrifugation at 4°C	NA	Acidic	10% MPA	MPA	[44] 2005
AA	Human lymphocytes	NA	Cold extraction autosampler 4°C	NA	About 2.0	PBS + 10% MPA pH 1.8 + 2 mM EDTA	MPA + EDTA	[62] 2005
AA(B_1, PP, B_6, B_5, B_9, B_{12}, B_2, B_8)	Polyvitaminated premixes	NA	Laboratory	NA	Neutral	NaOH 1 M phosphate buffer pH 5.5	–	[63] 2005
AA, GSH, GSSG, UA	Urinary bladder	NA	Laboratory extraction on ice	NA	Acidic	50 mM OPA or 5% MPA + 1 mM EDTA	2 mM EDTA	[75]* 2005
AA, carotenoids	Tomato, kiwi mango	NA	Laboratory	NA	Acidic	MeOH, 3% MPA acetic acid 8%	MPA	[77]* 2005
AA, DHA	Human milk	Protected from daylight	Laboratory centrifugation at 10°C	NA	Acidic	0.56% MPA	MPA	[71] 2006
AA (taurine, other vitamins) IS = hippuric acid	Multivitamin tablets	NA	Laboratory	NA	pH 7	Ammonia solution formic acid to adjust pH	–	[14] 2006

Table 2 (*continued*)

Determined substances	Matrix	Access of light	Temperature	Concentration	pH	Extraction agent/ treatment	Stabilization agent	Ref., year
AA	Wines	NA	Laboratory	NA	pH 3.2	Direct injection	–	[38] 2006
AA, AA-2G, other derivatives	Enzymatic hydrolysates	NA	Laboratory	NA	Neutral	10 mM sodium phosphate buffer pH 7.4	2 mg/ml DTT	[64] 2006
AA, DHA	Banana, papaya, mango, pineapple	Protected from daylight	Low temperature – 4°C	NA	Acidic	3% MPA, 8% acetic acid or 0.1% OXA	MPA OXA +1 mM TBHQ	[54] 2006
AA, iso-AA	Fortified food products	Amber glass	Laboratory	NA	Acidic	TCEP 250 μg/ml TCA 1%	TCA	[3] 2006
AA, sugars, organic acids	Blackberry	NA	Laboratory	NA	Acidic	3% MPA	MPA	[33] 2006
AA, acetaminophen DHA	Pharmaceuticals	NA	Laboratory	NA	Acidic	Buffer pH 3.7 + EDTA	EDTA	[45] 2007
AA	Strawberries tomatoes, apples	NA	Laboratory centrifugation at 4°C	NA	Acidic	4.5% MPA	MPA	[8] 2007
AA, iso-AA AA-2G, AA-2βG	Food beverages	NA	Laboratory	NA	NA	ACN-66.7 mM ammonium acetate buffer (85:15)	DTT	[59] 2007
L-AA, L-DHA	Foods	Protected from daylight	Laboratory	NA	Acidic neutral	1% MPA or 20% TCA + 0.3M NaOH	MPA or TCA	[73] 2007
AA, organic acids	*Castanea sativa*	NA	Laboratory sample prep. 40°C	NA	pH 2	MeOH, acid water 0.01 M sulphuric acid	HCl	[53] 2007
AA, IS = nicotinic acid	Pharmaceuticals cosmetics	NA	Laboratory	NA	Acidic	0.2% MPA:MeOH:ACN (90:8:2)	MPA	[15] 2007
AA, IS = iso-AA, UA	Plasma	NA	Sample prep. on ice centrifugation at 4°C	NA	Acidic	10% TCA + 0.54 mM EDTA	TCA+EDTA	[46] 2007
AA, DHA vitamin E, fatty acids	Human milk	Protected from daylight	Laboratory centrifugation at 10°C	NA	Acidic	0.56% MPA	MPA	[65] 2008 [66] 2008

substances			treatment					
AA, vitamins E and A iron, selenium	Infant milk-based powdered formula	Protected from daylight	Laboratory centrifugation at 10°C	NA	Acidic	0.56% MPA	MPA	[67] 2008
AA, IS = chlorogenic acid	Tablets	Amber glass	Studied at 4, 10, 25°C autosampler 4°C	Studied	Studied pH 6.8	10 mM OXA:ACN (78:22)	10 mM OXA or 5% OPA	[17] 2008

