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Biomarkers of exposure to vitamins A, C, and E and their relation to lipid and protein oxidation markers

Abstract Since antioxidant vitamins may affect an organism's capacity for defence against reactive oxygen species, biological markers of the dietary exposure to these vitamins is of importance. There is also a need of effect biomarkers for determining the ability of these and other antioxidants to increase the overall antioxidant capacity and decrease the oxidative damage occurring in biological samples. This review is concerned with exposure markers and markers of lipid- or protein damage following intervention with vitamins A, C and E. While there are several high quality exposure markers available it is not possible to identify functional markers of lipid or protein oxidation, which respond reliably to human dietary intervention with vitamins A, C or E.

Key words retinol – ascorbic acid – tocopherol – vitamin supplementation – lipid oxidation – protein oxidation

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Vitamin A: biomarkers for vitamin A in body fluids

The vitamin A (all-*trans*-retinol and its esters) level was originally determined by bioefficiency assay, a technique that was later superseded by various chromatographic and fluorescent techniques. Due to worldwide concern for vitamin A deficiency (VAD), the development of fast and simple methods for the determination of vitamin A status has long been given a high priority. Direct fluorescence methods for assessing the retinol level in plasma or in dried blood are feasible because of the high intensity of retinol fluorescence when it is bound to its transporter, the retinol binding protein (RBP) [27, 45]. With the advent of high performance liquid chromatography (HPLC), these techniques took over and today retinol can be determined in serum routinely by direct- [15, 16] or reversed-phase [44, 76, 85] liquid chromatography. The reversed-phase techniques are faster and smaller sample volumes suffice but they are generally unable to discriminate between the various isomers of vitamin A to the same extent as the direct-phase methods can, although reversed-phase methods able to separate certain of the retinol isomers have been published [77]. The direct-phase methods can also typically measure a large number of other lipid-soluble vitamin isomers in the same run, such as pro-vitamin A carotenoids, xanthophylls, tocopherols and tocotrienols, menadione and phylloquinones. The structure of vitamin A and pro-vitamin A carotenoids is shown in Fig. 1. The observation that RBP occurs in plasma in a virtually 1:1 ratio to retinol has prompted the development of radial diffusion assays and enzyme immunoassays for RBP as a surrogate marker for plasma or serum retinol [51, 78, 110]. The possibility of using dried blood spots, which is advantageous from a collection standpoint, has also been demonstrated [27]. A comparison of the various tests in terms of price, speed of performance and coefficient of intraindividual variability is shown in

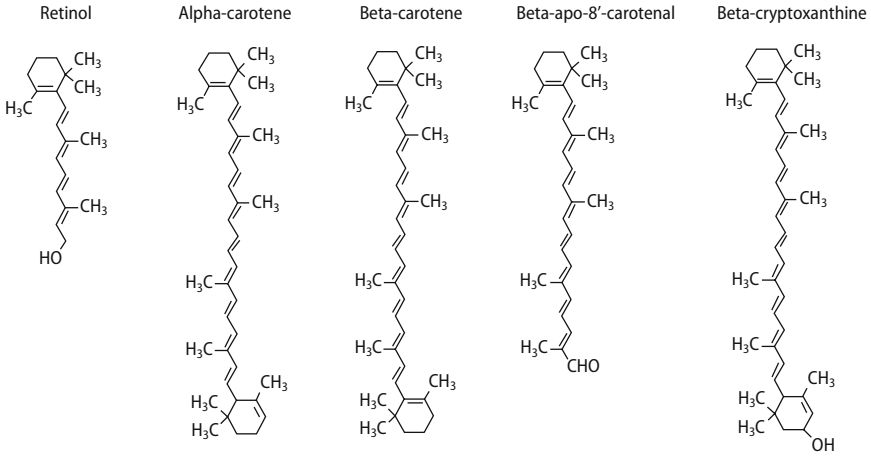

Fig. 1 Structures of retinol (vitamin A) and of the most important pro-vitamin A carotenoids

Table 1. Although the accuracy for each of the methods taken up in Table 1 is good (less than 5% deviation from the standards) and the interday variability is low, the correlation coefficient between the HPLC methods and ELISA is only around 0.8, probably due to differences in linearity. Since the latter methods are less demanding in terms of equipment they have considerable potential for screening purposes in the less developed countries where VAD is still causing blindness, growth retardation and decreased resistance to infections in large numbers of children.

Biomarkers of vitamin A related effects in the eye

VAD leads to dryness of the conjunctiva of the eye and moderate deficiency leads to decreased night vision due to interruption of light-sensitive chemical processes in the eye. Permanent blindness may ensue in severe cases. The WHO has compared the sensitivity of different methods for determining VAD (as modified in Table 2). Biological effects on the eye are still the only reliable means of detecting moderate to severe VAD, whereas the biochemical detection of retinol in blood samples is needed to identify mild cases and to be able to intervene at an early stage [120]. As already indicated, simple yet sensitive assays to determine the presence of this condition are thus still in demand. Histological markers that are employed include corneal cytology of the eye by direct visual inspection and by sampling a specimen of the conjunctiva for staining and microscopy. Functional markers include dark adaptation and the ability to see contrasts. The direct visual tests include staining with rose Bengal to visualize dry areas or to detect inflammation, but tests of this sort have been shown to be less reliable [38]. The standard today is the conjunctival impression cytology test which makes use of a vacuum pump to lift a small portion of the epithelium from the inferior temporal conjunctiva onto a filter paper disc, fix it in glacial acetic acid and stain it with periodic acid Schiff and haematoxylin for histological examination [73, 118].

Table 1 The performance of different methods for determining serum vitamin A

Test method	CV%	Cost/sample (relative)	Speed (samples/day)	Reference
Reversed-phase HPLC	4	20	25	[7]
Direct-phase HPLC	4	30[a]	20	[4]
Fluorescence	<10	2[a]	50–100[a]	[1]
ELISA (RBP)	9	1	150	[9]

[a]Estimates from the author's lab. This may change with new faster LC techniques

Table 2 Biological indicators of subclinical VAD

Indicator (cutoff)	Prevalence cutoffs for defining a public health problem and assessing its level of importance		
	Mild	Moderate	Severe
Functional tests			
Night blindness (age-specific)	>0% to <1%	≥1% to <5%	≥5%
Biochemical markers			
Serum retinol (≤0.70 μmol/l)	≥2% to <10%	≥10% to <20%	≥20%
Breast-milk retinol (≤1.05 μmol/l)	<10%	≥10% to <25%	≥25%
Histological markers			
Abnormal conjunctival impression cytology	<20%	≥20% to <40%	≥40%

Common biological indicators of subclinical VAD in children 6–71 month of age. A public health problem is considered to exist when the prevalence criteria of at least two of the above indicators of VA status are met (adapted from [27, 120]

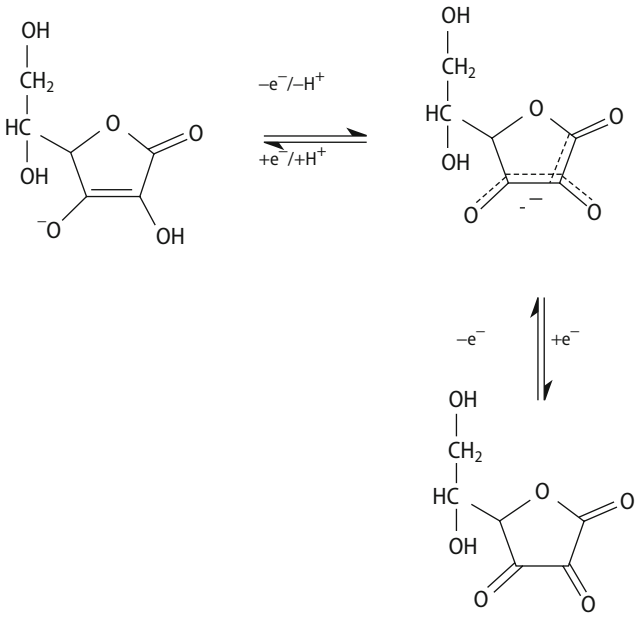

Fig. 2 Structures of ascorbate, the ascorbyl radical and dihydroascorbate, together constituting vitamin C

The dark adaptation tests measure the time needed to adapt to a defined level of limited illumination. A fast adaptation procedure involving the ability to discriminate between red and blue objects for field and for screening studies has been described [109]. When the eye shifts from cone-mediated day vision to rod-mediated night vision, the Purkinje shift occurs, the retinal peak light sensitivity shifting from red towards blue, blue objects being perceived then as lighter shades of gray than for red objects. Patients have to be taught use of the test, which must be repeated afterwards several times. In some studies the test results have been found to be more closely related to vitamin A intake by dietary assessment than to the plasma retinol level [38].

Biomarkers of oxidative stress after supplementation with vitamin A

There seem to be no studies reporting on markers of oxidative stress or oxidative status following intervention with use of increased doses of vitamin A. Neither retinol nor beta-carotene supplements to blood samples in vitro have been found able to affect a number of markers for antioxidant stability of the plasma and erythrocytes [70], which indicates that this vitamin may have a limited capacity to act as a scavenging antioxidant in vivo.

