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Antioxidant abilities of human plasma, buckwheat extracts and fractions, and quercetin metabolites in different biochemical assays

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In Dankbarkeit meinen Grosseltern Maria und Adam Moldan gewidmet

Dedicated gratefully to my grandparents Maria and Adam Moldan

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3'-mehylq3g:	3'-methylquercetin-3-glucoside
ABTS:	2,2'-azinobis(3-ethylbenzthiazoline-sulphonic acid)
ACC:	1-aminocyclopropane-1-carboxylic acid
ap7g:	Apigenin-7-glucoside
BSA:	Bovine serum albumin
B2:	Procyanidin B2
B5:	Procyanidin B5
C1:	Procyanidin C1
d:	Density
d7g:	Daidzein-7-glucoside
DMACA:	Dimethyl amino cinnamyl aldehyde
EDTA:	Ethylenediaminetetra-acidic acid
em:	Emission
$EMEM^+$:	Eagle's minimal essential medium with L-glutamine
EtOAc:	Ethyl acetate
EtOH:	Ethanol
ex:	Excitation
f. c.:	Final concentration
fcs:	Foetal calf serum
g7g:	Genistein-7-glucoside
H_2O_2:	Hydrogen peroxide
HDL:	High-density lipoprotein
HOCl:	Hypochlorite
HPLC:	High performance liquid chromatography
HSA:	Human serum albumin
irhm3glca:	Isorhamnetin-3-glucuronic acid
KMB:	α-keto-S-methylbutanoic acid
LDL:	Low-density lipoprotein
MeOH:	Methanol
MPO:	Myeloperoxidase
n7g:	Naringenin-7-glucoside
NEAA:	Non-essential amino acids
PBS:	Phosphate-buffered saline
PYC:	Pycnogenol
q3,4'dig:	Quercetin-3,4'-diglucoside
q3's:	Quercetin-3'-sulphate
q3g:	Quercetin-3-glucoside
q3glca:	Quercetin-3-glucuronic acid
q3r:	Quercetin-3-rutinoside
q4'g:	Quercetin-4'-glucoside
q4'glca:	Quercetin-4'-glucuronic acid
q7glca:	Quercetin-7-glucuronic acid
ROS:	Reactive oxygen species
Sin-1:	3-Morpholinosydnonimine
TLC:	Thin layer chromatography
Trp:	Tryptophan
UV:	Ultra violet
VLDL:	Very low-density lipoprotein
X:	Xanthine
XOD:	Xanthine oxidase

I. PREFACE

One aim of this thesis was to investigate the antioxidant properties of human plasma, a plasma pool from plasma of 12 volunteers and single-plasma samples of 24 volunteers as comparison. The assays used generate different kinds of reactive oxygen species mimicking certain diseases *in vitro* and are commonly used assays in our laboratory (BLAUROCK et al., 1992; VON KRUEDENER et al., 1995; HIPPELI et al., 1997a, b; SCHEMPP et al., 2000). The mimicked diseases are inflammation due to the activated leukocytes (ACC/HOCl, NADH/Diaphorase, Sin-1, peroxynitrite assays), lipid peroxidation (Rose Bengal, ABTS assays), ischaemia/reperfusion (X/XOD assay) and Fenton chemistry occurring through transition metal ions (Fenton assay). The plasma pool was studied in all our test systems whereas the comparison with the single-plasma samples was done with four out of the eight assays.

The antioxidant properties of different buckwheat extracts of the herb and kernels were determined with the mentioned *in vitro* assays. The fractionation of the buckwheat herb and its HPLC-analysis was done in co-operation with the Fachgebiet für Obstbau, to find out more about the compounds in the drug and possibly in the infusion of tea. These fractions were studied in four of our *in vitro* assays to determine their antioxidant properties. There was also an *in vivo* study with one volunteer who was first supplemented with buckwheat tea and then as comparison with Pycnogenol. The collected plasma was investigated in five of our test systems.

The last part of this thesis has been a Marie Curie Fellowship at the Institute of Food Research, Norwich, under the supervision of Geoff Plumb and Prof. Gary Williamson. The investigations during this fellowship were on the effect of quercetin metabolites on LDL oxidation and HSA binding. After an ingestion of a quercetin-rich meal, the naturally occurring quercetin and its glucosides can be found in the plasma of the volunteers as metabolites (glucuronides and sulphates). The main metabolites are quercetin-7-glucuronic acid, quercetin-3-glucuronic acid, quercetin-4'-glucuronic acid, quercetin-3'-sulphate and isorhamnetin-3-glucuronic acid (q7glca, q3glca, q4'glca, g3's and irhm3glca). These metabolites were tested in the copper-induced LDL oxidation and their binding properties studied with the tryptophan fluorescence of HSA. In addition, there were also studies done on the stability of quercetin and its metabolites in cultures of HepG2.

II. LITERATURE REVIEW

II.1. ROS and their relevance

Reactive oxygen species (ROS) are connected with several diseases. The types of ROS can be seen in the following list:

$O_2^{-\bullet}$	superoxide radical
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
$^1O_2$	singlet oxygen
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	peroxyl radical
ROOH	peroxide

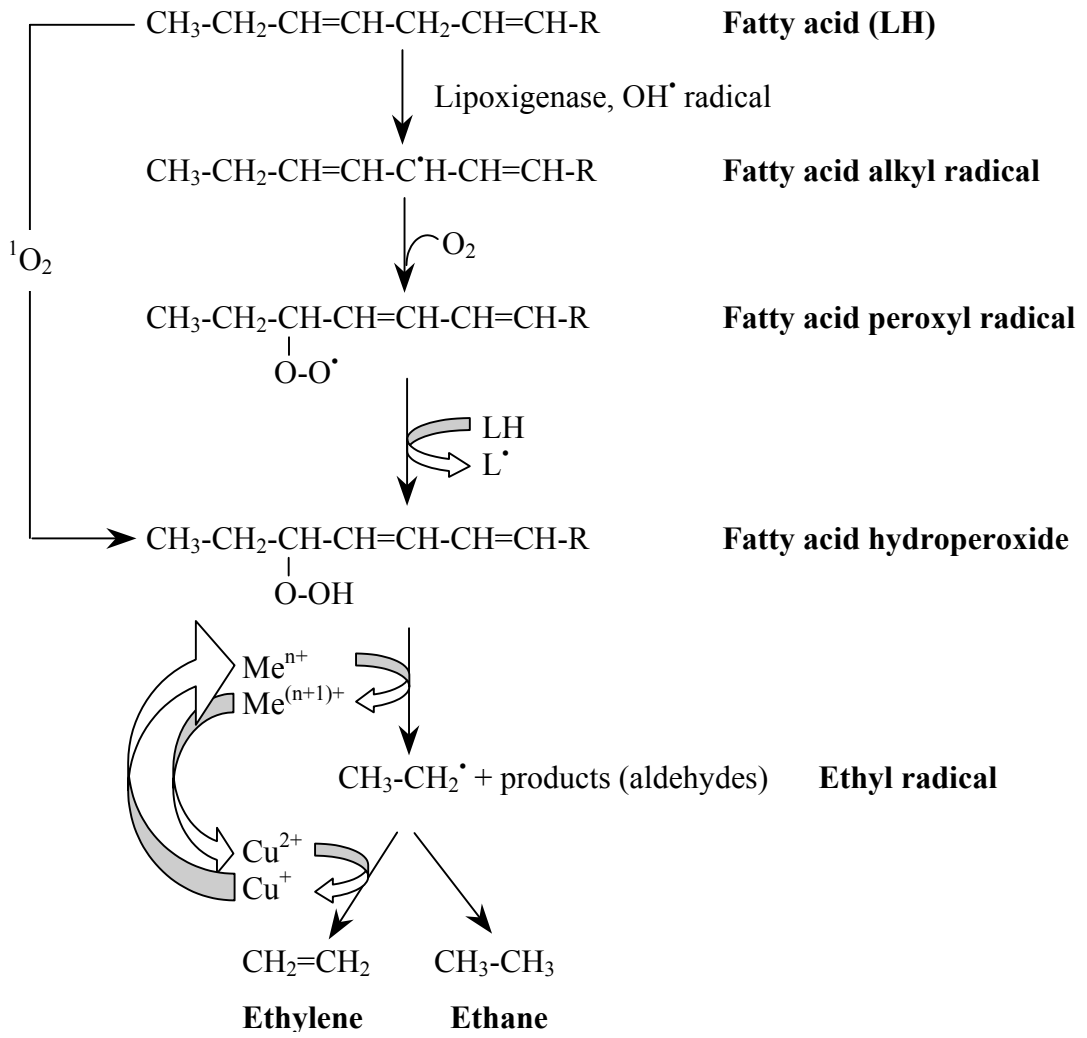
(ELSTNER, 1990; ELSTNER, 1993; HALLIWELL and GUTTERIDGE, 1999).

The transfer of one electron to molecular oxygen generates superoxide radicals. One potential place of the generation of superoxide radicals are mitochondria, in which oxygen is activated during the normal process of energy production in the respiratory chain. Superoxide radicals dismutate spontaneously to hydrogen peroxide and water at neutral to slightly acidic pH, whilst they are relatively stable at alkaline pH. Superoxide dismutases catalyze the dismutation independent of pH; this is important in the physiological range. Hydrogen peroxide can also arise via the transfer of two electrons to molecular oxygen, this reaction is carried out by oxidases in the peroxisomes. Catalases detoxify hydrogen peroxide to water and oxygen; peroxidases oxidize organic molecules with hydrogen peroxide. A further one-electron transfer to hydrogen peroxide produces the hydroxyl radical, which is the most reactive ROS and triggers chain reactions with organic molecules (ELSTNER, 1990; ELSTNER, 1993; ELSTNER et al., 1994; KARLSON et al., 1994; HALLIWELL and GUTTERIDGE, 1999).
Besides the formation of ROS during the healthy metabolism, they contribute to several diseases either in the defence of pathogens or as ‚side effects‘ in the progress of the disease. In the case of ischaemia-reperfusion due to the lack of oxygen, the xanthine dehydrogenase is transformed into the xanthine oxidase (XOD) and ATP degraded to

AMP, which leads to the accumulation of hypoxanthine. After reperfusion of the tissue, the XOD oxidizes the hypoxanthine to uric acid whilst reducing the oxygen. The superoxide radicals are the starting-point for the damage in ischaemia-reperfusion (ELSTNER, 1990; ELSTNER, 1993; HALLIWELL and GUTTERIDGE, 1999). ROS form likewise in the activated leukocyte. The attachment of bacteria to leukocytes triggers phagocytosis of the pathogens and activates over a signal transduction pathway of G-proteins, phospholipases, protein kinases and Ca^{2+} channels the membrane-bound NADPH oxidase, which generates superoxide radicals whilst consuming NADPH. SOD-catalysed hydrogen peroxide acts as bactericide and halogenated with chloride by myeloperoxidases forms hypochlorite, a strong oxidant and aggressive bactericide. The production of superoxide radicals happens not only inside the phagosome, but also at the outer membrane of the leukocyte. This leads to the release of ROS in the surrounding medium (ELSTNER, 1990; ELSTNER, 1993; KARLSON et al., 1994; HALLIWELL and GUTTERIDGE, 1999). Hydroxyl radicals, the strongest oxidants, are formed in the presence of transition metals like iron or copper via the 'Haber-Weiss' reaction:

$$O_2^{-\bullet} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$
$$O_2^{-\bullet} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$$

Hydroxyl radicals are the most reactive oxygen species. They react quickly with molecules in their surrounding e.g. DNA, proteins or fatty acids and trigger chain reactions; consequently, these molecules lose their functionality. In the case of lipid acids, the attack of hydroxyl radicals starts the lipid peroxidation as seen in scheme 01 (page 4). Further radicals like alkyl, peroxyl radicals and peroxides from fatty acids of membranes (scheme 01, page 4) arise during this chain reaction. Unsaturated fatty acids such as α-linolenic acid are particularly susceptible to the attack of hydroxyl radicals. The oxidation of the fatty acids results not only in the disintegration of membranes but also in the modification of LDL. This plays a crucial role in the progress of arteriosclerosis (see II.3.) (ELSTNER, 1990; ELSTNER, 1993; ELSTNER et al., 1994; HALLIWELL and GUTTERIDGE, 1999).