AA-2G, 2-O-α-D-glucopyranosyl-L-ascorbic acid; AA-2βG, 2-O-β-D-glucopyranosyl-L-ascorbic acid; DSA, Disodium succinate; GMP, Disodium guanosine-5'-monophosphate; GLT, Glutathione; GSH, Glutathione reduced; GSSG, Glutathione oxidized; IMP, Disodium inosine-5'-monophosphate; MPA, m-phosphoric acid; MSA, Monosodium L-aspartate; MSG, Monosodium L-glutamate; NA, Not available; OPA, o-phosphoric acid; OXA, Oxalic acid; PCA, Perchloric acid; SMB, Metabisulfite; TBHQ, *tert*-butylhydroquinone; TCA, Trichloroacetic acid; TCEP, Tris[2-carboxyethiyl]-phosphine; UA, Uric acid.

10-fold more sensitive than amperometric detectors [76].

3.4. Mass-spectrometry detection

As ion-pair reagents or non-volatile inorganic buffers are typically used for the determination of AA and DHA, very few assays employing MS as a detection technique have been described, in spite of its advantages in selectivity and sensitivity. MS methods have usually involved multi-component vitamin mixtures [14], using electrospray ionization (ESI) MS detection in negative mode or a combination of ESI-MS with DAD detection in series for the analysis of AA and phenolic compounds [69] or vitamin mixtures [70]. In some cases, MS has been a tool for identification using full-scan spectra [70]. AA together with carotenoids have also been determined by ESI-MS [77].

Generally, only AA has been monitored in negative ESI mode as $[M\text{-}H]^- = 175$, whereas there have been descriptions of some difficulties with MS determination of DHA. The mass spectra obtained from DHA in solutions were somewhat complicated. DHA formed a hydrated hemiketal during its solubilization and, depending on pH, it was prone to hydrolysis [78].

As these methods are very complex, commonly including many analytes, their selectivity and sensitivity are not focused on how to get the best results for AA. It would be convenient to develop selective and sensitive analysis using MS detection for the simultaneous determination of AA and DHA with the focus on the sensitivity and the selectivity for these two analytes. Such an approach has so far been missing from the scientific literature.

4. Stability of ascorbic and dehydroascorbic acid

Stability is a key problem of AA and DHA analysis, because the compounds are known to be very unstable in aqueous solution. There are lots of factors that negatively influence their stability (i.e. light, increased temperatures, increased pH, and the presence of oxygen or metal ions). It is therefore necessary to keep the influences of these variables to a minimum [10].

Many studies have been performed to find optimal conditions for AA analysis. Data about stability of DHA are generally missing. Stability of compounds in solution at various conditions (temperature, pH, light and presence of divalent cations) and with the addition of stabilizing agents has been part of many method-development and validation studies. Table 2 provides an overview of individual factors and stability precautions.

4.1. The influence of light

AA and DHA are known to be susceptible to degradation by light. This was studied by Iwase et al. [29]. The effect

of the natural light and of UV light (265 nm) on the stability of AA in solution was examined in order to optimize the choice of glassware. Periodic changes of AA stored in a brown flask and in a plug-free, transparent flask, which was exposed to UV light, were compared at room temperature.

The results of the experiment demonstrated that degradation of AA was affected by both natural and UV light. After 1 h of the experiments, the initial concentration of AA decreased to 79.7% under the influence of UV light. Under the influence of natural light, the initial concentration decreased to 84.2% in the transparent flask and to 95.6% in the brown flask [29].

The results from the experiment show that AA is apparently more stable when it is stored in brown volumetric flasks, which appear to protect it from natural light. Some approaches also recommended protection using aluminum foil. Generally, most articles have highlighted the importance of protecting AA solution from natural light (see Table 2).