Vitamin C: biomarkers of vitamin C in body fluids

Vitamin C (ascorbate and dihydroascorbate, Fig. 2) levels have been determined in plasma, serum, dialysates and other body fluids by colorimetric and fluorimetric techniques, by enzymatically based assays and by HPLC with or without post-column derivatisation. Since ascorbic acid is easily oxidized to dehydroascorbate, which can subsequently be degraded to diketogulonic acid, initial treatment by stabilizing acids such as metaphosphoric and perchloric acids has to be performed quickly after isolation of a sample for analysis. The effects of the anticoagulants used during sample collection has been compared in one study, heparin being found to result in only a minimal loss, EDTA in contrast giving rise to a significant loss of vitamin C within a 30 min period. Also, oxalate and citrate were found to be less efficient in stabilizing ascorbate than other anticoagulants were [25]. Storage time of the sample and storage conditions are important factors determining the stability of vitamin C. In one early study, the concentrations of ascorbate and dehydroascorbate were found to be unaffected in samples stored in the laboratory at a temperature of 12°C for up to 6 h [81], but in other studies considerable time- and temperature-dependent losses were found already from the first hour of storage onwards even after optimization of the collection conditions [25]. In another study the storage time and temperature were found to have no effect on loss of vitamin C during 2–14 day storage at either −25°C or −75°C, but a 3.5% loss due to freezing was observed [14]. In a third study, pre-treatment with metaphosphoric acid was compared with treatment by dithiotreitol, a commonly used laboratory antioxidant. The latter performed slightly better than the former since no loss of vitamin C was evident after storage at −80°C for 6 years, whereas the standard procedure involving the addition of metaphosphoric acid led to a small but significant mean loss of <1% per year [80]. However, since treatment with dithiotreitol is known to reduce dehydroascorbate to ascorbate, this procedure cannot be recommended if both compounds are to be determined separately. The normal range of plasma concentrations of dehydroascorbate is controversial and the observation of this compound in plasma may be a result of metal-catalysed oxidation following acidification [65]. If dehydroascorbate is physiologically present, its true concentration seems to be very low, around 0.1% of the total plasma vitamin C in non-smokers when the sample has been handled carefully, and about 1.8% under the same conditions in the case of smokers [75], possibly reflecting higher leakage of haem in this

Table 3 Typical performance of different methods for determination of plasma vitamin C

Test method	CV% AA interday	CV% DHAA interday	Limit of detection	Speed (samples/day)	Reference
Reversed phase HPLC	<4%	<6%	0.1 mg/l	40–50	[60]
Capillary electrophoresis	1.3%	n.d.	0.5 mg/l	40–50	[20]
Automated colorimetry	<5%	n.d.	3 mg/l	500	[12, 25]

group. There was a significant increase in the concentration of dehydroascorbate over time at low total vitamin C concentrations [75].

The colorimetric and fluorometric methods are generally based on redox-reactions, with ascorbate and dihydroascorbate leading to the formation of a chromophore or a fluorophore, which can be photometrically measured by use of manual or automated equipment. Most of these methods are quite unspecific [94], but a few of them use assay blanks produced by adding ascorbate oxidase to the sample, creating greater sensitivity with retention of speed. Some of these methodologies are very fast, allowing high sample handling rates to be achieved through automation [12]. Results in determining plasma ascorbate in this way correlate well with those obtained by use of chromatographic methods [25]. Dehydroascorbate is not readily determined by use of this approach.

The HPLC methods for detecting plasma ascorbate electrochemically give results similar to those using UV detection [72]. Postcolumn derivatization can be used to reduce dihydroascorbate so that it can be determined by use of an electrochemical detector; the stereoisomer of ascorbate, erythorbate, can be determined simultaneously [12, 60, 74]. A method for the simultaneous detection of ascorbate and uric acid by means of capillary zone electrophoresis (CZE) has also been described [20]. Recovery is better than 98% with use of the HPLC and CZE methods and good linearity is obtained even at low ascorbate concentrations.

In interlaboratory comparisons, quite disparate results have been obtained with use of these techniques. In a European study of laboratories carrying out population surveillance, a 13%–20% interlaboratory variation was found using plasma samples in the 'normal' range of 36–94 μmol/l in a second round after corrections had been instituted at several laboratories [10]. In another study an interlaboratory difference of about 15% was observed, whereas the intralaboratory variation was about 2 αmol/l, irrespective of the concentration, which led to relatively larger relative errors being registered at low concentrations [80]. The performance of different methods for the measurement of vitamin C in plasma is summarized in Table 3.

Vitamin C and lipid oxidation markers

Many different biomarkers of radical mediated lipid oxidation exist but for the purpose of this review the more commonly used assays appear adequate for comparing studies in this area. The assays employed to this end here are the following: plasma thiobarbituric acid reactive compounds (TBARS), ex vivo LDL oxidation, antioxidant capacity markers, plasma lipid hydroperoxides, and plasma or urinary isoprostanes. Only randomized study designs are included in this review.

The most commonly used marker is determination of TBARS, with or without calibration, to detect malondialdehyde. The method is based on the liberation of aldehydes from amino groups by acid or alkaline hydrolysis followed by a color reaction involving use of thiobarbituric acid. The product can be determined spectrophotometrically, either directly or online, following HPLC separation. This marker is highly controversial since it is variable both within and between laboratories, since it may partially measure aldehydes deriving from endogenous metabolism, and since there is no generally accepted assay procedure [49]. These flaws have caused some journals to generally regard the method as being invalid as a lipid oxidation biomarker [113]. The interday coefficient of variation (CV) for TBARS with use of HPLC is in the order of 10%–20%, depending on the method employed for hydrolysis, but as already mentioned this relatively simple method has an odd tendency of sometimes giving spurious results and of varying from one analytical series to another, also within the same laboratory.

In a randomized 2-months intervention study of 59 healthy smoking males MDA was found to increase significantly following daily doses of 250 mg ascorbate combined with 200 IU vitamin E, 30 mg beta-carotene and 100 μg organic selenium, given in a normal formulation, but MDA to be unaffected by a slow-release formulation as compared with placebo treatment [93]. In another, randomized double-blind crossover study the effect of vitamin C supplementation (6 weeks, 250 mg/day) was determined in 20 subjects each showing normal (67 μmol/l) or below average (32 μmol/l) plasma vitamin C concentration

at baseline. No differences between groups in plasma malondialdehyde concentrations were observed either before or after supplementation [119]. In another counterbalanced design, 25 males were exposed to vitamin C (500 or 1,000 mg/d) and/or to exercise. No effect of either treatment on plasma MDA was observed [48]. In a study comparing eight smoking women with eight controls during a 14-day period in which 1 g ascorbate was administered daily, plasma TBARS was found to not be affected [90]. In a larger study involving 56 smokers, the intake of 500 mg/d of vitamin C was found not to affect MDA as determined by HPLC [2]. In a third study of this sort, giving a combination of vitamin C (272 mg/d) and vitamin E (800 IU/d) compared to placebo was found to not affect plasma MDA in 77 smokers treated for 90 d [55]. Oxidative stress in 10 volunteers as determined by increased plasma MDA induced by infusion of free fatty acids was also found to be unaffected by high-dosage vitamin C infusion [11]. Infusion of large doses of vitamin C (5 g) in combination with EDTA treatment resulted initially in a marked increase in plasma MDA but in an overall decrease in this parameter after 16 repeated sessions [50]. Oxidative stress induced by Zn deficiency was found to respond to 250 mg/d vitamin C, given for 3 months, as compared with placebo treatment, by a decrease in plasma MDA concentration [17]. Overall it appears that the intake of vitamin C does not consistently affect plasma MDA but that significant increases may be observed following the infusion of large, acute doses.

Another controversial marker used by many laboratories is the ex vivo LDL oxidation assay, in which isolated LDL is exposed to copper chloride or to a semistable radical such as AAPH, the oxidative formation of conjugated dienes being followed spectrophotometrically at 234 nm [36]. The lag-time to oxidation and/or the slope of the oxidation curve are used as end points. The method is disputed because the outcome depends on the antioxidants present in the LDL and these may be lost during LDL isolation. A faster method applicable directly to a plasma or serum sample overcomes this problem by using a peroxide-sensitive fluorescent probe with high affinity for LDL [52]. The interday CV for this latter assay is less than 10%.

In a group of 48 middle-aged male and female participants in a 36-month intervention study receiving 500 mg/d of either vitamin C, vitamin C plus 182 mg/d dl-α-tocopherol, 182 mg/d dl-α-tocopherol alone, or a placebo in a parallel design, no effect was observed at 12 or at 36 months in the vitamin C group in terms of susceptibility of isolated LDL or VLDL to oxidation ex vivo. In addition, no change in whole plasma ex vivo oxidation was observed in this group [96]. In a smaller parallel study of vitamin C supplementation (1 g/d) versus placebo, in which 19 smokers participated for 4 weeks following a 2-week ascorbate depletion period (≤30 mg/d), there was a significant increase in ex vivo LDL oxidation lagtime in the vitamin-supplemented group [41]. In a subsequent study of 30 young smokers, no effect was observed after 8 weeks supplementation by 1 g/d of vitamin C [42]. In a study with 30 coronary artery disease patients there was no evidence after 6 months that random assignment to vitamin C (1 g/d) together with vitamin E (800 IU/d), or placebo, decreased LDL oxidation or antibodies to oxidized LDL [64]. A borderline effect on LDL oxidation was observed in a similar study with only 18 participants after a shorter period of 12 weeks [86]. No effect on LDL oxidation lag-times of 500 mg/d vitamin C supplementation for 4 weeks as compared with placebo was observed in 30 type II diabetics [111]. In a study without a control group, an increase in ex vivo LDL oxidation lag-time during a 12 week-period was observed in 20 volunteers receiving 260 mg vitamin C in combination with 14 mg iron/d. In another group, receiving only 60 mg vitamin C plus iron per day, no such increase was observed despite changes in plasma ascorbate [121]. Supplementation by 1 g/d ascorbate for 4 weeks in 11 healthy volunteers failed to change the plasma LDL oxidation lag-time as compared with nine controls [117]. Overall there seems to be no consistent effect of vitamin C supplementation on ex vivo LDL oxidation kinetics.

