Biological Properties of Dietary Micronutrients: Antioxidant Capacity and Structure-Activity Relationships

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To my parents / A mis padres

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Acronyms and conventional names

4-HNE	4-hydroxynonenal
5-HpETE	5-hydroperoxy-eicosatetraenoic acid
AAPH	2,2´-azobis(2-amidinopropane) hydrochloride
ABTS	2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
Ac-DEVD-pNA	acetyl-Asp-Glu-Val-Asp-*para*-nitroanilide
ATP	adenosine triphosphate
AUC	area under the curve
AVED	ataxia with vitamin E deficiency (familial isolated vitamin E deficiency)
BHT	2,6-di-tert-butyl-4-methylphenol
CE	cholesterol esters
CHD	coronary heart disease
CVD	cardiovascular disease
DCF	2´,7´-dichlorofluorescein
DCFH-DA	2´,7´-dichlorodihydrofluorescein diacetate
DEVD-CHO	Asp-Glu-Val-Asp-aldehyde
DiI	1,1´-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanine perchlorate
DMSO	dimethyl sulfoxide
DTNB	5,5´-dithio-bis(2-nitrobenzoic acid)
DTT	1,4-dithio-threitol
EC	(-)-epicatechin
EDTA	ethylene-diamine-tetraacetic acid
EGCG	epigallocatechin gallate
FCS	fetal calf serum
FRAP	ferric reducing ability of plasma
GAE	gallic acid equivalents
GJC	gap junction (intercellular) communication
GSH	reduced glutathione
GSSG	glutathione disulfide
HDL	high density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNBA	4-hydroxy-3-nitrobenzoic acid

IC_{50}	half-maximal inhibitory concentration
IDL	intermediate density lipoproteins
IHD	ischemic heart disease
Kpi	potassium phosphate buffer
LDL	low density lipoprotein
MAEC	(primary) mouse aorta endothelial cells
MDA	malondialdehyde
MI	myocardial infarction
MM-LDL	minimally-modified LDL
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
nLDL	native LDL
ORAC	oxygen radical absorption capacity
oxLDL	oxidized LDL
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluorid
PNA	*para*-nitroanilide
PUFA	polyunsaturated fatty acids
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SMC	smooth muscle cells
SSA	5-sulfosalicylic acid
TBARS	thiobarbituric acid-reactive substances
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TEAH	tetraethylammonium hydroxide
TG	triglycerides
TNB	5-thio-2-nitrobenzoic acid
TP	total phenols
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAP	total peroxyl radical trapping parameter
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
VLDL	very-low density lipoprotein

VP	2-vinyl-pyridine
α-CEHC	2,5,7,8-tetramethyl-2-(2´-carboxyethyl)-6-hydroxychroman
α-TTP	α-tocopherol transfer protein
γ-CEHC	2,7,8-trimethyl-2-(2´-carboxyethyl)-6-hydroxychroman

Reactive Species

H_2O_2	hydrogen peroxide
$^{\cdot}OH$	hydroxyl radical
NO	nitric oxide
$ONOO^-$	peroxynitrite
$O_2^{\cdot -}$	superoxide radical anion

Aminoacid Codes

Asp	aspartate
Glu	glutamate
Val	valine

1. INTRODUCTION

1. 1 Free Radicals, Reactive Oxygen Species and Disease

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in cells in the normal course of metabolism. Some, but not all, of these ROS/RNS are free radicals, i. e., entities which contain one or more unpaired electrons but are capable of independent existence. The main site for ROS production is the mitochondrial electron transport system, where the superoxide anion radical ($O_2^{\cdot -}$) is generated and, following its dismutation, hydrogen peroxide (H_2O_2) [Chance et al., 1979]. In the presence of transition elements like iron, hydroxyl radical ($^{\cdot}OH$) generation may follow as a result of the Fenton chemistry. Other sources of $O_2^{\cdot -}$ are the peroxisomal fatty acid metabolism and the detoxication from xenobiotics *via* microsomal P450 enzymes [Sies, 1986]. The latter are a minor source of ROS in physiological conditions, but may be an important generator of oxidants in some specific subjects (e.g. smokers).

Nitric oxide (NO) is the most important RNS *in vivo* as it is an endogenous regulator of several cellular functions (e. g. vasodilation, neurotransmission and roles in the immune system) [Ignarro, 1990]. Under physiological conditions, the generation of nitrosating species such as S-nitroso-glutathione or other S-nitrosothiols (RSNO) is likely and such compounds may participate in trans-nitrosation reactions [Al-Mustafa et al., 2001]. Key chemical reactions can, on the other hand, lead to more reactive species, potentially more toxic than NO. Reaction of NO with $O_2^{\cdot -}$ yields the highly toxic peroxynitrite ($ONOO^-$). Steady-state concentrations of $ONOO^-$ may be significant in the vicinity of activated macrophages, which generate NO and $O_2^{\cdot -}$ concomitantly [Fukuto, 1995].

Oxidative stress has been defined as an imbalance between oxidants (ROS/RNS) and antioxidants in favour of the former, leading to damage [Sies, 1985]. Experimental evidence largely supports a role for oxidative stress in the pathogenesis and/or progression of a number of diseases [Sies, 1991]. It is involved in tumor initiation, promotion, and progression, plays a role in reperfusion injury after ischemia, and, in inflammatory disorders such as rheumatoid arthritis, phagocytic cells (mainly neutrophils) are steadily activated and release $O_2^{\cdot -}$, H_2O_2 and the highly oxidising hypohalous acids.

Various cellular signaling pathways and the expression of certain genes are also oxidation-sensitive. ROS may thereby elicit changes in functional processes of the cell that ultimately lead to apoptosis, to increased proliferation, or to immune or other responses guiding to cellular malfunction. Redox-sensitive transcription factors in mammalian cells include activating protein (AP)-1 and nuclear factor (NF)-κB [Abate et al., 1990; Schreck et al., 1991]. Signaling pathways that are activated by stress stimuli including oxidative stress include the mitogen-activated protein kinases (MAPK) -comprising the extracellular signal-regulated kinases (ERK) 1 and 2, the p38 subgroup and the c-Jun-N-terminal kinases (JNK)- and the phosphoinositide-3 kinase (PI3K)/Akt pathway [Klotz, 2002]. In rat liver epithelial cells $ONOO^-$ induced an immediate activation of p38 and a progressive activation of ERK1/2 and JNK; activation was attenuated by selenite supplementation, suggesting a protective role of glutathione peroxidase *in vivo* [Schieke et al., 1999]. Besides, $ONOO^-$ elicited a decrease in gap junction intercellular communication (GJC) likely *via* ERK activation [Sharov et al., 1999]. Singlet oxygen ($^1O_2$), which may be generated photochemically by visible or UV light irradiation on photosensitizers such as porphyrins, was found to activate p38 and JNK in similar manner to UVA in skin fibroblasts [Klotz et al., 1999].

1. 2 The Oxidative Modification Hypothesis of Atherosclerosis

Atherosclerosis is a progressive occlusion of large and medium-sized arteries, basically consisting in the thickening of the intima (most inner layer of arteries) due to a combination of fibrosis, smooth muscle cell proliferation, and the development of an extracellular lipid core. In its more advanced stages all three coats of the arterial wall (*tunica intima*, *tunica media*, and *tunica adventitia*) become affected. The luminal side of the intima, a monolayer of endothelial cells (endothelium), loses its antithrombotic properties under this pathological state and the affected zone is prone to thrombus formation [Navab et al., 1995].

At present, atherosclerosis is accepted as the primary mechanism leading to cardiovascular diseases (CVD), the main cause of death in most developed countries. Various risk factors for atherosclerosis have been identified, such as fat-rich diets, sedentary lifestyles, hypercholesterolemia, hypertension, smoking, diabetes mellitus, and family history of premature coronary heart disease (CHD) [Vogel, 1997].

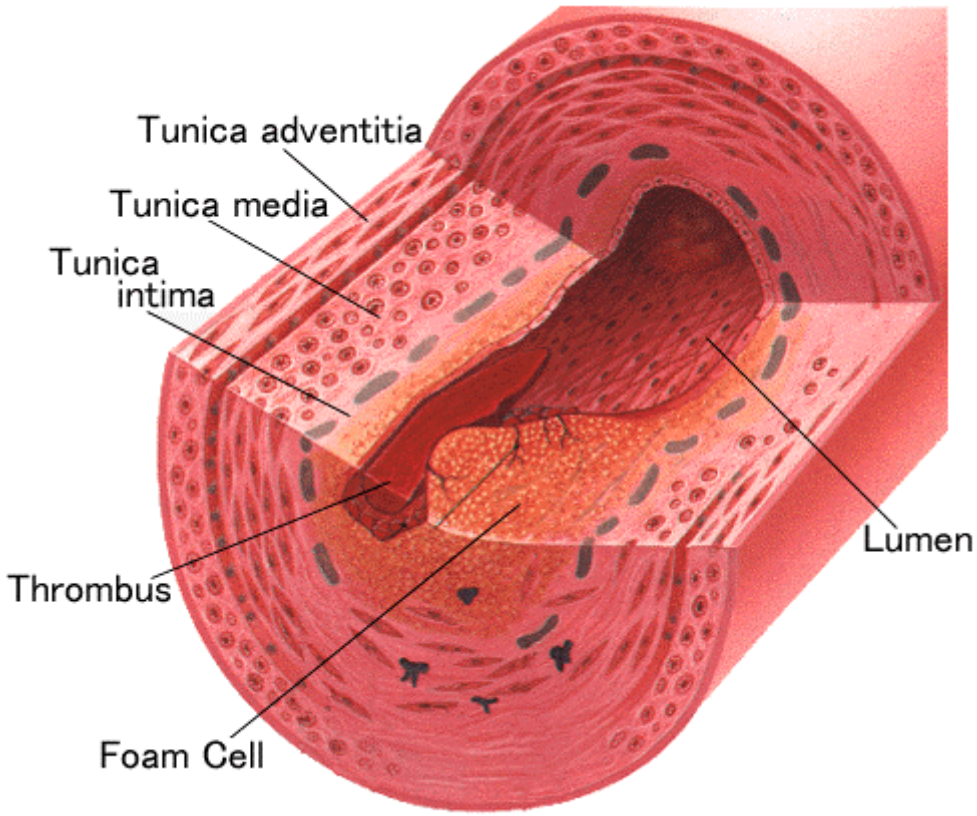


Figure 1. 1 Atherosclerotic artery showing intima thickening (due to foam cell and lipid accumulation) and thrombus formation.

The key determinants of the initiation of early lesions are [Schwartz et al., 1993]:
i) a local hemodynamic environment with domains of low shear and reverse flow; this enhances localised intimal influx and accumulation of plasma lipoproteins, mainly low-density lipoprotein (LDL) and lipoprotein a -Lp(a)-;
ii) increased net intimal oxidative stress;
iii) focal monocyte recruitment to the arterial intima;
iv) monocyte/macrophage activation in the intima leading to foam cell formation.

1. 2. 1 Oxidation of LDL

LDL is the fraction of human lipoproteins with a buoyant density between 1.019 and 1.063 kg/L. A particle of LDL has a mean molecular mass of $2.5 \cdot 10^6$ D and a mean diameter of 20 nm. Approximately 50% of the mass of LDL is cholesterol, making the particle the major sterol transporter in the circulation [Esterbauer at al., 1992]. 80% of the cholesterol is esterified, primarily with linoleic acid. The more

lipophilic core of the particle is formed by cholesterol esters (CE) and tryglicerides (TG), corresponding to 41 and 3% of the total LDL mass, respectively, and free cholesterol, phospholipids and proteins constitute the particle surface in direct contact with plasma (**Figure 1.2**). About 66% of the cholesterol carried by LDL is returned to the liver, the rest is transported to extrahepatic tissues. Uptake of LDL in all tissues is regulated by the LDL or apo B/E receptor, a 96 kD protein which appears in the cytoplasmic membranes of cells in response to their need for cholesterol. The major apolipoprotein in LDL, apo B_{100}, is located at the surface and recognized by the LDL receptor. LDL is then endocyted *via* so-called *coated pits* into vesicles formed by the protein clathrin, with subsequent fusion of endosomes and lysosomes, apolipoprotein digestion and cholesterol de-esterification [Löffler, 2002].

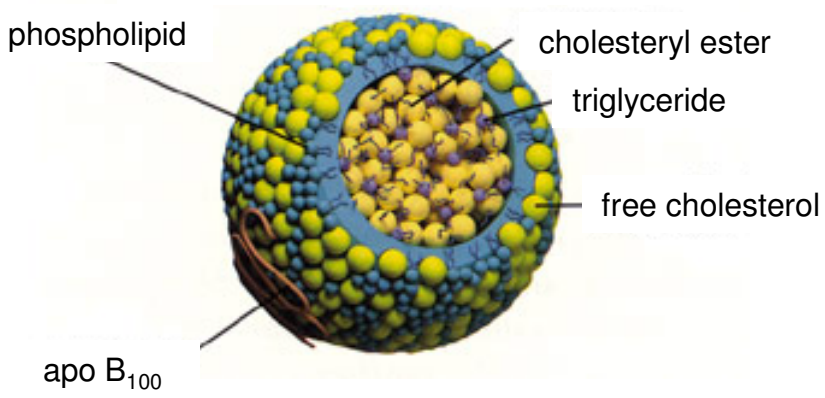


Figure 1. 2 Structure of an LDL particle showing the core and surface components

LDL passes readily across the endothelium. Within the subendothelial space, LDL gets trapped into extracellular matrix proteins and is exposed to radical-triggered oxidative alterations by surrounding cells, including endothelial cells, smooth muscle cells (SMC), and infiltrated macrophages. Candidate effectors of LDL oxidation in the artery wall are 15-lipoxygenase and enzymes present in neutrophils and endothelial cells, such as myeloperoxidase, NAD(P)H oxidase, and NO synthase, together with traces of metal ions [Esterbauer et al., 1992].

A large number of procedures have been applied to obtain LDL with the same oxidative modifications *in vitro*. LDL incubated in plain cell medium with 5 µmol/L copper (II) was found to have the same chemical and biological properties as the

LDL modified by incubation with rabbit aortic endothelial cells in the same medium (containing traces of iron and copper) for the same time period [Steinbrecher et al., 1984]. The process was inhibited by chelating agents, confirming the requirement of trace elements. Thus, LDL oxidated with copper (II) has been widely employed since then to assess biological activities of oxidatively-modified LDL in cellular and animal experiments.

In isolated LDL, peroxidation of lipids by copper (II) further required the presence of pre-formed lipid hydroperoxides [Thomas and Jackson, 1991]. Thus, the mechanism underlying this type of LDL oxidation appears to be the oxidative and reductive decomposition of peroxides by transition elements yielding peroxyl ($LOO^{\cdot}$) and alcoxyl ($LO^{\cdot}$) radical species [Rice-Evans et al., 1996]:

$$LOOH + Cu^{2+} \longrightarrow LOO^{\cdot} + Cu^{+} + H^{+}$$
$$LOOH + Cu^{+} \longrightarrow LO^{\cdot} + Cu^{2+} + OH^{-}$$

This leads to the propagation of radical attack on the polyunsaturated fatty acids (PUFA) yielding new peroxides, until all PUFA are consumed.

In vivo, cells may produce a range of redox reagents which can react with LDL directly or reduce any transition metal present, facilitating lipid peroxide decomposition and subsequent uncontrolled PUFA peroxidation. Injury to cells and tissues may enhance the toxicity of ROS by releasing metal ions from storage sites, decompartmentalized hem proteins or metalloproteins by interaction with accessible proteases or oxidants [Rive-Evans et al., 1996].

The first barrier preventing lipid peroxidation is that formed by the antioxidants within the LDL particle, including vitamin E and carotenoids. During a first lag phase, in which conjugated dienes are not detected and only minimal lipid peroxidation occurs, LDL becomes depleted of antioxidants, with α-tocopherol as the first and β-carotene as the last one. Conjugated dienes then result from oxidation of PUFA with isolated double bonds to PUFA hydroperoxides with conjugated double bonds, which may be detected by UV-spectrophotometry at 234 nm. Finally, lipid hydroperoxides decompose to aldehydes and other products [Esterbauer et al., 1992].

1. 2. 2 Degree of LDL Oxidation and Biological Activity

Oxidatively-modified LDLs have been categorized in minimally-modified LDL (MM-LDL) and extensively oxidized LDL (oxLDL) in view of a number of different biological activities attributed to each. However, it should be noted that up to date no consensus concerning the differentiation between both types of oxidized LDL has been reached, and even nomenclature differs in the literature.

From a functional aspect, properties such as moderate cytotoxicity, induction of smooth muscle cell (SMC) proliferation, increase of monocyte adhesion to endothelium, or stimulation of monocyte-chemotactic protein-1 (MCP-1) release from endothelial cells have been ascribed to MM-LDL. OxLDL has been shown to be chemotactic for circulating monocytes and SMC, but it diminishes the motility of macrophages, thus inhibiting egression of foam cell from the arterial lession. It is immunogenic, inducing antibody formation, highly cytotoxic, and induces the release of a number of pro-inflamatory cytokines and chemokines from endothelium [Esterbauer et al., 1997].

Chemically, this change of biological function has been attributed to oxidative modifications in apo B_{100}. Aldehydes generated as secondary products of lipid peroxidation react with aminoacid residues of apo B, mainly lysine, giving Schiff´s bases and Michael adducts. This usually occurs when thiobarbituric acid-reactive substances (TBARS) reach a threshold value of 25 mol/mol LDL [Esterbauer et al., 1990]. The ε- amino group of lysine residues is positively charged at physiological pH. Hence, the protein becomes more negatively charged after reaction with aldehydes. Such a degree of oxidation is reached after about 12 to 24 h of incubation with copper (II) depending on the conditions, and represents the conversion of minimally-modified LDL into oxLDL [Esterbauer et al., 1990]. The degree of modification may be followed by protein electrophoresis.

Modifications on apo B_{100} lead to the formation of new epitopes which are not recognized by the ubiquitous LDL receptor, but by other more tissue-specific so-called scavenger receptors [Jürgens, 1990]. Expression of some of the scavenger receptors has been found to be increased in the atherosclerotic lesion [Ozer at al., 2003]. SR-A, the major scavenger receptor known for macrophages, is responsible for massive oxLDL uptake leading to foam cell formation. CD-36 is expressed in monocytes/macrophages, capillary endothelial cells, SMC, adipocytes, and platelets. The scavenger receptor LOX-1 is most abundant in endothelial cells.

1. 3. Evidence for an Increased Oxidative Status in Atherosclerotic or CVD Patients

The understanding of the impact of oxLDL on the mechanism of atherogenesis and the underlying biochemical pathways is still limited. Nevertheless, if the oxidative-modification hypothesis is valid, one should be able to encounter proofs of oxidative damage *in vivo*. A broad range of approaches has been developed with this aim. However, appropriate biomarkers of *in vivo* oxidation in terms of specificity, stability and reproducibility are scarce. At present the concentration of F_2-isoprostanes, prostaglandin-like compounds formed from non-enzymatic free radical-dpendent peroxidation of long-chain PUFA, is thought to be the most reliable biomarker indicating lipid peroxidation.

Table 1. 3 Biomarkers of oxidative damage

Biomarker	Methodological characteristics
Lipid peroxidation	
TBARS and MDA	Not specific. Formation of artifacts possible. Not stable.
Conjugated dienes	Not specific. Interference with other components.
Lipid hydroperoxides, oxysterols (HPLC)	Specific. Lack of stability.
Pentane/ethane exhalation	Difficult sampling. Interference with isoprene.
Urine isoprostanes (HPLC - MS)	Specific, reproducible. Lack of stability (short half-life)
Antioxidant status	
Total antioxidant capacity as measured with LDL lag phase	Not specific for lipid peroxidation. *Ex vivo* experiments.
Antioxidant pattern	Specific and reproducible.

Adapted from Griffiths et al., 2002

Malondialdehyde-modified and oxidized LDL are formed in atherosclerotic lesions [Hammer et al., 1995]. Increased levels of MDA-modified LDL have been detected in the plasma of patients with ischemic heart disease (IHD) [Holvoet and Collen, 1998] and hypertension [Marchesi et al., 1996]. In addition, decreased lag phases of LDL

oxidation as compared with controls have been found in coronary artery disease [Cominacini, 1993] or hypertensive patients [Maggi et al., 1993], and evidence for an inverse relationship between oxidation resistance of LDL and both severity of myocardial infarction (MI) [Fainaru et al., 2002] and coronary estenosis [van de Vijver et al., 1998] has been provided. On the other hand, a number of human studies did not show any correlation between markers of lipid peroxidation or LDL modification and the risk of CVD.

1. 4 The Role of Antioxidants in the Prevention of Atherosclerosis; Human Studies

Many observational studies support a relationship between the intake of antioxidant-rich diets and a lower risk for CVD, and it may be affirmed that at least at this stage positive findings exceed indefinite or negative results. Results of the largest, most reliable prospective studies focussed on cardiovascular end-points state a consistent decrease in risk of CVD with increasing fruit and vegetable consumption [Lindsay and Astley, 2002]. Consumption of antioxidant-rich food (vegetables and fruits) with adequate frequency [Verlangieri et al., 1985] or diets low in fat and high in fruit and vegetables [Diaz et al., 1997; Gey et al., 1993] are inversely correlated with mortality due to CVD. Most promising results are related to the risk of IHD, which was 15% lower comparing the 90th and 10th centile of fruit and vegetable intake [Law and Morris, 1998]. A relative risk of 0.69 was found in the highest quintile of intake (5–6 servings per day), compared with the lowest quintile [Joshipura et al., 1999].

A number of observational studies examined the relationships between dietary intake of vitamin C, vitamin E, carotenoids, flavonoids and the incidence of CVD, on the assumption that these micronutrients exert *in vivo* the same protective action towards LDL oxidation as established *in vitro*.

a) Vitamin C: Three studies [Vollset and Bjelke, 1983; Enstrom et al., 1992; Gey et al., 1987 and 1993] found an inverse relationship between vitamin C intake and risk of CVD. In the EPIC-Norfolk study, plasma ascorbate levels were inversely correlated to mortality from all causes, including CVD and IHD in men and women [Khaw et al., 2001]. On the other hand, further prospective studies have found either no association between vitamin C intake and risk [Rim et al., 1993] or only a weak and non-significant trend [Stampfer et al., 1993; Knekt et al., 1994].

b) Vitamin E: The two largest prospective dietary intake studies so far [Rimm et al., 1993; Stampfer et al., 1993] found a significant inverse correlation between vitamin E intake and the incidence of CVD. Others such as the Basel Prospective Study [Gey et al., 1993; Gey and Puska, 1989], that followed 2,000 Swiss males with a mean age of 62 years for 12 years, showed that there was no relationship between the incidence of CVD or stroke and plasma vitamin E levels (probably because the Basel population already had high plasma α-tocopherol levels [median 34.6 μmol/L]).

c) β-Carotene: Subjects with low plasma β-carotene and vitamin C had the highest risk for IHD and stroke in the Basel study. In a case-control study [Kardinaal et al., 1993] which measured vitamin E and β-carotene in adipose tissue of patients who had an acute MI and in controls, the former had significantly lower plasma levels of β-carotene. A 75% lower risk for fatal MI was found in the highest quartile of carotene intake [Gaziano et al., 1995; van Poppel, 1996]. Some reviews of observational studies [Gaziano and Hennekens, 1993; Tavani and La Vecchia, 1999] also suggest that carotenoid or β-carotene-rich diets prevent CVD.

d) Polyphenols: One of the largest studies undertaken to examine the possible link between flavonoid intake and CVD has shown a significant lower incidence of coronary heart disease among 805 elderly men [Hertog et al., 1993]. A significant decrease in risk was found in men consuming more than 18.3 mg flavonoids/day (552 men over 15 years, with no previous stroke) [Keli et al., 1996]. Tea, onion and apple consumption showed similar effects [Knekt et al., 1996; Peters et al., 2001; Rimm et al., 1996; Yochum et al., 1999].

Some large-scale, controlled antioxidant supplementation trials specifically designed to evaluate the effect of vitamin E, vitamin C, or β-carotene on the incidence of CVD have been conducted as well and are listed in **Table 1.4**. The outcomes are less promising than those of observational studies, and to a certain extent contradictory. The interpretation of results is complex due to several reasons. Often surveys are not long enough and lack health-relevant end points, or the effects on chronic diseases that develop over the years are neglected. The ability of controlling compliance in long-term prospective studies is however quite limited. High-risk populations are often monitored, and results are thus not predictive for healthy subjects. Precise monitoring of food intake (e. g. through food frequency questionnaires) and regarding individual factors like age, gender, smoking, or physical activity in the evaluation of outcomes is indispensable.

Several theories have been proposed to explain the discrepancies between observational and intervention studies. It has been speculated that the dose of the vitamin supplements was too low for risk populations. It has also been suggested that the biological activity shown in observational studies was possibly assigned to wrong dietary constituents. Pro-oxidant effects of antioxidants, which are observed under certain *in vitro* conditions, cannot be excluded as the cause underlying failure of intervention [Mensink et al., 2003]. It should be noted that double-blind, placebo controlled studies with nutritionally relevant doses have not been undertaken yet.

Table 1. 4 Intervention studies examining cardiovascular disease end-points

Study		Antioxidant or Mixture	Effect	Reference
Heart Protection Study	20,536 with coronary/arterial disease or diabetes. 5 years	600 mg vitamin E; 250 mg vitamin C; 20 mg β-carotene	None	Heart Protection Study collaborative group, 2002
Cancer prevention study	29,584 men and women. 5.25 years	30 mg vitamin E; 15 mg β-carotene; 50 mg selenium	None	Blot et al., 1993
		Vitamin E		
Cambridge Heart Oxidation Study (CHAOS)	Studies on patients with pre-existing disease or who were smokers). 2,002 participants. 1.4 years	536 or 567 mg α–TE/day	Prevention. Improvement in risk of a second cardiovascular event but no improvement in overall mortality. 77% reduction in fatal MI 47% reduction in fatal and nonfatal MI	Stephens et al., 1996
α-Tocopherol, β-carotene Cancer Prevention Study (ATBC)	29,133 male smokers. 6 years	50 mg α–tocopherol	Small decrease of fatal CHD; increased risk of haemorrhagic stroke	Virtamo et al., 1998
Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico (GISSI)	11,324 patients with recent MI. 3.5 years	300 mg α–tocopherol and/or 1 g ω-3-PUFA	None	Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico (1999)
Heart Outcomes Prevention Evaluation (HOPE)	9,541 high-risk men and women. 4–6 years	400 IU vitamin E/day and/or 10 mg ramipril	None	Lonn et al., 2001
Primary Prevention Project (PPP)	4,495 participants	300 mg dl-α-tocopherol	53% reduction in peripheral artery disease only	Kochsiek et al., 2001

Table 1. 4 *(continued)*

		β-Carotene		
β-carotene and retinal efficacy trial (CARET)	14,254 individuals. Over 4 years	30 mg β-carotene; 25,000 IU retinyl palmitate	Increased risk of cardiac death	Goodman et al., 1996
Physicians Health Study	22,701 males, smokers and non-smokers. 12 years	β-carotene 50 mg alternate days	None	Hennekens et al., 1996
ATBC	29,584 males, 6 years	(a) 20 mg β-carotene; or (b) 20 mg β-carotene and 50 mg vit. A	No effect. Increased risk for those with previous myocardial infarction	Virtamo et al., 1998

Adapted from Lindsay and Astley, 2002

1. 5 Dietary Micronutrients with Antioxidant and Further Anti-atherogenic Properties

1. 5. 1 Vitamin E

Vitamin E is a generic term comprising a series of naturally occurring tocopherols and tocotrienols (α-, β-, γ-, δ-isomers) with three chiralic centres all having R-configuration. Intake of α- and γ-tocopherol greatly supersedes that of β- and δ-tocopherol. Main dietary sources of α-tocopherol are vegetable oils (olive, sunflower, rapeseed), foods containing or made of such oils (margarine, bakery products) and grains, legumes and dairy foods. γ-Tocopherol predominates in soy-, corn- and rapeseed oils and in cacao butter. Animal-derived products contain mainly α-tocopherol. The recommended dietary allowance (RDA) proposed in the newest report on dietary reference intakes for antioxidants in the USA is 15 mg/day of 2R-stereoisomeric forms of α-tocopherol [Standing Committee on the Scientific Evaluation of Dietary Reference, 2000], whereas estimated recommended intakes set by the German, Austrian and Swiss Nutrition Societies are 14 and 12 mg α-tocopherol equivalents for males and females, respectively [German, Austrian and Swiss Nutrition Societies, 2000]. α-Tocopherol equivalents involve the contribution of all eight naturaly occurring forms of vitamin E and of all seven stereoisomers of RRR-α-tocopherol, after adjustment for bioavailability using previously determined equivalence (e.g., γ-tocopherol is assumed to have 10% the availability of α-

tocopherol). α-Tocopherol plasma concentrations below 12 μmol/l are considered as indicative of vitamin E deficiency [Standing Committee on the Scientific Evaluation of Dietary Reference, 2000].

Absorption of tocopherols and tocotrienols takes place mainly in the proximal intestine [Muller et al., 1974], comprising the incorporation into mixed micelles and subsequent uptake by enterocytes via passive diffusion. Total absorption rates between 25 and 75% have been reported [Cohn, 1997], being dependent on a number of factors. For instance, in patients with cholestatic liver disease or pancreatitis where secretion of bile acids, pancreatic juice, or both, is severely diminished, a concurrent vitamin E malabsorption has been shown [Traber and Sies, 1996]. In addition, a pre-requisite for micelle formation is the simultaneous intake of fat, which is every time ingested in different amounts and thus stimulates bile flow and secretion of pancreatic enzymes distinctly. Micelle formation might be affected by dietary fibre or substances that help decrease fat absorption (orlistat, cholestyramines, stanol and sterol esters in margarines), which have various concomitant effects on vitamin E absorption [Melia et al., 1996; Gylling et al., 1999; Kersting et al., 2000]. The type of fat vitamin E is associated with is also important, e. g., absorption is much more efficient in the presence of medium-chain triglycerides rather than long-chain triglycerides [Gallo-Torres, 1980].

Enterocytes incorporate tocopherols into chylomicrons. These are transferred into the circulation via the lymphatic system [Traber and Sies, 1996]. At this stage, discrimination between different forms of vitamin E has been excluded [Traber and Kayden, 1989]. Before chylomicron remnants enter the liver, some vitamin E has already been transferred to other lipoproteins [Massey, 1984] and to tissues [Traber et al., 1985]; this is likely the major route by which tissues take up vitamin E forms other than RRR-α-tocopherol.