Scheme 01 modified from: Elstner, E.F. (1990). *Der Sauerstoff*

Transfer of light energy through photosensitizers (e.g. coumarines, dianthrons, and porphyrines) physically generates singlet oxygen. This plays a role in photosensitive reactions, at which UV radiation damages membranes. The formed singlet oxygen reacts quickly with organic molecules in the ground state. When endogenous antioxidants are depleted and/or the concentration of singlet oxygen is too high, lipid peroxidation (scheme 01) in the membranes sets on (SCHMIDT, 1997; ELSTNER, 1990).

II.2. Human plasma – a review

Plasma is the fluid component of blood forming the supernatant after removal of the solid components (erythrocytes, leukocytes, and thrombocytes) with centrifugation. About 90 % of its consistence is water; the remaining 10 % are the soluble compounds, which are transported via the blood system. Proteins are the main components of the soluble compounds, the total protein concentration ranges between 60 to 80 g/l. With 40 g/l albumin (for details see II.4.) forms the main group of proteins, the rest are globulins. Plasma proteins have a broad specificity; they function as reserve-proteins, buffers, hormones, enzymes, or enzyme inhibitors and maintain the colloid-osmotic pressure in the vessels. They are important factors in the blood coagulation and play a crucial role in the immune response (HINGHOFER-SZALKAY, 1994; SCHMIDT, 1999).

Electrolytes buffer the plasma and maintain the osmolarity; they are generally bound to carrier proteins. The main ion in plasma is sodium with an average concentration of 135-150 mM; further cations are calcium (2.15-2.75 mM), potassium (3.5-5.5 mM) and magnesium (0.6-1.0 mM). Chloride is the main anion with an average concentration of 98-110 mM, besides inorganic phosphate and sulphate as well as bicarbonate and organic acids. Metals are found in small amounts, they belong to the group of trace elements and have important functions in the metabolism, usually as parts of enzymes. The trace elements are iron (~ 20 µM), zinc (~ 18 µM), copper (~ 20 µM), manganese (~ 0.6-60 µg/l), molybdenum (~ 13 µg/l), fluorine, iodine, cobalt, chrome, and selenium. Further important compounds, which can be found in plasma, are vitamins. They have to be taken up with the diet and in many cases are coenzymes. Ascorbic acid (> 17 µM) and tocopherols (> 5.0 µM) serve as antioxidants, the water-soluble ascorbic acid in aqueous milieus, the lipophilic tocopherols in membranes. Further soluble components in plasma are organic compounds of the metabolism, e.g. glucose, uric acid, amino acids, bilirubin as well as absorbed xenobiotics like medicaments or drugs (GROSS et al., 1989; HINGHOFER-SZALKAY, 1994; KARLSON et al., 1994; REHNER and DANIEL, 1999; SCHMIDT, 1999; BURKHARDT, 2000).

II.3. Low-density lipoprotein (LDL) and arteriosclerosis

Due to their insolubility in aqueous solution, lipids are transported in plasma bound to proteins. These complexes are called lipoproteins (KARLSON et al., 1994). The different lipoproteins are classified by their density:

VLDL (d = 0.9-1.006 g/ml) with chylomicrones and very-low-density lipoproteins
LDL (d = 1.006-1.063 g/ml) with low-density lipoproteins
HDL (d = 1.063-1.21 g/ml) with high-density lipoproteins
(KARLSON et al., 1994; REHNER and DANIEL, 1999).

The LDL particle (see Fig. 01) is a spherical molecule (∅ = 15-25 nm) with a hydrophobic core of triglycerides, cholesterol and cholesterolesters, surrounded by a layer of polar phospholipids, which is interspersed with apolipoprotein B (= β-globulin). LDL contains endogenous antioxidants like α-tocopherol and β-carotene (KARLSON et al., 1994; REHNER and DANIEL, 1999; MENG, 2002). The molecular weight is about 2.5 x 10^6 Da (ESTERBAUER et al., 1992; MENG, 2002).

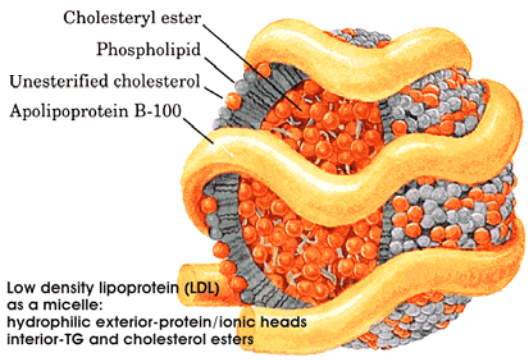


Fig. 01: LDL particle, (www.csmt.ewu.edu/csmt/chem/jcorkill/soap2000.html)

LDL transports about 60 % of the total cholesterol in the blood (KARLSON et al., 1994; REHNER and DANIEL, 1999; MENG, 2002).
An elevated LDL level is connected with an increased risk of arteriosclerosis (STEINBERG, 1997; BURKHARDT, 2000; LUSIS, 2000). Investigations showed that modified LDL triggers arteriosclerotic processes (ESTERBAUER et al., 1992; STEINBERG, 1997). During the process of arteriosclerosis, LDL accumulates in the

intima of the blood vessels due to the increased concentration. Preferred places of migration of LDL are diversions of arteries, at which, due to the shearing forces of the blood, the morphology of the endothelial cells is altered and an increased permeability for LDL was shown (STEINBERG, 1997; LUSIS, 2000; MENG, 2002). Within the intima the modification of LDL occurs through self aggregation of the LDL particle; immune complex formation; oxidation by cells such as endothelial cells, smooth muscle cells, monocytes; lipolysis and proteolysis (STEINBRECHER et al., 1984; ELSTNER, 1993; ESTERBAUER et al., 1992; STEINBERG, 1997; CHOPRA and THURNHAM, 1999; LUSIS, 2000). Transition metals likewise oxidize LDL; *in vitro* investigations take advantage of this fact (ESTERBAUER et al., 1992).

There are two different types of modified LDL: minimally modified LDL (MM-LDL) and oxidized LDL (OX-LDL). MM-LDL stimulates the release of adhesion proteins and growth factors (e.g. monocyte chemotactic protein-1, macrophage colony-stimulating factor) from endothelial cells; this effects the attraction of monocytes (STEINBERG, 1997; LUSIS, 2000; RUBBO, BATTHYANY and RADI, 2000). OX-LDL has a chemotactic effect towards T-cells and monocytes (STEINBERG, 1997) and several components of OX-LDL are toxic for endothelial cells (MENG, 2002).

In the intima migrated monocytes differentiate to tissue macrophages, which take up OX-LDL uncontrolled. The uncontrolled uptake of OX-LDL leads to the accumulation of cholesterol inside the macrophages, their transformation into foam cells and the development of fatty streaks (ESTERBAUER et al., 1992; STEINBERG, 1997; LUSIS, 2000; RUBBO, BATTHYANY and RADI, 2000). Arteriosclerotic plaques with a necrotic core develop from further accumulation of foam cells, lipids and cell debris of dying cells. A fibrous cap arises from migrated and proliferated smooth muscle cells and demarcates the plaques, which vault the artery wall (LUSIS, 2000; MENG, 2002). Calcification of the necrotic core, the burst of the endothelial layer, inflammation processes and thrombosis occur in the further progress (LUSIS, 2000; MENG, 2002).

II.4. Human serum albumin (HSA)

Serum albumin is the most abundant protein in plasma. It is synthesized and decomposed in the liver, its average lifetime is about 19 days. Its concentration in plasma is about 40 g/l and it contributes up to 90 % to the colloid-osmotic pressure in the vessels (PETERS, 1985; HE and CARTER, 1992; HINGHOFER-SZALKAY, 1994; SCHMIDT, 1999). Further important functions are anticoagulant effects, scavenging of free radicals, stabilisation of the concentration of free ligands in the blood stream due to their binding to HSA and the transport of the respective ligands to the tissue (PETERS, 1985; HE and CARTER, 1992; SCHMIDT, 1999). HSA (see Fig. 02) is a heart-shaped protein made of three homologue domains with two subdomains each (PETERS, 1985; HE and CARTER, 1992).

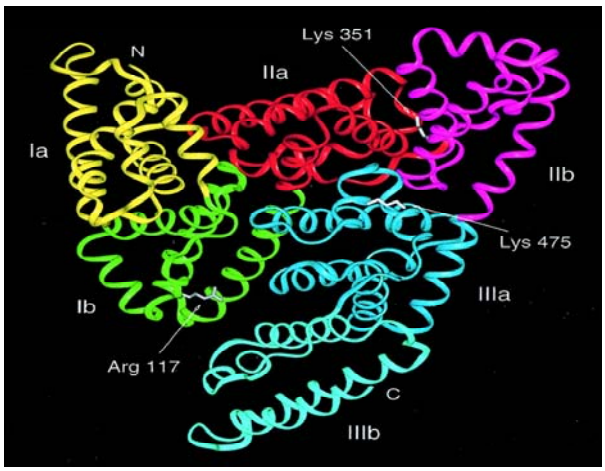


Fig. 02: HSA molecule (SUGIO et al., 1999)

HSA shows a broad specificity in the binding of ligands. Besides bilirubin it also binds cortisol and testosterone. Nearly half of the calcium is bound to albumin, as well as 10 % of the copper (~ 2 µM). Tyrosine and tryptophan are the sole amino acids that bind to HSA. 99 % of the fatty acids are bound to albumin. The flexibility of HSA to adapt its shape to the ligands seems to explain its ‘cosmopolitan taste’ (PETERS, 1985). The main regions for binding are hydrophobic pockets in subdomain IIA and IIIA. The amino acids tyrosine 411 in subdomain IIIA as well as lysine 199 and tryptophan 214 in subdomain IIA contribute to the binding involving hydrophobic interactions (LEE and MCMENAMY, 1980; PETERS, 1985; HE and CARTER, 1992).

II.5. Pycnogenol and buckwheat

Pycnogenol, a mixture of flavans and procyanidins, is obtained from the bark of the French pine *Pinus pinaster* and is patented as a registered trademark of Horphag Research, Geneva, Switzerland. The use of pine bark to treat inflammation and scurvy has been known for more than 2000 years (ROHDEWALD, 1998; PACKER, RIMBACH and VIRGILI, 1999). Pharmacologically important compounds are phenolic acids (e.g. caffeic acid, ferulic acid and p-hydroxybenzoic acid), flavans (e.g. catechin and epicatechin), flavanonols (e.g. taxifolin) and condensed polyphenols (e.g. procyanidins B1, B3, B7) (ROHDEWALD, 1998; PACKER et al., 1999; VIRGILI et al., 2000).