The oxidizability of LDL is inherently an antioxidant capacity assay for this particular compartment. A variety of antioxidant capacity markers exist for other blood compartments, especially for whole plasma. They are all ex vivo oxidation systems composed of some oxidizing system and some relatively simple marker of plasma oxidation, usually one leading to a change in visual or UV absorption of the test matrix [13, 47, 98, 102]. There are important differences between the methods, which call for caution when they are interpreted [101]. When applied to plasma samples some authors accordingly report poor correlation between methods [115] whereas others observed a high degree of correlation between some of the most commonly applied methods, the ferric reducing ability of plasma (FRAP) assay and the trolox equivalent antioxidant capacity (TEAC) assay which also correlated with ex vivo LDL oxidation [34, 35]. These methods generally have interday CVs of less than 10% for repeated measurements of the same sample, the size of the CVs depending on the exact wavelength used for determining the absorbance, the availability of a photometer with exact filters or one equipped with a narrow grid being required.

The infusion of 1,000 mg ascorbate for a 1 h period in ten healthy volunteers was found to result in an

increased antioxidant capacity as measured repeatedly by two different methods during both the infusion period and the hour following this [116]. Marked increases in antioxidant capacity in the plasma of elderly women during the 4 h period after a dose of 1,250 mg ascorbate was given, were also observed using three different antioxidant capacity measures [18]. However, only a limited response of this sort was observed with use of the FRAP assay during the 24-h period following a single dose of 500 mg ascorbate with or without 400 IU vitamin E [22]. At the same time, in a cross-over intervention study involving 48 non-smoking men and women, in which 0 mg, 60 mg or 6 g of vitamin C was administered daily during 14 day-periods separated by 6 weeks wash-out periods, the plasma antioxidant capacity of these persons was found to be significantly affected [5]. In a 14 d parallel study of 16 women who smoked, half of them treated with 1 g ascorbate daily and half of them given placebo, no effect on the antioxidant capacity of the plasma could be shown [90]. Neither was the plasma TEAC found to be affected by a 150 mg/d vitamin C supplement in a 2-week study involving 18 volunteers [107]. No effect of antioxidants on TEAC could be observed either in 39 lupus erythromatosis patients randomized to being given 500 mg vitamin C together with 800 IU vitamin E a day or a placebo for a 12-week period [105]. In a group of 48 middle-aged male and female participants in a 36-month intervention study in which 500 mg/d of vitamin C, vitamin C plus 182 mg/d dl-α-tocopherol, 182 mg/d dl-α-tocopherol alone, or placebo were given daily in a parallel design, no effect in either of the vitamin C groups on the plasma antioxidant capacity as determined by the TRAP assay was observed at either 12 or 36 months [96]. At the same time, the antioxidant capacity in six moderately trained males was found to be significantly affected, following 2.5 h of strenuous exercise stress, by the intake of a drink containing vitamin C (0.15%, approx. 200 mg ascorbate) [30]). In contrast, FRAP was not found to be affected by 1 h of hard exercise following the daily administration of 600 mg vitamin C in combination with a range of other micronutrients for a 7-day period [31]. In these various studies, the effect of vitamin C on antioxidant capacity measures could not always be explained simply by an increase in the plasma ascorbate concentration. It appears that, although vitamin C may increase the antioxidant capacity in normal, healthy individuals, the effect is more evident short-term and may be partially be due to indirect actions of unknown character.

Although plasma lipid hydroperoxides can be determined individually by HPLC together with electrochemical detection [43, 106] or collectively by means of hydroperoxide-sensitive photometric assays [82, 92, 108], in most published papers the determination of 'lipid hydroperoxides' is a euphemism for TBARS. The effect of ascorbate supplementation on the plasma lipid hydroperoxide concentration in humans under normal conditions has not been frequently reported. The formation of lipid hydroperoxides in LDL was not found to be affected by a 150 mg/d vitamin C supplement in a 2-week randomized study involving 18 volunteers [107]. The effect of an ascorbate supplement in reducing the plasma hydroperoxide level following various conditions of oxidative stress showed a significant effect on this marker. A 30% reduction in exercise-induced total lipid hydroperoxides was observed after acute supplementation of vitamin C [6]. Also vitamin C supplementation in conjunction with biweekly apheresis for 6 months in dyslipidemic and uremic patients markedly increased the efficiency of such treatment in reducing the plasma phosphatidylcholine hydroperoxide level as determined by HPLC [21]. Thus, vitamin C seems to affect lipid hydroperoxide formation in some oxidative stress conditions.

The least disputed lipid oxidation methods are the isoprostane assays. The determination of plasma isoprostanes can be performed by gas chromatography/mass spectrometry (GC–MS) using electron capture negative ionization (ECNI) or negative ion chemical ionization detection (NCI). The compounds need to be extracted from the sample and derivatized. The extraction has not always been straightforward and has involved multiple chromatographic steps, including thin layer chromatography, and has led to a poor overall recovery [8]. Improvements have included a combination of solid-phase extraction cartridges and HPLC [88, 114], two sequential solid-phase extractions [24] and most recently a single anion exchange cartridge [68]. The derivatization of the compounds is most often done by use of pentafluorobenzyl bromide followed by a silylation step employing bis-(trimethylsilyl) trifluoroacetamide [68]. The overall extraction efficiency is now around 70%, the interday analytical CV% varying from less than 1% to 5%–8 %, depending on extraction procedures. Since the interindividual variation is much larger, this method has considerable power for detecting the factors causing biological variation. An immunological method for the determination of 8-isoprostane $F_2\alpha$ having 5%–15% analytical variation also exists [9].

The formation of isoprostanes in plasma was found to not be affected by 150 mg/d vitamin C supplementation in a 2-week study involving 18 volunteers [107]. Using the chromatographic method on plasma samples, no change was observed after a daily dose of 272 mg vitamin C in combination with vitamin E (31 mg/d) and folate (400 μg/d) for a 90 day period in

Table 4 Performance of plasma lipid oxidation markers

Test method	Analytical CV%	Normal variation CV%	Speed[a] (samples/day)	Methods reference
TBARS (HPLC)	15–20	25	50	[67]
LDL ex vivo oxidation	5–10	20	10/50	[36, 52]
Antioxidant capacity	<10	20–25	>100	[47]
Lipid peroxides	<10	60–65	20	[92]
8-Isoprostane $F_{2\alpha}$ EIA	5–15	20–50	50–100	[9]
Isoprostanes GC–MS	<1–6	50	50	[24, 68]

[a]Estimates in the author's laboratory with use of standard semi automated equipment

Table 5 Summary of effects of vitamin C on plasma lipid oxidation markers

Treatment	MDA	LDL oxidation ex vivo	Antioxidant capacity	Lipid hydroperoxides	Isoprostanes
Vitamin C	–	–	+/–	–	–
Stress + vitamin C	–	–	+/–	+	nr[a]

[a]*nr* not reported

elderly male smokers and non-smokers [55]. A dose-response study of hospitalized women in which vitamin C (30–2,500 mg/d) was administered to them for 186 days following a short ascorbate-depletion period, gave no evidence of change in the levels of isoprostanes in the urine or plasma [71]. In a study of 30 coronary artery disease patients limited evidence was obtained after 6 months that random assignment to vitamin C (1 g/d) together with vitamin E (800 IU/d) versus placebo led to a decrease in plasma isoprostanes [64]. No studies of the effect of ascorbate supplementation on oxidative stress-induced isoprostanes have been reported. The performance of plasma lipid oxidation markers and the effects on them of vitamin C being administered is summarized in Tables 4 and 5. Overall, the effect of vitamin C on lipid oxidation markers seems limited to a possible effect in certain oxidative stress conditions.

Vitamin C and protein oxidation

Proteins have no natural carbonyl groups, so such groups are introduced into proteins by oxidative mechanisms, including the oxidative deamination of the α-amino group in lysine and the oxidation of carbons next to the secondary amine functions in proline and arginine. Other assays for oxidatively modified amino acid residues in proteins include the measurement of preformed hydroxytyrosine, dityrosine, and sulphoxides (e.g., methionine sulphoxide), and the loss of protein sulfhydryls. Oxidized proteins can denature, the denatured proteins being quickly chaperoned towards proteolytic degradation in vivo, but the oxidation may also be insufficient to denature the protein so that protein carbonyls are allowed to accumulate to some extent. The steady-state concentration of protein carbonyls and other oxidative modifications in the plasma or in other biological specimens may thus reflect the accumulated oxidative stress in the compartment in question during the lifetime of the protein, thereby providing a potentially valuable biomarker for low-level oxidative stress. Several approaches to developing biomarkers for protein oxidation have been taken, but only few of them have been applied to studying the effects of micronutrient supplementation.

The simplest assay is based on the reaction of carbonyl groups with primary amines to form semi-stable Schiff-bases. The reaction with 2,4-dinitrophenylhydrazine (DNPH) is commonly used for the photometric determination of carbonyls. In its simplest form, it involves the sample reacting with DNPH, precipitation and a washing procedure to remove the unspecifically bound DNPH. After washing, the protein is solubilized to determine the level of specific binding photometrically. This method has the advantage of being fast and of low technical demands. The disadvantages include difficulties in removing the unspecifically bound DNPH, leading to high and variable background readings, a variable loss of protein during washing, and a risk of introducing additional oxidative modification during the procedure, leading to binding of the excess DNPH. The first two disadvantages can be partly overcome by isolating a specific protein fraction prior to precipitation, which leads to a much greater loss of the unspecifically bound DNPH. It also introduces the additional advantage of selecting the specific protein for study, since oxidation can vary between proteins due to differences in the redox micro-environment surrounding the proteins. Another solution is to use an

immunoassay procedure involving specific antibodies to the DNPH-modified protein, thus avoiding the unspecifically bound DNPH and extensive washing.