In hepatocytes, the α-Tocopherol Transfer Protein (α-TTP) selectively enriches very low-density lipoproteins (VLDL) with the 2R-stereoisomers of α-tocopherol. Relative to RRR-α-tocopherol, the affinity of α-TTP is 38% towards β-tocopherol, 9% to γ-tocopherol, 2% to δ-tocopherol, and 12% to α-tocotrienol [Hosomi et al., 1997]. The absolute configuration at the C2-position is determinant for binding to the α-TTP: there is a marked preference for 2R isomers; after a single dose or long-term supplementation with RRR-α-tocopherol, twofold higher plasma concentrations are reached as compared to equal doses of all-rac-α-tocopherol [Burton et al., 1998].

Differences between plasma concentrations of α-tocopherol and those of other forms of vitamin E, between the stereoisomers of α-tocopherol, and to some extent between tissue concentrations of all vitamers reflect the differences in affinity of α-TTP. Thus, α-tocopherol accounts for 90% of the human body's tocopherol content, even though dietary intake of γ-tocopherol equals or supersedes that of α-tocopherol in USA [Cohn et al., 1992]. Plasma or serum concentrations of α-tocopherol are typically 20–35 µmol/L (or 4.5–6.0 µmol α-tocopherol/mmol cholesterol); γ-tocopherol concentrations are 5–15% those of α-tocopherol. Approximately half of the total α-tocopherol in blood is found in low-density lipoproteins (LDL), the major carriers for cholesterol and α-tocopherol, providing tissues with both via the LDL receptor; the other half is evenly distributed between VLDL and high-density lipoproteins (HDL) [Ribaya-Mercado et al., 1995].

Nevertheless, the large inter-individual differences in plasma vitamin E concentrations in response to vitamin E supplementation may only be explained as result of an interaction of diverse factors. Mutations in the α-TPP gene, genetic polymorphisms in apolipoproteins, lipid processing enzymes, and lipoprotein receptors seem to be involved. AVED (Ataxia with Vitamin E Deficiency) patients have extremely low plasma α-tocopherol levels due to a very low α-TPP activity [Schuelke et al., 2000]. Different phenotypes of apo E have an influence on vitamin E levels [Peroutka and Dreon, 2000]. α-Tocopherol plasma concentrations only increase to a plateau level of about 70–80 µmol/l after long-term supplementation with high doses, and α-tocopherol plasma concentrations linearly correlate with total plasma lipids [Schultz et al., 1995]. Thus, it has been speculated that plasma lipids determine the individual transport capacity for α-tocopherol in blood.

Recently, a 46-kDa α-tocopherol-associated protein (TAP) with a widespread distribution in human tissues has been identified [Stocker et al., 1999]. TAP has been suggested to play a central role in tocopherol transport, signaling, secretion and/or adjustment of the tocopherol composition of membranes [Zimmer et al., 2000] and has structural motifs that place it in a family of hydrophobic ligand-binding proteins.

The current knowledge on vitamin E metabolism presumes a hydroxylation (ω-oxidation) step and subsequent shortening of the phytyl side chain of tocopherols and tocotrienols, leaving the chroman ring intact, and resulting in the formation of water-soluble carboxy-ethyl-hydroxy-chromans (CEHC) (**Figure 1.5**), which have

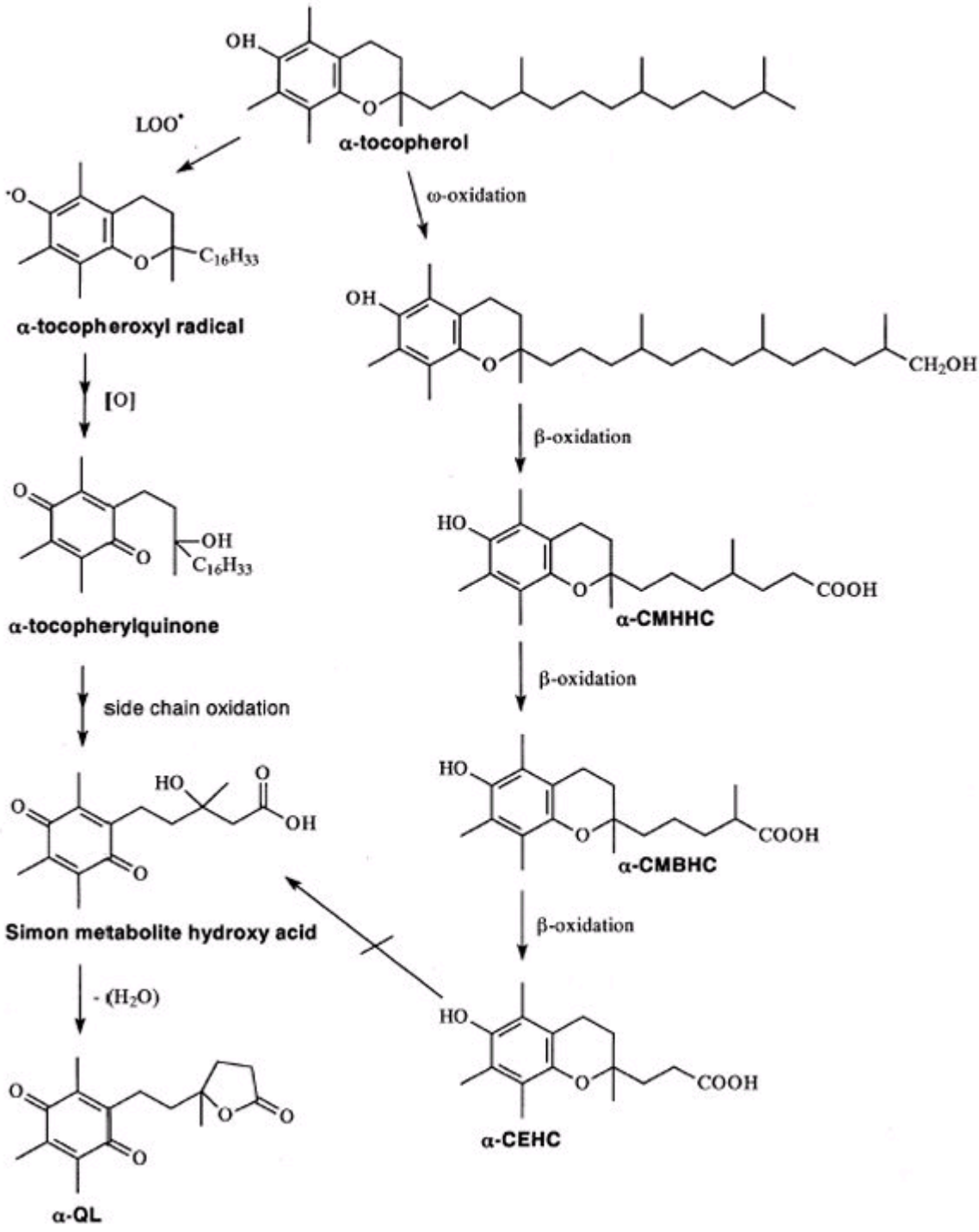


Figure 1. 5 α-Tocopherol degradation *in vivo* following two different metabolic pathways. Taken from Stahl et al., 2002.

been detected in blood and urine [Schultz et al., 1995; Stahl et al., 1999; Swanson et al., 1999]. Precursors of α- and γ-CEHC have been detected in urine and accordingly named α- and γ-CMBHC (Carboxy-Methyl-Butyl-Hydroxy-Chroman) or CPHC (Carboxy-Pentyl-Hydroxy-Chroman) [Schuelke et al., 2000; Parker et al., 2000].

Urinary α- and γ-CEHC appear as glucuronic acid and sulfate conjugates [Schultz et al., 1997]; in serum, about 30% of α-CEHC is present as glucuronide conjugate, while γ-CEHC does not appear to be conjugated [Stahl et al., 1999]. γ-CEHC has been shown to exhibit antioxidant activity [Appenroth et al., 2001] and other biological effects, such as inhibition of inflammatory reactions both at cellular level [Jiang et al., 2000] and in rats [Jiang et al., 2003], as well as acting as a natriuretic factor in rats [Wechter et al., 1996].

For α-CEHC, functions *in vivo* are unknown. It has been proposed as a marker for super-optimal vitamin E status, since its very low urine levels only increase significantly when α-tocopherol plasma concentrations reach a threshold of 30–50 μmol/L, corresponding to 7–9 μmol/g total lipids [Schultz et al., 1995].

Oxidation of α- and γ-tocopherol to α- and γ-CEHC has been shown to be mediated by either CYP3A, the major class of cytochrome P450 enzymes in mammalian liver [Birringer at al., 2001], and/or CYP4F2 [Sontag and Parker, 2002], which demonstrates the involvement of the xenobiotic-detoxicating system in vitamin E metabolism. Thus, more information in the area of genetic polymorphisms or inductive/inhibitory mechanisms of these isoenzymes might help to explain the constant individual but highly variable inter-individual response to repeated doses of vitamin E [Roxborough et al., 2000].

- Biological functions and anti-atherogenic properties.

Even though the first physiological function attributed to vitamin E was its role in fertility and foetal development, underlying mechanisms remain unclear [Hoppe and Krennrich, 2000]. Vitamin E acts as an antioxidant by scavenging free radicals, which can directly or indirectly initiate or propagate lipid peroxidation. Tocopherols also quench singlet oxygen and might protect membranes against this ROS. α-Tocopherol reacts slowly with superoxide and at an almost diffusion-controlled rate with hydroxyl radicals.

Tocopherols and tocotrienols inhibit lipid peroxidation, e. g. in LDL, largely because they scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side-chains or with membrane proteins (the rate constant for the reaction of α-tocopherol with peroxyl radicals is some four orders of magnitude higher than that for reaction of peroxyl radicals with lipids). In membranes,

vitamin E is anchored in the hydrocarbon part of the bilayer by the phytyl tail, positioning the chroman ring, which is responsible for the antioxidant activity, towards the membrane interface, probably rendering it capable of interacting both with free radicals in the aqueous phase and the membrane.

The formed tocopheroxyl radicals might react with a further peroxyl radical to give non-radical products (tocopherylquinones, see **Figure 1.5**). One tocopherol molecule is thus capable of terminating two peroxidation chains (stoichiometric factor of 2). Chemical reactivity towards peroxyl radicals differs considerably among the tocopherols, in the sequence α->γ->δ->β-. Some reducing agents such as ascorbate, cysteine and glutathione can regenerate vitamin E from the tocopheroxyl radical, which could explain the synergistic effects and inhibition of lipid peroxidation by vitamin E and ascorbate. However, there is still some doubt as to whether this reaction occurs *in vivo*.

The tocopherol quinone is subsequently transformed to both tocopheronic acid and the tocopheronolactone derived therefrom, the so-called Simon metabolites (**Figure 1.5**). Although these have been detected as glucuronides or sulfates in urine, some controversy exists concerning their authenticity, and it has been suggested that they are artefacts generated during sample preparation [Schultz et al., 1997]. Data from other groups propose that they are to some extent authentic [Pope et al., 2000]. Tocopherols can reduce Fe(III) to Fe(II) and Cu(II) to Cu(I) and thus may exert some pro-oxidant effects *in vitro*. The α-tocopheroxyl radical can abstract hydrogen from PUFA to yield alkenyl radicals [Carr et al., 2000], although the rate constant is about five orders of magnitude lower than the rate constant for the reaction of peroxyl radicals with PUFA. It is not clear if this pro-oxidant action has a functional meaning *in vivo*.

Apart from its antioxidant activity, further anti-atherogenic properties of α-tocopherol have been demonstrated *in vitro*. In several experiments on cell culture the four tocopherol isoforms were shown to exert different biological effects. Non-antioxidant functions unique to α-tocopherol have thus been postulated and later demonstrated experimentally. α-Tocopherol was found to induce vascular smooth muscle cell growth arrest [Boscoboinik et al., 1991]. Inhibtion of protein kinase -C (PKC) activity was found to be the basis of this effect and of further protective activities of α-tocopherol, e. g., the inhibition of thrombin-induced endothelin secretion from endothelial cells [Martin-Nizard et al., 1998] or the impaired assembly

of NAD(P)H oxidase in monocytes [Cachia et al., 1998]. α-Tocopherol inhibited the aggregation of human platelets by a PKC-dependent mechanism both *in vitro* and *in vivo*. α-Tocopherol was found to activate the phosphatase PP2A, which dephosphorylates PKC [Ricciarelli et al., 1998]. PKC is currently accepted as a common denominator in a number of cell events regulated by α-tocopherol, such as cell proliferation, cell adhesion, or enhancement of immune response [Ricciarelli et al., 2002].

There is also evidence of transcriptional regulation by α-tocopherol, e. g., it inhibits liver collagen α1(I) gene expression [Chojkier et al., 1998], and upregulates α-tropomyosin expression in rat vascular smooth muscle cells [Aratri et al., 1999]. In SMC and monocytes/macrophages, the oxLDL scavenger receptors SR-A and CD36 are down-regulated at transcriptional level by α-tocopherol [Teupser at al., 1999; Ricciarelli et al., 2000]. In rats, liver α-TPP and its mRNA are modulated by vitamin E deficiency [Shaw and Huang, 1998]. The involvement of PKC in these events has not always been examined.

1. 5. 2 Carotenoids

Carotenoids are a family of tetraterpenes (C40) with a central polyene chain differently substituted at the ends. At least 60 carotenoids occur in fruit and vegetables consumed by humans. Besides the provitamin A carotenoids (α- and β-carotene and β-cryptoxanthin), lycopene and the xanthophylls lutein and zeaxanthin are the major dietary carotenoids (**Figure 1. 6**). Their major roles in plants are light harvesting and quenching excited states of oxygen that might be formed during photosynthesis. There are no recommended daily intakes of the carotenoids, although a number of conversion factors have been suggested for the conversion of retinol precursors to vitamin A [Southgate, 2002]. The carotenoid profile in human plasma is determined by the variety of fruits and vegetables ingested with the diet.

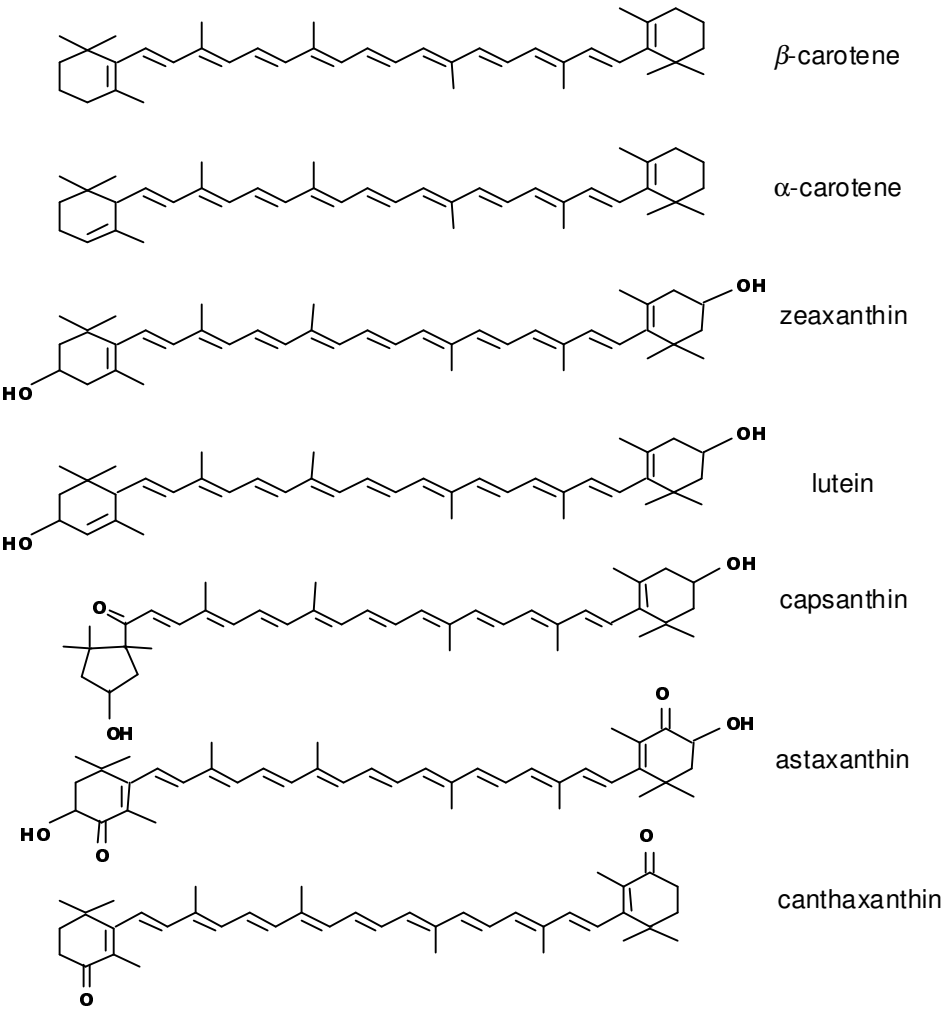


Figure 1. 6 Molecular structure of some dietary carotenoids.

Location and physical form of carotenoids in foods are important determinants of their bioavailability [Castenmiller and West, 1998; de Pee et al., 1998]. Disruption of cellular structure (e.g. during mastication) or the transfer of carotenoids to the lipid phase during processing (e.g. cooking with oil) are excellent facilitators of carotenoid absorption. Fatty acid esters of carotenoids are hydrolysed in the intestinal lumen before mucosal uptake, most likely by the carboxylic ester hydrolase secreted by the pancreas [Wingerath et al., 1995]. Co-ingestion of fat with the meal, and the type of dietary fat ingested are other factors with impact on bioavailability. Alcohol intake and smoking have been identified as having an adverse effect on serum carotenoid levels in several population-based studies [Albanes et al., 1997].

Incorporated in mixed micelles, carotenoids are absorbed by the mucosa of the small intestine, mainly in the duodenum. No active carrier or transporter proteins for carotenoids are known. In enterocytes, some cleavage to retinal/retinol occurs (see below) and both the remaining carotenoid fraction and the cleavage products are

incorporated into chylomicrons, secreted into the lymph and subsequently into the blood. In the liver, hepatocytes integrate most of the carotenoids into lipoproteins, which are then released into the systemic circulation [Stahl et al., 2002].

For humans, quantitative data on carotenoid absorption are scant. It is accepted that absorption efficiency decreases as the dose of the carotenoid increases, and the carotenoid plasma profile is dependent on the actual dietary carotenoid intake. Under normal dietary intake the hydrocarbon carotenoids (mainly β-carotene and lycopene) and the oxo-carotenoids (mainly lutein) are present at concentrations of 0.1 to 0.6 μmol/l in blood [Polidori et al. 2001]. Carotenes are mainly transported in LDL and oxo-carotenoids in HDL and LDL. It has been suggested that carotenoids are exchanged between lipoproteins, and apparently there is a greater exchange of xanthophylls than of carotenes [Stahl et al, 2002].

Not all factors controlling carotenoid uptake into tissues are yet identified [Stahl et al., 1992]. The enrichment of the human macula lutea specifically with lutein and zeaxanthin and the pronounced presence of β-carotene in the pineal gland and *corpus luteum* of cattle suggests a selective uptake. Lycopene concentrations are highest in testis and adrenal tissue [Clinton, 1998]. Hydrocarbon carotenoids accumulate in the adipose tissue [Su et al., 1998] and skin [Ribaya-Mercado et al., 1995]. Erythrocytes, leucocytes, and cell membranes also contain carotenoids [Fotouhi et al., 1996].

Pro-vitamin A carotenoids are partly cleaved to retinoid compounds in the enterocyte and, to a lesser extent, in the liver and other tissues. Central cleavage of the polyene chain is catalysed by β-carotene-15,15'-oxygenase, yielding two molecules of retinal. Isolation and characterization of this enzyme has confirmed a cytosolic location, not bound to membranes, and the requirement of ferrous iron [Redmond et al., 2001; von Lintig and Vogt, 2000; Wyss et al., 2000]. Excentric cleavage has been found to occur in vitro leading to apo-carotenals [Wang et al., 1991]. Non-enzymatic excentric oxidation might also occur under conditions of oxidative stress. The metabolism of non-pro-vitamin A carotenoids is less known. Two lycopene metabolites in cycled form [Khachik et al., 1997] and dehydration products of lutein have been found. Both central [Redmond et al., 2001] and excentric [Kiefer et al., 2001] cleavage of lycopene have been reported and found to be very limited.

In both the intestine and the liver, retinal is reduced to retinol and stored as retinyl-palmitate. Plasma retinoid-binding protein and lipoproteins are responsible for transport in serum and for the regulation of blood levels of retinoids.

- Antioxidant an other biological properties.

Carotenoids have been reported to react with ROS and virtually all radical species likely to be encountered in a biological system and thus protect cells from oxidative stress [Sies at al., 1992]. Mechanisms include:

- Quenching triplet-state sensitizers, such as flavins and porphyrins, which may either abstract a hydrogen atom or an electron from various molecules leading to further radical-catalyzed damage or react with ground-state oxygen to form singlet oxygen [Young et al., 1996; Sundquist et al., 1993].
- Quenching singlet oxygen directly, which results almost entirely from energy transfer and yields ground-state oxygen and a triplet excited carotenoid. The energy is then dissipated in the solvent as heat [Stahl and Sies, 1993]. The contribution of chemical quenching is thereby minor (<0.5% for β-carotene).
- Radical addition with adduct formation: a lipid peroxyl radical may add at any place along the carotenoid polyene chain, resulting in a carbon-centered radical, which is resonance-stabilized and interferes with the propagation step in lipid peroxidation [Sies at al., 1992]. It has been postulated that at high oxygen tensions this radical could react reversibly with molecular oxygen to form a new peroxyl radical. By cleavage of the resulting peroxyl bond this carotenoid peroxyl radical might generate additional radicals, resulting in a pro-oxidative effect [Truscott, 1996]. Recent investigation reported, however, that addition radicals would not react with molecular oxygen even at high pressures either in polar nor in non-polar solvents [El-Agamaey and Mc Garvey, 2003]. At low partial oxygen pressures β-carotene is an efficient antioxidant [Burton and Ingold, 1984], interrupting substrate oxidation by peroxyl radicals. At physiological levels of oxygen carotenoids have antioxidant activity.
- Electron transfer: these reactions result either in the formation of the cation radical $CAR^{+\cdot}$, the anion radical $CAR^{-\cdot}$ or in the generation of an alkyl radical $CAR^{\cdot}$ (Liebler, 1993)
- Hydrogen abstraction at the allylic position: Woodal et al. (1997) observed the formation of the 4-methoxy and 4, 4´-dimethoxy derivatives (non-radical products) of

β-carotene when reacted with peroxyl radical initiators in the presence of methanol, which is explained through this mechanism.

Some carotenoids have been shown to exert, beyond the role of provitamin A carotenoids in the vision process, non-antioxidant biological properties which may underlie beneficial effects observed in epidemiological studies in the field of prevention of neoplastic disorders. Lycopene inhibits growth of mammary, endometrial and lung cancer cells [Levy et al., 1995]. Lycopene, β-carotene [Prakash et al., 2001], and retinoic acid [Zhu et al., 1997] suppress growth of different lines of human breast cancer cells. Carotenes, lycopene, some xantophylls and their metabolites show a stimulatory effect on gap junctional intercellular communication [see Stahl et al., 2002 for review]. Besides, the role of retinoids in a large number of processes related to embryogenesis, morphogenesis, growth, differentiation and fertility is widely accepted [Hansen et al., 2000].

Anti-atherogenic properties of carotenoids or vitamin A have been explained almost exclusively through antioxidant activities. However, it has been suggested that retinoid signaling is involved in atherosclerosis since *in vivo* studies of retinoid administration after vascular injury have documented positive changes in vessel geometry, such as attenuation of neointimal mass or accelerated reendothelialization. A panel of retinoids with different selectivity for retinoid receptors was shown to inhibit dose-dependently platelet-derived growth factor (PDGF)/insulin-stimulated growth of human coronary artery SMC [Wakino et al., 2001]. Retinoic acid and synthetic retinoids were shown to regulate proliferation, migration and differentiation of SMC by a retinoic acid receptor (RAR)-α dependent signaling pathway [Neuville et al., 1999]. Furthermore, all-*trans*-retinoic acid was found to stimulate CD36 expression through RAR binding and thus increase uptake of oxLDL in macrophages [Wuttge et al., 2001].

1. 5. 3 Polyphenols

Polyphenols are a large variety of phenylpropanoid- or polyacetate-based compounds which are almost exclusive of higher vascular plants, including cinnamic acid derivatives (e. g. chlorogenic acid), diarylheptanoids (curcuminoids), flavonoids, and proanthocyanidins [Stahl et al., 2002]. Over 4000 different flavonoids have been isolated from plants (**Figure 1.7**). In the human diet, chlorogenic acid (coffee), ferulic

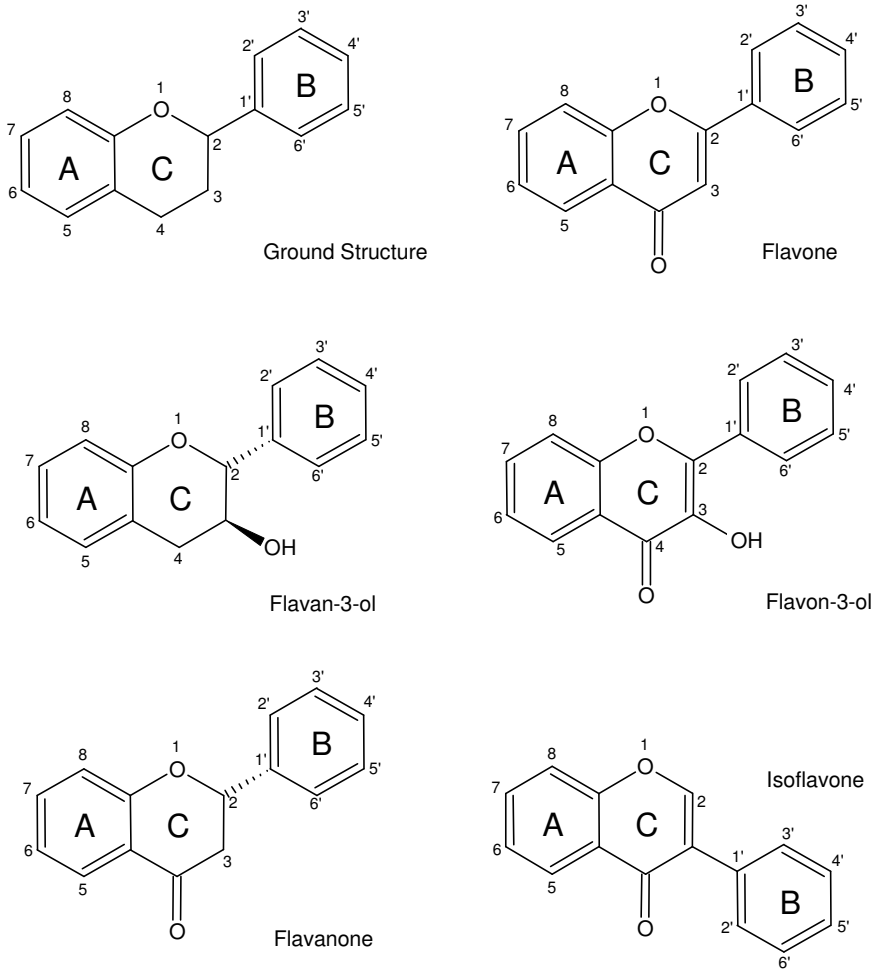


Figure 1. 7 Subclasses of the flavonoid family

acid (cereals), flavones and flavonols (onions, tea, apples), catechins and other flavan-3-ols (tea, grapes, cocoa), and isoflavones (soy and black beans) constitute the major classes. There is no recommended daily intake for these compounds. However, as a rough guide, the total polyphenol intake probably lies between 100 and 1000 mg / day.

In saliva, some degalloylation of the flavan-3-ol gallate esters, such as epigallocatechin gallate, already occurs [Yang et al., 1999]. In the stomach, flavonoid glycosides [Hollman and Katan, 1999] and hydroxycinnamate esters [Rechner et al., 2001] are not modified, but procyanidin oligomers have been shown to decompose on interaction with acidic gastric juice *ex vivo* essentially to epicatechin monomeric and dimeric units [Spencer et al., 2000]. The large amounts of epicatechin released can be absorbed by enterocytes.

Major factors influencing extent and rate of absorption of phenols are: (i) uptake and interactions in the small intestine, including conjugation; (ii) metabolism by the

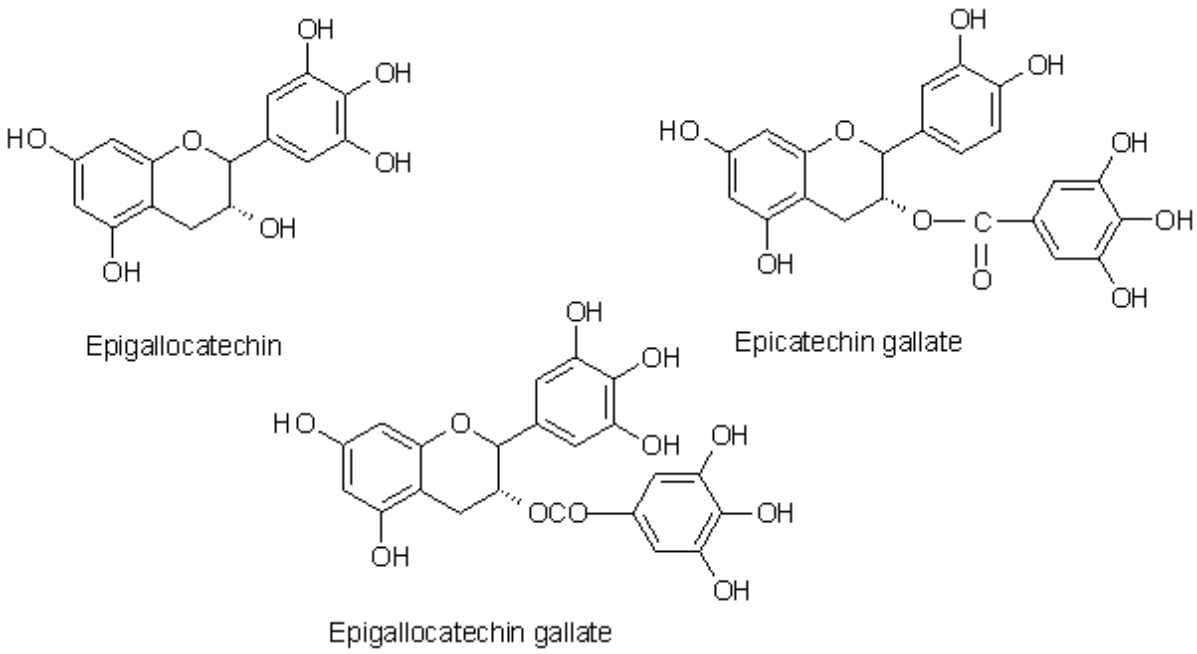


Figure 1. 8 Molecular structures of gallate derivatives of epicatechin

bacterial microflora of the colon and subsequent absorption of the resulting metabolites, and (iii) biliary excretion after hepatic biotransformation and/or conjugation [Lin et al., 1999].