The botany of buckwheat is as follows (KUHLMANN, 1998):

Class:	Magnoliatae
Order:	Polygonales
Family:	Polygonaceae
Genus:	*Fagopyrum*
Species:	*esculentum* MOENCH (synonymous: F. vulgare, F. sagittatum, F. sarracenium, F. cereale, Polygonum fagopyrum, P. cereale, Phegopyrum esculentum)
	tartaricum GAERTNER
Height:	15-60 cm, in culture up to 120 cm
Inflorescence:	thyrses in white, pink to red
Seed:	sharp three-angled fruit (achene); l = 5-6 mm, b = 3-4 mm

Formula of inflorescence: $G_{\underline{(3)}}\ A_8\ K_3\ C_5$

The home of the buckwheat is central and north Asia, but there are also several wild types in Russia (KUHLMANN, 1998). In Asia and Central Europe buckwheat substitutes rice and cereal (KREFT et al., 1994, KUHLMANN, 1998; DIETRYCH-

SZOSTAK and OLESZEK, 1999; IKEDA et al., 2001), whereas in Western Europe and Northern America the herb is used as a source of rutin (KREWSON and COUCH, 1949; GRIFFITH et al., 1995) and for medication at haemorrhages and capillary fragility (IHME et al., 1996; KIESEWETTER, 1998; SCHIMMEL, 1998; HAGELS et al., 2001). The compounds of the kernels are well known and investigated. The main carbohydrate in the kernel is starch, besides other polysaccharides like the polysaccharide A1 from the endosperm. 80 % of the proteins are water-soluble and are mainly globulins, albumins and the gluten proteins gliadin and glutenin. Buckwheat kernels contain up to 6.1 % lysine, together with L-2(2^1furoyl)alanine, fagonin and several other hydroxybenzylamines. Contrary to cereal buckwheat possesses less proline and glutamic acid, but is higher in aspartamic acid and arginine. Glucosidases (e.g. α-glucosidase, glucoamylase and α-amylase), proteases (e.g. thiol proteinase) and protease inhibitors were found in the kernels. Buckwheat oil contains 16-20 % saturated fatty acids, 30-45 % oleic acid and 31-41 % linoleic acid and does not differ in the composition to oils from rice, wheat, oat and millet (POMERANZ, 1985). LOUMING et al. (2001) determined the following concentrations of minerals: zinc 24.34 mg/kg, copper 12.10 mg/kg, manganese 17.42 mg/kg, calcium 472.30 mg/kg, iron 200-1000 mg/kg (depending on the cultivar). According to IKEDA et al. (2001) 100 g of buckwheat flour serves 13-88 % of the RDA. Another important dietary aspect is the high content of B-vitamins and tocopherol derivatives (alpha, beta, gamma) of the kernel (POMERANZ, 1985; KÖTTER, 1998; HONDA, 2001).

Polyphenols are found both in the kernels, though in small amounts, and the herb. Phenylpropanoids like coumaric acid, gallic acid, salicylic acid, cinnamic acid etc. are found in buckwheat (POMERANZ, 1985; KÖTTER, 1998; HAGELS et al., 2001). Besides the flavons vitexin, isovitexin, orientin and isoorientin (KÖTTER, 1998; DIETRYCH-SZOSTAK and OLESZEK, 1999) buckwheat also contains flavans like flavandiols and catechins as well as the flavonols quercitrin, hyperin and as the main compound rutin (POMERANZ, 1985; KÖTTER, 1998; HAGELS et al., 2001). The dianthron fagopyrin, which is related to hypericin, is only found in the buckwheat herb. Fagopyrin is not, according to KÖTTER (1998), found in the dried drug and the infusion of tea.

II.6. Flavonoids – properties, uptake and metabolism

Flavonoids belong to the secondary metabolites in plants and are found in all parts of the plants (SCHMIDT, 1997). The basic structure of flavonoids is the flavan structure (Fig. 03). Precursors for the flavonoid synthesis are three malonyl-CoA (ring A and C) and one 4-cumaroyl-CoA (ring B). Flavonoids are stored in the vacuole as glucosides connected by a β-glycosidic bond to mono- or disaccharides mainly at the positions C-3, C-5, C-7, C-3' and/or C-4' (RICHTER, 1996; SCHMIDT, 1997).

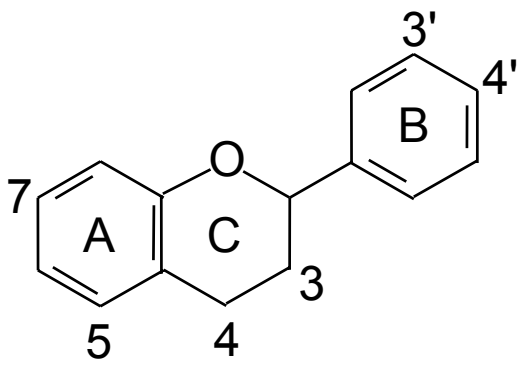


Fig. 03: Basic structure of flavan (SCHMIDT, 1997)

Quercetin is the main flavonoid in the diet and is consumed as quercetin-3-rutinoside (in black tea about 40 %), quercetin-4'-glucoside (45 % in onions) and quercetin-3-glucoside (mainly in tea, apples and tomatoes) (WATZL and LEITZMANN, 1999; MOON et al., 2000; OLTHOF et al., 2000).

Flavonoids exhibit several positive health aspects; they possess anti-carcinogenic, anti-mutagenic, antioxidant, anti-viral, immune-stimulating and oestrogen-active properties. They inhibit lipid peroxidation, LDL oxidation and chelate transition metals (RICE-EVANS et al., 1995; BORS et al., 1997; BROWN et al., 1998; PLUMB et al., 1998; PLUMB et al., 1999; WATZL and LEITZMANN, 1999; DISILVESTRO, 2001; HIDER et al., 2001).

The maximal scavenging properties are given by (see also Fig. 03):

- dihydroxyl groups at position 3' and 4' in ring B
- 2,3-double bond in combination with the 4-oxo group in ring C
- hydroxyl group at position 3 in ring C
- hydroxyl groups at position 5 and 7 in ring A

(BORS et al., 1990; RICE-EVANS et al., 1995; SOLEAS et al., 1997; HEIJNEN et al., 2002).

The knowledge about the bioavailability, uptake, and metabolism of flavonoids is important to judge their pharmacological significance. BRAVO (1998) showed that there are differences in the uptake of pure flavonoid supplements and 'complex food'. Non-soluble polyphenols (mainly polymer compounds) are not bio-available and bound to e.g. proteins or plant fibres in complex food. SOLEAS et al. (1997) stated that flavonoids in wine and tea exhibit a higher bioavailability then flavonoids of complex food.

Glucosides are not hydrolyzed in the acidic conditions in the stomach (GEE et al., 1998) and flavonoids are absorbed as aglycones as well as glucosides in the small intestine and colon, respectively. The group of HOLLMAN (HOLLMAN et al., 1996; 1997; 1999; OLTHOF et al., 2000) showed that the uptake of the glucosides depends upon the nature and position of the substitution. The highest concentration of quercetin in plasma was determined 0.5 h after a supplement of pure quercetin glucosides (q4'g, q3g) and 1-3 h after the consumption of a quercetin-rich meal (onions, apples). The short time of absorption suggests an uptake of the mono-glucosides via the sodium-dependent glucose/galactose transporter (SGLT1) in the small intestine. Investigations of GEE et al. (1998) confirmed the interaction of glucosides with SGLT1, by which an active uptake might be possible. Diffusion of the relative lipophilic aglycone is another possible form of absorption (GEE et al., 2000). Both the lactase/phlorizin hydrolase (LPH) and cytosolic β-glucosidase (CBG) in the brush border of the small intestine are able to hydrolyze glucosides (LPH: 3'-mehylq3g, d7g, g7g, q4'g, q3g and q3,4'dig; CBG: ap7g, d7g, g7g, q4'g, n7g; DAY et al., 1998; 2000a). Glucosides with di- and oligosaccharides (e.g. rutin) are metabolized and absorbed in the colon, this is confirmed with the later quercetin peak 9 h after a supplementation with rutin (HOLLMAN et al., 1997).

Cytolsolic β-glucosidases of cell-free liver extracts hydrolyze the same glucosides as CBG of the small intestine, though with a k_m-value half the value of the CBG of the small intestine (DAY et al., 1998).

Flavonoids are found as glucuronides, sulphates and methylates as well as ring fission products in the plasma (MANACH et al., 1998; DONOVAN et al., 1999; MOON et al., 2000; Day et al., 2001b; WITTIG et al., 2001), as they are hydrolyzed either before or after absorption. Enzymes of the detoxification metabolism (e.g. phase II enzymes, UDP-glucuronosyl transferases) glucuronate, sulphate and methylate absorbed xenobiotics to convert these compounds in water-soluble and higher-molecular metabolites (KARLSON et al., 1994). DAY et al. (2000) showed that quercetin actually is a substrate for UDP-glucuronosyl transferases of cell free liver extracts and the hydroxyl groups are conjugated according to their affinity as follows: 4' > 3' > 7 > 3. DAY et al. (2001b) detected q3glca, 3'-methylq3glca and q3's in the plasma of volunteers after consumption of onions, besides diglucuronides and mixed sulpho-glucuronides. The average concentration was 0.1 µM for 3'-methylquercetin and 0.5 µM for quercetin and is comparable to results of other studies (0.6 µM: HOLLMAN et al., 1996; MANACH et al., 1998; 0.74 µM: HOLLMAN et al., 1997).

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Instruments

▪ pH meter

Bachofer Orion Research 701A, pH 1-14

Mikroelektrode Ingold Model 402 M5

▪ UV/VIS Spectrophotometer

Kontron Uvikon Model 930/922

Thermostat 2209 Multitemp, Pharmacia, LKB Bromma

Beckman DU 640

▪ Fluorescence spectrophotometer

Perkins Elmer LS 50B

▪ HPLC

Instrument 1 (lipophilic antioxidants):

Solvent Delivery Module Type 112 with two pumps, Beckman

high pressure mixing chamber and injector (20 µl injecting volume) Altex 210A Valve, Beckman

Column thermostat: Waters

Detector Type 160, Beckman

Integrator Chromatopac C-R1B, Shimadzu

126 NM Solvent Module, Beckman

Software: System Gold

Column: Hypersil ODS 125 x 5 mm, 5 µm

Instrument 2 (flavonoid determination):

Beckman System Gold (20 µl injecting volume)

125 Solvent Module

168 Detector

Software: Gold Nouveau 1.7

Guard column: Hypersil ODS 50 x 4 mm, 5 µm

Column: Hypersil ODS 250 x 4 mm, 3 µm

▪ Gas chromatograph

Varian Aerograph 1400 with Shimadzu Integrator

Column: ⅛ in. x 60 cm aluminium oxide (ethylene determination)

Column: ⅛ in. x 80 cm aluminium oxide (ethane and ethylene determination)

Column temperature: 80 °C

Injector temperature: 80 °C

FID detector temperature: 225 °C

Carrier gas: N_2 (25 ml/min)

Burning gases: H_2 (25 ml/min), synthetic air (250 ml/min)