To assess oxidative stress by use of the simplest version of this assay, the influence of taking 500 mg/d versus 1 g/d of vitamin C for 2 weeks prior to 30 min of hard exercise was evaluated in 12 males using a counterbalance design. Vitamin C was found to decrease the exercise-induced increase in protein carbonyls in a dose-dependent manner [48]. Using the same method for protein carbonyls, the effect of taking 272 mg/d of vitamin C supplement in conjunction with vitamin E (800 IU/d) and folate (400 μg/d) for 90 d was assessed in 39 smokers and 38 non-smokers who habitually had a low intake of fruit and vegetables. No effect of the supplement on protein carbonyls was observed [55]. In a very small study, involving seven divers, no effect on the levels of plasma protein carbonyls as a results of diving apnea or of a 1 g/d supplement of vitamin C for a week preceeding a diving experiment was observed [104].

A more advanced approach with an isolated protein fraction was taken in a study of the effect of a 400 mg/d vitamin C supplement given to healthy volunteers for a 15-week period. The level of protein carbonyls in the immunoglobulins was found to decrease after both 10 and 15 weeks of supplementation, whereas no change in the total plasma sulfhydryls was detected [19]. The decrease was confined to individuals with a sub optimal intake of vitamin C.

A third method employed for assessment of protein oxidation takes advantage of the reaction of protein aldehydes with fluorescein and reduction of the product by cyanoborohydride to form a stable adducts. Following protein isolation by a rapid size-exclusion chromatographic step and complete acid hydrolysis, the fluoresceinamine adduct could be detected by HPLC through the use of UV, fluorescence or APCI-MS detection [29]. This procedure has the advantage of being specific for the products of lysine (2-aminoadipic semialdehyde (AAS)) or for proline and arginine (γ-glutamyl semialdehyde) oxidation and of avoiding any further oxidation during workup of the sample. The main disadvantage is the longer time required for analysis and the higher technical demands. The method has been used to study the effect of supplementation by 500 mg/d of vitamin C versus placebo for 30 days in 16 healthy volunteers. A marginal increase in plasma protein AAS was observed (Dragsted, unpublished data). Another line of evidence comes from studies of dietary fruit and vegetable depletion, showing a rapid depletion of plasma ascorbate and a concomitant decrease in AAS, indicating that the depletion of ascorbate might have an antioxidant effect. Use of this approach was found in one study to produce a significant time-dependent decrease in AAS during a 24-day period of fruit and vegetable depletion in the diet, whereas no change was observed after supplementation of the known nutrients from the fruits and vegetables, including 150 mg/d vitamin C. No effect of fruit, vegetables or nutrient supplements was found in that study on the levels of total plasma carbonyls or immunoglobulin carbonyls by the antibody approach in conjunction with Western blotting [35]. None of the volunteers had sub optimal vitamin C levels before intervention. Since vitamin C is a cofactor for the lysine oxidases that cross-bind cartilage in the body, the observed effect of vitamin C on this specific marker may reflect enzyme leakage from the cartilage rather than prooxidative stress.

Overall there appears to be certain but limited evidence for sub optimal vitamin C intake leading to an increase in protein oxidation in the plasma immunoglobulins. Evidence for the prevention of stress-induced carbonyl formation by increasing the intake of vitamin C is controversial and needs further confirmation.

Vitamin E: biomarkers for vitamin E in body fluids

Vitamin E is a collective term for alpha-, beta-, gamma- and delta-tocopherols as well as the tocotrienols, of which *RRR*-α-tocopherol has the highest vitamin E activity. The reference for the international unit (IU) is 1 mg of all-*rac*-tocopheryl acetate, 1 IU corresponding to 1.49 mg *RRR*-α-tocopherol. Since only the four R-forms of α-tocopherol (*d*-α-tocopherol) are recognized by the human vitamin E transporter in the body, none of the four S-forms of α-tocopherol and none of α-, α- and α-tocopherols or of the tocotrienols are thought to have any vitamin E activity in humans as opposed to the rat. This is still controversial, however, since in a study comparing the delivery of *RRR*-α-tocopherol and *all-rac*-α-tocopherol to lipoproteins in humans following 8 weeks of supplementation by 1,600 mg/d of either product, no difference in the total vitamin E content of LDL was observed [97]. The relative vitamin E activity of the various tocopherols and tocotrienols in the rat together with their structures are shown in Fig. 3. The vitamin E status in rats and possibly in other rodents can be determined as a function of all the active isomers, but in humans vitamin E status is preferably determined as the plasma, serum or erythrocyte concentration of *d*-α-tocopherol. Various functional tests have also been suggested, including the susceptibility of erythrocytes to lysis following an in vitro challenge with hydrogen peroxide [28, 37, 122], or the exhalation of breath pentane [69]. In one

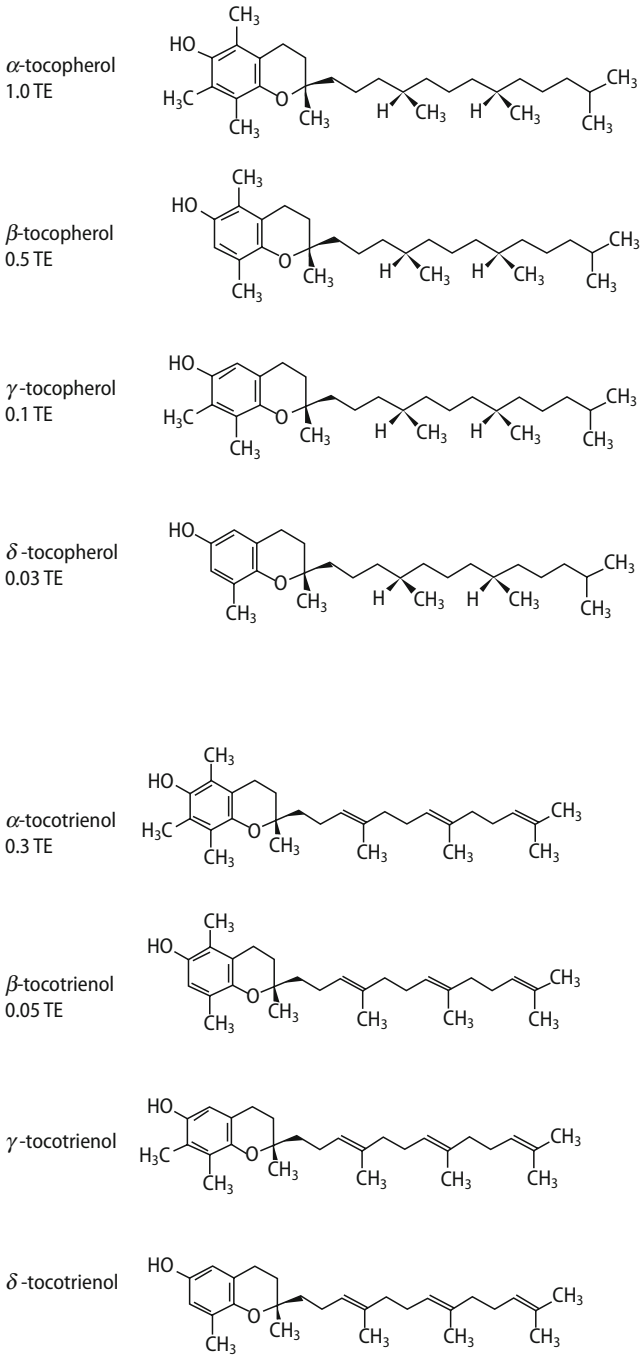

Fig. 3 Structures of tocopherols and tocotrienols and their relative potency as vitamin E in the rat (TE; *dl*-α-tocopherol equivalents). Redrawn from [53]

study no correlation was found between results of the erythrocyte membrane stability test and plasma or erythrocyte vitamin E concentrations [122]. Also, since these functional tests may be lipid oxidation markers, it seems more appropriate to evaluate the relationship of vitamin E supplementation to currently used lipid oxidation markers. Vitamin E has a physiological transporter in the human that only binds *d*-α-tocopherol, and deletion of this transporter leads to neurological symptoms [83].

Vitamin E can be routinely extracted from biological samples with hexane or heptane and determined by GC–MS or by direct or reversed phase HPLC using coulometric, fluorescence or mass detection [23, 46, 66, 89, 112]. The use of more simple methods often fails to discriminate between the active and inactive tocopherols [23, 59]. In a comparison between LC-MS and GC–MS methods, the former were found to perform slightly better in terms of interday analytical precision and variability [89].

Vitamin E and markers of lipid oxidation

This review concerns only randomized, controlled studies in which the effects of supplementation by vitamin E was investigated on peripheral blood samples using plasma or serum thiobarbituric acid reactive compounds (TBARS), ex vivo LDL oxidation, antioxidant capacity markers, lipid hydroperoxides, or isoprostanes (see desciption in the section on vitamin C above). In a study of 24 diabetic patients assigned to take a capsule each day containing 100 IU vitamin E or a placebo for a period of 3 months, a significant decrease in plasma MDA was found in the vitamin-supplemented group as determined by HPLC [56]. In a subsequent study of 24 diabetic children by use of the same design, there was a significant decrease in erythrocyte MDA in the vitamin-supplemented group [57]. In 49 diabetic patients given either 504 mg/d *d*-α-tocopherol or a placebo for a 6-month period a decrease in ex vivo induced TBARS in the erythrocyte membranes was found for the supplemented group [61]. In a study of 49 HIV patients randomized to supplementation with a placebo or with 800 IU/d vitamin E and 1,000 mg/d vitamin C for a 3-month period, a reduction in plasma MDA occurred in the group assigned vitamin supplements [3]. In a double-blind, controlled intervention study, 56 patients with congestive heart failure were given 335 mg/d of the natural *d*-α-tocopherol for a 12-week period and no effect on the plasma MDA level was detected [62]. In a randomized 2-months intervention study of 59 healthy smoking males, MDA was found to increase significantly following daily doses of 200 mg vitamin E combined with 250 mg ascorbate, 30 mg beta-carotene and 100 μg organic selenium, given in a normal formulation, but to be unaffected by a slow-release formulation as compared with placebo treatment [93]. In a study of the effects on 77 smokers of taking vitamins C (272 mg/d) and E (800 IU/d) or a placebo for 90 d, no effect on the plasma MDA was detected [55].