The sugar moiety plays an important role in the absorption of flavonol glycosides. Pharmacokinetic data suggest that quercetin glucoside is absorbed from the small intestine, whereas quercetin rutinoside was absorbed from the colon after deglycosylation [Hollman et al., 1999]. Glucosylated flavonoids might be carried into the small intestine enterocyte *via* active transport (intestinal Na^+-glucose cotransporter SGLT1), which has been shown *in vitro* for quercetin glucosides [Gee et al., 1998]. Alternatively, they might be hydrolysed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane [Day et al., 2000], and the aglycone absorbed by passive diffusion in the small intestine. On the other hand, in ileostomy patients, who lack a colon and thus bacterial flora, absorption of the quercetin glucosides from onions (52%) was better than that of the pure aglycone (24%) [Hollman et al., 1995], which confirms that some glycosides are absorbed without prior hydrolysis by microorganisms. Also in this model, it was found that 33% of chlorogenic acid, the major dietary hydroxycinnamic acid compound, was absorbed in the small intestine.

Conjugation of hydroxyl groups with glucuronic acid, sulfate or glycine or O-methylation of the catechols is reported is a crucial step in the metabolism of plant phenols. Flavonoids are substrates for small intestinal UDP-glucuronyl transferases and catechol-O-methyltransferases, whose presence in enterocytes has also been suggested [Okushio et al., 1999]. Conjugation reactions such as glucuronidation and methylation occur in the jejunal and ileal sections of the small intestine [Manach et al., 1998; Spencer et al., 1999]. Deglycosylation of glucosides can occur previously in the small intestine or afterwards in the liver, depending on the nature and position of the sugar residue. Further modificaons in the liver include methylation, sulfation and glucuronidation.

Catabolism and scission of the flavonoid rings and demethylation and dehydroxylation of the resulting phenolic acids, are, to a great extent, catalysed by enzymes present in intestinal microorganisms [Hackett, 1986]. Bacterial enzymes may catalyse hydrolysis of glucuronides, sulfates and glycosides, dehydroxylation, demethylation, reduction of double bonds, ring cleavage, and decarboxylation of some phenolic acids [Hollman and Katan 1998].

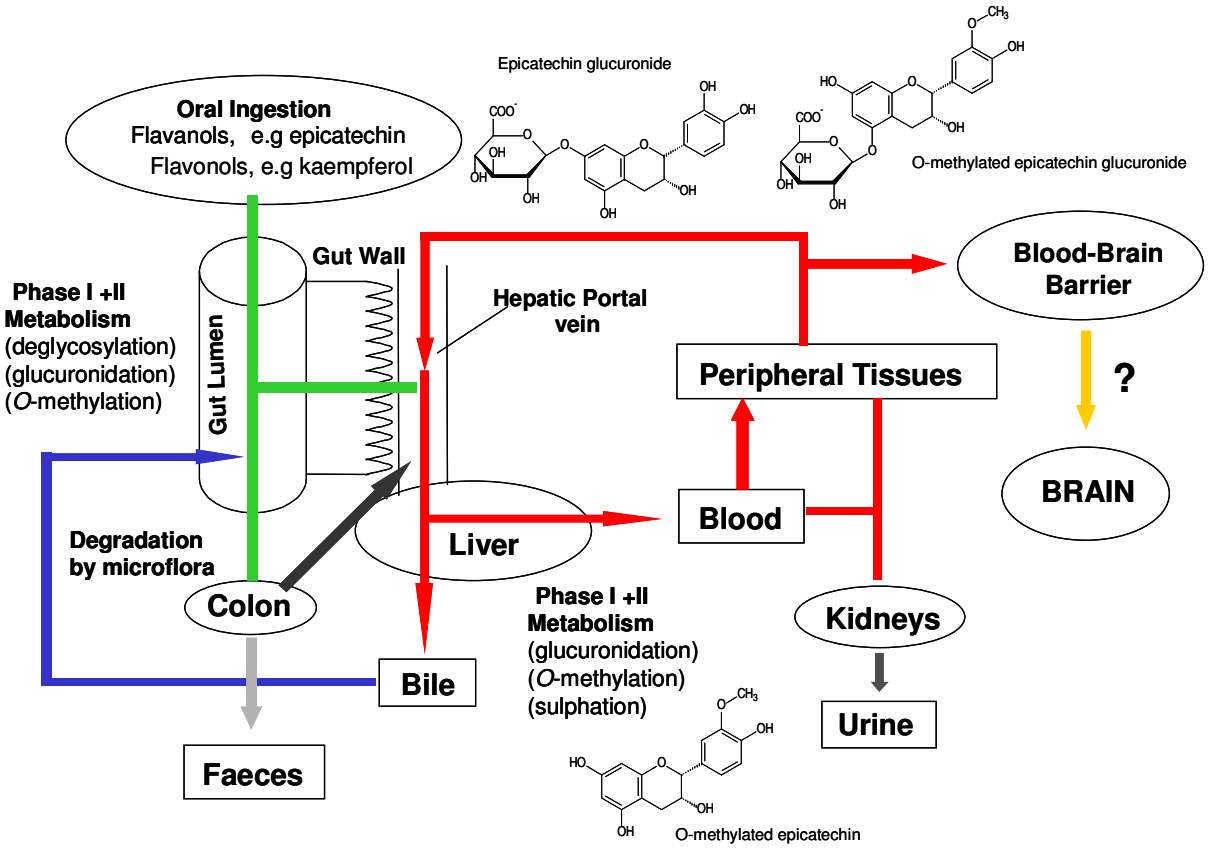


Figure 1. 9 Phenol absorption and metabolism. Changes in epicatechin structure are illustrated as an example. Taken from Stahl et al., 2002

As far as *flavonols* are concerned (**Figure 1.7**), evidence for the presence of quercetin conjugates in humans has been obtained. Quercetin aglycone could not be detected in plasma [Manach et al., 1998]. Major urinary and biliary conjugates are the glucuronides and sulfates of quercetin, 3′-*O*-methyl-quercetin and 4′-*O*-methyl-quercetin [Gross et al., 1996]. After consumption of onions by humans, the major plasma metabolites were quercetin 3′-sulfate and quercetin 3-glucuronide [Day et al., 2001]. Flavonols are degraded to a great extent by colonic microflora to phenylacetic and phenylpropionic acids. For quercetin, reported metabolites are 3-hydroxycinnamic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid) and 3-methoxy, 4-hydroxyphenylacetic acid (homovanillic acid) [Stahl et al., 2002].

Oral administration of *flavones* or *flavonones* leads to the formation of 3-hydroxyphenylpropionic acid, 3-hydroxycinnamic acid, and 4-hydroxy-3-methoxy-phenylpropionic acid [Booth et al., 1958; Scheline, 1991].

Regarding *flavan-3-ols*, especially epicatechin metabolites, the most predominant conjugates in human plasma and urine are glucuronides and sulfates of epicatechin and 3′-O-methyl epicatechin [Donovan et al., 1999]. Methylated and glucuronidated metabolites have not been detected in rat bile. The mechanism of flavan-3-ol metabolism is supposed to involve glucuronidation in the small intestine followed by O-methylation in the liver and kidney [Piskula and Terao, 1998]. Specific markers of catechin metabolism are the valerolactones, which can be detected in human plasma and urine after a single ingestion of green tea [Li et al., 2000].

Ferulic acid has long been determined as a biomarker for the absorption and metabolism of both dietary *caffeic* and *ferulic acid derivatives* [Booth et al., 1957]. The large intestine is the site of absorption and metabolism of quinic acid esters such as chlorogenic acid [Rechner et al., 2001]. Colonic microflora provides esterase activity [Plumb et al., 1999] and additionally could be, with the liver, responsible for O-methylation of caffeic acid to yield ferulic and isoferulic acids [Chesson et al., 1999].

- Antioxidant effects and further biological activities.

Plant phenols are antioxidants that can prevent lipid peroxidation and thus are potent inhibitors of the modifications of LDL. Mechanisms include [Briviba and Sies, 1994):

- scavenging initiating radicals such as $^{\cdot}OH$, and $O_2^{\cdot-}$
- binding metal ions
- scavenging lipid peroxyl radicals
- inhibiting enzymatic systems responsible for free radical production, such as cyclooxygenases, lipoxygenases, myeloperoxidase and xanthine oxidase.

The formation of 5-hydroperoxy-eicosatetraenoic acid (5-HpETE) by reaction of arachidonic acid with recombinant human 5-lipoxygenase was significantly inhibited by (-)-epicatechin in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) of 22 µmol/L. Among the procyanidin fractions isolated from the seeds of *Theobroma cacao*, only the dimer fraction and, to a lesser extent, the trimer through pentamer fractions exhibited comparable effects [Schewe et al., 2002]. Quercetin and other flavonoids were found to modulate the time course of the reaction of rabbit reticulocyte and soybean 15-lipoxygenases. The flavone luteolin turned out to be the most potent inhibitor of the mammalian enzyme with an IC_{50} of 0.6 µmol/L followed by baicalein (1 µmol/L) and fisetin (1.5 µmol/L) [Sadik et al., 2003].

Furthermore, experimental work in the last years has provided evidence of non-antioxidant functions of flavonoids which may be related to cancer preventive effects. Epicatechin exhibited stimulatory effects on GJC in WB-F344 rat liver epithelial cells after 24-72 h of incubation; inhibitory effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) on GJC were largely suppressed when epicatechin or genistein (40 µmol/L) were present during the incubation [Ale-Agha et al., 2002]. Other effects include: suppression of vascular smooth muscle cell proliferation by epigallocatechin, which partially inhibited the JNK/stress-activated protein kinase (SAPK) signal transduction pathway [Lu et al., 1998]; baicalein, a flavonoid from a Chinese herb (*Scuttelaria baicalensis*) was shown to exert an inhibitory effect on the proliferative response of PDGF on rabbit vascular smooth muscle cells [Huang et al., 1994]. In addition, several flavonoids, including quercetin, were able to improve the cell glutathione status in part through regulation of γ-glutamylcysteine synthetase gene expression [Myhrstad et al., 2002].

1. 6 Aim of the Study

(1) To assess the antioxidant activity of various types of dietary micronutrients and polyphenol-containing pharmaceutical preparations by challenging with different oxidative insults in model systems. The antioxidant activity of the α-tocopherol

metabolite α-CEHC and of compounds present in the preparations should be studied as well. The effect of solvent polarity, reaction conditions and nature of pro-oxidants applied will be considered.

(2) To investigate the effects of pre-treating vascular endothelial cells (primary mouse aorta endothelial cells) with selected antioxidants (α-tocopherol, α-CEHC, epicatechin and epigallocatechin gallate) on the subsequent incubation with oxidatively-modified LDL. Along with direct or indirect effects of antioxidant nature, intervention of these compounds at functional processes (oxLDL uptake, cell viability, apoptotic death) are to be determined.

(3) To monitor both the serum levels and the degree of biotransformation of α- and γ-tocopherol (by measuring the corresponding CEHC metabolites) in smoking and nonsmoking volunteers who are given a single oral dose of vitamin E, in order to compare outcomes between both groups. In smokers, the oxidative status is increased, and xenobiotic-detoxicating enzymes (such as cytochrome P450-dependent monoxygenases) may be induced. Those two factors may thus modify the physiological levels of certain dietary antioxidants in blood.

2. MATERIALS AND METHODS

2. 1 Chemicals

Following chemicals were used:

L-ascorbic acid	Merck, Darmstadt (Germany)
astaxanthin	BASF, Ludwigshafen (Germany)
2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS)	Sigma-Aldrich, Steinheim (Germany)
2,2´-azobis(2-amidinopropane) hydrochloride (AAPH)	Polysciences, Warrington (USA)
canthaxanthin	BASF
α-carotene	BASF
caspase-3 assay kit	Sigma-Aldrich
2-(rac)-α-CEHC	BASF
2-(rac)-γ-CEHC	BASF
2´,7´-dichlorodihydrofluorescein diacetate (DCFH-DA)	Sigma-Aldrich
chlorogenic acid	Sigma-Aldrich
copper (II) chloride ($CuCl_2$)	Sigma-Aldrich
curcumin	Extrasynthese, Lyon (France)
1,1´-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanine perchlorate (DiI)	Sigma-Aldrich
epicatechin	Sigma-Aldrich
ethylenediaminetetraacetic acid (EDTA)	Merck
Folin-Ciocalteau´s reagent 2N	Sigma-Aldrich
gallic acid	Sigma-Aldrich
glutathione disulfide (GSSG)	Sigma-Aldrich
harpagoside	Phytochem, Ichenhausen (Germany)
hydrochloric acid (HCl) 25%	Merck
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Boehringer-Mannheim, Mannheim (Germany)
4-hydroxy-3-nitrobenzoic acid (HNBA)	Sigma-Aldrich
kaempferol	Extrasynthese
lutein	BASF
luteolin	Sigma-Aldrich
dimethyl sulfoxide (DMSO)	Sigma-Aldrich
3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium (MTT)	Sigma-Aldrich
nicotinamide-adenine dinucleotide phosphate, reduced (NADPH)	Roche, Mannheim (Germany)
3-nitrotyrosine	Sigma-Aldrich
R-phycoerythrin from *Porphyra tenera*	Sigma-Aldrich
potassium bromide (KBr)	Merck
potassium di-hydrogen phosphate (KH_2PO_4)	Merck
di-potassium hydrogen phosphate (K_2HPO_4)	Merck
potassium peroxodisulfate (*persulfate*) ($K_2S_2O_8$)	Sigma-Aldrich
sodium bicarbonate ($NaHCO_3$)	Merck
sodium carbonate (Na_2CO_3)	Merck
sodium chloride (NaCl)	Merck
sodium dodecyl-sulfate (SDS)	Merck

sodium sulfate (Na_2SO_4)	Merck
5-sulfosalicylic acid (SSA)	Sigma-Aldrich
tetraethylammonium hydroxide (TEAH)	Sigma
5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich
1,4-dithio-threitol (DTT)	Boehringer-Mannheim
RRR-α-tocopherol	Cognis Deutschland, Düsseldorf (Germany)
RRR-γ-tocopherol	Cognis Deutschland
R-Trolox	Sigma-Aldrich
L-tyrosine	Sigma-Aldrich
2-vinyl-pyridine (VP)	Sigma-Aldrich

All solvents used were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). Minimum quality grade was p. A. Gaseous nitrogen purity grade 5.0 was provided by Linde (Höllriegelskreuth, Germany).

The following pharmaceutical trade products containing standardized natural extracts were provided by Truw Arzneimittel (Gütersloh, Germany):

- Curcu-Truw® capsules, containing ethanolic extract of turmeric rhizomes (dry drug-to-solvent ratio 13-25:1, 27% curcuminoids); Truw Arzneimittel (Gütersloh, Germany)
- Rheuma-Sern® capsules, containing aqueous extract of devil´s claw roots (dry drug-to-solvent ratio 2:1, 2.60% harpagoside); Truw Arzneimittel (Gütersloh, Germany)
- Hepar-SL® forte capsules, containing aqueous extract of artichoke leaves (dry drug-to-solvent ratio 4-6:1); Sertürner Arzneimittel, Berlin (Germany)
- Kwai®N coated tablets, containing fresh garlic extract (standardized to 1.0-1.4% alliin); Lichtwer Pharma, Berlin (Germany)
- Ameu® soft capsules, containing salmon oil concentrate (min. 35% n-3 fatty acids); Omega Pharma, Berlin (Germany)

The vitamin E capsules used for the intervention study were from Hermes (Munich, Germany).

2. 2 Instruments

a) UV/VIS and fluorescence spectroscopy

DU 530 Life Science UV/VIS Spectrometer (Beckman, Munich, Germany)

LS-5 Fluorescence/Luminiscence Spectrometer (Perkin-Elmer, Überlingen, Germany)

Victor 1420 Multilabel Counter (Wallac, Turku, Finland)

b) Microscopy

Axiovert 100TV (Zeiss, Oberkochen, Germany) coupled with a Hamamatsu Digital Camera (Hamamatsu Photonics, Hamamatsu, Japan)

c) Centrifuges and Rotors

Hettich Universal 30 RF (Hettich, Tuttlingen, Germany)

L7-55M Ultracentrifuge / Rotor SW 41 (Beckman)

d) High Performance Liquid Chromatography

Pump: La Chrom L-7100 (Merck-Hitachi, Darmstadt, Germany)

Integrator: D-7500 Integrator 7480 (Merck-Hitachi)

Analysis of 3-nitrotyrosine:

Autosampler: 655 A-40 Autosampler 7480 (Merck-Hitachi)

Column: 4.6 x 150 mm Lichrospher 100 RP 18 (Merck)

Detector: La Chrom UV/VIS Detector L-7420 (Merck-Hitachi)

Analysis of carotenoids:

Column: 4.1 x 250 mm Spherisorb ODS2 C-18 (Bischoff Chromatography, Leonberg, Germany)

Detector: La Chrom UV/VIS Detector L-7420 (Merck-Hitachi)

Analysis of serum α- and γ- CEHC:

Column: 4 x 250 mm Lichrospher 100 RP 18, endcapped (Merck-Hitachi)

Detector: Coulochem 5100A; Analytical Cell 5011; Conditioning Cell 5021 (ESA, Inc., Chelmsford, USA)

Analysis of α- and γ-tocopherol:

Column: 4.6 x 250 mm Suplex pKb-100 (Supelco, Bellefonte, USA)

Detector: La Chrom UV/VIS Detector L-7420 (Merck-Hitachi)

2. 3 Preparation of Stock Solutions

a) Pure Compounds

Stock solutions of micronutrients were prepared at concentrations ranging from 1 to 3 mmol/L. Flavonoids and hydroxycinnamates were dissolved in ethanol, methanol/water 50:50 or acetone; α-tocopherol in ethanol; carotenoids in ethanol and dichloromethane; harpagoside in water; α- and γ-CEHC in ethanol; Trolox and ascorbic acid in water, 75 mM phosphate buffer (pH 7.0) and ethanol.

b) Pharmaceutical Preparations

Content weight of each capsule of turmeric, devil´s claw, artichoke and salmon oil was calculated as the mean difference of weight between the whole capsule and the capsule cover (n=7).

Fractions A, B and C: Five entire garlic coated tablets and the contents of four to six capsules of turmeric, devil´s claw or artichoke extract were crushed in a mortar until homogeneity of particle size. The products were treated with solvents of different lipophilicity to obtain fractions for testing. To 10 to 100 mg of powder either 1 mL of water, methanol/water 70:30, or ethanol was added. After 24 h of incubation under agitation at 4°C in the dark, all suspensions were centrifuged at 5,000 rpm for 10 min and the supernatants collected. The pellets were washed with 0.5 mL solvent, left for two hours at 4°C in the dark and centrifuged. Supernatants from the same solvent were combined. The extracts were designated as fraction A (water), fraction B (methanol/water 70:30), and fraction C (ethanol). The clear supernatants and the solutions of the pure compounds were stored at –80°C until use. Storage time was less than 7 days.

Fraction D: Extraction of highly lipophilic compounds. A highly lipophilic fraction (Fraction D) was prepared as follows. 1 g of powder was suspended in 50 mL acetone/water (75:25 v/v) for two hours in the dark and vacuum-filtered through a Büchner funnel. The residue was extracted again until the filtrate was colourless. The filtrates were transferred to a decanting funnel; 150 mL of diethyl ether was added, and the funnel was shaken. Bidistilled water was added to separate the phases. The upper phase containing the lipophilic compounds was washed several times with water. The ether solution was filtered through a solid bed of Na_2SO_4 and dried in a

rotavapor. The residue was dissolved in acetone and named fraction D. Fraction D of salmon oil was a 200 mg/mL solution of the capsule contents in hexane.

2. 4 Antioxidant Activity of Dietary Micronutrients in Model Systems

2. 4. 1 Trolox Equivalent Antioxidant Capacity (TEAC)

a) Background

The TEAC assay is based on the ability of different compounds to scavenge a long-lived free radical generated by oxidation of 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) in aqueous solution with potassium persulfate. ABTS and potassium persulfate react following a stoichiometry of 2:1. The obtained stable radical has four absorption maxima in the VIS spectrum at 415, 645, 734 and 815 nm and is blue- green colored. The half-life of the radical in solution has been estimated to be 24 h.

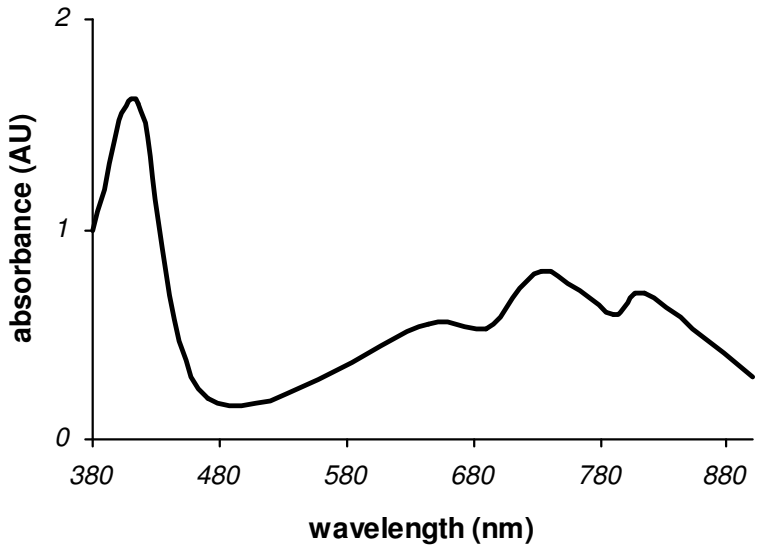
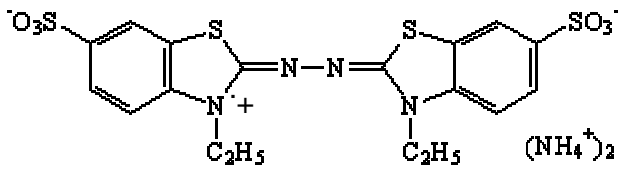


Figure 2. 1 Absorption spectrum of the ABTS radical

ABTS is a bivalent anion in solution. Thus, the net charge after one-electron donation is –1. However, the radical so obtained is commonly defined in the literature as *radical cation* or *ABTS*$^{\bullet+}$ and the use of the more accurate form ABTS$^{\bullet-}$ is rare. To avoid confusion, also in this work the radical is referred to as ABTS$^{\bullet+}$.



As a species capable of trapping one-electron, $ABTS^{\bullet+}$ was used to test the ability of compounds to one-electron donation, which results in partial bleaching of the blue-green color of the reaction mixture. Trolox, a water-soluble analogue of α-tocopherol, was used as a reference compound.

b) Experimental Procedure [Re et al., 1999]

Following substances were tested:

Pure compounds*:*

- *carotenoids*: β-carotene, α-carotene, zeaxanthin, lutein, capsorubin, capsanthin, canthaxanthin and astaxanthin.
- *flavonoids*: epicatechin, luteolin and kaempferol.
- *others*: α-CEHC, RRR-α-tocopherol, ascorbic acid, curcumin, chlorogenic acid and harpagoside.

Pharmaceutical preparations:

- turmeric, artichoke, devil´s claw and garlic: *Fractions A to D.*
- salmon oil: *Fraction D.*

A fresh stock solution of ABTS radical was prepared every day by adding 14 mmol/L ABTS to 4.9 mmol/L potassium persulfate; oxidation of ABTS was thus incomplete. The maximal intensity due to oxidation was measured after 6 hours.

At the beginning of the experiment the $ABTS^{\bullet+}$ solution was diluted to yield an absorbance of 0.7 at 734 nm, either in ultra-pure water or in ethanol depending on the polarity of the solvent used for the stock solutions of the samples. The extinction coefficients of $ABTS^{\bullet+}$ at 734 nm in water and ethanol are very similar: 1.5×10^4 $M^{-1}cm^{-1}$ and 1.6×10^4 $M^{-1}cm^{-1}$, respectively.

10 µL of sample was added to 1 mL of the $ABTS^{\bullet+}$ solution. The mixtures were vortexed briefly and absorbance at 734 nm was measured 1, 3, 6 and 9 min after addition. For the pure compounds, the final concentrations tested were 2.5, 5, 10 and 15 µmol/L; each concentration was tested in triplicate. Of every extract fraction five replicates were prepared and at least four dilutions of each were tested. A solvent control was run in each series.

The percentage loss of absorbance determined after 6 or 9 min was plotted as a function of the concentration of the samples. Absorbance values generally remained constant after the first minute.

The antioxidant activity of a compound or mixture using the TEAC assay was quantified by dividing the slope or gradient of the plots of percentage loss of absorbance vs. concentration for each sample by the slope of the curve for Trolox. Thus, the TEAC assay assumes a value of 1 for Trolox per definition and the results corresponding to the samples are expressed as Trolox Equivalents (TE).

c) Determination of Total Phenols (TP)

The amount of TP in the fractions of pharmaceutical preparations was determined following the Folin-Ciocalteau method [Singleton and Rossi, 1965], based on a colorimetric oxidation/reduction reaction between the phenols and the Folin-Ciocalteau´s reagent. The Folin-Ciocalteau´s reagent is a complex oxidizing agent comprised of heteropolyphosphotungstate-molybdate; upon reaction with phenols, a blue-coloured product is obtained consisting of a mixture of the 1-, 2-, 4-, and 6-electron reduction products of the tungstate reagents, and the 2-, 4-, and 6-electron reduction products of the molybdate reagents. It should be noted that all phenolic molecules with no differentiation between phenol monomers, dimers and larger phenolic compounds are determined in this assay.

0.1 mL sample was diluted with 5.4 mL bi-distilled water and mixed with 4 mL of 7.5% sodium carbonate and 0.5 mL of 2 N Folin-Ciocalteau´s reagent. After 2 h incubation in the dark, absorbance was measured at 765 nm. A gallic acid standard curve was plotted and results were expressed as gallic acid equivalents (GAE).

d) Carotenoid Analysis by HPLC

Aliquotes of the Fraction D of the natural extracts were taken (volumes ranging from 10 to 100 µL, depending on the dilution of the dry material), then dried under nitrogen in the absence of light and injected into the HPLC system described above. UV/VIS detection was set at 450 nm. The mobile phase comprised a binary gradient at a constant flow of 1.5 mL/min. The initial composition of the eluent (acetone/water 75:25) was held for 5 min. Then, a linear gradient was applied for 5 min to yield a final composition of acetone/water of 95:5, which was held for 7 min. Finally, the column was washed for 3 min with acetone [Mínguez-Mosquera and Hornero-Méndez, 1993]. For quantification, the external standard mode was applied. Carotenoids not listed in 2. 1 were isolated from natural sources such as paprika oleoresine by HPLC.

2. 4. 2 Oxygen Radical Absorption Capacity (ORAC)

a) Background

Phycobiliproteins are accessory photosynthetic proteins occurring in cyanobacteria (blue-green algae) and in two groups of eukaryotic algae, the red algae and the cryptomonads. R-phycoerythrin is a phycobiliprotein occurring in various red algae. R- and B-phycoerythrin are the most remarkable phycobiliproteins with respect to quaternary structure stability and to spectroscopic features. Despite their complex multisubunit structure $(\alpha\beta)_6\gamma$, no dissociation is observed at concentrations as low as 10^{-15} mol/L. Besides, R-phycoerythrin carries 34 covalently attached tetrapyrrole prosthetic groups which are resposible for the fluorescence properties of the macromolecule. The intensity of fluorescence is highly sensitive to conformation and chemical integrity of the protein and of the prostetic groups. Phycoerythrins rapidly lose fluorescence when subjected to peroxyl or hydroxyl radical attack. This may be in turn retarded in the presence of radical scavengers.

In the present study, AAPH was used as a radical generator to initiate R-phycoerythrin loss of integrity and loss of fluorescence. AAPH is a diazo-compound which decomposes in aqueous oxygenated solutions in a temperature-dependent manner to yield peroxyl radicals:

$$A - N = N - A \longrightarrow A^{\cdot} + N_2 + A^{\cdot}$$
$$A + O_2 \longrightarrow AOO^{\cdot}$$

where A = HCl · HN = C (NH_2) C (CH_3)$_2$ – .

b) Experimental Procedure

The Oxygen Radical Absorption Capacity (ORAC), e. g. the ability to retard R-phycoerythrin complete loss of fluorescence due to peroxyl radical attack, was determined as described by [Cao and Prior, 1999] for α-CEHC, RRR-α-tocopherol, ascorbate and epicatechin

0.1 mL of test substance was mixed in a fluorescence cuvette with 0.1 mL of 3.4 mg/L R-phycoerythrin (final concentration) and 1.75 mL 75 mmol/L phosphate buffer (pH 7.0). The mixture was equilibrated for 10 min at 37°C and then fluorescence was measured at 540 nm (excitation) and 565 nm (emission) wavelenghts. This value was used as baseline and referred to as 100% fluorescence. After addition of 50 µL of 4 mmol/L AAPH, the cuvette was vortexed briefly. Fluorescence was first recorded exactly 15 seconds after AAPH addition and then every 6 min until the fluorescence was less than 5% of the starting value. The rack containing the cuvettes was kept at 37°C in a water bath. Appropriate solvent controls as well as a standard containing 0.1 mL of 1 µM Trolox were run in each assay. Each compound was tested in triplicate at four concentrations.

Final results were expressed as ORAC values using the formula proposed by Cao *et al*. (15):

$$\text{ORAC value (µmol/L)} = 20 \times k \times (S_{sample}\text{-}S_{control})/(S_{Trolox}\text{-}S_{control})$$

k is the dilution factor of the sample (usually 1/20), and S the area under the curve (AUC) of fluorescence intensity vs. time. Thus, ORAC values depend on the inhibition rate as well as the inhibition time caused by the presence of antioxidants as both parameters are enclosed in the AUC. According to the formula, the ORAC value for Trolox is 1.