Calibration gas: ethane, ethylene

▪ Centrifuges

Biofuge A, Haereus Christ

Sigma 4 K10

▪ Ultracentrifuge

LE-80, Beckman

Rotor 70.1 Ti

▪ Balance

Model 2474, Sartorius

▪ Desalting columns

EconoPac DG-10, BioRad

▪ Thin layer chromatography

Silica gel-plates, Merck

Cellulose-plates, Merck

Glass chambers

▪ Sephadex Column

Sephadex LH-20

Column: l = 28 cm, Ø = 4.5 cm

▪ Speedvac

Vacuum rotary evaporator RVC2-18, Christ

▪ Centrifuge tubes

Centrifuge tubes, Beckman, No. 344322

▪ Light bath

Light strength 500 mE/cm*s, temperature 37 °C

▪ Rotary evaporator

▪ Soxhlet extractor

▪ Quartz cuvettes

Hellma Quartz cuvettes, 1.5 ml volume

Hellma Fluorescence cuvettes, 1.5 ml volume

▪ Pipettes

Gilson Pipetman 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl

III.1.2. Chemicals

Name	Formula	M (g/mol)	Company	Art. No
(±)-Catechin	$C_{15}H_{14}O_6$	290.3	Sigma	C-1788
1-aminocyclopropane-1-carboxylic acid	ACC	101.1	Sigma	A-3903
1-Butanol	$CH_3(CH_2)_3OH$	74.12	Merck	101990
1-methyl-2-phenyl-indol	MPI	207.3	Sigma	40,488-8
2,2'-azinobis(3-ethylbenz-thiazoline-sulphonic acid)	ABTS	548.7	Sigma	A-1888
3-Morpholinosydnonimine	Sin-1	206.7	Hoechst	
Acetic acid	CH_3COOH	60.05	Merck	818755
Acetone	CH_3COCH_3	58.08	Merck	159005
Acetonitrile	CH_3CN	41.05	Merck	100029
Ammonium hydroxide (ca. 30 % NH_3)	NH_4OH	35.05	Sigma	A-6899
Ascorbic acid	$C_6H_8O_6$	172.13	Sigma	A-5960
Borax	$Na_2B_4O_7$ x 10 H_2O	381.4	Sigma	B-9876
Boric acid	H_3BO_3	61.8	Merck	1.00165.
Bovine serum albumin	BSA	~ 66000	Sigma	A-2153
Buckwheat kernels			Neuform international	
Calcium chloride dihydrate	$CaCl_2$ x 2 H_2O	147.0	Merck	159104
Cibachrom Blue 3GA Type 3000-CL			Sigma	C-1535
Coomassie Protein Assay			BioRad	500-0006
Cupric sulphate pentahydrate	$CuSO_4$ x 5 H_2O	249.7	Merck	2790.
Desferal	$C_{25}H_{48}N_6O_8$*CH_4O_3S	656.8	Sigma	D-9533
Diaphorase from pig heart		134 U/mg Protein	Sigma	D-3752
Dichloromethane	CH_2Cl_2		Merck	106044
Epicatechin	$C_{15}H_{14}O_6$	290.3	Sigma	E-1753

Ethanol (d = 79 kg/l)	C_2H_5OH	46.1	Merck	1.00983.
Ethyl acetate	$C_4H_8O_2$	88.11	Sigma	15,485-7
Ethylenediamine-tetraacidic acid	$C_{10}H_{16}N_2O_8$	372.2	Merck	1.08417.
Fagorutin Tea (100 % buckwheat herb)			SmithKline Beecham	
Ferrous sulphate heptahydrate	$FeSO_4$ x 7 H_2O	278.0	Sigma	F-7002
Formic acid	HCOOH	46.03	Merck	100264
Human serum albumin	HSA	66439	Sigma	A-1887
Hydrochloric acid (32 %, ca. 12.5 M)	HCl	36.5	Merck	1.00317.
Hydrogen peroxide 30 %	H_2O_2	34.01	Merck	1.08597
Isopropanol	$(CH_3)_2CHOH$	60.10	Merck	818766
Isoquercitrin	$C_{21}H_{20}O_{11}$	464.38	Apin	03804q
Kaempferol	$C_{15}H_{10}O_6$	286.24	Sigma	K-0133
Lycopene	$C_{40}H_{56}$	536.85	Sigma	L-9879
Magnesium chloride hexahydrate	$MgCl_2$ x 6 H_2O	203.3	Sigma	M-9272
Methanesulfonic acid	CH_3SO_3H	96.11	Sigma	M-4141
Methanol	CH_3OH	32.04	Merck	1.006009.
Myoglobin		18800	Sigma	M-1882
n-Hexane	C_6H_{14}	86.18	Merck	1.04367.
Nicotinamid adenine dinucleotid, reduced form	β-NADH	709.4	Sigma	N-8129
Petroleum ether			Merck	159542
Potassium bromide	KBr	119.0	Sigma	P-5912
Potassium chloride	KCl	74.6	Merck	1.04936.
Potassium phosphate, monobasic	KH_2PO_4	136.1	Sigma	P-5379
Pycnogenol			Isarlab-systems	
Quercetin dihydrate	$C_{15}H_{10}O_7$ x 2 H_2O	338.27	Sigma	Q-0125

Quercitrin	$C_{21}H_{20}O_{11}$	448.4	Sigma	Q-3001
Rose Bengal	$C_{20}H_2Cl_4I_4Na_2O_5$	1017.6	Sigma	R-3877
Rutin trihydrat	$C_{27}H_{30}O_{16}$ x 3 H_2O	664.6	Sigma	R-5143
Saccharose	$C_{12}H_{12}O_{11}$	342.3	Merck	1.07687.
Sodium bicarbonate	$NaHCO_3$	84.0	Merck	1.06329.
Sodium borohydride	$NaBH_4$	37.83	Sigma	S-9125
Sodium chloride	NaCl	58.4	Merck	1.06404.
Sodium hypochlorite (ca. 13 % active chloride)	NaOCl	74.4	Merck	1.05614.
Sodium phosphate, dibasic	Na_2HPO_4 x 2 H_2O	177.99	Merck	1.06580.
Sodium phosphate, monobasic	NaH_2PO_4 x H_2O	137.99	Merck	1.06346.
Tocopherol acetate	$C_{31}H_{54}O_5$	472.8	Sigma	T-3001
Toluol			Merck	
Trizma Base	$C_4H_{11}NO_3$	121.1	Sigma	T-1503
Tween®20 (d = 1.11 kg/l)			Merck	8.22184.
Ubichinone	$C_{59}H_{90}O_4$	863.4	Aquanova	
Xanthine	$C_5H_4N_4O_2$	152.1	Sigma	X-4002
Xanthine oxidase from milk	XOD	20 U/13.5 mg Protein	Sigma	X-4875
α-Keto-S-methylbutanoic acid	KMB	170.2	Sigma	K-6000
α-Tocopherol	$C_{29}H_{50}O_2$	430.7	Sigma	T-4389
α–Linolenic acid C18:3 (d = 0.92 g/ml)	$C_{18}H_{30}O_2$	278.4	Sigma	L-2376
β-Carotene	$C_{40}H_{56}$	536.85	Sigma	C-4582

Naturstoffreagens A and DMACA for the thin layer chromatography were from Fachgebiet für Obstbau, TUM-Weihenstephan, Freising, Germany.

Quercetin-3-glucuronic acid, quercetin-7-glucuronic acid, quercetin-4'-glucuronic acid, quercetin-3'-sulfate and isorhamnetin-3-glucuronic acid as well as the HepG2 cells were from Institute of Food Research, Norwich, UK.

III.1.3. Buffers, media, extracts

III.1.3.1. Phosphate buffer 0.2 M

Solution A: Na_2HPO_4 x 2 H_2O 27.6 g/l
Solution B: NaH_2PO_4 x H_2O 35.6 g/l
Adjust solution B to pH of 7.4 with solution A.

III.1.3.2. Phosphate buffer 5 mM

Solution A: Na_2HPO_4 x 2 H_2O 0.69 g/l
Solution B: NaH_2PO_4 x H_2O 0.89 g/l
Adjust solution A to pH 7.2 with solution B.

III.1.3.3. HUGO buffer

Dissolve in 900 ml *aqua bidest*:

NaCl	8 g/l
KCl	0.2 g/l
Na_2HPO_4 x 2 H_2O	1.442 g/l
KH_2PO_4	0.2 g/l
$MgCl_2$ x 6 H_2O	0.1 g/l

Dissolve 0.1 g/l $CaCl_2$ x 2 H_2O in 100 ml *aqua bidest* and add as last. Adjust the pH with HCl/NaOH to 7.2.

III.1.3.4. PBS buffer

Fill 100 ml phosphate buffer 0.2 M pH 7.4 and 9 g NaCl up to 1 l using *aqua bidest*.

III.1.3.5. Isotonic salt solution

Dissolve 9 g NaCl in 1 l *aqua bidest*.

III.1.3.6. Borate buffer 0.2 M

Solution A: boric acid 12.4 g/l
Solution B: borax 76.3 g/l
Adjust solution A to pH 9.0 with solution B.

III.1.3.7. Eluting buffer 0.01 M Tris-HCl with 1.5 M NaCl

Dissolve 1.21 g Trizma Base in 800 ml *aqua bidest*. Adjust the pH to 7.5 with HCl, add 87.88 g NaCl (= 1.5 M) and make up to 1 l with *aqua bidest*.

III.1.3.8. $EMEM^+$ media with foetal calf serum

A stock solution of $EMEM^+$ media contains:

Aqua bidest	1.518 ml
10 x $EMEM^+$	200 ml
7.5 % $NaHCO_3$	55 ml
Penicillin (100 U/ml) +	
Streptomycin (100 µg/ml)	20 ml
100 x NEAA	2 ml

Adjust the pH to 7.2-7.4 with 1 N NaOH and store at 4 °C. The foetal calf serum (final concentration 10 %) will be added before use.

III.1.3.9. Trypsin/EDTA solution

Trypsin	0.5 %
EDTA	5.3 mM

The solution is stored as 4 ml aliquots at -70 °C prior to use.

III.1.3.10. Density solution

Solution D: d = 1.006 g/ml

11.4 g NaCl

0.1 g EDTA

1 ml 1 M NaOH

make up to 1 l using *aqua bidest*

III.1.3.11. Buffers for thin layer chromatography

BAW = butanol - acetic acid - *aqua bidest* (4:1:2.2)

TAA = toluol - acetone - formic acid (30:60:10)

5 % formic acid

III.1.3.12. Buffers for HPLC

Determination of lipophilic antioxidants:

acetonitrile – methanol - dichloromethane (4.5:4.5:1)

Determination of flavonoids:

Buffer A: 5 % formic acid

Buffer B: methanol (gradient grade)

III.1.3.13. Fatty acid preparation

0.1 ml Tween®20 and 2.0 ml borate buffer 0.2 M pH 9.0 were mixed and slowly added to 100 mg linolenic acid until an emulsion developed. Stir the solution after adding 0.26 ml 1 N NaOH until it clears up. Adjust to a final volume of 25 ml with borate buffer 0.2 M pH 9.0.

The resulting 8 x concentrated fatty acid (~ 71.84 mM) can be stored at -20 °C for several months.

III.1.3.14. Peroxynitrite preparation

The following

5 ml 0.7 M H_2O_2 (in 0.6 M HCl)

5 ml 0.6 M $NaNO_2$

5 ml 1.2 M NaOH

were mixed quickly on ice. The yellow solution was frozen overnight at -20 °C; an intense-yellow layer develops on the surface. This layer was retained after thawing and measured at 302 nm after dilution (1:3000) with 1 N NaOH.