In a trial involving 80 men showing an increase in plasma lipid oxidation from exposure to either olive oil or menhaden oil, giving a 900 IU dose of vitamin E was found to be no better than a placebo in decreasing MDA [4]. In a trial of lipid oxidation induced in 18 untrained men by three sessions of resistance exercises, those assigned to treatment with vitamin E (1,200 IU/d starting 7 days before the exercises began) were found to not differ from the placebo group in the levels of plasma MDA [7]. In another study, of a group of 14 runners assigned to either a placebo or a 1,200 IU/d dose of vitamin E starting 4 weeks before and continuing during 6 days of increased running training, a decrease in serum MDA was found in the group given vitamin E [54]. In a cross-over intervention study involving 4-week periods of either taking 500 or 1,000 IU of vitamin E together with 500 or 1,000 mg of vitamin C or taking placebo, no effect on exercise-induced oxidative stress could be shown by serum MDA [32]. In another study, 16 young and 16 older male subjects were first given eccentric exercises for 45 min and were then placed on 1,000 IU/d of vitamin E supplementation for 12 weeks before being given a second round of exercises. No change in plasma MDA either within or between the two groups could be shown [100].

In a three parallel groups of 16 middle-aged male and female participants, who were given either 500 mg/d of vitamin C together with 182 mg/d *dl*-α-tocopherol, 182 mg/d *dl*-α-tocopherol alone, or placebo, no effect on plasma antioxidant capacity was observed at 12 or at 36 months [96].

In a double-blind controlled intervention study of 56 patients with congestive heart failure, treatment with 335 mg/d of natural *d*-α-tocopherol for 12 weeks was found to have no effect on the level of breath pentane exhalation [62]. In a trial involving 80 men who had an increase in plasma lipid oxidation following exposure to olive oil or to menhaden oil, a dosage of 900 IU vitamin E was found to equal placebo in reducing the level of lipid hydroperoxides in the plasma or the exhalation of breath pentane [4]. In a study of 49 HIV patients randomized for supplementation with a placebo or with 800 IU/d of vitamin E and 1,000 mg/d of vitamin C over a 3-month period, there was found to be a reduction in plasma lipid peroxides and in breath pentane exhalation in the group to which a vitamin supplement was assigned [3]. In a study without a parallel control group, a 1,000 IU/d vitamin E supplement for a 10 d period was found to reduce the exhalation of breath pentane [69].

In a dose-response study of 40 healthy men assigned doses of 60–1,200 IU of vitamin E for an 8-week period, vitamin E given at doses higher than 200 IU/d was found to affect the kinetics of ex vivo oxidation of LDL [58]. In a group of 45 randomized healthy males and females, the effects of giving mixed supplements of 200 mg/d vitamin E, 900 mg/d vitamin C and 18 mg/d beta-carotene for 6 months were compared with those of a placebo. Non-induced lipoprotein oxidation ex vivo was found to be delayed in the group given the vitamin supplement, and the strength of this effect was correlated with the level of plasma α-tocopherol [1]. In a group of 48 middle-aged male and female participants in a 36-month intervention study in which either 500 mg/d of vitamin C, this together with 182 mg/d *dl*-α-tocopherol, 182 mg/d *dl*-α-tocopherol alone, or a placebo in a parallel design, a significant increase in the susceptibility of isolated LDL or VLDL to oxidation ex vivo was observed at 12 and at 36 months in the group given only vitamin E and in group given the combined dosage. A significant change in these groups in whole plasma ex vivo oxidation at 36 weeks was likewise observed [96]. In a study comparing the delivery of *RRR*-α-tocopherol and *all-rac*-α-tocopherol to lipoproteins in humans following 8 weeks of supplementation with 1,600 mg/d of either product, no difference was observed in LDL-MDA, LDL-dienes, or LDL-hydroperoxides induced ex vivo [97].

In 49 diabetic patients given 504 mg/d *d*-α-tocopherol or a placebo for 6 months, no change in the group receiving the supplement was detected in the antioxidant capacity of the erythrocytes as determined on the basis of the glutathione concentration and the glutathione peroxidase activity [61]. In a double-blind controlled intervention study of 56 patients with congestive heart failure, treatment with 335 mg/d of the natural *d*-α-tocopherol for 12 weeks was found to have no effect on the level of activity of plasma glutathione peroxidase [62].

In a blinded intervention study of 33 triathletes participating in a world championship, who were given 800 IU/d vitamin E or a placebo for 2 months prior to the race, the treatment with vitamin E was found to significantly increase the level of plasma F2-isoprostanes as well as of several markers of inflammation, including IL-6 [91]. In another study, 16 young and 16 older male subjects were given 45 min of eccentric exercise and were then given a 1,000 IU/d supplement of vitamin E for a 12-week period before being given a second round of exercises. Following vitamin E supplementation a significant lowering of the plasma isoprostane level both before and 24 h after the exercise was found for the older men. This was not observed for the young men. No other significant differences were observed between the two groups [100]. The study design could not control for period effects, which may have caused the difference. In a third study of the effects of vitamin E on oxidative damage induced by exercise, 21 ultra-

marathon runners were given either a combination of 300 mg/d *d*-α-tocopherol and 1,000 mg/d vitamin C, or a placebo during the 6 weeks prior to the race. Running as such was found to increase the plasma isoprostane level, particularly in the males, and the vitamin supplement was found to prevent this [84]. The inflammatory markers were also affected by running, but this was not changed by the vitamin supplements. In a study of the combined effects of giving either vitamin C (500 mg/d) together with *d*-α-tocopherol (400 mg/d), vitamin C (500 mg/d) together with *d*-α-tocopherol (290 mg/d) and *d*-α-tocopherol (130 mg/d), or a placebo during a 28 d period on the plasma isoprostane concentrations induced by exercise, no effect of either vitamin treatment was observed, although the treatment by α-tocopherol was found to affect the exercise-induced increase in plasma and muscle heat shock protein (HSP72) and also the expression of HSP72 in muscle [40]. In an intervention study of 46 healthy smokers given 0, 300, 600 or 1,200 IU/d of vitamin E for 3 weeks, no effect on the excretion of F2-isoprostane could be observed for any of these interventions [95]. In a double blind controlled intervention study of 56 congestive heart failure patients, treatment with 335 mg/d of natural *d*-α-tocopherol for a period of 12 weeks was found to have no effect on the plasma F2-isoprostane level [62]. In another double-blind placebo-controlled trial with a crossover design, no effect on the plasma F2-isoprostane level of taking 1,000 IU/d of vitamin E was found for 20 patients with endothelial dysfunction [103]. In a group of 33 sclerosis patients, 10 of them were given 500 mg/d and 10 of them 1,000 mg/d vitamin E for a period of 3 weeks, the rest being given a placebo. No effect on urinary excretion of F2-isoprostanes was observed [26]. In 43 hypercholesterolemic patients randomized to taking simvastatin, simvastatin together with 600 mg/d vitamin E or a placebo for 2 months, the adding of vitamin E was found to have no further effect on the urinary excretion of F2-isoprostanes [33]. In a small non-blind study of cirrhotic patients, the nine patients receiving standard medication together with 600 mg/d vitamin E for a 30-day period showed lower urinary excretion of F2-isoprostanes than five controls given only standard medication [39].

In a dose-response study, groups of five healthy individuals received either 0, 200, 400, 800, 1,200 or 2,000 IU/d of vitamin E for a period of 8 weeks, followed by 8 weeks of washout. No significant change in the plasma F2-isoprostane level could be detected by GC–MS at any time point, irrespective of the treatment [87]. In a study without a control group, isoprostane excretion was found to be decreased at the end of a 2-month period in a group of 15 healthy individuals receiving 400 IU/d of vitamin E [79].

Effects of vitamin E on protein oxidation

In a study without a control group, the plasma carbonyl content in 15 healthy individuals was found to be unaffected by 400 IU/d of vitamin E for a period of 2 months [79].

Other markers related to vitamin E effect

Intervention trials involving ingestion of synthetic vitamin E either alone or in combination with vitamin C have been performed to assess their effects on various conditions assumed to be caused by oxidative stress. Sperm counts and sperm motility are thought to be partially influenced by oxidative stress. Vitamin E deficiency is also known to cause semen abnormalities and infertility in rats. In two randomized studies, each with 30 men, little effect of taking 600–800 mg/d of vitamin E for 2–3 months was shown. In one study, a significant increase in the binding ratio to the zona pellucida of the unfertilized oocyte in a competitive binding assay was observed, but this parameter was not assessed in the other study [63, 99]. No evidence was presented that this effect was related to antioxidation.

Summary and conclusions on markers of vitamin A, C and E

Serum retinol is still the most reliable indicator of vitamin A deficiency. HPLC methods are the most sensitive, but they are not useful for large screenings or for field studies in poor areas of the world where deficiency is a common problem. Although simpler tests using fluorescence, as well as immunological techniques possessing good accuracy and high precision exist, there is still a need of methodology, which is simpler, faster and cheaper yet, and requiring no complicated sample treatment or use of complex equipment. Present day HPLC and GC–MS methodology has also high precision and accuracy in the detection of vitamin E analogues in plasma. Since plasma vitamin E levels are not always correlated with the stability of the erythrocyte membrane or with the exhalation of breath pentane, these proposed functional tests for vitamin E must be regarded as obsolete. Total plasma vitamin C can readily be determined by automated colorimetric assays or HPLC. HPLC has the advantage of having lower detection limits, permitting simultaneous determination of ascorbate and dehydroascorbate, and possibly possessing higher accuracy, whereas the colorimetric assays have higher throughput. The handling and

storage of plasma for vitamin C determination has a strong effect on accuracy.