2. 4. 3 Inhibition of Nitration of Tyrosine by Peroxynitrite

Protection against peroxynitrite-triggered nitration of tyrosine was performed as described by [Pannala et al., 1997] with minor modifications. A 200 µmol/L tyrosine stock solution was prepared in 100 mmol/L phosphate buffer/0.1 mmol/L EDTA (pH 7.3) and the concentration was confirmed spectrophotometrically at 274 nm (ε= 1405 M^{-1} cm^{-1}). 350 µmol/L peroxynitrite was added by bolus addition to 100 µmol/L tyrosine under vortexing at 25°C. The potential effects of cosolution of α-CEHC, Trolox or (-)-epicatechin (0-40 µmol/L) with tyrosine were examined.

50 µL of each sample containing 100 µM 4-hydroxy-3-nitrobenzoic acid (HNBA) as an internal standard was then injected into a C-18 reverse-phase column using an autosampler. The eluent used for separation was a mixture of 50 mmol/L potassium phosphate buffer (pH 7):acetonitrile (95:5) subject to the next step gradient (1.0 mL/min flow rate):

		5 min		8 min		13 min	
buffer:acetonitrile	95:5	⟶	50:50	⟶	95:5	⟶	95:5

3-Nitrotyrosine was detected at 430 nm. Ratios of peak areas of 3-nitrotyrosine standard vs. internal standard were used for calibration and quantification.

2. 5 Cell Culture Study on Anti-atherogenic Effects of Dietary Micronutrients and α-CEHC

RRR-α-tocopherol, α-CEHC, epicatechin and epigallocatechin gallate were the antioxidants selected to explore potential protective effects on endothelial cells which were stressed with oxidatively-modified LDL. Stock solutions were prepared in ethanol (3 to 5 mmol/L).

2. 5. 1 LDL Preparation and Oxidation

Blood from 2 healthy non-smoking volunteers was obtained by venipuncture, allowed to clot for 10 min and centrifuged for 15 min at 4,500 rpm to obtain the serum. The LDL fraction of serum was isolated using the short-run ultracentrifugation method [Kleinveld et al., 1992]. For that purpose, 1.87 mL of the so-called “heavy solution” (354 g KBr, 153 g NaCl, and 100 µg EDTA in 1 L ultra-pure water) was added to 0.9 mL serum in a polyallomer tube (Beckman, Munich, Germany) to reach a final density of 1.33 g/mL. The mixture was then overlayed with 0.15 mol/L NaCl up to the tube edge and 6 tubes were centrifuged together at 4°C / 120,000 g for 2 h to allow separation of lipoprotein bands.

The orange-colored LDL bands were collected, combined and stored with 100 µmol/L EDTA under nitrogen at 4°C for up to 3 days. Salts and EDTA were removed by gel-filtration over a Sephadex G-25 separation column (modell PD-10, Amersham Pharmacia Biotech, Freiburg, Germany).

LDL concentration was determined indirectly by measuring total cholesterol with an enzymatic colorimetric test (CHOD-PAP, Boehringer Mannheim, Germany) and applying a conversion factor of 2200 ± 163 mol cholesterol/mol LDL [Esterbauer, H. et al., 1990] .

100 nmol/L purified LDL in PBS was incubated with 5 µmol/L $CuCl_2$ at 37°C and the extent of oxidation was followed by measuring conjugated diene formation at 234 nm every 10 min. Oxidation was stopped at a level of 250 nmol conjugated dienes/mg protein (approx. 80 min) by placing the reaction vessel on ice. Oxidized LDL were used immediately in cell culture experiments to avoid further extensive oxidation before incorporation into the cells. Oxidized LDL will be abbreviated with the term oxLDL; however, note that under these conditions and time of oxidation a so-called minimally-modified LDL is obtained (see Introduction).

2. 5. 2 Cell Culture

Primary murine aorta endothelial cells (MAEC) were kindly provided by Dr. Cristoph V. Suschek, Heinrich-Heine-University Düsseldorf. Cells were grown in 550 mL culture flasks (Greiner, Frickenhausen) using RPMI-1640 culture medium (Sigma-

Aldrich, Steinheim) to which 10% fetal calf serum (Greiner), 2 mmol/L L-glutamine (Sigma) and 20 mg/mL penicilline/streptomycine (Sigma) were added. Cells were incubated in a Binder CO_2-Incubator Line CB (Tuttlingen, Germany) incubator (set at 37°C/ 5% carbon dioxide/ water-vapour saturation) until confluence, washed with PBS and subsequently detached from the flask ground using 3 mL of 0.5 mg/L trypsin/PBS (2:1 v/v). The isolated cells were resuspended in growth medium and divided into new culture flasks at dilutions ranging from 1:5 to 1:10. For experiments, cells were allowed to grow on 6 cm dishes or 24-well plates (Greiner) until 90% confluence and then cultured for 24 hours in serum-deprived RPMI-1640 medium.

2. 5. 3 Cytotoxicity

Cytotoxic effects of samples on MAEC were studied using a spectrophotometric test for cell viability, the MTT test. This assay is based on the physiological ability of mitochondria to oxidize 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium to formazan, which takes place in intact and early apoptotic cells but not in dead cells.

MAEC were grown on 24-well culture plates until 90% confluence, washed once in PBS and subsequently treated according to one of these three series of experiments:

a) Cytotoxicity of Test Compounds: RRR-α-tocopherol, α-CEHC, epicatechin and epigallocatechin gallate stocks were diluted to 30 and 15 µmol/L in RPMI-1640 medium containing 0.5% FCS. Incubation with MAEC for 24 h followed. A control containing 1% ethanol was run in each series.

b) Cytotoxicity of Native and Oxidatively-modified LDL: nLDL (200 nmol/L) or oxLDL (12.5, 25, 50, 75, 100 and 200 nmol/L) in RPMI-1640 medium containing 0.5% FCS were pipetted onto the cell monolayers and effects were determined after 24 h. A control comprising only 0.5% FCS was run in each assay.

c) Cytotoxicity of Native and Oxidatively-modified LDL on Antioxidant-preloaded Cells: After 20 h cell preincubation with either 15 µmol/L epigallocatechin gallate or 30 µmol/L α-tocopherol, α-CEHC or epicatechin, cells were washed twice with PBS and incubated further 32 h with 30 nmol/L moLDL in RPMI-1640 medium containing 0.5% FCS. The concentrations of antioxidants and oxLDL were selected in

accordance to the results of the experiments detailed under *a)* and *b)*. Appropriate controls without antioxidants and/or oxLDL were applied.

MTT stock was prepared by dissolving 5 mg in 1 mL PBS. Working solutions consisted of a 1:10 dilution in FCS-free culture medium without phenol red. After treatment, 200 µL MTT working solution was added to each well and cells were incubated under usual culture conditions further 3 h. The reaction was stopped by adding 200 µL SDS/0.1 mol/L HCl (10% w/v). After overnight incubation at 37°C, formazan release was measured at 570 nm.

2. 5. 4 Uptake of oxLDL

The time course of uptake of nLDL and oxLDL by MAEC was followed fluorometrically.

2 mL of 1.5 µM nLDL was incubated overnight with the lipophilic fluorescent probe 1,1´-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanine perchlorate (DiI, 50 µL of a 3 mg/mL solution in DMSO) under agitation, then isolated using the short-run ultracentrifugation mode, filtered over a PD-10 column and finally diluted to 0.1 µmol/L in phenol-red free RPMI-1640 medium 0% FCS. A part was stored as labelled nLDL fraction, the rest was oxidized with 1 µmol/L $CuCl_2$ up to 250 nmol conjugated dienes/mg protein. MAEC were incubated with either labelled nLDL or labelled oxLDL (100 nmol/L) and uptake was followed using one of these approaches:

a) *Semiquantitative Method*, by observing the cells under a confocal fluorescent microscope (excitation filter 450-490 nm, emmision filter 570-610 nm).

b) *Quantitative Method*. Confluent cells grown on 6 cm dishes were incubated with the DiI-labelled LDL, washed in PBS at different time points and collected in 500 µL Tris-HCl (10 mmol/L, pH 7) using a plastic cell scraper (Greiner). 3 mL of ice-cold chloroform/methanol 1:2 (v/v) was then added to the cell suspensions, the mixture was vortexed for 30 s and sonicated for 10 s using a Branson Sonifier 250 (Branson Ultrasonics, Danburg, USA). Phase separation was achieved by addition of 2 mL chloroform and 1 mL KCl 1 mol/L and centrifugation at 2000 rpm for 5 min. Fluorescence of the lower chloroform phase was measured in a fluorescence

spectrometer at 549 and 565 nm absorption and emission wavelenghts, respectively; if needed, samples were reconcentrated under a mild stream of nitrogen.

2. 5. 5 Short-term Variations of the Redox State

The approach to assess the cell redox state within 10 h after addition of oxLDL was based on the fluorogenic properties of 2´,7´-dichlorodihydrofluorescein diacetate (DCDHF-DA), a lipophilic probe which is readily taken up by cells, stored mainly at the internal cell membrane compartment and subsequently deesterified by cellular esterases. Only the deacetylated form (DCF) is fluorescent, in contrast to the parent compound.

Cells were treated with test compounds and/or LDL as described in **2. 5. 3**. Cells were washed once with PBS 2, 4, 6, 8, and 10 h after starting incubation with either nLDL or oxLDL, and 100 µmol/L DCDHF-DA in 0% FCS culture medium was added. After 30 min incubation at usual culture conditions, cells were washed once again with PBS and subsequently 0.5 mL phenol red-free medium was added. Finally, fluorescence was measured in a plate reader (excitation 485 nm, emission 535 nm).

2. 5. 6 Long-term Variations of the Redox State

Overall redox state during the 24 h following oxLDL addition was monitored as changes in levels of total free thiols and glutathione. Free thiols including reduced glutathione (GSH) are oxidized by 5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB) to produce disulfides under stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). The amount of TNB formed can be followed at 405 or 412 nm.

a) *Measurement of Total Free Thiols*: After being stressed for 0, 2, 6, 10 and 24 h with oxLDL, cells were collected in 500 µL PBS containing 2 mmol/L PMSF and 1 µg/mL leupeptin, shortly sonicated and centrifuged to obtain the supernatants. Supernatants were derivatized with DTNB (50 nmol dissolved in $NaHCO_3$ 10 mg/mL per mg protein), and TNB release followed at 412 nm. Supernatants of cells treated with nLDL were run as controls.

b) *Measurement of Glutathione*: Glutathione was measured using the DTNB-glutathione disulfide (GSSG) reductase recycling procedure [Anderson, 1985] in its

two main applications, for non-bound total glutathione and for GSSG alone. Confluent cells on 24-well plates were collected in 250 µL ice-cold HCl 0.01 N and immediately frozen at –80°C. Measurements were performed within the 2 following days. After thawing out on ice, cells were sonicated for 5 s and lysates centrifuged at 15,000 rpm for 3 min. The supernatants were collected and deproteinized by adding 5-sulfosalycilic acid (SSA, 5% w/v final concentration) followed by vortexing and recentrifuging at 15,000 rpm for 3 min. The composition of the solutions used is detailed below:

Stock solutions (prepared daily)

Derivatization reagent	*NADPH*
39.6 mg DTNB	6.25 NADPH
100 mg $NaHCO_3$	1 mL bi-distilled water
10 mL PBS / 6 mmol/L EDTA	

Working solution (for 10 samples)

480 µL derivatization reagent
100 µL NADPH stock solution
9.42 mL PBS / 6 mmol/L EDTA

For single measurements, 900 µL working solution was mixed with 75 µL sample in a cuvette and absorbance at 412 nm was set at 0. 25 µL Glutathione reductase (final activity: 2.66 U/mL) was added and reaction kinetics was followed for 3 min. Reaction rates (dA/min) in the linear range were used for calculations.

i) Total Non-bound Glutathione: Working samples were obtained as detailed above, without ulterior modifications. Thus, all GSSG is converted into GSH and both GSH and GSSG are measured as GSH equivalents, according to the following reactions:

$$2\ GSH + DTNB \longrightarrow GSSG + TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{Glutathione Reductase}} 2\ GSH + NADP^+$$

A sample blank lacking GSSG was used to determine the background rate. Appropiate GSSG standards also containing SSA were run and a standard curve was plotted.

ii) GSSG: To 100 µL of the protein-free supernatant, 3 µL 2-vinyl-pyridine (VP) and 58 µL triethanolamine/water 1:10 (v/v) were added. VP at the concentrations used reacts with GSH without inhibiting the glutathione reductase. After 1 h derivatisation, 75 µL samples were used as detailed above. GSSG standards and a sample blank containing VP were run.

2. 5. 7 Apoptotic Cell Death

a) Involvement of Apoptosis in oxLDL-induced Cytotoxicity was assessed by:

- evaluating morphological changes using confocal microscopy during 48 h treatment with oxLDL and
- incubating cells with the cell-permeable caspase-3 inhibitor Asp-Glu-Val-Asp-aldehyde (DEVD-CHO, 100 µmol/L) for 30 min, then stressing with oxLDL for 24 h and finally performing the MTT assay for cell viability as detailed above. A control without inhibitor was run simultaneously.

b) Caspase-3 Activity

Caspase-3 activity was determined using a colorimetric caspase-3 assay kit (Sigma) on a 96-well plate. The reaction mixture (100 µL) contained 30 µL cell lysate and 10 µL of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA, 200 µmol/L final concentration) in assay buffer (20 mmol/L HEPES, pH 7.4, 0.1% CHAPS, 5 mmol/L DTT, 2 mmol/L EDTA). When the substrate is cleaved, the yellow compound *p*-nitroanilide (pNA) is released and can be measured at 405 nm. A control containing also 10 µL of DEVD-CHO (20 µmol/L final concentration) was carried out to account for non-specific hydrolysis of the substrate. Besides, a caspase-3 positive control comprising both substrate and caspase-3 (0.1 µg/mL final concentration) was performed.

2. 5. 8 Protein Measurement

Protein concentration in cell lysates was determined using the Lowry-based RC-DC protein assay (Bio-Rad, Hercules, USA).

2. 6 Human Intervention Study on the Effects of Smoking on Vitamin E Metabolism after a Single Dose of Vitamin E

2. 6. 1 Study Design

The intervention study presented here is an extension of the work initiated by Dragan Radosavac. For a better interpretation of the outcomes, data corresponding to former volunteers were added to novel data produced for the present work.

Twenty-nine healthy, free-living adults (males and females) were recruited for the study. 17 volunteers were smokers, 12 non-smokers. None of the participants ingested vitamin supplements during the study. Women were not pregnant or in the lactation phase. None of the participants suffered from lipid malabsorption. Written, informed consent was obtained from each volunteer.

All subjects received a single oral dose of vitamin E (Hermes, Munich) containing 306 mg of RRR-α-tocopherol and 1.77 mg of γ-tocopherol. Capsules were taken together with a meal.

Blood was obtained by venipuncture before ingestion of the capsule (baseline level) and 2, 6, 12, 24, 35, 50 and 74 h after intake of vitamin E. Blood was allowed to clot for 10 min and centrifuged for 15 min at 4,500 rpm to obtain the serum. Samples were stored at –40 °C until analysis.

2. 6. 2 Determination of α- and γ-CEHC in Serum [Stahl et al., 1999]

a) Sample Preparation

Extraction Solvent A: To a stock solution of 100 mL hexane/dichloromethane 5:2, 350 µL of 3 mg/mL α-tocopherol in ethanol was added freshly every day.

Extraction Solvent B: 350 µL of 3 mg/mL α-tocopherol in ethanol was added freshly to 100 mL hexane/dichloromethane 1:1 every day.

500 µL serum was pipetted into a glass tube and 25 µL of 10 mg/mL ascorbate in water was added. Lipids were removed from serum by addition of 5 mL of extraction solvent A, 1 min vortexing and centrifugation for phase separation at 15°C for 5 min at 5000 rpm. The upper organic phase was discarded and the remaining aqueous phase acidified with 20 µL of acetic acid (100%) and extracted twice by addition of 5 mL of extraction solvent B, 1 min vortexing and subsequent centrifugation at 15°C for 5 min at 5000 rpm. The two organic layers obtained were combined and the solvent was evaporated under a gentle stream of nitrogen at room temperature. For HPLC analysis the dry residue was dissolved in 80 µL of acetonitrile containing 12.5 µg α-tocopherol/mL to avoid prompt oxidation. The solution was diluted to 200 µL with bi-distilled water and filtered through a 0.45 µm polyvinylidene fluoride filter (Lida Manufacturing Co., Kenosha, USA). 100 µL sample was injected.

b) HPLC Analysis

For the mobile phase, 25 mL of tetraethylammonium hydroxide (TEAH, 20% in water) and 186 mg EDTA were added to 620 mL water and the pH adjusted to 4.5 with approx. 5 mL acetic acid (100%). After filtration through a 0.22 µm hydrophilic Millipore Type GV filter, 350 mL acetonitrile was added and the mixture degassed with helium for about 4 min. The eluent was left to recirculate in the HPLC system for about 12 h with the detector switched on to stabilize the baseline response.

For analysis of the samples the system was also used in recirculating mode and the flow was set at 1 mL/min. For coulometric detection, the analytical cell was set at +0.35 V and the conditioning cell at –0.1 V.

External standard mode was used for calculation. Stock solutions of α- and γ-CEHC were prepared in ethanol and their concentrations calculated using the extinction coefficients of $\varepsilon_{289\ nm}$= 3230 $M^{-1}\cdot cm^{-1}$ and $\varepsilon_{297\ nm}$= 3620 $M^{-1}\cdot cm^{-1}$ respectively. Stock solutions were diluted in acetonitrile/water 40:60 and 100 µL sample was injected.

2. 6. 3 Determination of α- and γ-Tocopherol in Serum [Aust et al., 2001]

a) Sample Preparation

200 µl Serum was added to 1 mL KPi-Puffer (2 mmol/L KPi, pH 7,2 , 250 mg/L EDTA, 250 mg/L ascorbate) in a polypropylene tube, and 20 µL of an ethanolic solution of δ-tocopherol was added. Proteins were precipitated by addition of 1 mL ethanol and vortexing. 6 mL of an extraction solution (n-hexane/dichloromethane, 5:1 v/v, 0,01 % BHT) were added. After 1 min agitation and 10 min sonication, samples were centrifuged for phase separation for 3 min at 3,000 rpm.

5 mL of the organic phase was then given to one new polypropylene tube and dried under nitrogen stream. Residues were subsequently resolved in di-ethylether and dried again under nitrogen. After reconstitution with 20 µL dichloromethane, 180 µL of mobile phase A was added and 50 µL was injected into the HPLC system.

b) HPLC Analysis

The mobile phase was a mixture of following solutions:

A methanol/acetonitril/2-propanol, 54:44:2, v/v/v,

B methanol/acetonitril/water/2-propanol, 46:37:15:2, v/v/v/v

Following gradient was followed at 1 mL/min:

		5 min		10 min		12 min		5 min	
A:B	64:36	⟶	64:36	⟶	100:0	⟶	100:0	⟶	64:36

Tocopherols were detected at 292 nm.

2. 7 Statistics

Statistical analysis was performed using the STATISTICA 5.5 software package for Windows and Microsoft Excel 2000.

In the study of *in vitro* antioxidant activities of single compounds and in cell culture experiments, parametric tests were performed (1 or 2-way variance analysis

followed by Tuckey´s HSD test; *t*-Student test). Levels of significance chosen were: (*) or (§) $p \leq 0.05$; (**) or (§§) $p \leq 0.001$.

For parameters corresponding to the different fractions of the pharmaceutical preparations (TEAC and content of total phenols) data were analysed using non-parametric tests (Friedman´s test; individual values were compared using the Mann-Whitney U test). The effect of sample concentration on $ABTS^{\bullet+}$ reduction was assessed using the Kruskal-Wallis test. Significant differences were considered at $p \leq 0.01$ and expressed by different small letters or (*).

For the intervention study, non-parametric tests were performed, including the Wilcoxon rank-sum test for comparison between time points and the Friedman´s test.

All data are presented as mean ± standard deviation (SD).

3 RESULTS

3. 1 Antioxidant Activity of Dietary Micronutrients in Model Systems

The first aim of this work was to characterise antioxidant properties of different classes of micronutrients which are consumed with the diet at a regular basis. For this section, the antioxidant activity was regarded as the ability of a substance to act as a radical scavenger or reducing species *in vitro* in the absence of cells. Test substances included vitamin E, carotenoids, flavonoids and hydroxycinnamic acid derivatives. α-CEHC, a metabolite of α-tocopherol and α-tocotrienol in human blood and urine, was assessed for the first time for antioxidant capacity. Not only single compounds but complex mixtures in form of plant extracts were also investigated.

3. 1. 1 Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay gives a first approximate measure of the antioxidant activity of compounds (see Discussion), determined by the decolorization of the $ABTS^{\bullet+}$. The reduction of the radical is related to the decrease of absorbance at 734 nm. Precision of the methodology was calculated in terms of within-day and day-to-day coefficients of variation (c.v.) for all substances, related to the TEAC values obtained. Within-day coefficients of variation coefficients ranged from 5.2% to 11.8%. Inter-day c.v. varied between 0.4% and 5.7%.

a) Single Compounds

The reaction of the stable radical with most of the compounds tested was completed after 1 min, the first time point studied. Main exceptions were carotenoids and curcumin, which showed a further small inhibitory effect up to 6 min of reaction time. Also, a good linear correlation between the concentrations used and the percentage $ABTS^{\bullet+}$ reduction was found for all single compounds studied, with the exception of curcumin. The linear response range of curcumin ranged only from 0 to approximately 4 µmol/L.

Table 3. 1 Trolox Equivalent Antioxidant Capacity (TEAC) of single compounds.

	test compound	**TEAC (µmol Trolox equivalents)***
flavonoids-	epicatechin	1.45 ± 0.03
hydroxycinnamates	epigallocatechin gallate	2.45 ± 0.13
	luteolin	2.18 ± 0.10
	kaempferol	1.39 ± 0.09
	chlorogenic acid	1.20 ± 0.08
	curcumin	1.92 ± 0.09
monophenols	α-tocopherol	0.97 ± 0.04
	α-CEHC	0.96 ± 0.02
carotenes	β-carotene	3.56 ± 0.42
	α-carotene	2.32 ± 0.67
xanthophylls-	zeaxanthin	3.28 ± 0.37
ketocarotenoids	lutein	1.58 ± 0.38
	capsorubin	5.05 ± 0.30
	capsanthin	1.61 ± 0.20
	canthaxanthin	0.59 ± 0.16
	astaxanthin	2.61 ± 0.71
others	harpagoside	0.04 ± 0.02
	ascorbic acid	1.26 ± 0.07
	Trolox	**1**

*µmolar equivalents of Trolox with the same antioxidant activity as 1 µmol of test compound.
Compounds were tested in triplicate at four different concentrations.

The TEAC values were calculated by dividing the slope of the concentration-response curves for each test compound by the slope of the concentration-response curve of Trolox (**Figure 3. 2**). Curcumin, luteolin, kaempferol, chlorogenic acid, epicatechin and epigallocatechin gallate exhibited an antioxidant activity superior to Trolox (1 per definition). Among them, epigallocatechin gallate showed the most pronounced ability to reduce $ABTS^{\bullet+}$. α-Tocopherol and α-CEHC showed about the same activity as Trolox. Carotenoids covered a broad range of antioxidant activity levels. Harpagoside showed only 4% of the Trolox activity. Ascorbic acid was a slightly better reducing agent compared to Trolox.

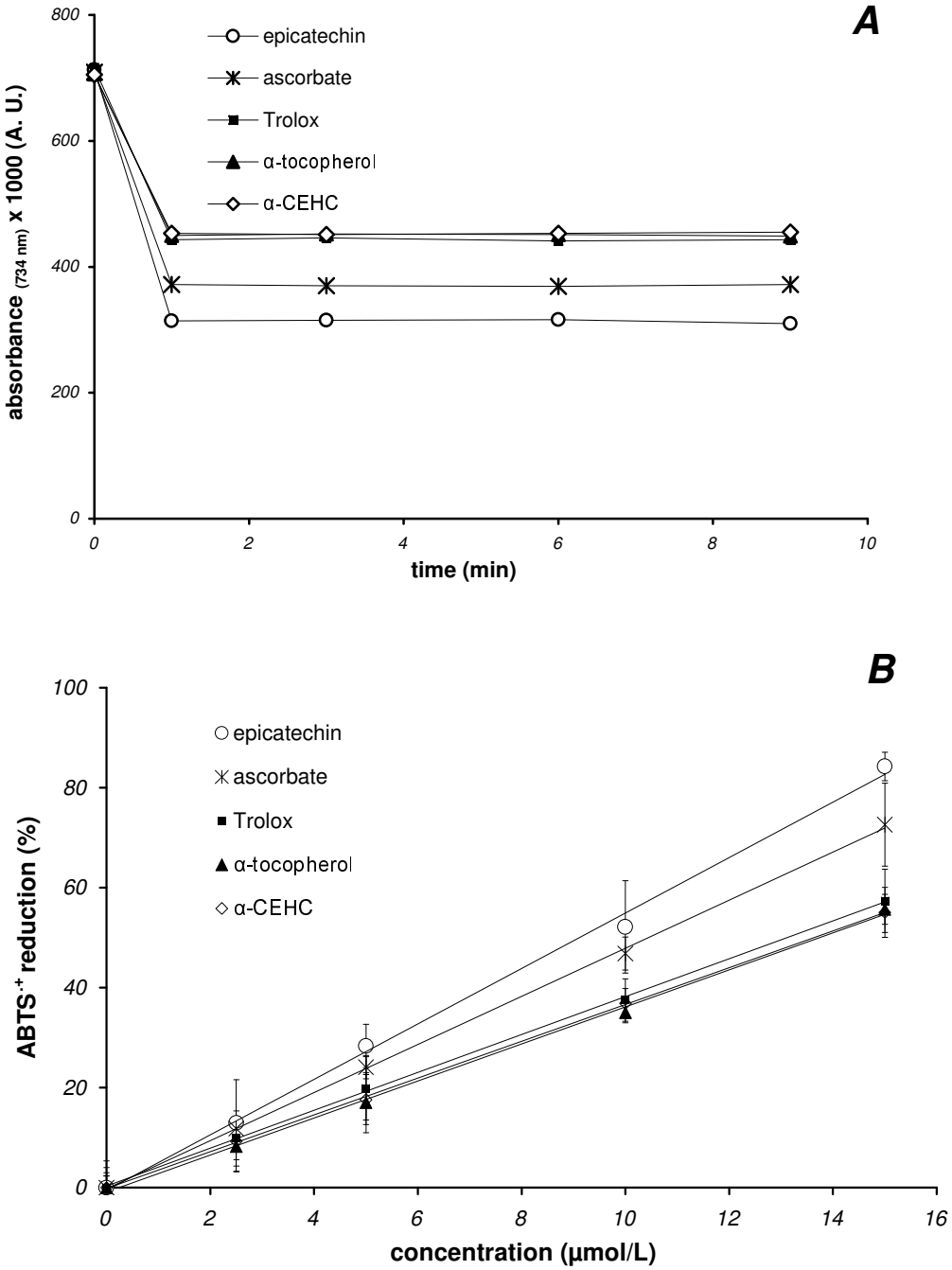


Figure 3. 2 (*A*) Reaction of the $ABTS^{\cdot+}$ solution with test compounds (10 µmol/L); (*B*) Dose – response curves for test compounds at $ABTS^{\cdot+}$ reduction. n = 3

b) Pharmaceutical Preparations

It has been suggested that dietary supplements or other pharmaceutical preparations which are rich in specific antioxidants may be useful to improve the antioxidant status of the organism and contribute to the preventive effects associated

with an increased antioxidant intake. Reactive oxygen species generated endogenously in excess play a role in inflammatory processes and may trigger the pathogenesis of several degenerative diseases such as atherosclerosis, cancer, or cataract [Halliwell and Gutteridge, 1999]; antioxidants supplied with the normal diet or with special pharmaceutical formulations scavenge these intermediates protecting the organism at the cellular and molecular level and may thus contribute to the prevention of such diseases [Sies et al., 1992]. Therefore, the antioxidant capacity of five common pharmaceutical preparations derived from turmeric, artichoke, devil´s claw, garlic and salmon was studied using the TEAC assay. In order to estimate the chemical properties of the dietary constituents responsible for the major contribution to the antioxidant activity, four different fractions with different lipophilicity were obtained by solid/liquid extraction (one unique liquid/liquid extraction in the case of salmon oil) according to this scheme (details in **2. 3**):

Turmeric, artichoke, devil´s claw and garlic preparations

fraction A: water

fraction B: methanol/water 70:30

fraction C: ethanol

fraction D: acetone/water 75:25 followed by diethyl ether

Samon oil preparation

fraction D: hexane

The outcomes were expressed as µmol Trolox equivalents (TE) per gram of dry matter (referred to the contents of the capsule or the entire garlic coated tablet):

Table 3. 3 Trolox Equivalent Antioxidant Capacity of fractions of pharmaceutical preparations

		TEAC (µmol Trolox equivalents / g)			
preparation	***n***	*fraction A**	*fraction B*	*fraction C*	*fraction D*
turmeric	5	375 ± 55^{a}	506 ± 63^{b}	1257 ± 82^{c}	599 ± 59^{b}
artichoke	5	193 ± 39^{a}	302 ± 46^{b}	3.7 ± 0.7^{c}	0.1 ± 0.1^{d}
devil´s claw	5	394 ± 24^{a}	302 ± 57^{b}	4.6 ± 1.1^{c}	2.2 ± 0.6^{d}
garlic	5	14.4 ± 2.7^{a}	5.6 ± 1.0^{b}	0.1 ± 0.0^{c}	0.0 ± 0.0^{c}
salmon oil	5	---	---	---	3.9 ± 0.6

* each fraction was tested at least at four concentrations.

$^{a, b, c, d}$ different small letters indicate significant differences between two fractions within each preparation; $p \leq 0.01$.