Formula for peroxynitrite concentration:

$$c = E_{302} / (\varepsilon * d)$$

c = concentration in [M]

E_{302} = extinction at 302 nm

d = 1 cm

ε = extinction coefficient (= 1670 $M^{-1}cm^{-1}$)

III.1.3.15. Buckwheat extracts

a) aqueous extract (SCHNEIDER-LEUKEL, PAPER and FRANZ, 1992)
1.8 g sample material was extracted in 125 ml *aqua bidest* at 100 °C for 30 min in a soxhlet extractor and centrifuged at 3300 g for 15 min afterwards. The supernatant was, if required, made up to the volume at the beginning and stored in aliquots at -70 °C.

b) alkalic-ethanolic extract (KREFT, KNAPP and KREFT, 1999)
1.8 g sample material was extracted in 36 ml $H_2O/EtOH/NH_4OH$ (35:60:5) for 3 h at 30 °C. The sample was centrifuged at 3300 g for 15 min, the supernatant (made up to the volume at the beginning, if required), was dried as 1 ml aliquots in a speedvac at 40 °C and stored at -70 °C.

c) ethanolic extract (WATANABE, 1998)
1.8 g sample material was extracted 3 times with 9 ml ethanol each time at 80 °C for 1 h in a soxhlet extractor and centrifuged at 3300 g for 15 min after uniting. The supernatant (made up to the volume at the beginning, if required), was dried as 1 ml aliquots in a speedvac at 40 °C and stored at -70 °C.

The sample material:
A = Buckwheat herb
B = Buckwheat kernels whole
C = Buckwheat kernels ground

The lyophilised ethanolic and alkalic-ethanolic extracts were dissolved in 1 ml *aqua bidest* for the investigations in the different test assays shortly before use.

III.2. METHODS

III.2.1. Plasma preparation

The plasma for the plasma pool was obtained from the blood of 12 healthy volunteers (n = 12; ♀ = 5, ♂ = 7; aged 25-40 years); the plasma for the single-plasma samples was from blood of 24 healthy volunteers (n = 24; ♀ = 14; ♂ = 10; aged 25-50 years).

4 ml EDTA solution (25 mg/ml) was added to 100 ml blood, corresponding to an EDTA amount of 1 mg per ml blood. Blood samples were centrifuged at 1500 g for 20 min at 10 °C; the blood samples were not mixed for the single-plasma samples. The plasma (= yellow-orange supernatant) was obtained without any cells (= pellet), which were discarded. For the plasma pool, the plasma was therefore pooled. 1 ml of a 60 % saccharose solution was added to 100 ml plasma, gently mixed and the plasma aliquoted. The samples were stratified with N_2 prior to storage at -70 °C for several months.

III.2.2. LDL preparation

LDL was isolated by isopycnic ultra centrifugation (personal communications; GARY BRETT, 2002) for the test with quercetin metabolites:

Plasma (d = 1.000 g/ml) was adjusted to a density of 0.3265 g/ml with solid KBr and stratified with density solution D. The samples were centrifuged at 377856 g at 7 °C for 3-4 h and the following layers are visible above the plasma (increasing order): HDL, LDL, VLDL with chylomicrons. LDL was obtained, filtered (0.22 µm, Millex-GP, Millipore) and EDTA added to give a final EDTA concentration of 1 mg/ml prior to storage at 4 °C.

Prior to use, the EDTA was removed from the LDL samples using an Econo-Paq DG-10 desalting column.

III.2.3. Protein determination

The protein content of the single-plasma samples and of the LDL samples was determined using the Coomassie Blue protein assay. The assay is based on the complex of the proteins with the Coomassie Blue solution; this results in a shift of the absorption from 465 nm to 595 nm. The quantification is done with a BSA standard curve (Fig. 04).

The assay is as follows:

Aqua bidest	790 µl
Coomassie Blue solution undiluted	200 µl
BSA standard or sample	10 µl

The absorption is measured at 595 nm after 10 min.

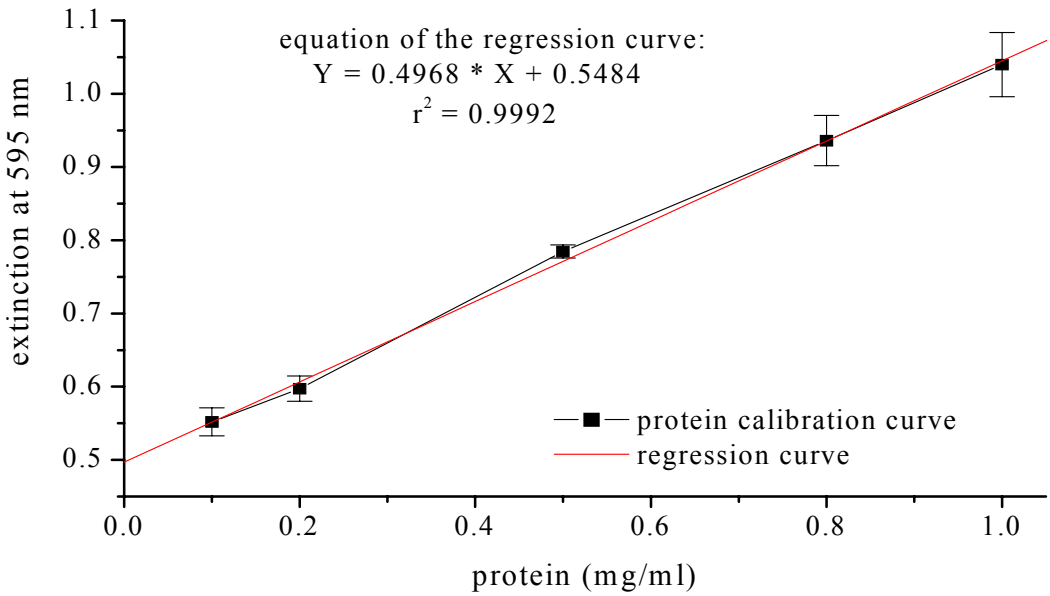


Fig. 04: Protein calibration curve with the equation of the regression curve; n = 3

III.2.4. Diene conjugation (copper-induced LDL oxidation)

The copper-induced LDL oxidation is a commonly used method (ESTERBAUER et al., 1989) to investigate protective properties. Conjugated dienes, accumulated via oxidation, were photometrically monitored and the protective properties evaluated with the resulting curve progression as seen in Fig 05. An elongation of the lag phase indicates higher protective abilities. This is due to either the regeneration of the endogenous antioxidants via the *in vitro* added test substance or the later onset of the oxidation of the endogenous antioxidants after the consumption of the test substance. The diene formation reaches its maximum in the propagation phase, which is followed by the decomposition phase with the oxidized substrates degrading to aldehydes, epoxides etc.

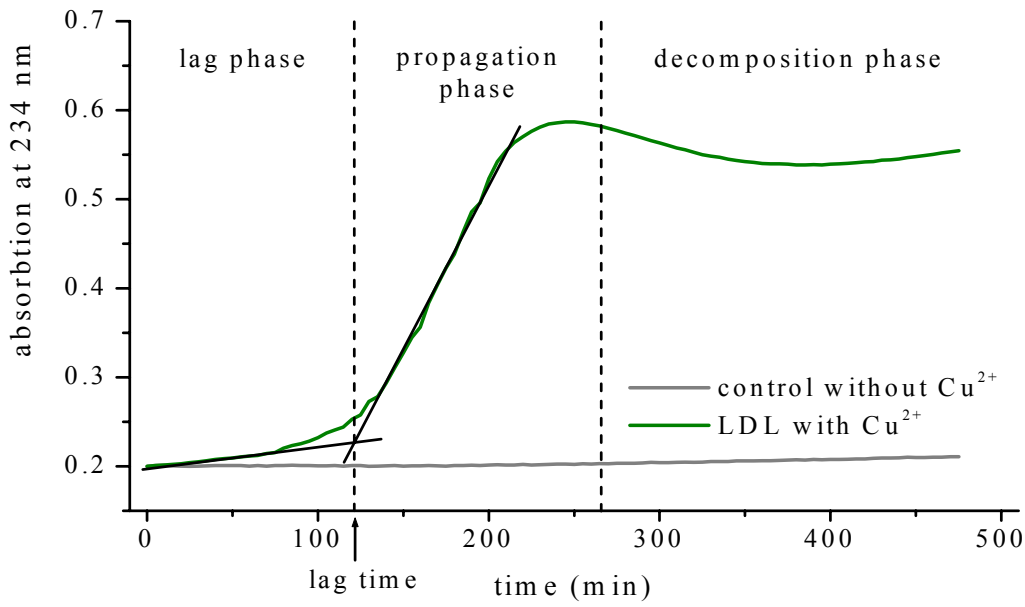


Fig. 05: Graph of the LDL oxidation

Formula for amount of LDL for the assay:

25 µg/protein content (µg/µl) = amount (µl)

The assay was performed as follows:

PBS buffer pH 7.2	add up to 1 ml
LDL	25 µg protein f. c.
Sample	10 µl
$CuSO_4$ (1,67 µM f. c.)	50 µl

The assay was measured at 234 nm at 37 °C for 500 min (every 5 min) and 1000 min (every 10 min), respectively.

III.2.5. HPLC methods

III.2.5.1. Determination of lipophilic antioxidants in plasma

The lipophilic antioxidants were extracted from plasma and determined with HPLC. To a 500 µl plasma aliquot 100 µl internal standard (= tocopherol acetate in ethanol, c = 250 µM) and 400 µl ethanol were added to precipitate the protein. Then 1 ml hexane was added followed by mixing for 1 min. From the hexane phase 800 µl was retained after centrifugation at 4000 rpm for 3 min. After adding a further 800 µl fresh hexane, the mixing and centrifuge step was repeated. Another 800 µl was taken from the hexane phase and combined with the further 800 µl. The hexane was evaporated from the sample and the residue dissolved in 100 µl running buffer and 20 µl injected in the HPLC for analysis.

HPLC settings (for instrument 1 see I.1.1):

Flow rate:	1 ml/min
Time:	25 min
Temperature:	35 °C
Running buffer:	acetonitrile – methanol - dichloromethane (4.5:4.5:1)
Program:	isocratic

Retention times of the single substances:

α-Tocopherol	~ 5.4 min
Tocopherol acetate	~ 6.3 min
Lycopene	~ 7.5 min
β-Carotene	~ 11.2 min
$Ubiquinone_{red}$	~ 13.1 min
$Ubiquinone_{ox}$	~ 19.3 min

The concentration of the lipophilic antioxidants in plasma samples is calculated with the aid of standard curves (Fig. 06-11) and with consideration of the dilution factor.