Vitamin A appears to have only limited capacity as a direct antioxidant in vitro and its effects on biomarkers of oxidative damage or antioxidant effects in vivo are not well known. Likewise, there is only limited evidence for a protective effect of vitamin E supplements on markers of lipid oxidation even after doses of 200–2,000 IU/d, and supposed effects of high levels of vitamin E supplementation either on exercise-induced lipid oxidation as determined by isoprostanes or on inflammatory markers are still controversial. Vitamin E intervention has not been found to decrease the oxidation of plasma proteins.

In human intervention studies to assess the effects of vitamin C supplementation most of the currently available markers for assessing lipid or protein oxidation in humans have been tested. There is only limited evidence that plasma ascorbate is a functional antioxidant in the body as assessed by means of these markers and very limited evidence that high dosages of vitamin C may decrease oxidative stress. No evidence for the antioxidant effect of vitamin C in the dose range of 60–2,500 mg/d in connection with mild ascorbate depletion was obtained using the most widely accepted lipid oxidation marker available, that of the formation of plasma isoprostanes. Clear short-term pro-oxidant effects on lipid oxidation were observed following high-dose vitamin C infusion into the bloodstream. There are no studies currently available on the relationship between lipid or protein oxidation markers, vitamin C and chronic disease.

Although it is well known that vitamin C and E have antioxidant actions in challenge tests with plasma or serum ex vivo and also that humans spend energy on the regeneration of reduced ascorbate in plasma, the currently available markers of oxidative damage seem to be unable to pick up antioxidant effects of supplementation with ascorbate and/or tocopherols after dietary interventions. While there is an ample supply of exposure markers for these vitamins it is still not possible to recommend any functional marker test in humans for their actions as antioxidants.

Conflict of interest None.

References

1. Abbey M, Nestel PJ, Baghurst PA (1993) Antioxidant vitamins and low-density-lipoprotein oxidation. Am J Clin Nutr 58:525–532
2. Aghdassi E, Royall D, Allard JP (1999) Oxidative stress in smokers supplemented with vitamin C. Int J Vitam Nutr Res 69:45–51
3. Allard JP, Aghdassi E, Chau J, Tam C, Kovacs CM, Salit IE, Walmsley SL (1998) Effects of vitamin E and C supplementation on oxidative stress and viral load in HIV-infected subjects. AIDS 12:1653–1659
4. Allard JP, Kurian R, Aghdassi E, Muggli R, Royall D (1997) Lipid peroxidation during n-3 fatty acid and vitamin E supplementation in humans. Lipids 32:535–541
5. Anderson D, Phillips BJ, Yu TW, Edwards AJ, Ayesh R, Butterworth KR (1997) The effects of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with "low" or "high" cholesterol levels. Environ Mol Mutagen 30:161–174
6. Ashton T, Young IS, Peters JR, Jones E, Jackson SK, Davies B, Rowlands CC (1999) Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. J Appl Physiol 87:2032–2036
7. Avery NG, Kaiser JL, Sharman MJ, Scheett TP, Barnes DM, Gomez AL, Kraemer WJ, Volek JS (2003) Effects of vitamin E supplementation on recovery from repeated bouts of resistance exercise. J Strength Cond Res 17:801–809
8. Awad JA, Morrow JD, Takahashi K, Roberts LJ (1993) Identification of non-cyclooxygenase-derived prostanoid (F2-isoprostane) metabolites in human urine and plasma. J Biol Chem 268:4161–4169
9. Basu S (1998) Radioimmunoassay of 8-iso-prostaglandin F2alpha: an index for oxidative injury via free radical catalysed lipid peroxidation. Prostaglandins Leukot Essent Fatty Acids 58:319–325
10. Bates CJ (1994) Plasma vitamin C assays: a European experience. EC FLAIR concerted action no. 10: micronutrient measurement, absorption and status. Int J Vitam Nutr Res 64:283–287
11. Bayerle-Eder M, Pleiner J, Mittermayer F, Schaller G, Roden M, Waldhausl W, Bieglmayer C, Wolzt M (2004) Effect of systemic vitamin C on free fatty acid-induced lipid peroxidation. Diabetes Metab 30:433–439
12. Benzie IF (1996) An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). Clin Biochem 29:111–116
13. Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239:70–76
14. Bobrowicz E, Naskalski JW, Siedlecki A (2001) Preanalytical factors in human plasma ascorbate assay. Clin Chim Acta 314:237–239
15. Breinholt V, Lauridsen ST, Daneshvar B, Jakobsen J (2000) Dose-response effects of lycopene on selected drug-metabolizing and antioxidant enzymes in the rat. Cancer Lett 154:201–210
16. Bui MH (1994) Simple determination of retinol, alpha-tocopherol and carotenoids (lutein, all-trans-lycopene, alpha- and beta-carotenes) in human plasma by isocratic liquid chromatography. J Chromatogr B Biomed Appl 654:129–133
17. Candan F, Gultekin F, Candan F (2002) Effect of vitamin C and zinc on osmotic fragility and lipid peroxidation in zinc-deficient haemodialysis patients. Cell Biochem Funct 20:95–98

18. Cao G, Russell RM, Lischner N, Prior RL (1998) Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. J Nutr 128:2383–2390
19. Carty JL, Bevan R, Waller H, Mistry N, Cooke M, Lunec J, Griffiths HR (2000) The effects of vitamin C supplementation on protein oxidation in healthy volunteers. Biochem Biophys Res Commun 273:729–735
20. Cheng ML, Liu TZ, Lu FJ, Chiu DT (1999) Simultaneous detection of vitamin C and uric acid by capillary electrophoresis in plasma of diabetes and in aqueous humor in acute anterior uveitis. Clin Biochem 32:473–476
21. Chien CT, Chang WT, Chen HW, Wang TD, Liou SY, Chen TJ, Chang YL, Lee YT, Hsu SM (2004) Ascorbate supplement reduces oxidative stress in dyslipidemic patients undergoing apheresis. Arterioscler Thromb Vasc Biol 24:1111–1117
22. Choi SW, Benzie IF, Collins AR, Hannigan BM, Strain JJ (2004) Vitamins C and E: acute interactive effects on biomarkers of antioxidant defence and oxidative stress. Mutat Res 551:109–117
23. Chou PP, Jaynes PK, Bailey JL (1985) Determination of vitamin-e in microsamples of serum by liquid-chromatography with electrochemical detection. Clin Chem 31:880–882
24. Chu KO, Wang CC, Rogers MS, Pang CP (2003) Quantifying F2-isoprostanes in umbilical cord blood of newborn by gas chromatography-mass spectrometry. Anal Biochem 316:111–117
25. Chung WY, Chung JK, Szeto YT, Tomlinson B, Benzie IF (2001) Plasma ascorbic acid: measurement, stability and clinical utility revisited. Clin Biochem 34:623–627
26. Cracowski JL, Girolet S, Imbert B, Seinturier C, Stanke-Labesque F, Bessard J, Boignard A, Bessard G, Carpentier PH (2005) Effects of short-term treatment with vitamin E in systemic sclerosis: a double blind, randomized, controlled clinical trial of efficacy based on urinary isoprostane measurement. Free Radic Biol Med 38:98–103
27. Craft NE (2001) Innovative approaches to vitamin A assessment. J Nutr 131:1626S–1630S
28. Cynamon HA, Isenberg JN, Nguyen CH (1985) Erythrocyte malondialdehyde release in vitro: a functional measure of vitamin E status. Clin Chim Acta 151:169–176
29. Daneshvar B, Frandsen H, Autrup H, Dragsted LO (1997) Gamma-glutamyl semialdehyde and 2-amino-adipic semialdehyde: biomarkers of oxidative damage to proteins. Biomarkers 2:117–123
30. Davison G, Gleeseon M (2005) Influence of acute vitamin C and/or carbohydrate ingestion on hormonal, cytokine, and immune responses to prolonged exercise. Int J Sport Nutr Exerc Metab 15:465–479
31. Davison GW, Hughes CM, Bell RA (2005) Exercise and mononuclear cell DNA damage: the effects of antioxidant supplementation. Int J Sport Nutr Exerc Metab 15:480–492
32. Dawson B, Henry GJ, Goodman C, Gillam I, Beilby JR, Ching S, Fabian V, Dasig D, Morling P, Kakulus BA (2002) Effect of Vitamin C and E supplementation on biochemical and ultrastructural indices of muscle damage after a 21 km run. Int J Sports Med 23:10–15
33. De Caterina R, Cipollone F, Filardo FP, Zimarino M, Bernini W, Lazzerini G, Bucciarelli T, Falco A, Marchesani P, Muraro R, Mezzetti A, Ciabattoni G (2002) Low-density lipoprotein level reduction by the 3-hydroxy-3-methylglutaryl coenzyme-A inhibitor simvastatin is accompanied by a related reduction of F2-isoprostane formation in hypercholesterolemic subjects: no further effect of vitamin E. Circulation 106:2543–2549
34. Dragsted LO, Haren GR, Pedersen A, Hermetter A, Basu S, Hansen M, Kall M, Breinholt V, Castenmiller JJ, Stagsted J, Jakobsen J, Skibsted L, Rasmussen SE, Loft S, Sandstrom B (2005) Answer to Serafini et al. Am J Clin Nutr 81:532–534
35. Dragsted LO, Pedersen A, Hermetter A, Basu S, Hansen M, Haren GR, Kall M, Breinholt V, Castenmiller JJ, Stagsted J, Jakobsen J, Skibsted L, Rasmussen SE, Loft S, Sandstrom B (2004) The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. Am J Clin Nutr 79:1060–1072
36. Esterbauer H, Striegl G, Puhl J, Rothender M (1989) Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radi Res Commun 6:67–75
37. Farrell PM, Mischler EH, Gutcher GR (1982) Evaluation of vitamin E deficiency in children with lung disease. Ann NY Acad Sci 393:96–108
38. Favaro RM, de Souza NV, Vannucchi H, Desai ID, de Oliveira JE (1986) Evaluation of rose bengal staining test and rapid dark-adaptation test for the field assessment of vitamin A status of preschool children in Southern Brazil. Am J Clin Nutr 43:940–945
39. Ferro D, Basili S, Pratico D, Iuliano L, FitzGerald GA, Violi F (1999) Vitamin E reduces monocyte tissue factor expression in cirrhotic patients. Blood 93:2945–2950
40. Fischer CP, Hiscock NJ, Basu S, Vessby B, Kallner A, Sjoberg LB, Febbraio MA, Pedersen BK (2006) Vitamin E isoform-specific inhibition of the exercise-induced heat-shock protein 72 expression in humans. J Appl Physiol 100:1679–1687
41. Fuller CJ, Grundy SM, Norkus EP, Jialal I (1996) Effect of ascorbate supplementation on low density lipoprotein oxidation in smokers. Atherosclerosis 119:139–150
42. Fuller CJ, May MA, Martin KJ (2000) The effect of vitamin E and vitamin C supplementation on LDL oxidizability and neutrophil respiratory burst in young smokers. J Am Coll Nutr 19:361–369
43. Funk MO (1987) The electroanalytical approach to lipid peroxide determinations. Free Radic Biol Med 3:319–321
44. Furr HC, Bawa AB, Olson JA (1994) Analytical methods. In: Sporn MB, Roberts AB, Goodman DS (eds) The retinoids: biology, chemistry, and medicine. 2nd edn. Raven Press Ltd., New York, pp 179–209
45. Futterman S, Swanson D, Kalina RE (1975) A new, rapid fluorometric determination of retinol in serum. Invest Ophthalmol 14:125–130
46. Gawlik MT, Gawlik MB, Gorka A, Brandys J (2003) Optimization and validation of a high-performance liquid chromatographic method for determination of alpha- and gamma-tocopherol in rat plasma and erythrocytes. Acta Chromatogr 13:185–195
47. Ghiselli A, Serafini M, Natella F, Scaccini C (2000) Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radic Biol Med 29:1106–1114
48. Goldfarb AH, Patrick SW, Bryer S, You T (2005) Vitamin C supplementation affects oxidative-stress blood markers in response to a 30-minute run at 75% VO2max. Int J Sport Nutr Exerc Metab 15:279–290

49. Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, Cooke M, Coolen S, Haenen G, Hoberg AM, Loft S, Lunec J, Olinski R, Parry J, Pompella A, Poulsen H, Verhagen H, Astley SB (2002) Biomarkers. Mol Aspects Med 23:101–208
50. Hininger I, Waters R, Osman M, Garrel C, Fernholz K, Roussel AM, Anderson RA (2005) Acute prooxidant effects of vitamin C in EDTA chelation therapy and long-term antioxidant benefits of therapy. Free Radic Biol Med 38:1565–1570
51. Hix J, Martinez C, Buchanan I, Morgan J, Tam M, Shankar A (2004) Development of a rapid enzyme immunoassay for the detection of retinol-binding protein. Am J Clin Nutr 79:93–98
52. Hofer G, Lichtenberg D, Hermetter A (1995) A new fluorescence method for the continuous determination of surface lipid oxidation in lipoproteins and plasma. Free Radic Res 23:317–327
53. Institute of Medicine, National Academy of Sciences USA (2000) Dietary reference intakes for vitamin C, vitamin E, selenium, carotenoids, National Academy Press, Washington, D.C, pp 186–283
54. Itoh H, Ohkuwa T, Yamazaki Y, Shimoda T, Wakayama A, Tamura S, Yamamoto T, Sato Y, Miyamura M (2000) Vitamin E supplementation attenuates leakage of enzymes following 6 successive days of running training. Int J Sports Med 21:369–374
55. Jacob RA, Aiello GM, Stephensen CB, Blumberg JB, Milbury PE, Wallock LM, Ames BN (2003) Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. J Nutr 133:740–743
56. Jain SK, Krueger KS, McVie R, Jaramillo JJ, Palmer M, Smith T (1998) Relationship of blood thromboxane-B2 (TxB2) with lipid peroxides and effect of vitamin E and placebo supplementation on TxB2 and lipid peroxide levels in type 1 diabetic patients. Diabetes Care 21:1511–1516
57. Jain SK, McVie R, Smith T (2000) Vitamin E supplementation restores glutathione and malondialdehyde to normal concentrations in erythrocytes of type 1 diabetic children. Diabetes Care 23:1389–1394
58. Jialal I, Fuller CJ, Huet BA (1995) The effect of alpha-tocopherol supplementation on LDL oxidation. A dose-response study. Arterioscler Thromb Vasc Biol 15:190–198
59. Julianto T, Yuen KH, Noor AM (1999) Simple high-performance liquid chromatographic method for determination of alpha-tocopherol in human plasma. J Chromatogr B Biomed Sci Appl 732:227–231
60. Kall MA, Andersen C (1999) Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. J Chromatogr B Biomed Sci Appl 730:101–111
61. Keenoy B, Shen H, Engelen W, Vertommen J, Van Dessel G, Lagrou A, De LI (2001) Long-term pharmacologic doses of vitamin E only moderately affect the erythrocytes of patients with type 1 diabetes mellitus. J Nutr 131:1723–1730
62. Keith ME, Jeejeebhoy KN, Langer A, Kurian R, Barr A, O'Kelly B, Sole MJ (2001) A controlled clinical trial of vitamin E supplementation in patients with congestive heart failure. Am J Clin Nutr 73:219–224
63. Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, Barratt CL (1995) A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. Fertil Steril 64:825–831
64. Kinlay S, Behrendt D, Fang JC, Delagrange D, Morrow J, Witztum JL, Rifai N, Selwyn AP, Creager MA, Ganz P (2004) Long-term effect of combined vitamins E and C on coronary and peripheral endothelial function. J Am Coll Cardiol 43:629–634
65. Koshiishi I, Mamura Y, Liu J, Imanari T (1998) Evaluation of an acidic deproteinization for the measurement of ascorbate and dehydroascorbate in plasma samples. Clin Chem 44:863–868
66. Lauridsen C, Leonard SW, Griffin DA, Liebler DC, McClure TD, Traber MG (2001) Quantitative analysis by liquid chromatography tandem mass spectrometry of deuterium-labeled and unlabeled vitamin E in biological samples. Anal Biochem 289:89–95
67. Lauridsen ST, Mortensen A (1999) Probucol selectively increases oxidation of atherogenid lipoproteins in cholesterol-fed mice and in Watanable heritable hyperlipidemic rabbits. Atherosclerosis 142:169–178
68. Lee CY, Jenner AM, Halliwell B (2004) Rapid preparation of human urine and plasma samples for analysis of F2-isoprostanes by gas chromatography-mass spectrometry. Biochem Biophys Res Commun 320:696–702
69. Lemoyne M, Van Gossum A, Kurian R, Ostro M, Axler J, Jeejeebhoy KN (1987) Breath pentane analysis as an index of lipid peroxidation: a functional test of vitamin E status. Am J Clin Nutr 46:267–272
70. Lesgards JF, Lehucher-Michel MP, Vidal N, Prost M, Stocker P (2005) Assessment of antioxidative activity of lipid- and water-soluble vitamins in human whole blood. Comparative analysis between a biological test and chemical methods. Int J Vitam Nutr Res 75:11–18
71. Levine M, Wang Y, Padayatty SJ, Morrow J (2001) A new recommended dietary allowance of vitamin C for healthy young women. Proc Natl Acad Sci USA 98:9842–9846
72. Liau LS, Lee BL, New AL, Ong CN (1993) Determination of plasma ascorbic acid by high-performance liquid chromatography with ultraviolet and electrochemical detection. J Chromatogr 612:63–70
73. Lietman TM, Dhital SP, Dean D (1998) Conjunctival impression cytology for vitamin A deficiency in the presence of infectious trachoma. Br J Ophthalmol 82:1139–1142
74. Lykkesfeldt J, Bolbjerg ML, Poulsen HE (2003) Effect of smoking on erythorbic acid pharmacokinetics. Br J Nutr 89:667–671
75. Lykkesfeldt J, Loft S, Nielsen JB, Poulsen HE (1997) Ascorbic acid and dehydroascorbic acid as biomarkers of oxidative stress caused by smoking. Am J Clin Nutr 65:959–963
76. MacCrehan WA, Schonberger E (1987) Determination of retinol, alpha-tocopherol, and beta-carotene in serum by liquid chromatography with absorbance and electrochemical detection. Clin Chem 33:1585–1592
77. MacCrehan WA, Schonberger E (1987) Reversed-phase high-performance liquid chromatographic separation and electrochemical detection of retinol and its isomers. J Chromatogr 417:65–78
78. Malvy DJ, Poveda JD, Debruyne M, Burtschy B, Dostalova L, Amedee-Manesme O (1993) Immunonephelometry and radial immunodiffusion compared for measuring serum retinol-binding protein. Eur J Clin Chem Clin Biochem 31:47–48
79. Marangon K, Devaraj S, Tirosh O, Packer L, Jialal I (1999) Comparison of the effect of alpha-lipoic acid and alpha-tocopherol supplementation on measures of oxidative stress. Free Radic Biol Med 27:1114–1121