4.2. The influence of temperature

Temperature has been described as one of the key factors, which significantly influences the stability of AA and DHA in solution. The effect of the temperature on the stability of AA has been studied by many research groups [17,29,42,43]. Generally, temperatures of 4°C, 10°C and 25°C were used in experiments. Decreasing temperature (e.g., in modern auto-samplers) has enabled sample cooling during analysis up to 4°C to improve stability. Stability studies at higher temperatures confirmed a large degree of degradation of AA. The concentrations of AA at 60°C and 80°C were decreased within 1 h to less than 20% of initial concentration; at 40°C, the concentration decreased to 75% [29]. The solution of AA at laboratory temperature was stable for 1 h.

Most analytical methods do not sufficiently highlight the temperature aspect. The sample-preparation step and analysis have generally been performed at laboratory temperature (see Table 2). Sometimes, centrifugation was performed at low temperature of 4°C or the auto-sampler was set to this temperature.

Sample extraction on ice seems to be a very convenient sample-preparation step [46,75].

4.3. The influence of pH

AA exhibits higher stability in solution under acidic conditions. At these conditions, the formation of ascorbate, the main degradation product, is not favored (see Fig. 1). Most analytical methods included sample-preparation extraction at acidic conditions (see Table 2). Some studies involved a comparison of extraction agents of various pH and their impact to AA stability [17,29,43]. Generally, acidic pH around 2.1 was useful for sample preparation, ensuring sufficient stability and recovery of AA. M-phosphoric acid (MPA) was the most widely used extractant [8,33,44,65–67,71], sometimes in combination with EDTA [62,75] or with some organic additives (e.g., methanol [77] or methanol and acetonitrile [15]). In some cases, extraction was performed at neutral pH using various buffers [63,64]. Under HILIC conditions, it was very important to keep the organic modifier at high concentration, and extraction was performed using ammonium-acetate buffer in order to provide similarity to the HILIC mobile phase [17,59].

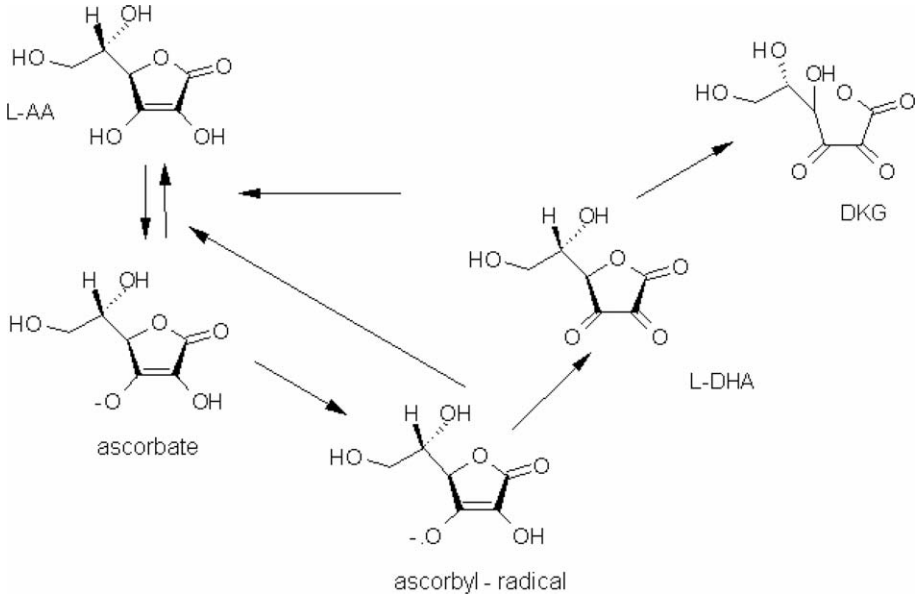

Figure 1. Structure of ascorbic acid and its degradation products.