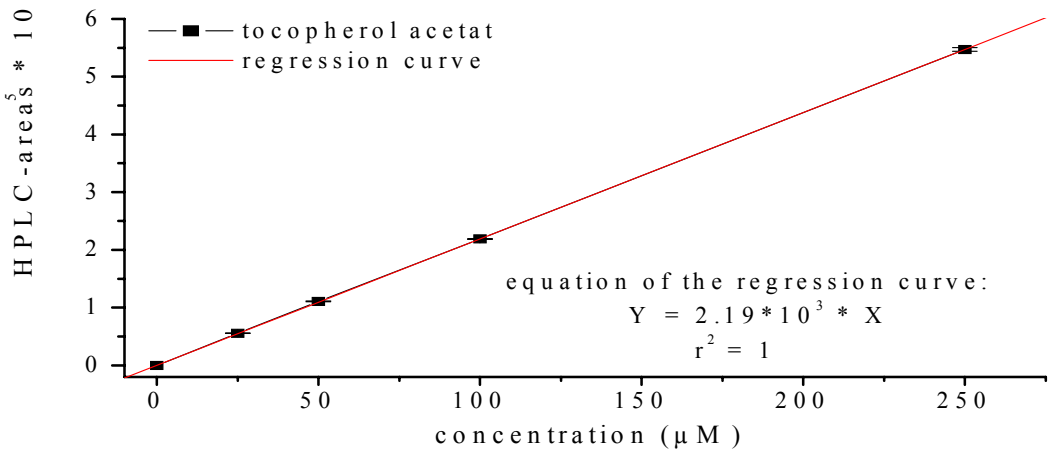


Fig. 06: Standard curve for tocopherol acetate, n = 3

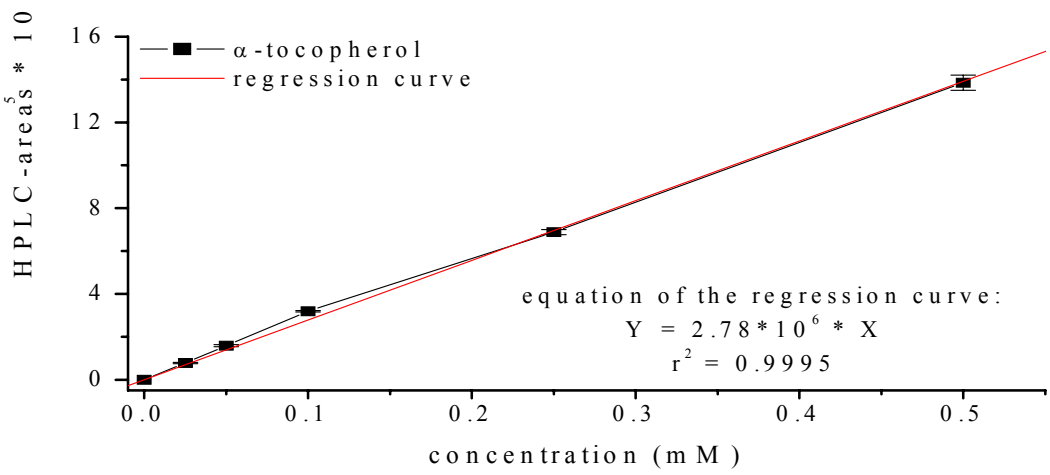


Fig. 07: Standard curve for α-tocopherol, n = 3

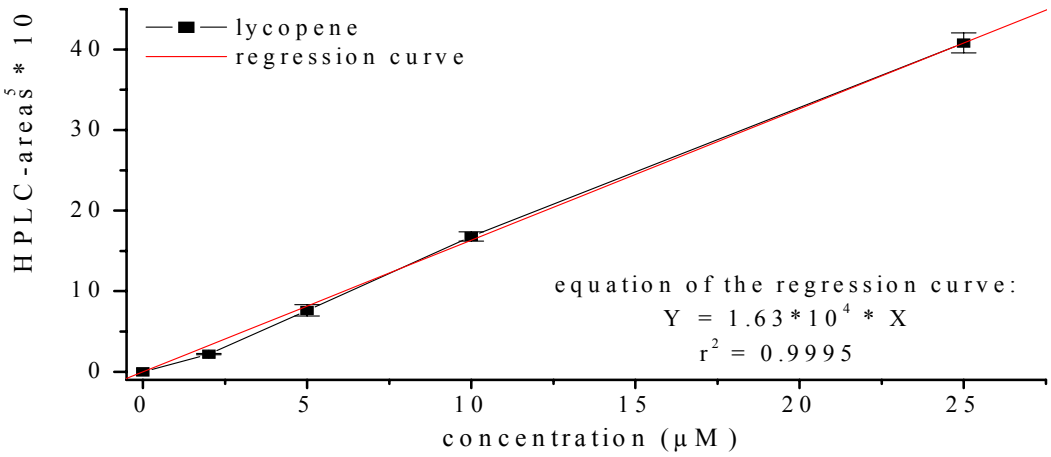


Fig. 08: Standard curve for lycopene, n = 3

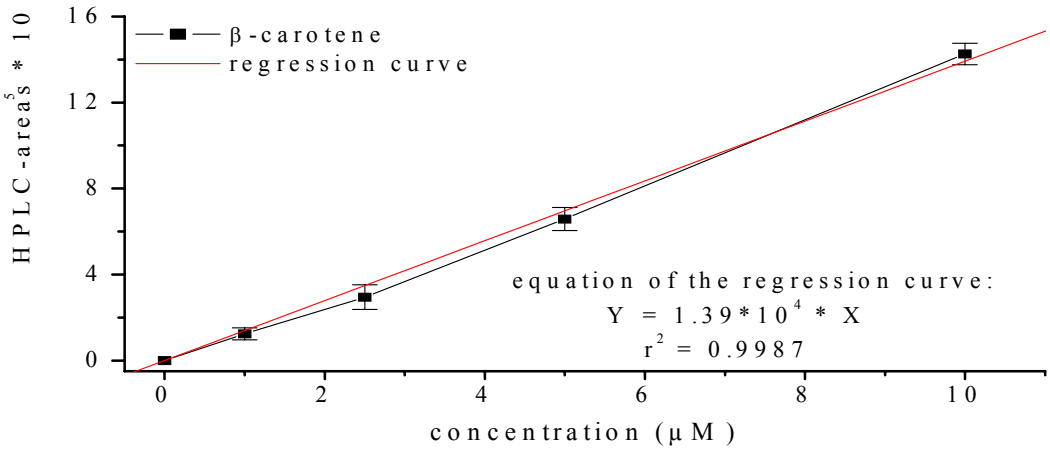


Fig. 09: Standard curve for β-carotene, n = 3

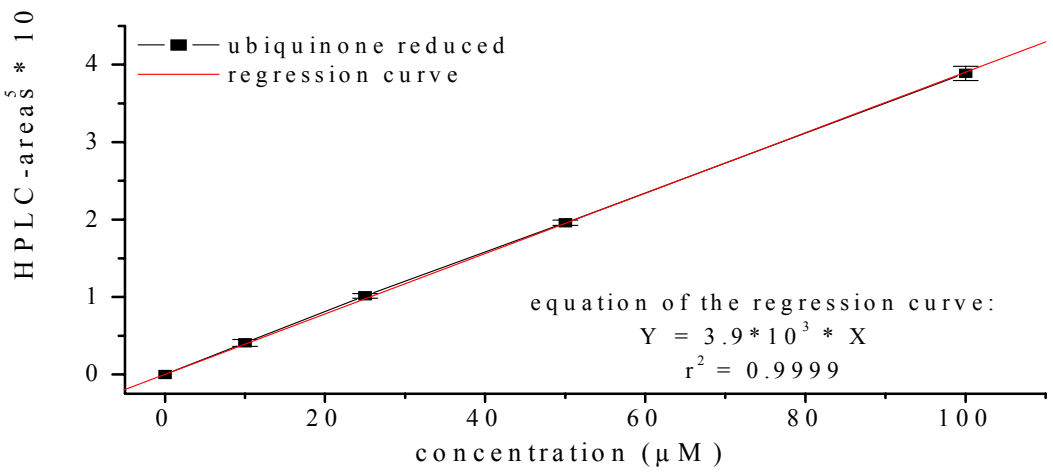


Fig. 10: Standard curve for ubiquinone$_{red}$, n = 3

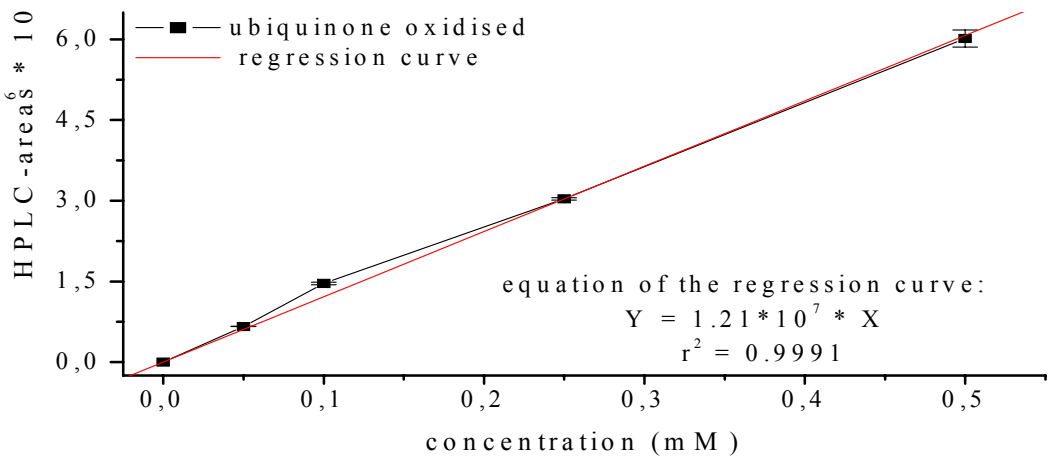


Fig. 11: Standard curve for ubiquinone$_{ox}$, n = 3

III.2.5.2. Determination of the main flavonoids in buckwheat extracts

For the determination of the main flavonoids in buckwheat extracts (see III.1.3.15) a modified method of LUX-ENDRICH (1998) was used.

The aqueous extracts were diluted 1:2 for analysis; the dried ethanolic and alkalic-ethanolic extracts were dissolved in 200 µl methanol (≡ 5 times concentrated) and all samples were centrifuged at 3000 rpm for 10 min.

HPLC settings (for instrument 2 see III.1.1.):

Flow rate:	0.5 ml/min
Time:	66.5 min
Temperature:	RT
Running buffer:	5 % formic acid - methanol
Program:	gradient program (see Tab. 01)

Tab. 01: HPLC gradient program to determine the main flavonoids in buckwheat extracts

Time (min)	% B (methanol)
0-3.5	20
3.5-7.3	20-25
7.3-15	25-30
15-20.1	30-40
20.1-23.9	40-50
26.9-30.7	50-60
33.7-37.5	60-70
40.5-44.3	70-80
47.3-51.1	80-90
56.2-60.0	90-20
60.0-66.5	20

Retention times of the substances:

Catechin	~ 7.8 min
Epicatechin	~ 12.9 min
Rutin	~ 27.8 min
Quercitrin	~ 30.3 min
Quercetin	~ 34.2 min
Kaempferol	~ 38.2 min

The concentration of the flavonoids in the extracts is estimated with the standard curves of the substances (Fig. 12-17) and with consideration of the dilution or concentrate.