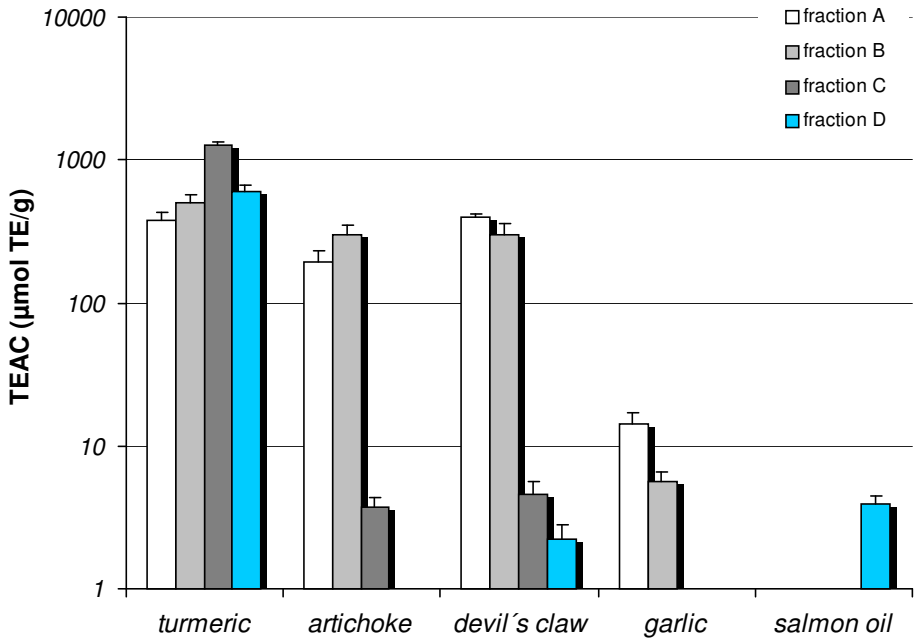


Figure 3. 4 Trolox Equivalent Antioxidant Capacity of fractions of pharmaceutical preparations represented in a logarithmic scale. n = 5 (each fraction was tested at least at four concentrations).

A linear relationship for concentration vs. response was found for all fractions of each preparation except in fractions B, C and D of the turmeric extract preparation, where a bi-phasic response was observed (**Figure 3.5**). Thus, the TEAC values corresponding to curcumin and the turmeric extract fractions were calculated using the slope in the linear range.

Turmeric (*Curcuma longa* L., Zingiberaceae) rhizome extracts are rich in the yellow-reddish pigment curcumin and other curcuminoids, derivatives of hydroxycinnamic acid. The main medical indication for turmeric extract is the treatment of disorders of liver and gallbladder. Curcumin has been shown to exhibit anti-carcinogenic activity and possess anti-inflammatory properties (Hänsel et al., 1997).

Fractions C and D of the turmeric preparation exhibited 100% reduction at 0.15 and 0.5 mg dry matter/mL, respectively, whereas fractions A and B were less efficient. The concentration-dependent effect of all fractions was found to be significant ($p \leq 0.05$) using the Kruskall-Wallis test.

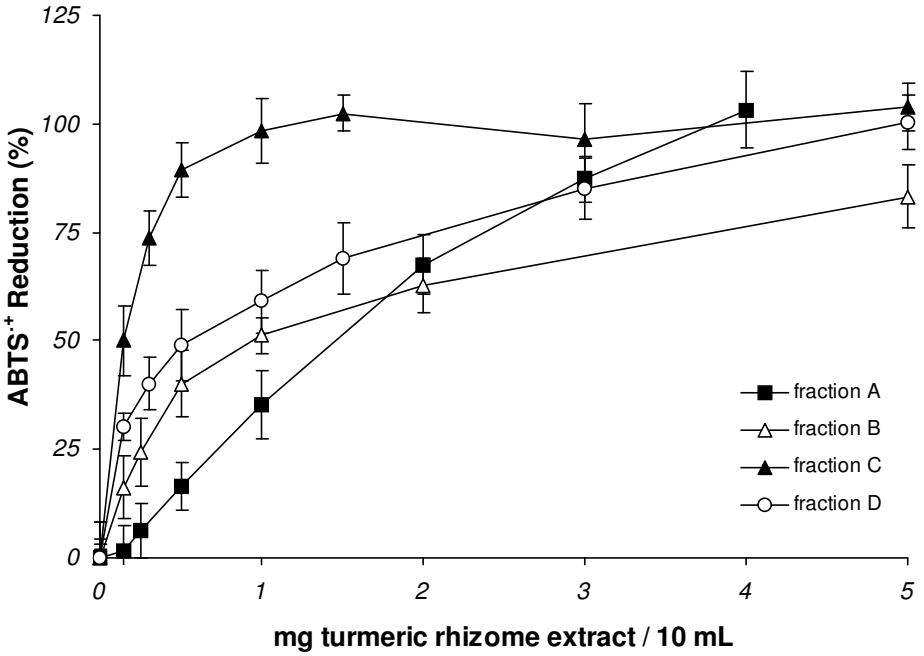


Figure 3. 5 Reduction of the ABTS radical cation in the presence of fractions A to D of the turmeric preparation. Concentration is expressed as mg dry matter equivalents per 10 mL solvent; n = 3 - 5.

Curcumin is the major, almost exclusive, polyphenol in the ethanolic extract of turmeric. The VIS spectrum obtained for fraction C of the turmeric preparation was similar to that of curcumin in ethanol, showing a maximum at 424 nm. On the basis of the content of curcuminoids provided by the manufacturer, and the TEAC value determined for curcumin, the antioxidant activity of fraction C (1257 ± 82 µmol TE/g) could be largely ascribed to the presence of curcumin (to 90%). In fraction D, where carotenoids should be most solublę, only β-carotene was detected by HPLC at a level corresponding to 0.69 µg / g dry matter, implying that the contribution of carotenoids to the antioxidant activity of the product is of minor importance.

Pharmacological properties of **artichoke** (*Cynara scolymus* L.*,* Compositae) leaf extracts are attributed to caffeoylquinic acids, such as chlorogenic acid, and their derivatives, mainly cynarin, but also to the variety of glycosides of the flavone luteolin. Artichoke extracts are used to treat disorders of liver and gallbladder, as well as dyspepsia, atherosclerosis or diabetes [Wegener and Fintelmann, 1999].

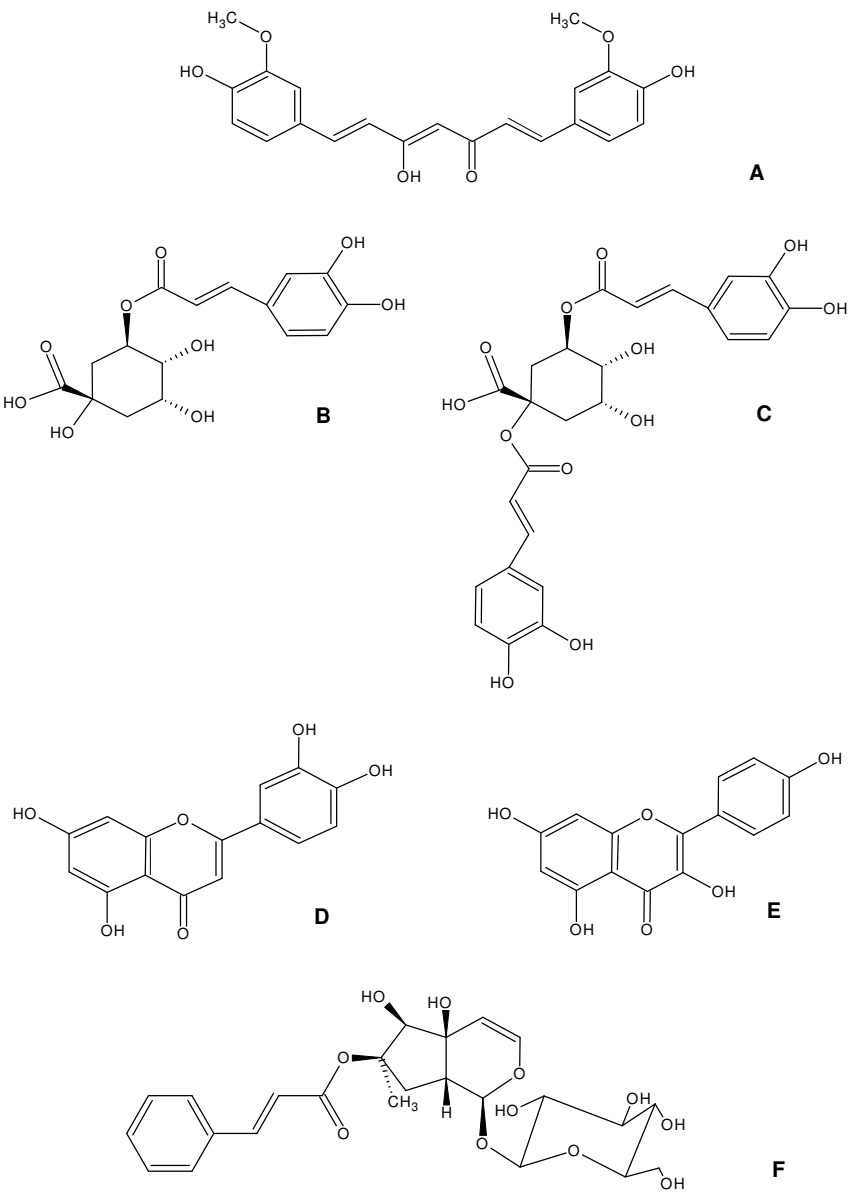


Figure 3. 6 Chemical structure of expected constituents in the pharmaceutical preparations: (A) curcumin; (B) chlorogenic acid; (C) cynarin; (D) luteolin; (E) kaempferol; (F) harpagoside

For the artichoke leaf-based product, the highest TEAC was found in fraction B, corresponding to 302 ± 46 µmol TE/g. Fraction A exhibited somewhat less activity and very little antioxidant activity was found in fractions C and D.

The antioxidant activities measured as TEAC for two abundant compounds in artichoke leaves, luteolin and chlorogenic acid, were 2.18 ± 0.10 and 1.20 ± 0.08 µmol TE/µmol respectively (**Table 3.1**). This is in accordance with the fact that these compounds are very good soluble in methanol/water 70:30 and somewhat less

soluble in water alone. No carotenoids nor significant antioxidant activity were found in fraction D.

Extracts of **devil´s claw** (*Harpagophytum procumbens* [BURCH.] D.C. ex MEISSN., Pedaliaceae) roots are used in the treatment of arthritis and other inflammatory processes [Chantre et al 2000]. Iridoid-glycosides, among them harpagoside, harpagide and procumbide, are the most abundant compounds in the roots; pharmacological activity is tentatively ascribed to them. Luteolin and kaempferol are also present in small amounts.

The devil´s claw extract tested here is particularly rich in water-soluble antioxidants, since the major TEAC was found in fractions A and B. Pure harpagoside was very good soluble in water but showed very poor antioxidant activity in the TEAC assay (**Table 3.1**). In Fractions C and D, very low activity was detected. Traces of β-carotene (2.34 µg/g) and β-cryptoxanthin (less than 0.1 µg/g) were found in fraction D. The antioxidant activity of kaempferol, a frequent flavonol in devil´s claw roots, was 1.39 ± 0,09 µmol TE/µmol.

Garlic (*Allium sativum* L.*,* Amaryllidaceae) exhibits cardioprotective, lipid-lowering, and anti-thrombotic effects [Borek 2001]. The protective effect appears to be related to the presence of organosulfur compounds [Bianchini and Vainio, 2001]. Fractions A and B of the garlic coated tablets exerted little antioxidant activity in the TEAC assay, whereas almost no effect was detected for fractions C and D. No carotenoids were found in fraction D.

Salmon oil concentrate is a suitable source of polyunsaturated n-3 fatty acids, such as eicosapentaenoic or docosahexaenoic acids. Dietary intervention trials provide evidence that an increased intake of n-3 fatty acids is correlated with a lower incidence of coronary heart disease [Harris and Isley 2001]. The fatty acid-free (non-saponifiable) fraction of the salmon oil containing vitamin E (added for stabilization by the manufacturer) was obtained. Fraction D, corresponding to the oil dissolved in hexane, showed an antioxidant activity very similar to that of the non-saponifiable fraction (3.9 µmol TE/g); α-tocopherol concentration was 4.3 µmol/g oil as determined photometrically. Thus, the antioxidant activity of the salmon oil product could be wholly ascribed to α-tocopherol (TEAC for α-tocopherol was 0.97 ± 0.04 TE). No carotenoids were found in fraction D.

b) Determination of Total Phenols (TP)

The concentration of Folin-active compounds was particularly high in the turmeric fractions, followed by devil´s claw and artichoke. Good coefficients of linear regression between TEAC values and contents of TP were found for all test preparations.

The results confirm the observations that moderately water-soluble polyphenols are responsible for the antioxidant activity of artichoke leaf extract and that polyphenols of high hydrophilicity account for the antioxidant properties of devil´s claw root extract. Three different curcuminoids (curcumin, monodemethoxy-curcumin and bisdemethoxycurcumin), covering the large solubility spectrum from methanol to ether screened here, are present in *Curcuma longa* rhizomes and may well account for the high activities found in fractions B to D. It should be noted that organosulfur compounds may interfere in the results for garlic extract as some are Folin-active.

Table 3. 7 Total phenols in fractions of pharmaceutical preparations and correlation with the antioxidant activity.

		TP (mg GAE / g dry matter)				$r_{TEAC/TP}$
preparation	***n***	*fraction A*	*fraction B*	*fraction C*	*fraction D*	
turmeric	5	30.1 ± 2.8^{a}	46.0 ± 4.1^{b}	124.7 ± 6.0^{c}	46.2 ± 4.6^{d}	0.983*
artichoke	5	14.8 ± 3.1^{a}	25.5 ± 6.9^{b}	1.0 ± 0.5^{c}	0.0 ± 0.0^{d}	0.973*
devil´s claw	5	37.4 ± 4.5^{a}	29.9 ± 7.1^{a}	0.7 ± 0.2^{b}	0.3 ± 0.1^{c}	0.986*
garlic	5	0.8 ± 0.4^{a}	0.9 ± 0.3^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.688*
salmon	5	---	---	---	0.5 ± 0.1	---

TP = Total Phenols
GAE = Gallic Acid Equivalents
$^{a, b, c, d}$ different small letters indicate significant differences between two fractions within one preparation; $p \leq 0.05$.
r is the coefficient of linear regression between TEAC and TP within each preparation; (*) $p \leq 0.01$.

3. 1. 2 Oxygen Radical Absorption Capacity (ORAC)

The ORAC assay was performed as a method complementary to the TEAC to gain further information on the radical scavenging activities of compounds of interest which were used in subsequent experiments in cell culture, e. g., α-CEHC, α-tocopherol and epicatechin.

In **Figure 3.8**, the decay in fluorescence of R-phycoerythrin when exposed to peroxyl radicals generated by AAPH in the absence or presence of radical scavengers is shown. Each antioxidant caused a delay in the loss of fluorescence (a so-called *lag phase*), and an increase in the area under the curve "*AUC*" [% fluorescence intensity of the initial value · minute] which was proportional to the concentration of the radical scavenger in the reaction mixture at time zero. A linear correlation between ORAC values and concentrations (0-4 µmol/L) for all the substances tested was found ($r^2 > 0{,}9972$). Within-day coefficients of variation ranged from 2.0 to 9.6%. In order to calculate day-to-day coefficient of variations, data were collected over three days. The corresponding results ranged between 4.3 and 16.7%. α-CEHC exerted a slightly higher peroxyl quenching activity than Trolox but without significant difference. Epicatechin showed the best protective effects of the compounds tested. Ascorbate was included as an alternative positive control in all series of experiments; even though it was active, the ORAC value for ascorbate was much lower than the corresponding TEAC value, and was about one half the score for Trolox, indicating a different reactivity of ascorbate towards peroxyl radicals in comparison to $ABTS^{\bullet+}$. In the case of α-tocopherol, the necessary presence of high amounts of ethanol as organic co-solvent modified the reaction kinetics, which was interpreted as an impairment of protein integrity. Indeed, a major restriction inherent to this assay are the problems associated with the testing of highly lipophilic compounds or those of intermediate polarity.

Oxygen Radical Absorbance Capacity of single compounds

	Trolox	*epicatechin*	*α-CEHC*	*α-tocopherol*	*ascorbate*
ORAC (µmol TE)	1.00 ± 0.34	2.64 ± 0.25**	1.42 ± 0.35	n. p.	0.58 ± 0.39*

t-Student test was applied to determine significant differences with Trolox; (*) $p \leq 0.05$; (**) $p \leq 0.001$. Each compound was tested in triplicate at four concentrations. n. p. = not practicable

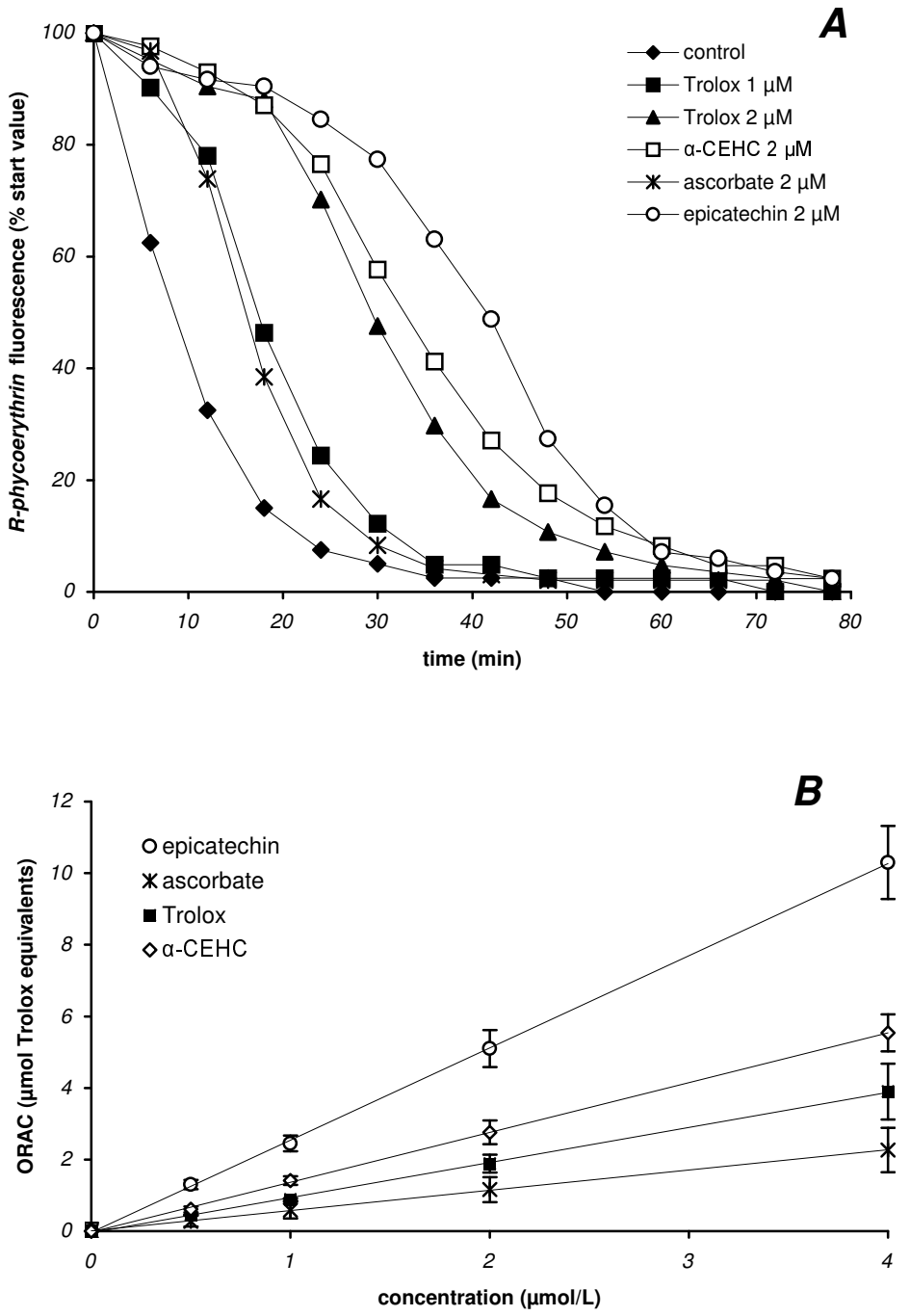


Figure 3. 8 (*A*) Fluorescence of R-phycoerythrin after addition of AAPH and extension of the lag phase with antioxidants at different concentrations. Fluorescence was recorded at 37°C every 6 min until intensity was less than 5% of the first reading. Each compound was tested in triplicate at four different concentrations. (*B*) Dose-response curve by test compounds in the ORAC assay.

3. 1. 3 Inhibition of Tyrosine Nitration by Peroxynitrite

a) Analysis and Chromatography

The amount of initial tyrosine was checked spectrophotometrically before the experiments; the amount of 3-nitrotyrosine formed was determined from calibration curves plotted using authentic 3-nitrotyrosine. 4-Hydroxy-3-nitrobenzoic acid (HNBA) was used as an internal standard. Retention time for 3-nitrotyrosine was 4.3 min and for HNBA 5.7 min. Mobile phase was allowed to flow up to 26 min according to the gradient specified in **2. 4. 3** to elute the test compounds and their reaction products. For calibration plots, peak area ratios of 3-nitrotyrosine:internal standard were plotted against the injected concentration of both compounds. Linear relation was given with

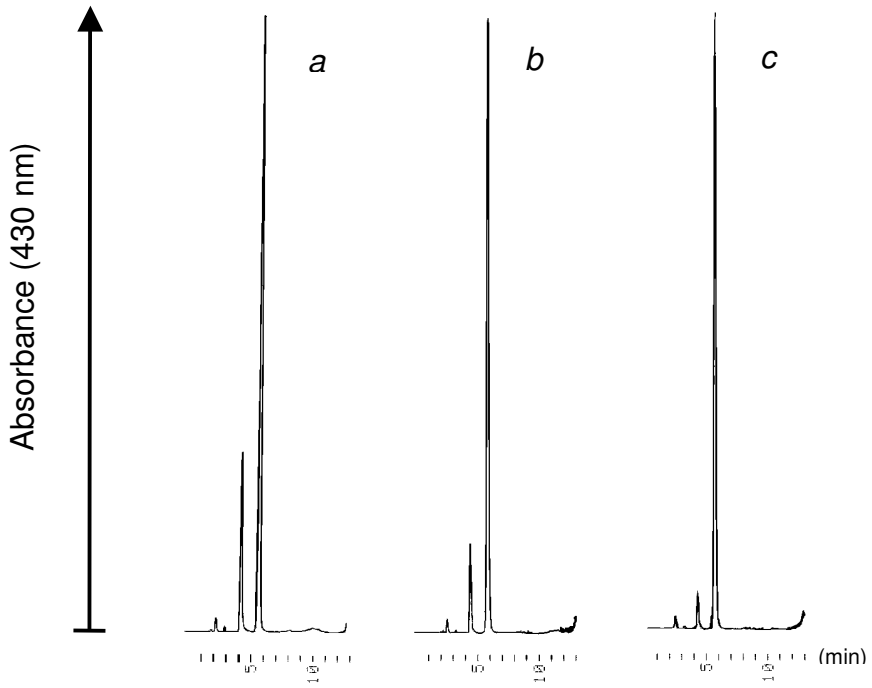


Fig 3. 9 HPLC chromatograms showing (a) maximal amount of 3-nitrotyrosine formed when 350 µmol/L peroxynitrite were added to 100 µmol/L tyrosine, corresponding to 22.3 µmol/L 3-nitrotyrosine; (b) effect of adding 10 µmol/L epicatechin to the reaction mixture; (c) effect of adding 20 µmol/L epicatechin. Peak at 4.3 min corresponds to 3-nitrotyrosine and peak at 5.7 min to 4-hydroxy-3-nitrobenzoic acid (internal standard).

r^2 > 0.995. Limit of quantification for 3-nitrotyrosine was 1.5 µmol/L. Within-day and day-to-day coefficients of variation were less than 5%.

b) Results

As shown in **Figure 3.10**, α-CEHC and Trolox showed a comparable inhibitory capacity against the nitrating activity of peroxynitrite. Epicatechin was the most efficient inhibitor of tyrosine nitration. Reaction with α-tocopherol was not viable.

In the absence of test compound, but in the presence of vehicle (0.4% methanol), the amount of 3-nitrotyrosine generated was 21.6 ± 1.9 µmol/L, which was considered as 100% yield of 3-nitrotyrosine or 0% inhibition of tyrosine nitration. Half-maximal inhibitory concentrations (IC_{50}) of epicatechin, α-CEHC and Trolox were 12, 46 and 52 µmol/L respectively.

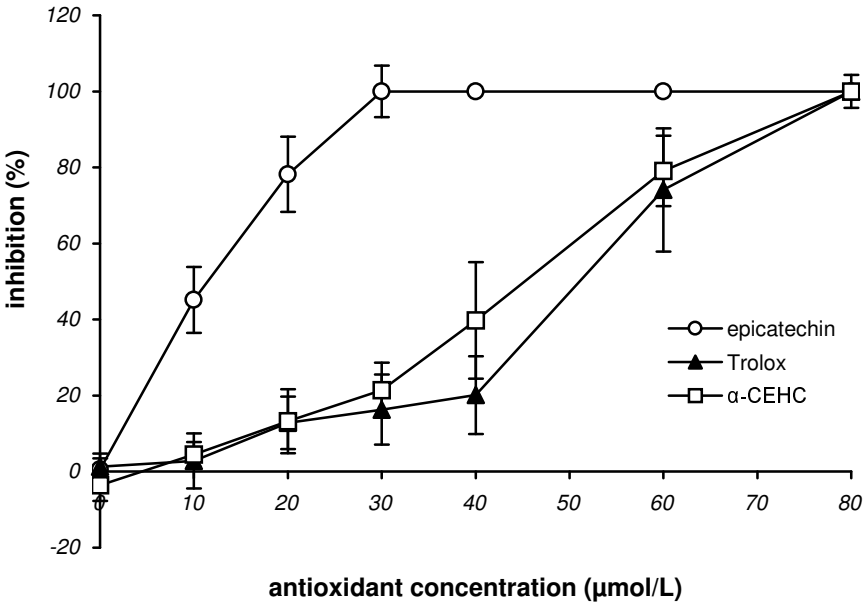


Figure 3. 10 Inhibition of peroxynitrite-mediated formation of 3-nitrotyrosine. n = 3.

3. 2 Cell Culture Study on the Anti-atherogenic Effects of Dietary Micronutrients and α-CEHC

In the former section, a structurally broad range of dietary constituents and several natural extracts were characterised for the antioxidant activity *in vitro*. In the case of the pharmaceutical preparations, the contribution of specific groups of natural occurring compounds to the antioxidant capacity was elucidated. Of the oxidative challenges applied in the former experiments, $ABTS^{\bullet+}$ unquestionably helps identifying the electron-donating ability of substances due to its spectroscopic features and enormous stability, but is an artificial radical not occurring in living organisms. On the other hand, peroxyl radicals (ORAC) and peroxynitrite are reactive species being constantly formed *in vivo* and are produced in excess in pathological conditions where oxidative imbalance plays a crucial role.

For the present section, the antioxidant activity of α-tocopherol and α-CEHC as well as of epicatechin and epigallocatechin gallate, two hydrophilic compounds with high TEAC scores, was assessed in mouse aorta endothelial cell (MAEC) culture exposed to oxidatively-modified human LDL. A novel perspective was approached: *1*. Test compounds were given to cells *before* incubation with the oxidized LDL to get information of intracellular effects alone. *2*. The relation to indicators of cytotoxicity, redox state, and cell death by apoptosis was studied.

3. 2. 1 Cytotoxicity

It is known that LDL oxidized *in vitro* by means of various prooxidants causes damage to endothelial cells which can lead to death depending on the concentration and time of incubation applied. Thus, the first issues to be explored were whether the antioxidants alone have some effect on endothelial cell survival, or if loading the cells with those antioxidants before exposure to oxLDL enhances cell survival.

Using the MTT test, treatment with antioxidant micronutrients (15 or 30 µmol/L) for 24 h was found to have no significant effect on cell viability. Cell morphology was not changed as observed by optical microscopy. The only exception was epigallocatechin gallate at 30 µmol/L. Treatment led to a 40% decay of in mitochondrial function (MTT assay) as compared to solvent control. Consequently,

concentrations selected for later preincubation experiments were: 30 µmol/L for α-tocopherol, α-CEHC and epicatechin, and 15 µmol/L for epigallocatechin gallate.

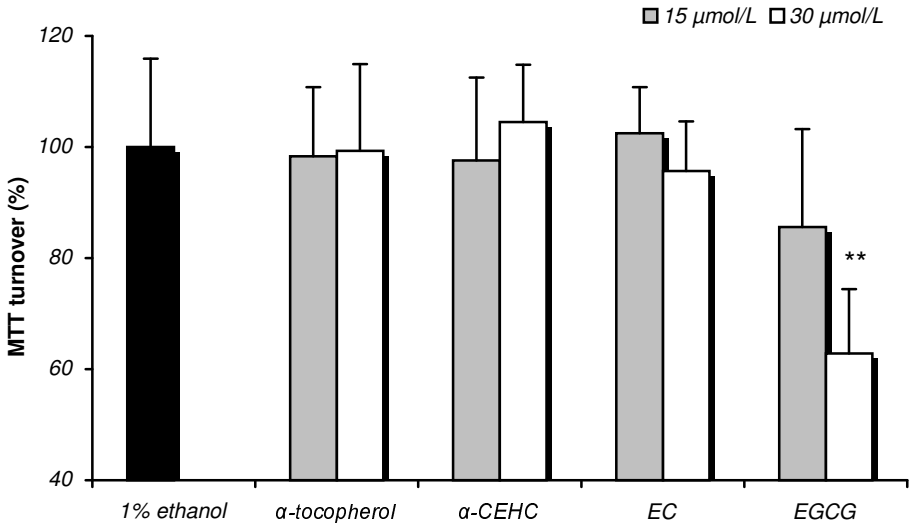


Figure 3. 11 Cell viability after 24 h treatment with the different antioxidants. Viability was measured as MTT oxidation to the blue-coloured compound formazan and referred to the solvent control; n = 4. Significant differences to the control were calculated: (**) $p \leq 0.001$. EC = epicatechin; EGCG = epigallocatechin gallate

Incubation of mouse aorta endothelial cells (MAEC) with 200 nmol/L nLDL over 24 h did not induce mitochondrial disfunction (MTT Test) nor abnormal cell structure patterns were detected. However, stressing with oxLDL for the same time elicited a concentration-dependent decrease of cell viability, as shown in **Figure 3.12**.