Change in concentration of the organic modifier could significantly influence the partition equilibrium in HILIC and could lead to the shift of retention times and irregular peak shapes. Oxalic acid [54], its mixture with MPA [39] or trichloroacetic acid [46,73] has been among the extractants used for the sample treatment before analysis of AA and DHA.

4.4. The influence of concentration

Concentration of AA and DHA in solution could also influence stability. Rumelin et al. [60], Iwase et al. [29] and Novakova et al. [17] studied the stability of AA in solutions of different concentrations. It was shown that the higher the concentration, the better the stability. The stability was found to decrease significantly at concentration below 0.1 mg/l [17].

4.5. The influence of stabilization

Stabilizing agents have often been used to improve the stability of AA and DHA. Typically, MPA was able to fulfill the roles of extractant and stabilizer [8,33,44,65–67,71]. Its combination with EDTA was also found to be efficient [62,40]. Among other stabilizers, trichloroacetic acid [73], o-phosphoric acid [17,33], homocysteine [79], oxalic acid [17], EDTA [60], trifluoroacetic acid [70], dithiotreitol [64] or their combinations TCA + EDTA [46], metabisulfite/glutathione [80] or combination of citric acid/pyrogallol [41] also proved to prolong the stability of AA/DHA in solutions. Iwase et al. tested less common stabilizing agents {e.g., L-cysteinene [72], L-methionine [31], monosodium L-glutamate (MSG, amino acid) [31] and guanosine-5- monophosphate (nucleic acid) [30]}.

4.6. The influence of metal ions

The presence of metal ions has also been described as one of the factors that could decrease the stability of AA/DHA in solutions. For this reason, EDTA, as a chelating agent, can improve stability. Iwase et al. [29] examined the effect of Cu^{2+} and Fe^{3+} on the stability of AA in standard solution. As chelating agents, EDTA and MSG were found to be able to assure the stability of AA in the standard solution sufficiently.

Müller et al. [41] examined the influence of Cu^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} metal ions, where the amount of each element corresponded to the amount added to multivitamin preparations. Only Cu^{2+} was found to influence the content of AA significantly.

5. Conclusion

We reviewed HPLC methods for the determination of AA and DHA in order to provide an overview of current trends and problems in the analysis of these two compounds. Although the determination of such simple molecules could seem easy, it is a great analytical challenge. The main difficulties, which we discussed, are the choice of the analytical separation method, especially the mechanism of separation, and the selection of detection technique, which must be suitable for both compounds and the instability of analytes.

Concerning the mode of separation, the HILIC approach seems to be very convenient for the analysis of these two compounds. Ion-exclusion chromatography was also found to be suitable.

However, simultaneous detection of both compounds is still an unresolved problem. There is always a need for redox reaction in order to change one of the analytes into the other and subsequent subtraction, if simultaneous analysis of AA and DHA using one detection technique needs to be performed.

Whereas AA could easily be detected by UV or ED, DHA must be reduced to AA in a preliminary step and the analysis has to be performed twice – once to determine total content of AA (DHA and AA together) after the reduction of DHA, and second to determine the content of AA. DHA is then calculated by the subtraction approach.

By contrast FD enables determination of DHA, but a derivatization step is needed prior to analysis and again, in this case, AA had to be transformed to DHA in order to react with the fluorescent derivatization agent. The subtraction approach is needed to calculate the content of both AA and DHA.

There is not enough of information about MS-detection approaches for both AA and DHA in one analytical run.

New approaches for the simultaneous detection of AA and DHA are still needed and this problem is a great analytical challenge. The elegant solution could be, e.g., to use universal kinds of detector that are independent of physico-chemical properties [e.g., charged aerosol detection (CAD)].

Stability problems of AA and DHA in solutions have been well resolved by decreasing temperature (i.e., sample extraction on ice and analysis at 4°C), protecting samples from light, and adding various stabilizing agents, among which m-phosphoric acid, EDTA and oxalic acid have been the most widely used.

Acknowledgements

The authors gratefully acknowledge the financial support of GAČR 203/07/P730, MSM 0021620822 and Research Project MZO 00179906.

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