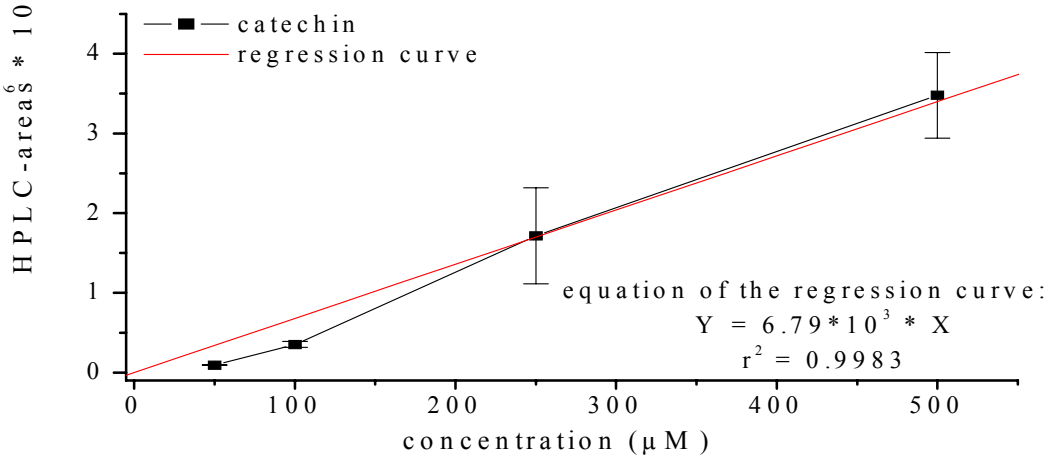


Fig. 12: Standard curve of catechin, n = 3

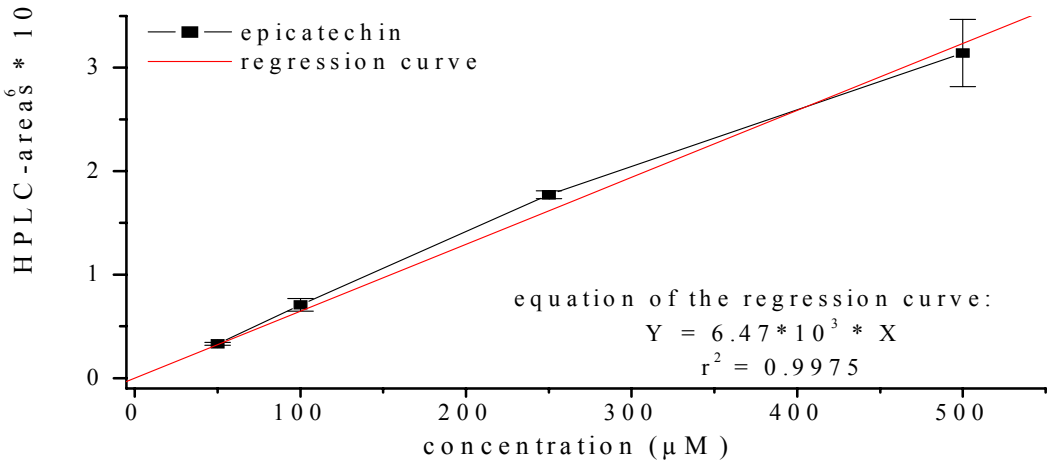


Fig. 13: Standard curve of epicatechin, n = 3

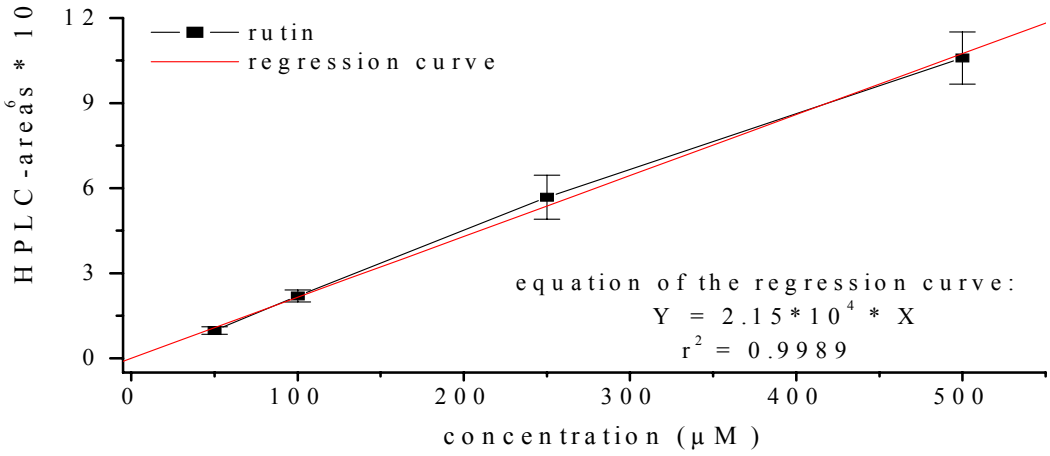


Fig. 14: Standard curve of rutin, n = 3

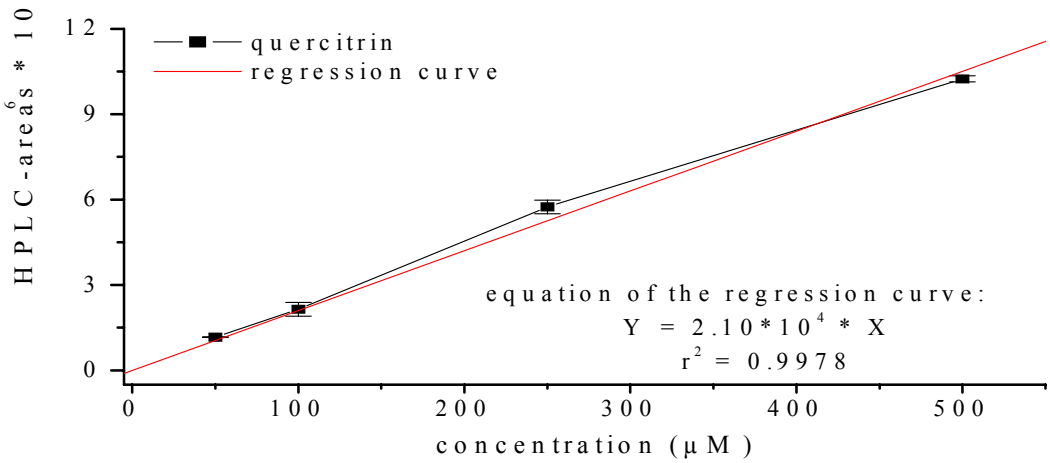


Fig. 15: Standard curve of quercitrin, n = 3

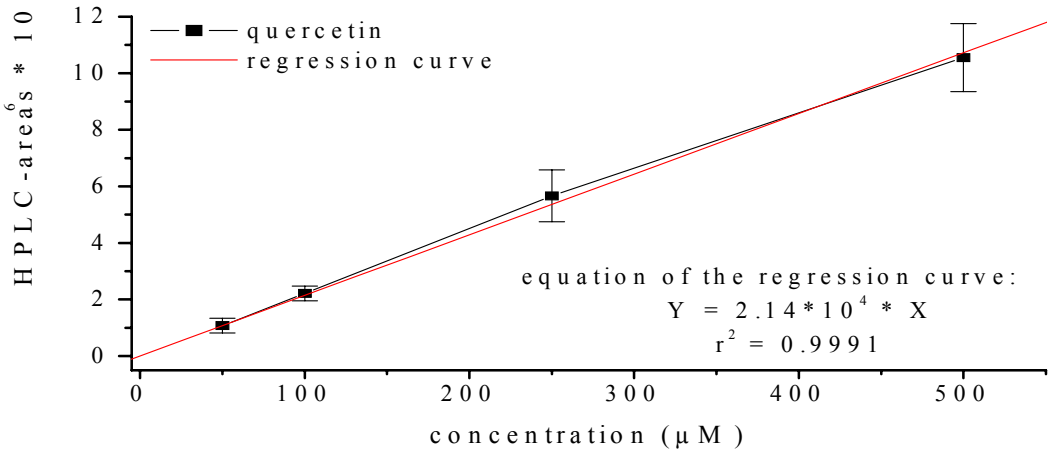


Fig. 16: Standard curve of quercetin, n = 3

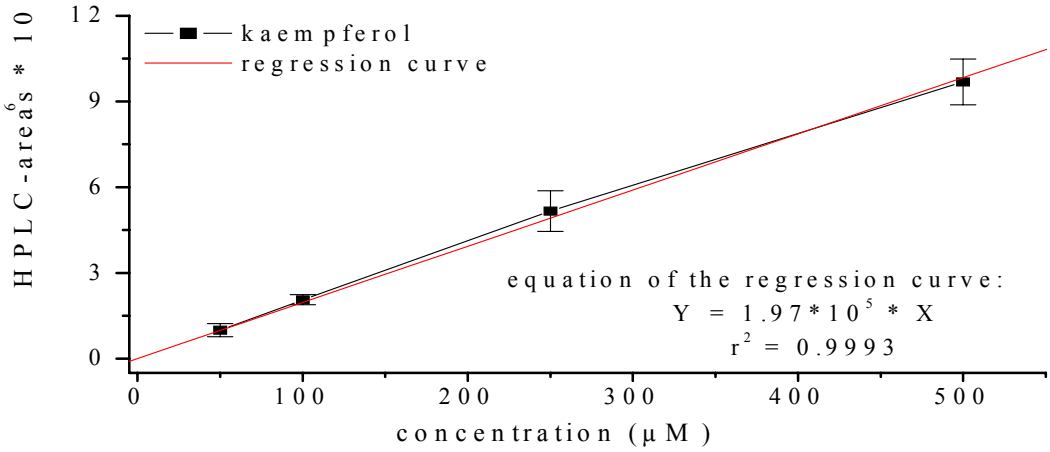


Fig. 17: Standard curve of kaempferol, n = 3

III.2.6. Ethylene production via KMB or ACC fragmentation with ROS

α-Keto-S-methylbuturonic acid (KMB), the transamination product of the essential amino acid methionine, is fragmented into ethylene and other fission products via the attack of reactive oxygen species (ROS). A stimulation of the ethylene formation due to the added test substances indicates an increase in the ROS production and pro-oxidative properties. Contrary, from a decrease in the ethylene formation, it can be concluded that the test substances possess scavenging properties or, depending on the assay, are able to inhibit enzymes.

The release of ethylene from 1-aminocyclopropane-1-carboxylic acid (ACC) is known from plant physiology. In contrast to KMB, only hypochlorite (HOCl) is able to fragment ACC to ethylene and products. A decrease in the ethylene production indicates an interaction of the test substance with HOCl and therefore a decreasing ACC fragmentation (VON KRUEDENER et al., 1995).

The assays were done in gas-tight test tubes with known volumes and 1 ml of the headspace was withdrawn with gas-tight syringes after an incubation of 30 min. The amount of ethylene was evaluated gas-chromatographically with ethylene calibration gas as standard.

III.2.7. Assays

III.2.7.1. ABTS assay (MILLER, RICE-EVANS and DAVIES, 1993):

The ABTS assay simulates the lipid peroxidation in an aqueous enviroment (RICE-EVANS and MILLER, 1996). Hydrogen peroxide and myoglobin convert ABTS, measurable with an increase in the extinction at 734 nm, to a nitrogen-centred cation radical ($ABTS^{+\bullet}$), which can be neutralised with H- and e^- -donors (RICE-EVANS and MILLER, 1996). Antioxidants as well as reductants such as sulphite lead to a decrease of the extinction (Fig. 18). This decrease is due to either the scavenging of the radical or the inhibition of the POD-like complex myoglobin forms with H_2O_2; both effects induce a decrease in the cation radical development.