80. Margolis SA, Duewer DL (1996) Measurement of ascorbic acid in human plasma and serum: stability, intralaboratory repeatability, and interlaboratory reproducibility. Clin Chem 42:1257–1262
81. Margolis SA, Ziegler RG, Helzlsouer KJ (1991) Ascorbic and dehydroascorbic acid measurement in human serum and plasma. Am J Clin Nutr 54:1315S–1318S
82. Marshall PJ, Warso MA, Lands WEM (1985) Selective Microdetermination of Lipid Hydroperoxides. Anal Biochem 145:192–199
83. Marzouki N, Benomar A, Yahyaoui M, Birouk N, Elouazzani M, Chkili T, Benlemlih M (2005) Vitamin E deficiency ataxia with (744 del A) mutation on alpha-TTP gene: genetic and clinical peculiarities in Moroccan patients. Eur J Med Genet 48:21–28
84. Mastaloudis A, Morrow JD, Hopkins DW, Devaraj S, Traber MG (2004) Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. Free Radic Biol Med 36:1329–1341
85. McClean SW, Ruddel ME, Gross EG, DeGiovanna JJ, Peck GL (1982) Liquid-chromatographic assay for retinol (vitamin A) and retinol analogs in therapeutic trials. Clin Chem 28:693–696
86. McKechnie R, Rubenfire M, Mosca L (2002) Antioxidant nutrient supplementation and brachial reactivity in patients with coronary artery disease. J Lab Clin Med 139:133–139
87. Meagher EA, Barry OP, Lawson JA, Rokach J, FitzGerald GA (2001) Effects of vitamin E on lipid peroxidation in healthy persons. JAMA 285:1178–1182
88. Mori TA, Croft KD, Puddey IB, Beilin LJ (1999) An improved method for the measurement of urinary and plasma F2-isoprostanes using gas chromatography-mass spectrometry. Anal Biochem 268:117–125
89. Mottier P, Gremaud E, Guy PA, Turesky RJ (2002) Comparison of gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry methods to quantify alpha-tocopherol and alpha-tocopherolquinone levels in human plasma. Anal Biochem 301:128–135
90. Mulholland CW, Strain JJ, Trinick TR (1996) Serum antioxidant potential, and lipoprotein oxidation in female smokers following vitamin C supplementation. Int J Food Sci Nutr 47:227–231
91. Nieman DC, Henson DA, McAnulty SR, McAnulty LS, Morrow JD, Ahmed A, Heward CB (2004) Vitamin E and immunity after the Kona Triathlon World Championship. Med Sci Sports Exerc 36:1328–1335
92. Nourooz-Zadeh J, Tajaddini-Sarmadi J, Wolff SP (1994) Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal Biochem 220:403–409
93. Nyyssonen K, Poulsen HE, Hayn M, Agerbo P, Porkkala-Sarataho E, Kaikkonen J, Salonen R, Salonen JT (1997) Effect of supplementation of smoking men with plain or slow release ascorbic acid on lipoprotein oxidation. Eur J Clin Nutr 51:154–163
94. Pachla LA, Reynolds DL, Kissinger PT (1985) Analytical methods for determining ascorbic-acid in biological samples, food-products, and pharmaceuticals. J Assoc Off Anal Chem 68:1–12
95. Patrignani P, Panara MR, Tacconelli S, Seta F, Bucciarelli T, Ciabattoni G, Alessandrini P, Mezzetti A, Santini G, Sciulli MG, Cipollone F, Davi G, Gallina P, Bon GB, Patrono C (2000) Effects of vitamin E supplementation on F(2)-isoprostane and thromboxane biosynthesis in healthy cigarette smokers. Circulation 102:539–545
96. Porkkala-Sarataho E, Salonen JT, Nyyssonen K, Kaikkonen J, Salonen R, Ristonmaa U, Diczfalusy U, Brigelius-Flohe R, Loft S, Poulsen HE (2000) Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arterioscler Thromb Vasc Biol 20:2087–2093
97. Reaven PD, Witztum JL (1993) Comparison of supplementation of RRR-alpha-tocopherol and racemic alpha-tocopherol in humans. Effects on lipid levels and lipoprotein susceptibility to oxidation. Arterioscler Thromb 13:601–608
98. Rice-Evans C, Miller N (1997) Measurement of the antioxidant status of dietary constituents, low density lipoproteins and plasma. Prostaglandins Leukot Essent Fatty Acids 57:499–505
99. Rolf C, Cooper TG, Yeung CH, Nieschlag E (1999) Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study. Hum Reprod 14:1028–1033
100. Sacheck JM, Milbury PE, Cannon JG, Roubenoff R, Blumberg JB (2003) Effect of vitamin E and eccentric exercise on selected biomarkers of oxidative stress in young and elderly men. Free Radic Biol Med 34:1575–1588
101. Serafini M, Del Rio D (2004) Understanding the association between dietary antioxidants, redox status and disease: is the total antioxidant capacity the right tool? Redox Rep 9:145–152
102. Serafini M, Ghiselli A, Ferro LA (1996) In vivo antioxidant effect of green and black tea in man. Eur J Clin Nutr 50:28–32
103. Simons LA, von Konigsmark M, Simons J, Stocker R, Celermajer DS (1999) Vitamin E ingestion does not improve arterial endothelial dysfunction in older adults. Atherosclerosis 143:193–199
104. Sureda A, Batle JM, Tauler P, Aguilo A, Cases N, Tur JA, Pons A (2004) Hypoxia/reoxygenation and vitamin C intake influence NO synthesis and antioxidant defenses of neutrophils. Free Radic Biol Med 37:1744–1755
105. Tam LS, Li EK, Leung VY, Griffith JF, Benzie IF, Lim PL, Whitney B, Lee VW, Lee KK, Thomas GN, Tomlinson B (2005) Effects of vitamins C and E on oxidative stress markers and endothelial function in patients with systemic lupus erythematosus: a double blind, placebo controlled pilot study. J Rheumatol 32:275–282
106. Terao J, Shibata SS, Matsushita S (1988) Selective quantification of arachidonic acid hydroperoxides and their hydroxy derivatives in reverse-phase high performance liquid chromatography. Anal Biochem 169:415–423
107. Tesoriere L, Butera D, Pintaudi AM, Allegra M, Livrea MA (2004) Supplementation with cactus pear (Opuntia ficus-indica) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C. Am J Clin Nutr 80:391–395
108. Thomas SM, Jessup W, Gebicki JM, Dean RT (1989) A continuous-flow automated-assay for iodometric estimation of hydroperoxides. Anal Biochem 176:353–359
109. Thornton SP (1977) A rapid test for dark adaptation. Ann Ophthalmol 9:731–734
110. Tomlinson PA, Dalton RN, Turner C, Chantler C (1990) Measurement of beta 2-microglobulin, retinol-binding protein, alpha 1-microglobulin and urine protein 1 in healthy children using enzyme-linked immunosorbent assay. Clin Chim Acta 192:99–106

111. Upritchard JE, Sutherland WH, Mann JI (2000) Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. Diabetes Care 23:733–738
112. Van Pelt CK, Haggarty P, Brenna JT (1998) Quantitative subfemtomole analysis of alpha-tocopherol and deuterated isotopomers in plasma using tabletop GC/MS/MS. Anal Chem 70:4369–4375
113. Verhagen H, Aruoma OI, van Delft JH, Dragsted LO, Ferguson LR, Knasmuller S, Pool-Zobel BL, Poulsen HE, Williamson G, Yannai S (2003) The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo. Food Chem Toxicol 41:603–610
114. Walter MF, Blumberg JB, Dolnikowski GG, Handelman GJ (2000) Streamlined F2-isoprostane analysis in plasma and urine with high-performance liquid chromatography and gas chromatography/mass spectroscopy. Anal Biochem 280:73–79
115. Waring WS, Mishra V, Maxwell SR (2003) Comparison of spectrophotometric and enhanced chemiluminescent assays of serum antioxidant capacity. Clin Chim Acta 338:67–71
116. Waring WS, Webb DJ, Maxwell SR (2001) Systemic uric acid administration increases serum antioxidant capacity in healthy volunteers. J Cardiovasc Pharmacol 38:365–371
117. Wen Y, Cooke T, Feely J (1997) The effect of pharmacological supplementation with vitamin C on low-density lipoprotein oxidation. Br J Clin Pharmacol 44:94–97
118. Wittpenn JR, Natadisastra G, Mele L, Sommer A (1988) Reproducibility of determining vitamin A status by impression cytology. Ophthalmic Surg 19:559–561
119. Woollard KJ, Loryman CJ, Meredith E, Bevan R, Shaw JA, Lunec J, Griffiths HR (2002) Effects of oral vitamin C on monocyte: endothelial cell adhesion in healthy subjects. Biochem Biophys Res Commun 294:1161–1168
120. World Health Organization (1994) World Health Organization Indicators for Assessing Vitamin A Deficiency, Their Application in Monitoring and Evaluating Intervention Programmes. WHO/NUT/941. Geneva, Switzerland. Ref Type: Report
121. Yang M, Collis CS, Kelly M, Diplock AT, Rice-Evans C (1999) Do iron and vitamin C co-supplementation influence platelet function or LDL oxidizability in healthy volunteers? Eur J Clin Nutr 53:367–374
122. Zoeren-Grobben D, Jacobs NJ, Houdkamp E, Lindeman JH, Drejer DF, Berger HM (1998) Vitamin E status in preterm infants: assessment by plasma and erythrocyte vitamin E-lipid ratios and hemolysis tests. J Pediatr Gastroenterol Nutr 26:73–79