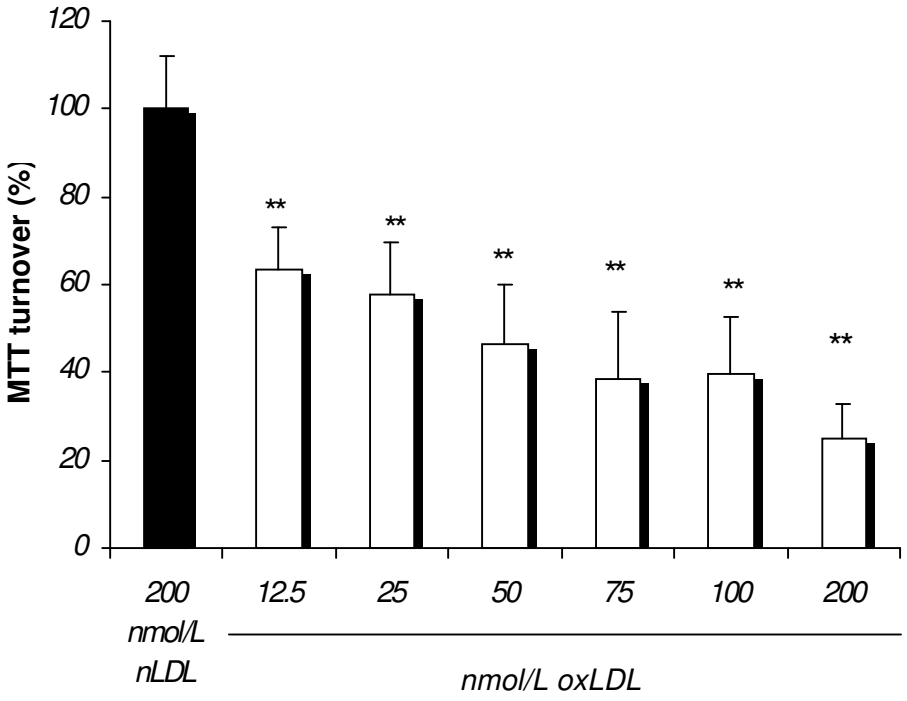


Figure 3. 12 Cell viability after 24 h exposure to oxLDL. Viability was measured as MTT transformation to the blue-coloured formazan and referred to the cells treated with 200 nmol/L nLDL (100%); n = 5. Significant differences to the control were calculated. (**) $p \leq 0.001$.

30 nmol/L (causing approx. 50% cell death) was the amount of oxLDL selected for subsequent experiments. At this level, first detectable microscopic changes in cell morphology occurred after 20 h incubation. Cell bodies were shrunken, cell nuclei and chromatin appeared condensed. Later, blebbing of plasma membrane became more evident and cell detachment from the flask surface followed. **Figure 3.13** shows a typical picture of changes in cell morphology after 24 h incubation with nLDL (30 nmol/L) in comparison to oxLDL. Furthermore, cell blebbing and suspension in the medium after stressing for 48 h with oxLDL is shown.

The most interesting result of the viability tests is that the gradual decay due to incubation with oxLDL (30 nmol/L) could be prevented in part when cells were pre-treated with epigallocatechin gallate (15 µmol/L), epicatechin (30 µmol/L) or α-tocopherol (30 µmol/L). Differences to cells treated with oxLDL alone were statistically significant at 6 h and afterwards. However, pre-incubating cells with α-CEHC had no impact on cell survival (**Figure 3.14**). Therefore, successive expe-

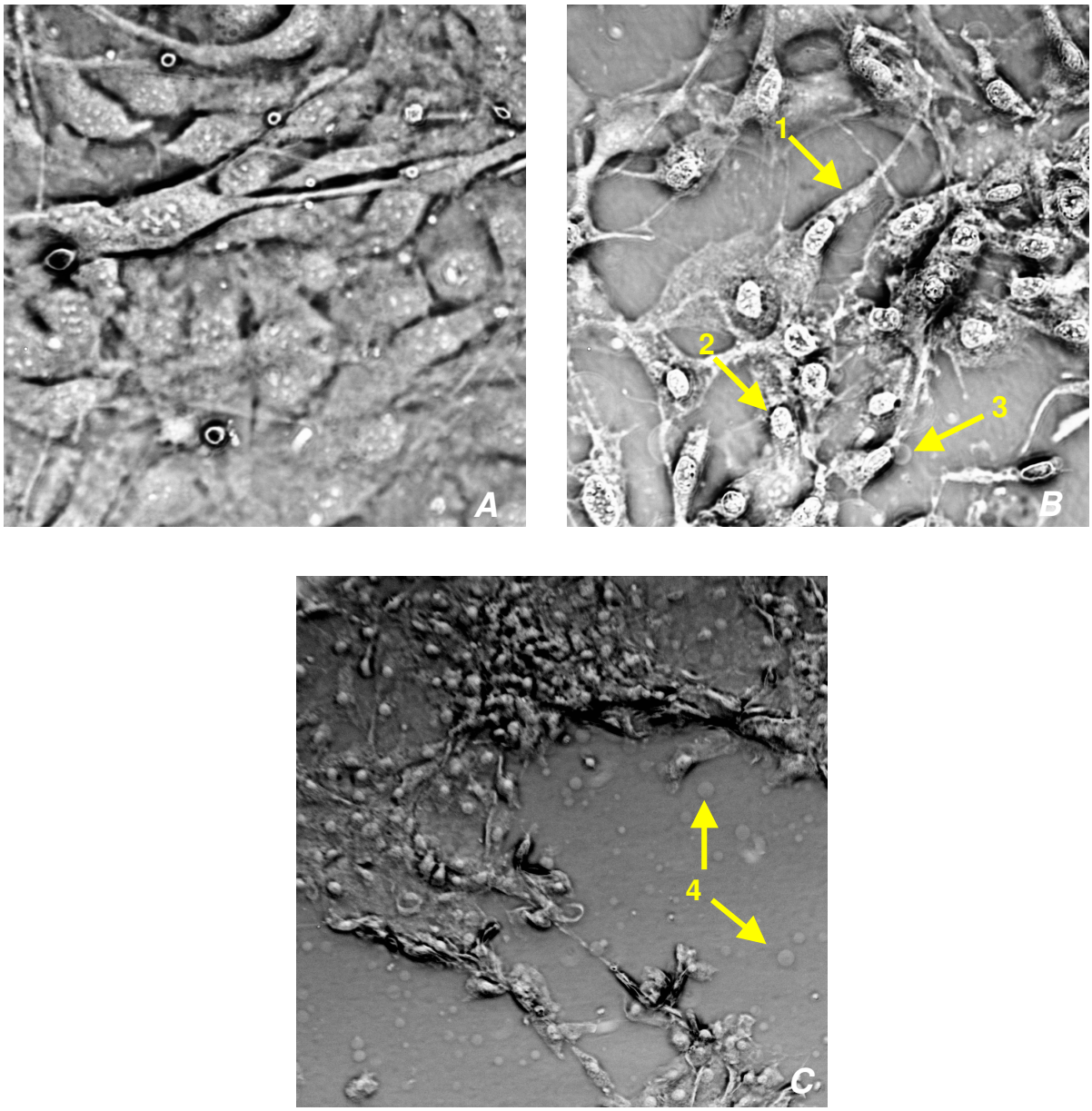


Figure 3. 13 Morphology of MAEC under following conditions: (*A*) 30 nmol/L nLDL, 24 h; (*B*) 30 nmol/L oxLDL, 24h; (*C*) 30 nmol/L oxLDL, 48 h. Yellow arrows show: (1) cell body shrinkage; (2) nucleus and chromatin condensation; (3) cell membrane blebbing, and (4) cell detachment from the culture surface.

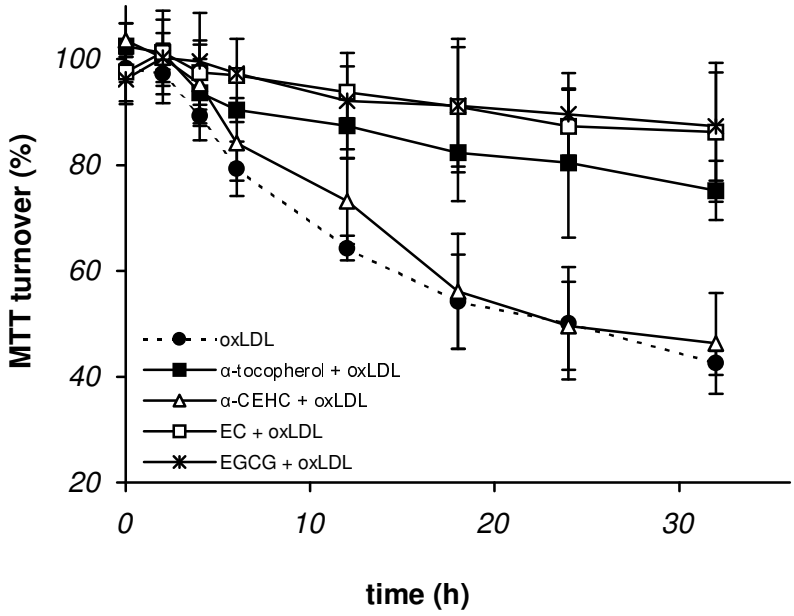


Figure 3. 14 Cell viability after stressing with 30 nmol/L oxLDL. Cells were pre-treated for 20 h with the antioxidants specified in the legend. Concentrations were: 30 µmol/L α-CEHC, α-tocopherol and EC or 15 µmol/L EGCG. n = 3. EC = epicatechin; EGCG = epigallocatechin gallate.

riments were performed in order to elucidate the mechanisms underlying prolonged survival of cells exposed to oxLDL after pre-treatment with the test antioxidant species.

3. 2. 2 Uptake of oxLDL

The first question to be addressed was whether pre-loading the cells with antioxidants would retard or diminish cellular uptake of oxLDL and thereby prevent loss of viability. For that purpose, nLDL and oxLDL were labelled with the fluorescent probe DiI and uptake was followed as described in **2. 5. 4**. DiI-labelled nLDL and oxLDL were taken up by MAEC at a very similar rate as followed by confocal fluorescence microscopy. For both types of LDL, subcellular distribution of fluorescence was identical. Fluorescent images were superimposed to those taken with visible light microscopy. Results are shown in **Figure 3.15**; images of cells treated with nLDL are shown on the left side and those treated with oxLDL are shown on the right. Pictures A and B were taken after 2 h, C and D after 5 h and E and F af-

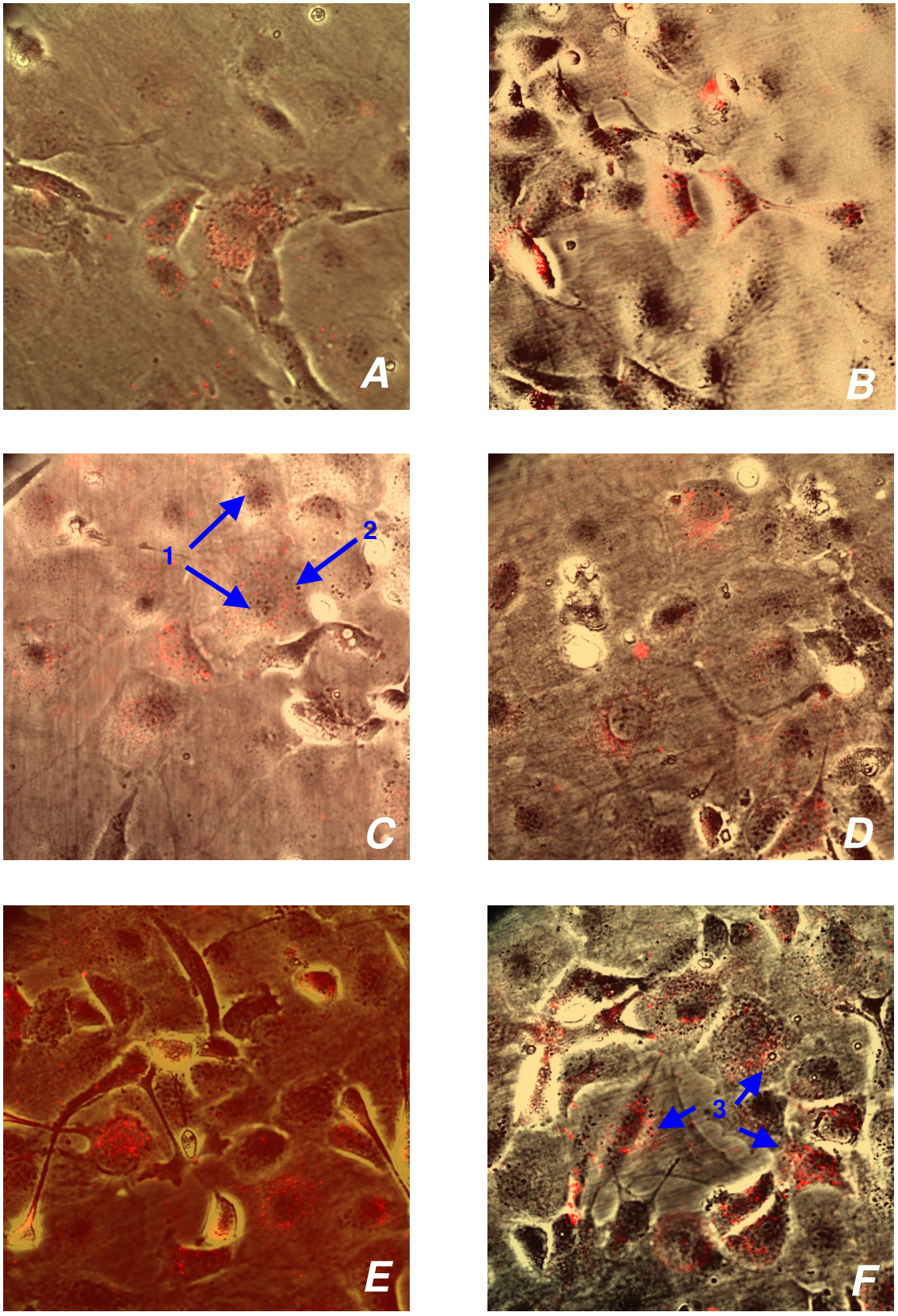


Figure 3. 15 Over-time cellular uptake of DiI-labelled nLDL (left) and oxLDL (right). (*A*) and (*B*) were taken after 2 h, (*C*) and (*D*) after 5 h and (*E*) and (*F*) after 8 h incubation. Blue arrows show: (1) cell nuclei; (2) cytoplasmic localisation of fluorescence, and (3) increase of intracellular fluorescence and of the total number of fluorescent cells over time.

ter 8 h incubation. Over-time increase of intracellular fluorescence and exclusive cytoplasmic distribution of fluorescence with no accumulation in the cell nuclei are evident. Besides, the number of fluorescence emitting cells increased over-time. Changes in cell morphology were not detected even after 8 h of incubation.

In **Table 3.16**, uptake of oxLDL is presented as a function of fluorescence intensity associated with the probe DiI in the chloroform phase obtained by extraction of the cell lysates. Even when cells were pre-incubated with antioxidants, oxLDL-treated cells showed a similar amount of uptake as compared to those treated with nLDL, which served as a control.

Table 3. 16 Uptake of DiI-labelled oxLDL

	Relative uptake of DiI-labelled oxLDL (%)*					
	control	*1% ethanol*	*30 µmol/L α-tocopherol*	*30 µmol/L α-CEHC*	*30 µmol/L EC*	*15 µmol/L EGCG*
6 h	100 ± 9.3	94.3 ± 10.3	103.5 ± 12.6	94.6 ± 11.3	97.6 ± 17.1	104.6 ± 11.7
10 h	100 ± 11.4	99.4 ± 9.6	93.2 ± 15.6	105.3 ± 14.1	105.3 ± 6.8	106.2 ± 7.8

* percentage amount of intracellular DiI after incubation with 100 nmol/L DiI-labelled oxLDL for 6 or 10 h, relative to the control (= 100%; cells treated with the same concentration of DiI-labelled nLDL). n = 5. No significant differences with the control were found.

3. 2. 3 Short-term Variations of the Redox State

In the first experiments performed to assess if the test compounds were acting as antioxidants inside the cells, short-term alterations of the cellular redox state were followed. Intracellular fluorescence due to DCDHF-DA oxidation to DCF is correlated with the amount of reactive oxygen species (ROS) present. Thus, when cells were exposed to oxLDL a rise of ROS was observed; however, it was not perceptible after treatment with nLDL (both 30 nmol/L). On the other hand, pre-treatment with α-tocopherol (30 µmol/L) counteracted this effect, with an inhibition of 30% at the point of greatest fluorescence intensity (6 h). Pre-incubation with the other antioxidants had no effect. It should be pointed out that inhibition effects observed using this

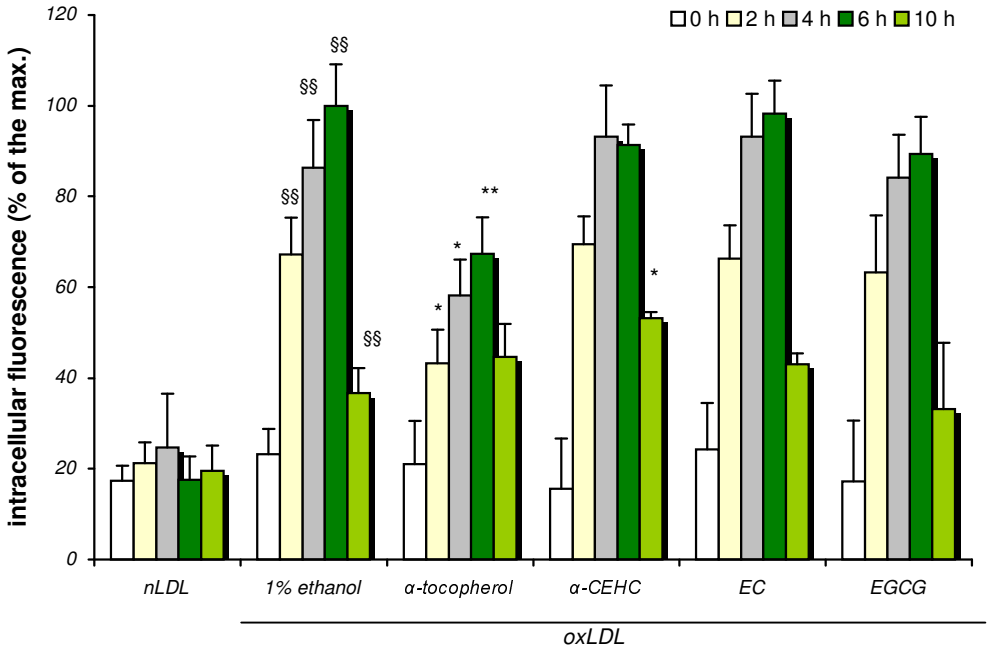


Figure 3. 17 Intracellular amount of ROS calculated as DCF-associated fluorescence (% of the maximal value measured). Cells were pre-treated with either 30 µmol/L α-CEHC, α-tocopherol or epicatechin (EC); 15 µmol/L epigallocatechin gallate (EGCG); or 1% ethanol and subsequently incubated with 30 nmol/L oxLDL for up to 10 h. Cells incubated with 30 nmol/L nLDL were run in each series. n = 3. (§§) indicates significant differences between oxLDL-treated and nLDL-treated cells; $p \leq 0.001$. (*) indicates significant differences between antioxidant-pre-treated cells and cells pre-treated with 1% ethanol and then exposed to 30 nmol/L oxLDL; $p \leq 0.05$. (**) $p \leq 0.001$.

assay cannot be ascribed categorically to ROS scavenging, since modulation of ROS production by several antioxidants cannot be excluded.

3. 2. 4 Long-term Variations of the Redox State

a) Measurement of Total Free Thiols

Total free thiols in the cell include not only GSH but also sulfhydryl moieties in other molecules such as proteins, where cysteinyl residues involved and not involved in disulfide bonds coexist. The balance of the thiol pool of a cell is a highly regulated process where glutathione reductase, glutathione peroxidase and NADPH produced in the pentose-phosphate route play an important role; ROS including lipid peroxides from oxLDL readily oxidize sulfhydryl groups and in excess they may impair the thiol protective system.

Exposure to 30 nmol/L nLDL for up to 24 h did not affect the free thiol pool of MAEC (data not shown). In combination with the results exposed for cell viability, morphology and ROS production, it is clear that natural oxidation of lipoprotein constituents inside the cell did not cause any detectable changes within the first 24 h of incubation.

However, stressing with oxLDL lowered the level of thiols by 50% after 10 h and by 65% after 24 h indicating a striking deterioration of the cell redox sate. Pre-treatment with α-tocopherol, epicatechin (both 30 µmol/L) or epigallocatechin gallate (15 µmol/L) was able to diminish this effect in a significant manner after 6 h incubation with oxLDL, as shown in **Figure 3.18**; α-CEHC showed no significant protective activity. Cells pre-treated with catechins were best protected with thiol amounts at 24 h twofold higher than those of oxLDL-stressed cells at the same time point.

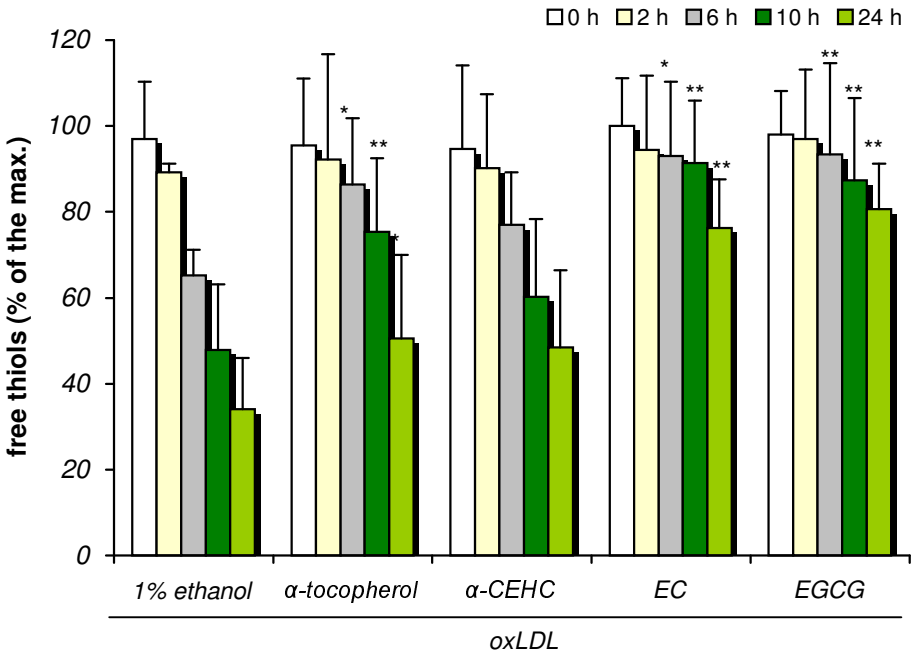


Figure 3. 18 Amount of free thiols measured over time using derivatization with DTNB. Cells were pre-treated with either 30 µmol/L α-CEHC, α-tocopherol or epicatechin (EC); 15 µmol/L epigallocatechin gallate (EGCG); or 1% ethanol, and subsequently exposed to 30 nmol/L oxLDL up to 24 h. Cells incubated with 30 nmol/L nLDL were run in each series. n = 3. (*) indicates significant differences between antioxidant-pre-treated cells and cells pre-treated with 1% ethanol and then challenged with 30 nmol/L oxLDL; $p \leq 0.05$. (**) $p \leq 0.001$.

b) Measurement of Glutathione

In the DTNB-GSSG reductase recycling procedure, *total* glutathione is measured as the sum of GSH plus twice the amount of GSSG. However, it should be noted that other forms of glutathione exist in the cell, e. g., mixed disulfides or thioethers. A decrease in the levels of total glutathione may result from processes other than GSH oxidation to GSSG, such as from conjugation to other molecules, inhibition of its synthesis *de novo*, or leakage of GSSG into the medium, a well known phenomenon.

Cells which were treated with antioxidants alone for 24 h did not undergo any significant changes in GSSG or total glutathione levels when compared to the solvent control (data not shown).

Table 3. 19 Intracellular level of glutathione upon incubation with nLDL or oxLDL.

		total glutathione (nmol / mg protein)		**glutathione disulfide (nmol / mg protein)**	
	n	*3 h*	*24 h*	*3 h*	*24 h*
*control**	3	34.7 ± 4.5	39.8 ± 4.5	1.3 ± 1.0	1.2 ± 1.0
nLDL	3	35.8 ± 4.8	34.7 ± 4.8	1.4 ± 1.2	2.0 ± 1.2
oxLDL	3	31.6 ± 7.2	14.2 ± 7.2	9.7 ± 0.7	12.2 ± 0.7
α-tocopherol[§]	3	37.3 ± 6.3	28.7 ± 6.3	2.0 ± 0.9	22.6 ± 6.3
α-CEHC[§]	3	32.5 ± 5.0	13.5 ± 5.0	7.7 ± 2.3	11.3 ± 2.3
EC[§]	3	36.9 ± .8	17.6 ± 1.8	8.6 ± 0.4	5.3 ± 0.4
EGCG[§§]	3	29.6 ± 5.0	14.3 ± 5.0	13.3 ± 1.9	4.0 ± 1.9

* cells incubated with 1% ethanol (instead of antioxidants) for 24 h and kept subsequently in serum-free medium (instead of LDL) for the time frame specified.

[§] cells pre-incubated with 30 µmol/L of the corresponding antioxidant for 24 h and challenged subsequently with 30 nmol/L oxLDL for the time frame specified.

[§§] cells pre-incubated with 15 µmol/L of EGCG for 24 h and challenged subsequently with 30 nmol/L oxLDL for the time frame specified.

EC = epicatechin; EGCG = epigallocatechin gallate

When cells were challenged with 30 nmol/L oxLDL for 3 h, a non-significant significant decrease in total glutathione from 35.8 ± 4.8 to and a significant increase in GSSG levels were observed in relation to the control and the nLDL-treated cells (**Figure 3.20**). After 24 h, total glutathione was diminished from 31.6 ± 7.2 at 3 h to 14.2 ± 7.2 (**Figure 3.21**), and GSSG increased from 9.7 ± 0.7 to 12.2 ± 0.7.

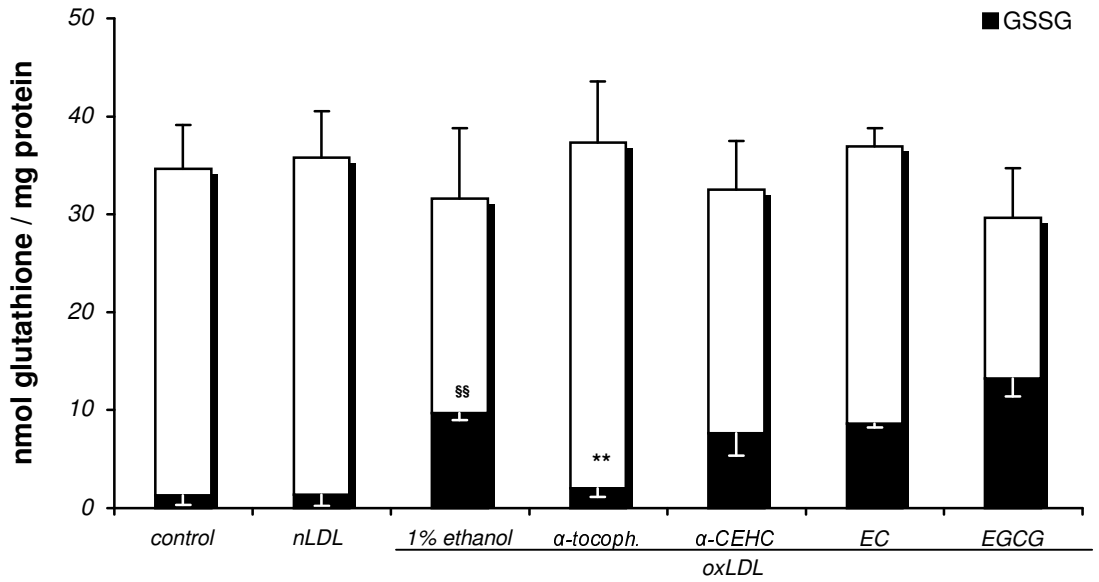


Figure 3. 20 Total non-bound glutathione and GSSG using the DTNB-glutathione reductase recycling method. Cells were pre-treated for 24 h with 30 µmol/L α-CEHC, α-tocopherol and epicatechin (EC); 15 µmol/L epigallocatechin gallate (EGCG); or 1% ethanol, and subsequently incubated with 30 nmol/L oxLDL for 3 h. Cells incubated with 30 nmol/L nLDL were run in each series. n = 3. (§§) indicates significant differences between oxLDL-treated and nLDL-treated cells for GSSG; $p \leq 0.001$. (**) indicates significant differences between antioxidant-pre-treated cells and those pre-treated with 1% ethanol and then exposed to 30 nmol/L oxLDL (oxLDL balk) for GSSG; $p \leq 0.001$.

Test compounds showed no protective effects at 3 h. The only exception was α-tocopherol, for which the GSSG/GSH ratio was comparable with that of the nLDL-treated cells. At 24 h (**Figure 3. 21**), all compounds except α-CEHC exerted positive actions of two distinct kinds: α-tocopherol was the most effective in terms of protecting total non-bound glutathione, whereas epicatechin and epigallocatechin gallate contributed by keeping the GSSG level significantly lower than that of cells only treated with oxLDL.

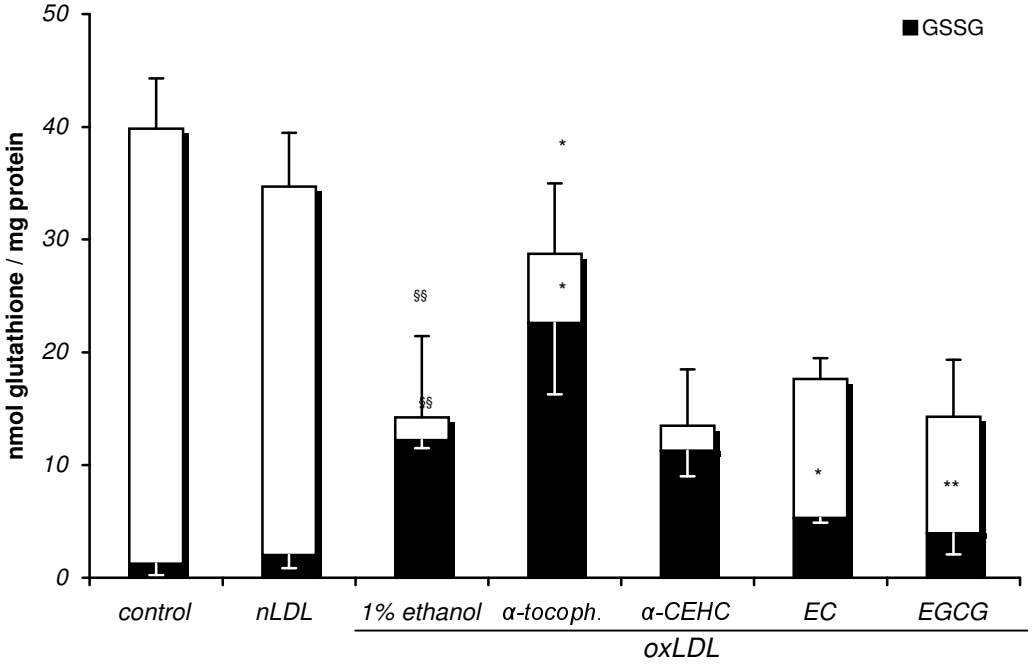


Fig 3. 21 Total non-bound glutathione and GSSG at 24 h. n = 3. (§§) indicates significant differences between oxLDL-treated and nLDL-treated cells for GSSG and total glutathione; $p \leq 0.001$. (*) indicates significant differences between antioxidant-pre-treated cells and those pre-treated with 1% ethanol and then 30 nmol/L oxLDL; $p \leq 0.05$. (**) $p \leq 0.001$. EC = epicatechin; EGCG = epigallocatechin gallate.

3. 2. 5 Apoptotic Cell Death

Figure 3.13 showed that apoptosis is an important component of cell death caused by oxLDL at the concentrations and level of oxidation applied. Cell body shrinkage, nuclei condensation and plasma membrane blebbing are considered markers of apoptotic cell death. However, an indicator functioning at a molecular level is more specific and may facilitate the quantification of relative contribution of apoptosis to cell death.

Caspases (cysteine-requiring aspartate proteases) are a family of proteases that mediate cell death by apoptosis. Caspase-3, an effector caspase, can specifically cleave most of caspase related substrates known to date, including key proteins such as the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP) or others important for the integrity of the cytoskeleton. In addition, caspase-3 plays a central role in mediating nuclear apoptosis including chromatin condensation, DNA fragmentation and cell blebbing.

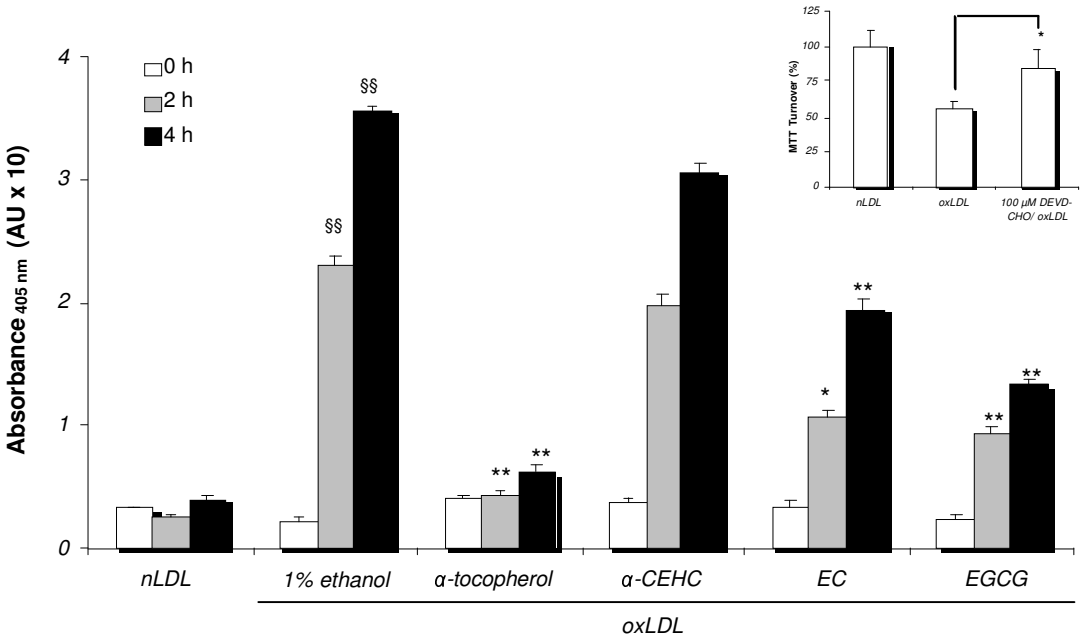


Fig. 3. 22 Caspase 3 activity 0, 2 and 4 h after addition of 30 nmol/L nLDL or oxLDL and effect of pre-incubation with antioxidants. n = 5. (§§) indicates significant differences between oxLDL-treated and nLDL-treated cells; $p \leq 0.001$. (*) indicates significant differences between antioxidant-pre-treated cells and those pre-treated with 1% ethanol and then 30 nmol/L oxLDL; $p \leq 0.05$. (**) $p \leq 0.001$. Upper graph: caspase 3 contribution to cell death triggered by incubating with 30 nmol/L oxLDL for 24 h. Cells were pre-incubated with (left column) or without (middle column) a caspase-3 specific inhibitor for 30 min prior to oxidative challenge. EC = epicatechin; EGCG = epigallocatechin gallate.

Involvement of caspase-3 in the cytotoxic effects of oxLDL was demonstrated. Addition of the caspase-3 inhibitor DEVD-CHO before incubation with oxLDL increased cell viability by 50% after 24 h when compared with cells only treated with oxLDL. About 50% cell death after 24 h could be thus directly attributed to caspase-3 activation (small panel in **Figure 3.22**).

Caspase 3 activity rapidly increased within the first 4 h in cells which were pre-incubated with 1% ethanol or α-CEHC; with the flavonols, caspase 3 increase was moderate, and with α-tocopherol it was extremely low.

3. 3 Human Intervention Study on the Effects of Smoking on Vitamin E Metabolism after a Single Dose of Vitamin E

α- and γ-CEHC are metabolites of α- and γ-tocopherol and -tocotrienol in human blood; γ-CEHC (also called LLU-α) has been shown by others to possess antioxidant activity and effects at cellular level such as prevention of inflammatory responses on in macrophages and epithelial cells or growth arrest of a prostata carcinoma cell line; for α-CEHC, functions *in vivo* are unknown.

In this work, α-CEHC has been shown to act as an antioxidant in aqueous solution, being able to scavenge $ABTS^{\bullet+}$, peroxyl radicals and peroxynitrite with an efficacy comparable to Trolox. Thus, so far it may be affirmed that α- and γ-CEHC contribute as antioxidants to the antioxidant defense system; in contrast to their parent compounds, which are active in lipoproteins and cell membranes, they likely act in the aqueous phase. Therefore, the question was addressed whether conditions of oxidative stress may influence the levels of α- and γ-CEHC *in vivo*. Smoking is a situation where oxidative stress is increased [Polidori et al., 2001; Polidori et al., 2003] and was investigated in this study.

3. 3. 1 HPLC Analysis of α- and γ-CEHC in Serum

Under the conditions of analysis (for details see **2. 6. 2)**, recovery of α-CEHC was 99.6 ± 1.5% (n = 4) and that of γ-CEHC was 87.9 ± 2.8% (n = 3). Addition of antioxidants (vitamin E, vitamin C) during extraction was necessary to avoid decomposition of the analytes.

A chromatogram of a human serum sample is shown in **Figure 3.23**. The analytes are clearly separated and eluted with retention times of 12.3 min for α-CEHC and 8.6 min for γ-CEHC. The limit of quantification in serum was 1.5 nmol/L for both compounds; the detection limit was 0.75 nmol/L at a signal to noise ratio of 3/1. For both compounds the signal was linear in the range of 0.75-750 nmol/L, related to the final concentration in the sample. Linear regression coefficients were 0.9997 for α-CEHC and 0.9999 for γ-CEHC. Coefficients of variation for within-day precision in three independent experiments ranged from 2.6 to 5.1% for α-CEHC and

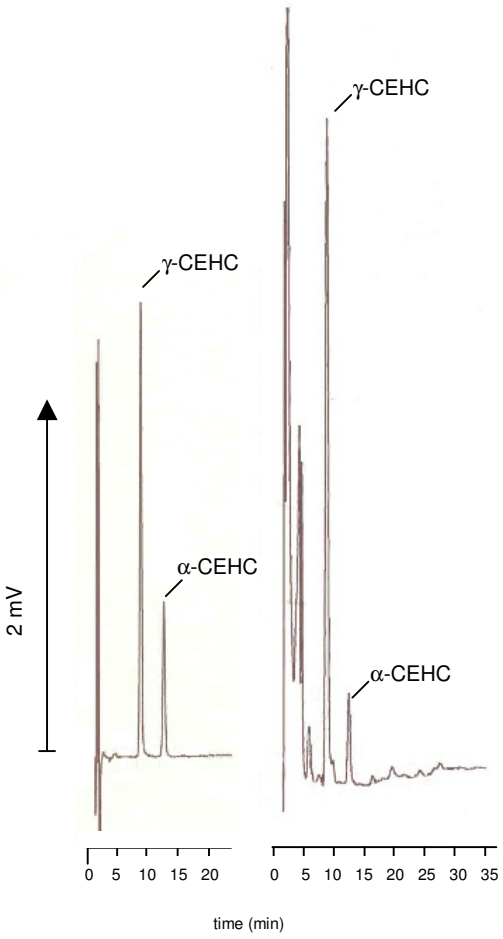


Fig 3. 23 HPLC chromatogram of standards (left); γ-CEHC standard (201.5 nmol/L) eluted at 8.6 min, α-CEHC standard (177.3 nmol/L) eluted at 12.3 min. The chromatogram on the right shows a human serum sample.

from 2.8 to 3.9% for γ-CEHC. Inter-day precision was calculated with coefficients of variation of 2.7% for α-CEHC and 1.8% for γ-CEHC.

3. 3. 2 HPLC Analysis of α- and γ-Tocopherol

Reference compounds were used for tocopherol identification. Pool serum was regularly analysed and served as quality controls for tocopherol analysis. δ-tocopherol (2 µg/sample) was used as an internal standard. Recovery of the internal standard was reproducible during the study. Coefficient of variation corresponding to the height of the internal standard signal was 15%. For quantitation, the ratio between peak height of the analyte and that of δ-tocopherol was used; based on a regression curve calculated from calibration samples ($r > 0.998$), the concentration of the analytes in the samples was calculated.

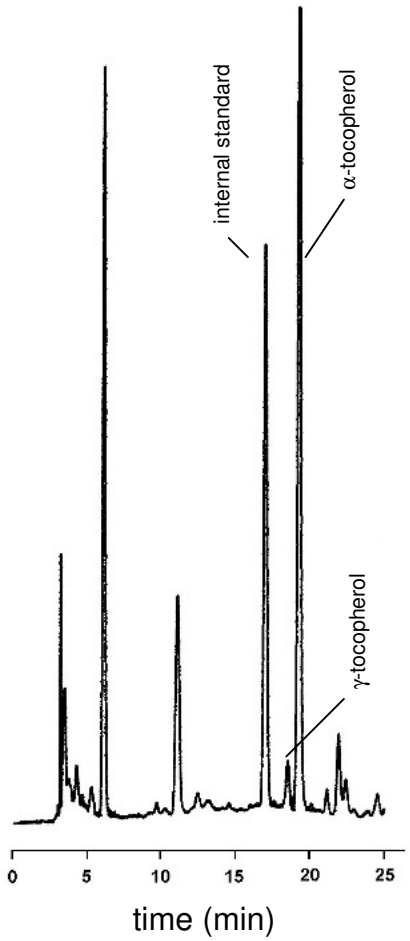


Fig 3. 24 Typical HPLC chromatogram from tocopherol analysis in human serum under the conditions specified in 2. 6. 3.

3. 3. 3 General Remarks

There was a pronounced inter-individual variability in the uptake and biotransformation of the orally applied α-tocopherol (306 mg), which was independent of factors like smoking or gender. This phenomenon is already known not only for α-tocopherol but also for other isomers of vitamin E. Using the non-parametric Wilcoxon rank-sum test, over-time changes of all parameters were significant ($p < 0.001$). Besides, certain trends of interest deserve special attention.

In **Figure 3.25**, individual responses for α-tocopherol serum levels in smoking and non-smoking subjects are shown. The maximal concentrations of α-tocopherol occur between 6 and 12 h after supplementation. From **Figure 3.26**, where mean values and standard deviations are presented, a net average increase of 8.87 µmol/L for smokers and 12.90 µmol/L for nonsmokers was calculated at the t_{max} (12

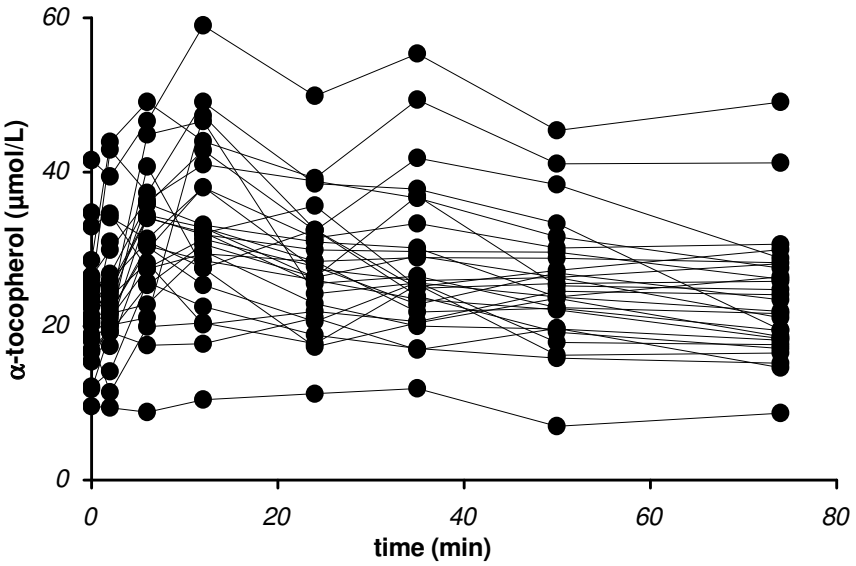


Figure 3. 25 α-tocopherol levels in all subjects after supplementation with one single dose of vitamin E.

h). Although absolute bioavailability in the classical pharmacokinetic approach cannot be calculated (since a concentration/time curve after intravenous application of the same dose is needed), from the C_{max} and assuming an average blood volume of 5 L in the adult body it is obvious that only a small portion of the total oral dose was transferred into the blood. This suggests a limited intestinal absorption and/or a pronounced first-pass metabolism (immediate liver processing) of α-tocopherol.

While α-tocopherol levels decreased between time points 12 and 24 h in all subjects with only 5 exceptions (**Figure 3.25**), mean α-tocopherol levels are very similar at 24 h and 35 h, which shows a counteraction of the process of elimination started after 12 h. New intake of α-tocopherol with the diet over the day or re-absorption of some of the α–tocopherol ingested with the oral supplement due to an enterohepatic cycle may account for this phenomenon. Biliary excretion and intestinal reabsorption of α-tocopherol has already been described [Stahl et al., 2002].

γ-Tocopherol concentrations decreased up to 24 h in both groups after intake of the vitamin E capsule. This level was still detected 72 h after supplementation (**Figure 3.27**). Even though the capsule contained 1.77 mg of γ-tocopherol, an increase of this parameter was not expected due to the co-ingestion with a much larger amount of α-tocopherol and the known metabolic interferences between these two vitamers, which will be discussed later.

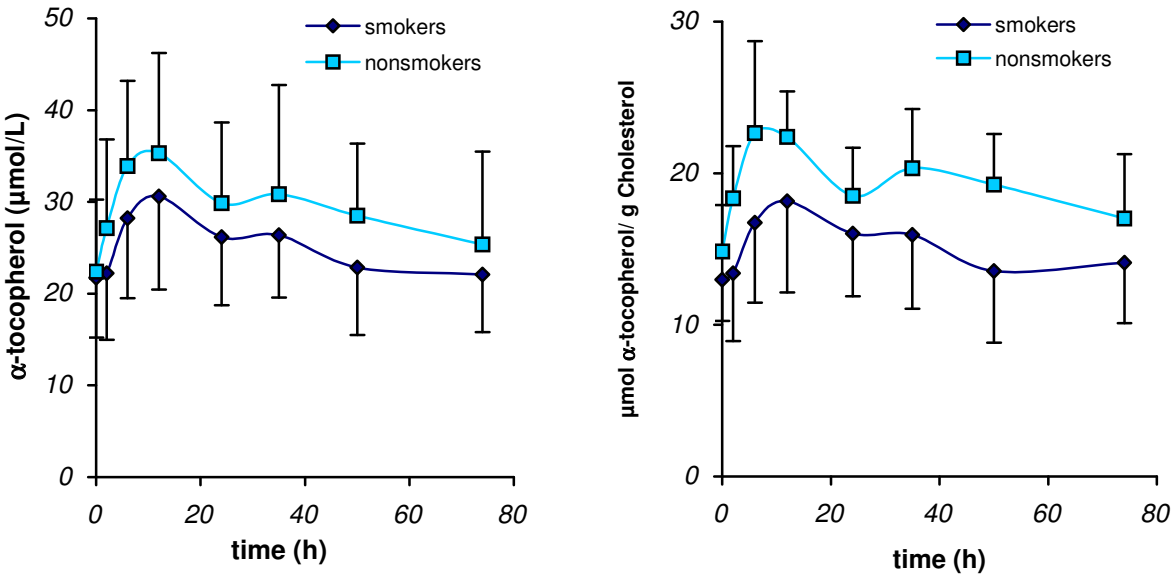


Figure 3. 26 α-Tocopherol serum concentrations in smokers and nonsmokers after supplementation with vitamin E. Concentrations in the right graph are corrected for cholesterol.

It should be noted that baseline α-tocopherol levels corresponding to both groups together but not those of γ-tocopherol were significantly correlated with baseline cholesterol levels ($r = 0.680$; $p \leq 0.001$). Hence, α-tocopherol was also expressed per g serum cholesterol, the corrected values were plotted to search for differences with the former values and are shown in **Figure 3.26**. It is obvious that kinetic patterns are the same for cholesterol-normalized or uncorrected α-tocopherol, however, differences between smokers and nonsmokers are more obvious. Also noteworthy is the fact that baseline levels of α-tocopherol were about 10 fold those of γ-tocopherol, irrespective of smoking and gender, whereas no linear correlations between baseline α-tocopherol and γ-tocopherol levels were found.

α-Tocopherol concentrations were 2000-fold higher than those of its metabolite α-CEHC; γ-tocopherol levels were only 17 to 19 times higher than those corresponding to γ-CEHC. This is a striking indicator of the different metabolism of both vitamers in humans. On the other hand, no significant correlations between baseline α-tocopherol and α-CEHC levels nor between baseline γ-tocopherol and γ-CEHC levels were found. Thus, under unsupplemented conditions, the fates of α- and γ-tocopherol rather depend on inter-individual variability in the metabolic machinery than on blood tocopherol concentrations.

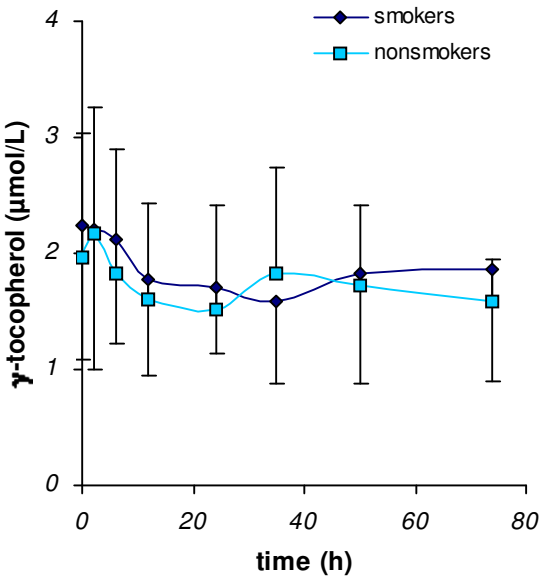


Figure 3. 27 γ-tocopherol serum concentrations after a single oral dose of vitamin E.

3. 3. 4 Differences between Smokers and Nonsmokers in Vitamin E Metabolism

Baseline levels of α-tocopherol were similar for smokers and nonsmokers (21.74 ± 6.51 and 22.40 ± 7.84 µmol/L, respectively). However, the increment was proportionally higher in nonsmokers (59% at the t_{max}) when compared to smokers (only 38%). Despite sharing a comparable kinetics, mean values for nonsmokers were higher than those for smokers at all time points. The direct consequence was that smokers reached baseline levels of α-tocopherol faster (at 50 h, vs. 72 h or later for nonsmokers).

Differences in serum levels of α-CEHC between both groups were almost undetectable. However, the trend was inverse to that detailed above for α-tocopherol, showing smokers slightly higher values (**Figure 3. 28**).

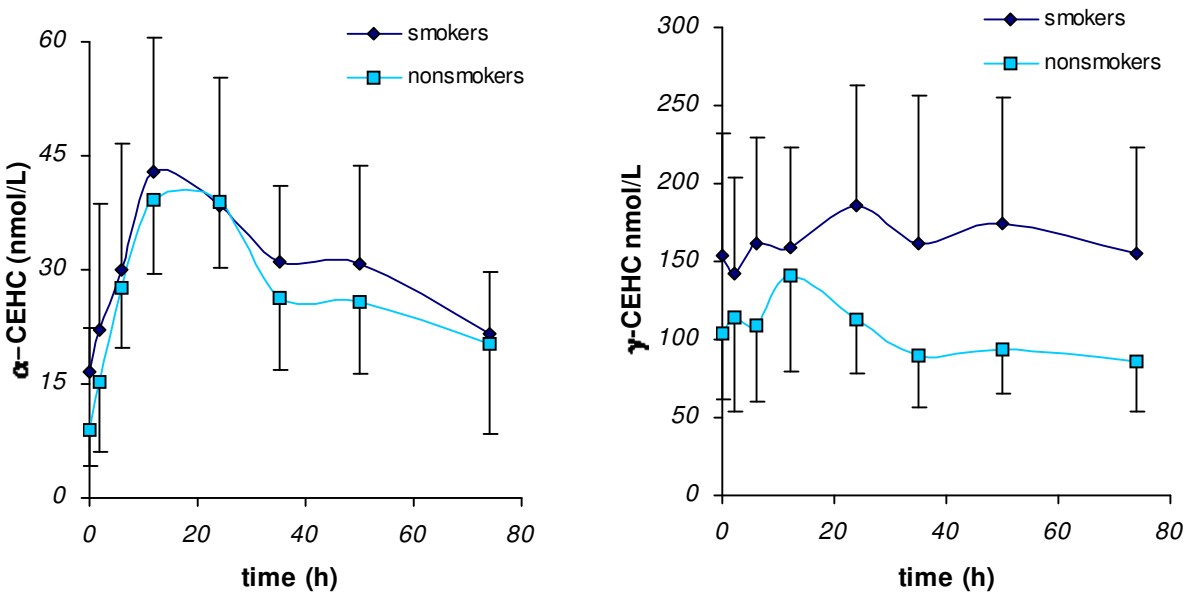


Figure 3. 28 α-CEHC (left) and γ-CEHC (right) levels in smokers and nonsmokers after a single dose of vitamin E.

The most evident differences between both groups were found in γ-CEHC levels. The mean baseline level of γ-CEHC in nonsmokers is 1.5-fold higher than that of smokers. This difference is maintained or even amplified over time (after a short convergence of the values) upon supplementation. In view of the data shown here, it is clear that a single dose of α-tocopherol accelerates the degradation of endogenous γ-tocopherol for a short period of time in smokers, but it is uncertain in which way it affects γ-tocopherol metabolism in smokers. However, it may be affirmed that a larger amount of γ-tocopherol is continually transformed to γ-CEHC in smokers, irrespective of the supplementation with α-tocopherol.

3. 3. 5 Further data of relevance

Very few intervention studies on vitamin E metabolism in humans involving blood levels of CEHC´s have been performed up to date, and the number of subjects

investigated is still very low. Hence, normal or reference values of those metabolites are missing. So far, it seems clear that the enormous range of responses in blood tocopherol levels among individuals after supplementation is also reflected in CEHC levels.

In our study, two volunteers were found for whom values or orders of magnitude of some parameters clearly differ from median and mean value of their corresponding group.

Subject 1 was a healthy 26-year-old male smoker with a body mass index (BMI) of 22.89; subject 2 was also a healthy male smoker, aged 33, with a BMI of 19.79. Subject 1 was slightly hypercholesterolemic. Baseline data of these volunteers as compared to the mean ± standard deviation corresponding to all smokers studied are presented in **Table 3.29**:

Table 3. 29 Baseline serum concentrations of subjects 1 and 2 in comparison to baseline levels of smokers.

	cholesterol (g/L)	**α-tocopherol (µmol/L·g chol)**	**γ-tocopherol (µmol/L)**	**α-CEHC (nmol/L)**	**γ-CEHC (nmol/L)**
n	13	13	17	14	14
subject 1	2.13 (2)	7.9 (13)	1.7 (10)	36.7 (1)	1096 (2)
subject 2	1.44 (9)	16.6 (2)	4.9 (1)	13.7 (7)	1099 (1)
smokers:					
median	1.58	12.7	2.1	14.5	130
mean ± SD	1.64 ± 0.36	13.0 ± 2.7	2.3 ± 1.2	16.5 ± 8.3	153 ± 79

Ranks of the subjects for each parameter are presented in brackets. (1) = the highest.

In both subjects, overall γ-CEHC levels were 6 to 7 times higher than the mean for smokers. In subject 1, overall α-CEHC levels were the highest (2 to 5 times higher than the mean for smokers) and overall α-tocopherol (including non-normalized values) was the lowest among smokers. Subject 2 was normocholesterolemic and showed very high overall levels of α- and γ-tocopherol. His α-CEHC levels after the baseline were about one half the values corresponding to the mean for smokers.

An *apparent* metabolic ratio was calculated for every time point by dividing the concentration of each metabolite (α- or γ-CEHC, nmol/L) by the concentration of the corresponding tocopherol (µmol/L).The word *apparent* is added to clarify that:

a) This parameter does not correspond to the *metabolic ratio* used in formal pharmacokinetics, for which urine amounts of the metabolite are needed. Thus, the major factor neglected is renal excretion of the metabolite; it should be pointed out, though, that renal clearance in healthy subjects mainly depends on the solubility and molecular weight of the substance excreted itself.

b) It is assumed that α- and γ-CEHC are the major final metabolites of α- and γ-tocopherol, respectively.

From **Figure 3.30**, it is evident that the increase in α-CEHC levels following vitamin E intake greatly exceeded the increase in α-tocopherol levels, since the apparent metabolic ratio increased to two-fold of the baseline at 12/24 h. This reflects that acute loads of α-tocopherol are subject to a rapid and massive degradation so that the pool of α-tocopherol already present is preserved.

Putting the data from **Table 3.29** and **Figure 3.30** together, it may be affirmed that subject 1 is an extensive metabolizer of both vitameres; subject 2 holds a large endogenous pool of vitamin E, metabolizes γ-tocopherol extraordinarily fast and is a slow metabolizer of newly-ingested α-tocopherol.

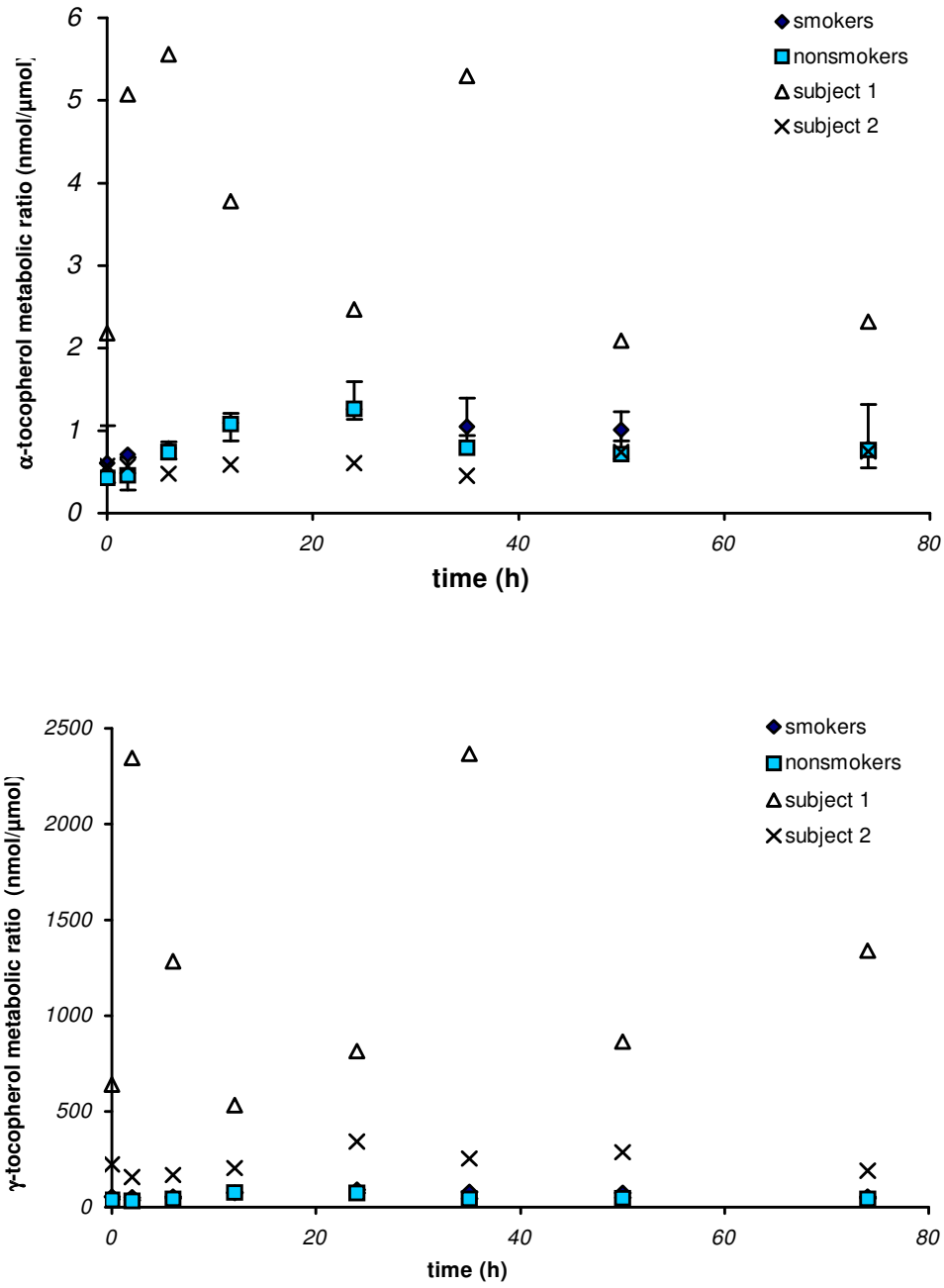


Figure 3. 30 Over-time α-tocopherol and γ-tocopherol *apparent* metabolic ratios. These were calculated by dividing concentrations of each metabolite (CEHC, in nmol/L) by the levels of the corresponding parent compound (tocopherol, in µmol/L) at all time points. Plotted values represent mean ± SD for all smokers or all nonsmokers; single ratios for two volunteers are also presented (see 3. 3. 5).

4. DISCUSSION

4. 1 Antioxidant Activity of Dietary Micronutrients in Model Systems

Nearly all single dietary micronutrients were active in the systems investigated. A potential antioxidant activity of harpagoside, due to the presence of hydroxyl groups in the bicyclic ring (see **Figure 3.6**), was not confirmed, however. In general, the ranking of antioxidant activities of the single compounds obtained by applying the TEAC assay (see **Table 3.1**) could be explained by specific structural features (see below), and the activities of dietary extract fractions were strictly related to the contents of polyphenols (**Table 3.7**).

4. 1. 1 TEAC and ORAC Assays

- *Polyphenols – phenylpropanoids*

Among flavonoids, the flavonol kaempferol, containing a single 4´-hydroxyl group on the B ring (**Figure 3.6**), exerted the lowest activity measured in the TEAC assay (**Table 3.1**). Both the flavan-3-ol epicatechin and the flavone luteolin, which share a catechol group as B ring showed intermediate activities. Epigallocatechin gallate, with three neighbouring hydroxyl groups on the B ring (**Figure 1.8**), exhibited a much better antioxidant activity than epicatechin. It is thus evident that the electron-donating capacity of the molecule becomes enhanced by increasing the number of adjacent hydroxyl groups on the B ring. It should be pointed out that among flavonoids with a 4´-monohydroxyl group (e. g. apigenin, pelargonidin), kaempferol has been identified as the best antioxidant due to the following reasons: *1)* the B ring is conjugated with the C ring (2, 3 positions and the 4-carbonyl group) and *2)* the 3-hydroxyl group enhances the potential for conjugation [Pannala et al., 2001]. This should be the rationale for the TEAC value of kaempferol being so close to that of epicatechin.

In cholorogenic acid, the electron delocalization over the catechol ring is expanded over the propenoate chain (**Figure 3.6**). In view of the TEAC values, however, the antioxidant activity is higher when a chromanol ring is bound to the catechol as in flavonoids.

Both catechol rings of curcumin are partially *hindered* by methylation of one of the hydroxyl groups (**Figure 3.6**). Nonetheless, the introduction of a methoxy group

ortho to phenol increases the antioxidant activity of unsubstituted monophenols by probable two mechanisms: *1)* it enhances the stability of the phenoxyl radical by an inductive effect, and *2)* steric hindrance disturbs the formation of hydrogen bonds to the medium, thus enhancing the transfer of the hydrogen atom (in a manner comparable to the methyl groups in α-tocopherol). It seems that curcuminoids act as classical chain-breaking antioxidants like α-tocopherol; their antioxidant activity decreases in hydrogen bond forming media and synthetic, non-phenolic curcuminoids are mostly inactive [Barclay et al., 2000]. It is noteworthy that curcumin is stabilized by a keto-enol tautomeric form, which confers the molecule its characteristic color, under formation of an intramolecular hydrogen bond so that hydrogen abstraction from the central carbon is unlikely and should not contribute to the antioxidant properties.

Two distinct patterns have been described concerning the general mechanisms underlying the antioxidant activity of phenylpropanoids. It appears that the oxidation of catechol-containing flavonoids consists of a two-electron donation process yielding a semiquinone and successively an ortho-quinone [Jovanovic et al., 1998, Pannala et al., 2001]. On the other hand, hindered and single phenols react *via* formation of phenoxyl radicals, of which a part dimerise and another fraction may continue to act as antioxidants yielding phenolates. The biphasic responses obtained for curcumin and the turmeric fractions B, C and D (Figure **3.5**) are hence explained as a result of curcumin radicals being formed and coupled in the reaction mixture to yield insoluble dimers and eventually larger polymers that precipitate [Masuda et al., 1999]. Such reactions are favoured in the present system at curcumin concentrations above 4 µmol/L.

- *Monophenolic compounds*

α-Tocopherol and α-CEHC exhibited similar antioxidant capacity as Trolox in the TEAC assay due to their similar molecular structure. α-Tocopherol could not be tested in the ORAC assay (see below).

- *Carotenoids*

All the carotenoids induced a decrease in the radical cation absorption. However, results demonstrate substantial differences between the various types of carotenoids.

The higher scores of β-carotene and zeaxanthin (β-rings only) with respect to α-carotene and lutein (one β-ring plus one ε-ring) are explained by the fact that the double bond in ε-rings is not conjugated with the polyene chain, and electron delocalisation is thus more restricted (**Figure 1.6**). Besides, the negative charge density on C-4 of the ε-ring is clearly higher than that on C-4 of the β-ring [Hernández-Blanco et al., in preparation]. In xanthophylls, the charge distribution is further affected by the presence of hydroxyl groups. In the 3´-hydroxyl-ε-ring (lutein), the charge skip between carbon 3´ –with a net positive charge in all compounds- and 4´ is maximised. These differences in charge distribution likely affect reactions involving terminal rings, such as electron abstraction. The stability of the carotenoid radical generated by such mechanism when an ε-ring is present is lower and may account for the lower TEAC value of ε-ring-containing compounds.

It is remarkable that the presence of a hydroxyl group decreases the antioxidant activity in the case of lutein, when compared with α-carotene, but not that of zeaxanthin in comparison with β-carotene (non-significant difference between TEAC values). It has been speculated that the 3´-position on ε-rings is a reactive one (e. g., for hydrogen abstraction and/or hydroxylation), and might be hindered by the hydroxyl groups as in lutein or isozeaxanthin [Palozza et al., 1995; El-Tinay et al., 1970]. This effect has also been observed in the reaction of different carotenoids with AMVN and AIBN [Woodall et al., 1997].

Capsorubin exerted the highest radical quenching activity. This ketocarotenoid has two 3-hydroxyl-6-oxo-κ end groups. The carbonyl groups cause a pronounced electronic charge skip between C-6 of the κ-groups and the polyene chain [Hernández-Blanco et al., in preparation], and strongly stabilise the polyene chain by an electron withdrawal effect, which is most appropriate for the delocalisation of the unpaired electron of the carotenoid radical intermediates generated by addition. Even though charge distribution over the central part of the molecule is the same in capsorubin and capsanthin, a very different distribution is observed at the end groups due to the asymmetry of the latter, which accounts for its lower antioxidant ability with respect to capsorubin. This effect has been reported previously by other authors [Terao, 1989; Martin et al., 1999; Pérez-Gálvez and Mínguez-Mosquera, 2002].

Astaxanthin has been reported to be a good antioxidant in comparison with other carotenes and xanthophylls [Terao, 1989; Miki, 1991]. A symmetrical charge

distribution along the molecule is observed in canthaxanthin and astaxanthin, with high charge skips close to the hydroxyl and keto groups, but not immediate to the polyene chain as in the case of capsorubin [Hernández-Blanco et al., in preparation].

As addressed in the introduction, antioxidants supplied with the diet or with dietary supplements undergo a complex process until they reach the target tissue, involving liberation from the food matrix, absorption through the gastro-intestinal tract and distribution into different organs via blood circulation. Partial degradation of micronutrients to inactive compounds may occur even before target tissues are reached, e. g. due to colonic microorganisms or immediate biotrans-formation in the liver after gut absorption. Thus, it should be pointed out that the antioxidant activity of micronutrients *in vivo* is influenced by a number of factors not contemplated in conditions of experimentation *in vitro*.

- *Pharmaceutical Preparations*

In the **turmeric** preparation, curcuminoids were the most abundant constituents. The contribution of β-carotene, as single carotenoid detected, to the antioxidant capacity was less than 0.1% of the absolute amount. This indicates that further antioxidant compounds must be present, since the water-soluble fraction A shows a high activity. Since this fraction is rich in phenols (**Table 3.7**) but curcumin is insoluble in water, phenolic decomposition products of curcumin, such as ferulic acid, vanillin or vanillic acid (Masuda et al 1999) may account for this effect.

The major antioxidant capacity of the **artichoke** leaf preparation was found in fractions A and B coinciding with the presence of phenols. Most flavonoid glucosides as well as hydroxycinnamates are soluble in these solvents [Macheix and Fleuriet 1998]. Artichoke leaves are particularly rich in caffeic acid and caffeic acid esters with quinic acid (e. g. chlorogenic acid and the diester cynarin, which is almost exclusively found in artichokes). Since water is the extraction solvent used in the manufacture of the commercial product, the low antioxidant properties found in the more lipophilic fractions C and D are realistic.

Results for **devil´s claw** preparation are consistent with the fact that water-extractable substances account for about 70% the dry weight of the roots. In view of the weak antioxidant activity of harpagoside, contribution by other iridoids, such as harpagide or procumbide, to the antioxidant activity, is excluded because of their high

structural similarity. According to linear regression data, phenols significantly contribute to the antioxidant activity. Major phenols in devil´s claw roots are glycated forms of flavonoids like those of kaempferol or luteolin.

The **garlic** tablets rendered low antioxidant activities for all fractions. There are more water-soluble than lipid-soluble antioxidants present in garlic extract. TEAC and total phenols correlated among treatments but other compounds present in garlic extract are also Folin-active, particularly those containing sulfhydryl and disulfide groups.

Results for the **salmon** oil reveal that all the antioxidant activity found in the oil in solution is related to α-tocopherol, added to the product for stabilization.

It should be noted that the results obtained for each fraction of each preparation are not additive, as some of the antioxidant constituents dissolve in more than one medium.

Today, 59% of the population in USA report taking dietary supplements on a regular basis. Of these regular supplement users, almost half (46%) take a multiple vitamin/mineral product on a daily basis and more than a third (35%) take single vitamins, such as vitamin C, vitamin E, or vitamin B complex. Almost 23% regularly consume herb/plant and specialty supplements (such as glucosamine/chondroitin). 15% adults currently consume such plant supplements as garlic, ginseng and St. John's Wort [Dietary Supplement Education Alliance, 2001].

Even though the manufacturer´s claims on health benefits of dietary supplements become increasingly regulated, such statistics constitute a rationale for the chemical evaluation of those products and the closer examination of potential biological activities derived therefrom. The total antioxidant capacity of widespread dietary supplements and the relationship to their antioxidant contents was assessed in this work as contribution to that concern.

4. 1. 2 Methodological Aspects

The ranks of antioxidant capacity were dependent on the assay applied (e. g. for ascorbic acid), yet for some of the compounds consistent with already published values [Pannala et al., 1991; Cao and Pryor, 1999; Re at al., 1999]. The diversity of nature of the radicals (or radical generators) and discrepancy in the redox potential of

the oxidant/reductant pair account for this effect. Testing antioxidant activities or capacities in more than one system is thus indispensable.

There has been an intense debate on the question which measure for *Total Antioxidant Capacity* is most appropriate from the increasing number of methods being developed, especially in order to prospect biological fluids such as blood plasma [Duthie, 1999; Prior and Cao, 1999; Rice-Evans, 2000]. Prior and Cao repeatedly claimed the superiority of their method, the ORAC assay, over others like the *TRAP* or the *FRAP* assays, for being able to determine both inhibition time and inhibition percentage and unique in taking the reaction to completion, which enables the use of an "area under the curve (AUC)" technique for quantification [Cao and Prior, 1999]. However, later improvements made on the original TEAC assay (the ferryl myoglobin/ABTS procedure) [Miller et al., 1993], by substituting H_2O_2/metmyoglobin by potassium persulfate [Re et al., 1999] –the procedure applied in this work-, provided the TEAC assay in practice with the same advantages which the ORAC has.

To be tested in the ORAC assay, α-tocopherol needed a high amount of organic co-solvent, thereby changing the habitual reaction kinetics, which led to assume that the presence of ethanol had affected the stability of the protein. The ORAC value for α-tocopherol has been reported to be 1.0 by making the score for the more hydrosoluble α-tocopheryl succinate applicable for α-tocopherol (Cao et al., 1993) – however, the peroxyl radical scavenging capacity of α-tocopherol resides in the free 6-hydroxyl moiety of the chroman ring, which is protected and slowly released in α-tocopherol succinate. Likewise, an ORAC value of 1 was assigned to α-tocopherol under the consideration of Trolox being the water- soluble analogue of α-tocopherol [Pieri at al., 1994], even though such a statement is tentative.

Intra- and inter-day variability concedes the TEAC assay a relative superiority. It is noteworthy that coefficients of variability obtained in the ORAC assay for this work are much higher than those corresponding to experiments which were performed with an automated analyser [Cao et al., 1995]. On the other hand, neither the TEAC nor the ORAC assay at their actual state of development are reliable methods to prospect the total antioxidant capacity of biological fluids [Rice-Evans, 2000].

In favour of the ORAC assay count both a *more physiological* reaction with peroxyl radicals and the use of a biological target (protein) as the marker of oxidative damage; the novel TEAC assay is nevertheless much less time-consuming and

applicable to lipophilic compounds such as α-tocopherol. Both may be performed as complementary methods to screen the antioxidant activity of single compounds or mixtures except for biological fluids as plasma.

4. 1. 3 Inhibition of Peroxynitrite-mediated Tyrosine Nitration

Epicatechin was the most efficient inhibitor of tyrosine nitration. α-CEHC and Trolox showed a comparable inhibitory capacity.

In Trolox and α-CEHC, the benzene moiety of the chromanol ring is completely substituted like in α-tocopherol so that nitration is not possible, and pure electron donation must be responsible for the protective effects shown. Recent data for α-tocopherol suggest that the reaction occurs via two-electron transfer yielding a stable α-tocopherol-quinone which would not be reduced back to α-tocopherol e. g. by ascorbic acid [Hogg et al., 1994]. Vitamin E has been subject of profound research to elucidate its role as RNS scavenger [Christen at al., 1997; Goss at al., 1999].

In epicatechin, the possibility of nitration on the cromanol ring is also low since the two hydroxyl groups are located *meta* to each other and π-electron delocalisation is weakened. However, positions *ortho* to the hydroxyl groups on the catechol ring (2´and 5´) are strongly activated by an electron resonance effect (+M). This should enable the formation of stable nitro- adducts in those positions upon reaction with peroxynitrite. It is likely that epicatechin thereby competes with an intermediate tyrosine radical, since epicatechin´s IC_{50} against peroxynitrite-mediated dityrosine formation has been shown to be comparable to its IC_{50} of 3-nitrotyrosine generation [Schröder et al., 2001]

The antioxidant properties of α-CEHC were examined in the three model systems with positive results. A number of synthetic water-soluble vitamin E analogues are being prospected for advantages over the lipophilic tocopherols in the via of administration (i.v. *vs.* p.o.) and/or in the ability to offset sudden rises of reactive species as occurring in reperfusion after ischemia [Abadie at al., 1993], radical-mediated hepatotoxicity [Campo et al., 2001], myocardial infarction [Campo et al., 1994] and possibly other types of CVD [Campo et al., 1997]. These compounds share an intact or acetylated chroman ring but vary in the type of functional groups or length of the side chain. Some have succeeded at animal experimentation and in initial intervention trials [Chowienczyk et al., 2000]. Furthermore Trolox, a further

water-soluble α-tocopherol analogue, is widely employed in *in vitro*, cell or animal model studies. Trolox significantly enhanced cardiac recovery after ischaemia /reperfusion, both when it was perfused in isolated hearts and after its oral administration to rats, and its activity was better than that of vitamin E [Sagach et al., 2002]. Since the solubility and structural features of such analogues are shared by α-CEHC, with the plus of being a natural-occurring compound, α-CEHC should be tested for similar applications.

4. 2 Cell Culture Study on the Anti-atherogenic Effects of Dietary Micro-nutrients and α-CEHC

Findings from the cellular *pro-atherogenic* system suggest that:

- pre-treatment with α-tocopherol, epicatechin and epigallocatechin gallate at physiological concentrations is able to transiently prevent cell damage and significantly prolong cell survival, and
- pre-incubation with α-CEHC has neither impact on cell damage nor can enlarge cell viability even at super-physiological levels.

Only α-tocopherol was able to scavenge efficiently intracellular ROS that were generated as an early event in response to oxLDL (**Figure 3.17**). Membrane-bound NAD(P)H oxidase has been found to be responsible for the bulk of ROS (mainly $O_2^{\cdot-}$) produced in oxLDL-stressed endothelial cells [Heinloth et al., 2000] which is in turn activated through the LOX-1 receptor [Cominacini et al., 2000] NOS impairment occurs concomitantly and NO production is drastically diminished [Galle et al., 2000]. α-Tocopherol is mainly positioned within membranes and thus closer to the site of $O_2^{\cdot-}$ generation, which may account for its superiority in ROS scavenging.

Since the amounts of DiI-labelled oxLDL and DiI-labelled nLDL taken up by MAEC were similar at each point studied (**Figures 3.15** and **3.16**), it appears that the *uptake rates* for both lipoprotein types were similar. Scavenger receptors (mainly LOX-1 in endothelial cells) take up highly oxidized LDL at a faster rate than the nLDL receptor is able of taking up mildly oxidized LDL. Thus, one could assume that the labelled oxLDL used here (obtained at conditions of low oxidation) was recognized and taken up mainly by the LDL receptor.

OxLDL at the mild level of oxidation applied in our experiments caused a significant decrease of total levels of glutathione and a deterioration of the

glutathione oxidative status by increasing GSSG levels. The three dietary antioxidants prevented glutathione depletion by two apparently different mechanisms. α-Tocopherol showed a protective effect on total glutathione but could not restrain GSSG levels over a long time. Both catechins were able to maintain levels of GSSG significantly lower to those of cells without pre-treatment with antioxidants, but had no positive effect on total glutathione, at least in view of the data corresponding to 24 h (**Figures 3.20** and **3. 21**).

Comparable effects were observed in another study where bovine endothelial cells were challenged with mildly oxidized LDL. α-Tocopherol was shown to prevent glutathione and ATP depletion as well as to delay the cytotoxic effects. Rutin exerted a similar protection of intracellular glutathione and ATP, however, it did not prevent membrane lipid peroxidation efficiently, whereas α-tocopherol did (for 48 h) [Schmitt et al., 1995]. Thus, it can be postulated that the preserving effect on total glutathione by α-tocopherol (**Figure 3.21**) may be related to its membrane protective action which hampers leakage of glutathione to the medium. Catechins likely act dissolved in the cytosol thus preventing glutathione oxidation more efficiently than α-tocopherol (**Figure 3.21**) but might not effectively prevent glutathione leakage caused by membrane damage.

The results for total free thiols reflect the effects of the test compounds on glutathione preservation. Only the active compounds α-tocopherol, epicatechin and epigalocatechin gallate were also active in protecting the thiol pool over time, whereas α-CEHC was inactive in both sets of experiments (**Figures 3.18** to **3.21**). Reduced glutathione is responsible for sustaining the thiol pool in the cell, including free sulfhydryl groups of enzymes which may be crucial for their activity. Ranks of test compounds in mantaining levels of reduced glutathione after 24 h (epigallocatechin gallate = epicatechin > α-tocopherol > α-CEHC, **Figure 3.21**) were the same as ranks corresponding to the ability of keeping levels of total free thiols at the same time point (**Figure 3.18**).

oxLDLs have been reported to have opposite effects on glutathione levels of cultured cells. In macrophages, they were found to induce a rapid depletion followed by an adaptive increase triggered by a rise of γ–glutamylcysteine synthetase (γ–GCS) heavy subunit levels [Shen and Sevanian, 2001]. In human vascular endothelial cells, such compensating response was observed after 24 h incubation with oxLDL [Cho et al., 1999]. In another study, different lipid fractions from oxLDL caused partial

glutathione depletion without a later balancing rise [Therond et al., 2000], as also shown here.

There is substantial evidence that oxLDL activates multiple apoptotic signaling pathways in different cell types. Mildly oxidized LDL induced apoptosis in primary cultures of human endothelial cells and SMC. Signals were mediated by intramembrane domains of both Fas and tumor necrosis factor (TNF) receptors I and II, accompanied by an increase of proapototic and a decrease in antiapoptotic proteins of the Bcl-2 family, and resulted in marked activation of class I and II caspases [Napoli et al., 2000]. In the human endothelial cell line ECV-304, LDLs oxidized under mild conditions induced apoptosis by a mechanism involving both derivatization of cell proteins with 4-hydroxynonenal (4-HNE) and ubiquitination, and late inhibition of endogenous proteolysis by the proteasome pathway [Vieira et al., 2000].

A role for apoptosis in cell death triggered by oxLDL was also observed in our experiments, in view of the changes in cell morphology and caspase-3 activation. The three dietary micronutrients were able to diminish this effect, in the order α-tocopherol > epigallocatechin gallate > epicatechin (**Figure 3.22**). Even though this was a relative early event, obviously it must play a role in the decrease of late-onset oxLDL-mediated cytotoxicity, which is observable in **Figure 3.14**.

4. 3 Human Intervention Study on the Effects of Smoking on Vitamin E Metabolism after a Single Dose of Vitamin E

Results of the intervention study indicate differences in tocopherol metabolism between smokers and nonsmokers. Moreover, even though virtually all vitamin E in the oral dose was RRR-α-tocopherol, divergences were more obvious in γ-tocopherol metabolism. The decrease in γ-tocopherol levels after intake of a high dose of α-tocopherol (**Figure 3.27**) is accounted for by the fact that newly ingested α-tocopherol is readily sorted out by the liver α-TPP and released to the circulation within VLDLs, whereby larger amounts of endogenous γ-tocopherol are metabolized. Nevertheless, this effect appears to be irrespective of smoking and cannot explain the overall significantly higher levels of γ-CEHC for smokers on its own (**Figure 3.28**).

In preceding studies, responses (AUC) to the administration of a single dose of deuterated α-tocopherol were consistently and significantly lower in smokers than in

non-smokers [Munro et al., 1997], and smoking was found to enhance plasma α-tocopherol disappearance [Traber et al., 2001]. There is substantial evidence that the oxidative status in smokers is increased. Arterial tissue vitamin E and C are significantly lower in smokers [Mezzetti et al. 1995]; significantly lower plasma carotenoid and vitamin C levels were found in male smokers as compared to nonsmokers [Ross et al., 1995]; and quitting cigarette smoking increases plasma levels of a number of antioxidant micronutrients and improves plasma resistance towards oxidative challenge [Polidori et al., 2003]. Thus, an increased oxidative stress has been made in part responsible for the lower response and higher clearance of vitamin E in smokers [Traber at al., 2001].

Recent contributions in the field of vitamin E metabolism have assigned a role for two different cytochrome P450-dependent isoenzymes, CYP3A and CYP4F, at the initial stage of tocopherol degradation (ω-hydroxylation of the phytyl chain) [Birringer at al., 2001; Sontag and Parker, 2002]. A role for Phase I enzymes suggests that vitamin E metabolism may be a process of relatively high complexity, due to the large number of isoenzymes, their different degrees of activation among tissues such as liver, lung or kidney, and the well-known variety of inter-individual responses associated with inherited and environmental factors [Guengerich, 2001].

The effects of smoking on gene regulation of such enzymes have not been completely elucidated yet. Smoking is thought to induce mainly CYP1A1 and CYP2E1. This effect has been observed in liver and lung of “smoking” mice [Villard et al., 1994; Villard at al., 1998]. The role of CYP3A is still unclear, however. CYP3A has been shown to be induced in liver microsomes but not in lung of “smoking” mice [Villard et al., 1994], and also results for human smokers confer a relative importance to this isoenzyme [Wanwimolruk et al., 1993 and 1995]. Therefore, it may be speculated that induction either of CYP3A or of other cytochrome P450-dependent isoenzymes latently involved in tocopherol metabolism accounts for the higher rate of γ-tocopherol metabolism in smokers.

In addition, it has been postulated that smoking may have an induction effect on postpandrial lipolysis of trigliceride-rich lipoproteins (mainly chylomicrons) [Zaratin et al., 2001]. The transfer of vitamin E between circulating lipoproteins depends not only upon α-TPP selectivity/activity, but also upon lipolysis of VLDL (mainly by lipoprotein lipase) in the bloodstream [Parks et al., 2000], so it is reasonable that this effect might increase α-tocopherol distribution and thus accelerate clearance from plasma.

α- and γ-CEHC serum levels in humans have been measured in three studies up to date and the number of subjects examined is still very limited. The levels found in the present work for most of all volunteers are very similar to those already published [Stahl et al., 1999, Galli et al., 2002, Radosavac et al., 2002]. Nonetheless, two of the smokers rendered unexpectedly high levels (**Table 3. 29**).

In subject 2, a normocholesterolemic man with very high baseline levels of both tocopherols, biotransformation of γ-tocopherol to γ-CEHC was greatly enhanced, but that of α-tocopherol to α-CEHC was considerably diminished (**Figure 3.30**). It may be postulated that, in the case of simultaneous super-optimal levels of α- and γ-tocopherol, α-tocopherol is especially preserved, since the more α-tocopherol is bound to α-TPP -which is by far more selective for α-tocopherol –, the larger amount of γ-tocopherol is available for metabolism. α-CEHC has been related to a super-optimal α-tocopherol status [Schultz et al., 1995]. α-CEHC was only detected in urine when a threshold level of α-tocopherol in blood was reached, which was interpreted as a result of α-TPP becoming saturated and remaining α-tocopherol being metabolized. It seems feasible that γ-CEHC is the metabolite which is over-produced when both the α-TPP capacity is saturated and endogenous levels of γ-tocopherol are high, since the metabolic enzymes CYP3A and CYP4F2 exert a notably higher catalytic activity on γ-tocopherol in comparison with α-tocopherol [Sontag and Parker, 2002; Birringer et al., 2002].

Tocopherol levels in subject 1 were the lowest among all smokers. Also overall α-tocopherol corrected by cholesterol was very low even after α-tocopherol intake, showing that the binding capacity for α-tocopherol of cholesterol-rich lipoproteins (mainly LDL) was under-utilized. Thus, the rationale for those data must be different from that exposed for subject 2, even though CEHC levels in subject 1 were also extremely high. A similar situation is found in the literature concerning AVED patients [Schuelke et al., 2000]. A broad range of mutations in the α-TPP gene of those patients lead to an extreme decrease in α-TPP´s binding capacity for α-tocopherol, which results in blood α-tocopherol levels below 10 µmol/L and very high amounts of α-CEHC in urine. Even though subject 1 is completely healthy, his serum levels of α-tocopherol were not far above the plasma level which is considered as indicative of vitamin E deficiency (12 µmol/L) [Standing Committee on the Scientific Evaluation of Dietary Reference, 2000]. It is thus suggested that there may be (still unknown)

mutations of the α-TPP gene which compromise α-TPP´s activity in a less dramatic manner than in AVED patients and thus do not result in any clinical outcome.

In summary, vitamin E metabolism appears to be as variable among individuals as its absorption is. The limited number of subjects studied up to date is not enough to establish normal values of serum CEHC. The existence of healthy subjects with very outlying concentrations as shown here should be further investigated as this could provide for closer understanding of activation/induction patterns.

5. SUMMARY

The antioxidant properties of selected micronutrients (vitamin E, vitamin E derivatives, carotenoids, and polyphenolic compounds) were assessed. Studies were performed at three different experimental levels:

(1) in cell-free model systems. The *Trolox Equivalent Antioxidant Capacity* (TEAC) was measured for all compounds and for pharmaceutical preparations with a broad range of therapeutic indications (derived from turmeric rhizome, devil´s claw root, artichoke leaf, garlic, and salmon oil); for selected compounds both the *Oxygen Radical Absorbance Capacity* (ORAC) and the half-maximal inhibitory concentration (IC_{50}) against peroxynitrite-mediated nitration of tyrosine were determined.

(2) at the cellular level, under pro-atherogenic conditions. Primary mouse aorta endothelial cells were incubated with oxidatively-modified low density lipoproteins (oxLDL) in different time frames. The effects of pre-treating cells with selected dietary micronutrients or 2,5,7,8-tetramethyl-2(2´-carboxyethyl)-6-hydroxychroman (α-CEHC) on indicators of cell viability, redox state, amount of oxLDL uptake, and apoptosis were investigated.

(3) in a human intervention trial. In smokers, oxidative stress is increased and xenobiotic-detoxicating enzymes, e. g. cytochrome P450-dependent monoxygenases, are induced. Both factors may modify the levels of certain antioxidants in blood and affect their metabolism. 29 smoking and nonsmoking volunteers received a single oral dose of vitamin E. Both the serum levels of α- and γ-tocopherol and their major metabolites (α- and γ-CEHC) were monitored for 72 h and both groups were compared.

- to (1): Most of the test micronutrients and α-CEHC exhibited antioxidant activities in the TEAC and ORAC assays. The differences in activity observed in the TEAC assay were assigned to particular structural features of each compound: in flavonoids, the number of neighbouring hydroxyl groups on ring B was most important; in carotenoids, molecular symmetry, the position of double bonds and absence/presence of hydroxyl groups in the terminal rings were the most decisive factors; the length of the side chain had no influence on the antioxidant activity of α-tocopherol, its structural analogue Trolox, and α-CEHC. The turmeric extract was rich in lipophilic and hydrophilic antioxidants. The antioxidant capacity of artichoke and devil´s claw extracts was considerably lower and was significantly correlated with the

polyphenol content. Garlic and salmon oil preparations were almost inactive in the TEAC assay.

- to (2): α-Tocopherol and catechin compounds counteracted oxLDL-induced cell damage to some extent over a period of 24 h. Involved mechanisms comprise ROS scavenging, maintenance of glutathione and the total thiol redox status, and inhibition/repression of caspase-3.
- to (3): In smokers, overall levels of the γ-tocopherol metabolite γ-CEHC were significantly higher and those of α-CEHC non-significantly higher than in nonsmokers, even though this effect appeared to be independent of α-tocopherol supplementation. Since γ-tocopherol is preferentially metabolized compared to α-tocopherol, it may be speculated that biotransformation is enhanced in smokers due to induction of vitamin E degrading enzymes, e. g. cytochrome P450-dependent monoxygenases.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation eigenständig und ohne unerlaubte Hilfe angefertigt und diese in der vorliegenden oder in ähnlicher Form noch bei keiner anderen Institution eingereicht habe.

(Alejandro José Betancor Fernández)

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