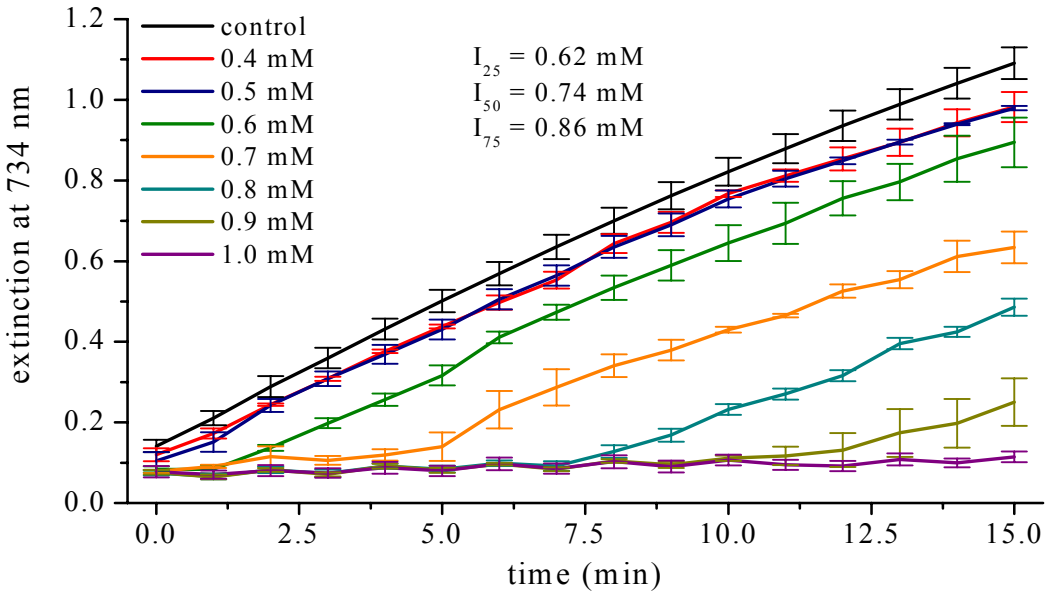


Fig. **18**: ABTS assay with sulphite and determined I-values; n = 3

The different curve progressions (Fig. 19) caused by various test substances indicate diverse mechanisms in their protective effect.

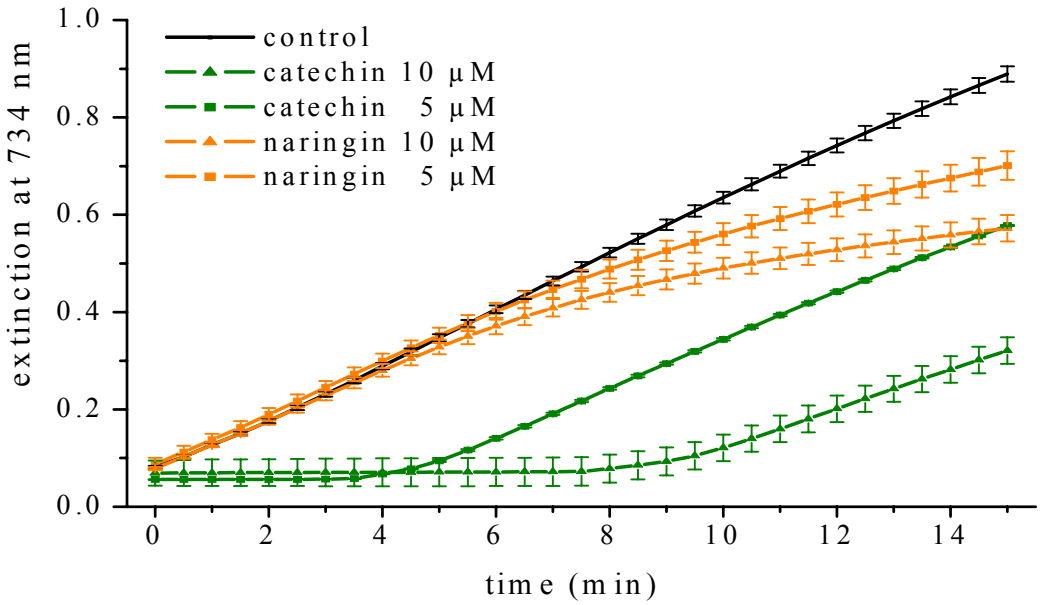


Fig. 19: ABTS assay with catechin and naringin; n = 3

Due to the fact that reductants like sulphite exhibit an "antioxidant" effect in this assay (Fig 18), the ABTS assay was only used to determine the antioxidant potentials of plasma.

The assay was as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
ABTS	150 µM	0.1 ml
Myoglobin	2.5 µM	0.1 ml
Plasma	IV.1.	0.1 ml
H_2O_2	100 µM	0.1 ml

Absorbance measured at 734 nm at 30 °C every 30 s for 15 min, the measurement starts with addition of hydrogen peroxide.

III.2.7.2. ACC/HOCl assay (VON KRUEDENER et al., 1995)

The ACC/HOCl assay simplifies the process in activated leukocytes. During degranulation the activated leukocyte excretes myeloperoxidase (MPO) in the extra-cellular medium of the phagosome, where the MPO catalyses the strong oxidant hypochlorite (HOCl) from the existing NaCl and H_2O_2. Hypochlorite inactivates important biological molecules in the further progress of pathogen defence. As explained in III.2.5. ACC is fragmented to ethylene by HOCl and is the indicator in this assay. Samples, which lead to a decrease in ethylene production after a pre-incubation with HOCl, possess protective capacity towards HOCl (see III.2.5.).

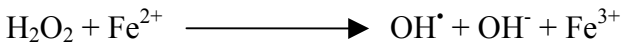
The assay was as follows:

	Final concentration	**Amount**
HUGO buffer pH 7.2		1 ml
Aqua bidest		add up to 2 ml
Plasma	IV.1.	0.1 ml
HOCl	25 µM	0.1 ml
ACC (in HUGO buffer)	1 mM	0.1 ml

After a pre-incubation of 30 s at 37 °C, ACC was added and the assay incubated for 30 min at 37 °C. The ethylene sampling was done as described in III.2.5.

III.2.7.3. Fenton assay (VON KRUEDENER et al., 1995)

The formation of ROS occurs in various disease processes. In most cases reduced transition metals, which promote Fenton-type reactions, are found inside the cell:



$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

The resulting $OH^{\bullet}$ radicals react rapidly with surrounding organic molecules and cause as the strongest oxidant severe cell damage.

Fe^{2+} is chelated with EDTA in this assay to increase the $OH^{\bullet}$ production and therefore the ethylene production.

The added samples ought to scavenge the produced hydroxyl radicals or inactivate the iron through chelating and accordingly lead to a decrease in ethylene production (see III.2.5.).

The assay was performed as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
KMB	1 mM	0.1 ml
Plasma/sample	IV.1,2.,4.,5.	0.1 ml
EDTA	50 µM	0.1 ml
$Fe(SO_4)$	10 µM	0.1 ml
H_2O_2	10 µM	0.1 ml

The ethylene sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C.

III.2.7.4. Sin-1 and peroxynitrite (HIPPELI et al., 1997; HIPPELI and ELSTNER, 1997)

The production of NO occurs in the activation process of granulocytes. It reacts spontaneously with the likewise formed $O_2^{-\bullet}$ to form ONOOH. Peroxynitrite as a strong oxidant attacks protein thiols, iron/sulphur centres and a variety of other biological molecules (BECKMANN and KOPPENOL, 1996).
In physiological conditions 3-morpholinosydnonimine (Sin-1) cleaves spontaneous to superoxide and nitrogen oxide (BOHR and SCHÖNAFINGER, 1989; FEELISCH et al., 1989) and forms therefore ONOOH.
The dissolved Sin-1 must be kept on ice for 5 min and used within 20 min.

The assay was performed as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
KMB	1 mM	0.1 ml
Plasma/sample	IV.1.,2.,4.,5.	0.1 ml
Sin-1	10 µM	0.1 ml

The ethylene sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C.

Instead of Sin-1 the use of a pure peroxynitrite solution (see III.1.3.11.) can be indicated, as Sin-1 does not produce solely ONOOH, but also $O_2^{-\bullet}$, NO and $OH^{\bullet}$.

The assay modifications were as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
KMB	1 mM	0.1 ml
Plasma/sample	IV.1.,5.	0.1 ml
Peroxynitrite	5 µM	0.1 ml

The ethylene sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C.

III.2.7.5. NADH/diaphorase assay (HIPPELI et al., 1997)

The NADH/diaphorase assay is synonymous with the membrane-bound NADPH oxidase, the starting point for the *oxidative burst* in the leukocytes.
In this assay the antioxidant capacity of the samples is due to either their scavenging properties, their ability to inhibit the enzyme or an addition of both mechanisms.

The assay was as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
Plasma/sample	IV.1.,5.	0.1 ml
NADH	75 µM	0.1 ml
KMB	1 mM	0.1 ml
Diaphorase	2.2 U	0.1 ml

The ethylene sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C.

III.2.7.6. Xanthine/xanthine oxidase (X/XOD) assay (BLAUROCK et al., 1992)

This assay mimics ischaemia/reperfusion, in which xanthine oxidase (XOD), usually present as xanthine dehydrogenase, catalyzes the step from hypoxanthine to uric acid with the simultaneous formation of superoxide, hydrogen peroxide and hydroxyl radicals.
The antioxidant capacity of the samples is, as mentioned III.2.6.5., due to either their scavenging properties, their ability to inhibit the enzyme or an addition of both mechanisms.

The assay was performed as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
KMB	1 mM	0.1 ml
Plasma/sample	IV.1.,2.,4.,5.	0.1 ml
Xanthine	0.5 mM	0.1 ml
XOD	0.016 U	0.1 ml

The ethylene sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C.

III.2.7.7. Rose Bengal assay

With the photosensitizer Rose Bengal singlet oxygen can be generated physically by light energy as seen in scheme 02:

$$^{0}RB \xrightarrow{h*v} RB^{*} \longrightarrow {}^{1}RB \longrightarrow {}^{3}RB + {}^{3}O_2 \longrightarrow {}^{0}RB + {}^{1}O_2$$

Scheme 02 from: E. F. Elstner, *Der Sauerstoff*, 1990

In buffered systems up to 20 % $O_2^{-\bullet}$ is produced besides singlet oxygen due to the presence of electron donators. Ethane/ethylene as well as malonylaldehyde

(MDA)/aldehyde generation from lipid peroxidation of α-linolenic acid is used as indicator in this assay. The lipid peroxidation is shown in scheme 01 on page 4.
As seen in scheme 01 there are different sites for test substances to interfere with the lipid peroxidation. Chelating agents can chelate the transition metals; this leads to a decrease in ethylene production and to a possible decrease in the ethane production. The content of MDA declines while the aldehyde content remains the same or increases compared to the control. A break in the radical chain is indicated by a decreased ethane/ethylene production and a decline in MDA/aldehyde content or an unchanged aldehyde content. As seen in scheme 01, a substance can interfere at different sites making Rose Bengal a complex system when evaluating the mode of action of the test substances.

The assay for Rose Bengal (RB) was as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7.4	0.1 M	1 ml
Aqua bidest		add up to 2 ml
Fatty acid	3.592 mM	0.1 ml
$CuSO_4$	5 µM	0.1 ml
Sample	IV.2.,4.	0.1 ml
Rose Bengal	20 µM	0.1 ml

The gas sampling (see III.2.5.) was done after an incubation of 30 min at 37 °C and 500 mE/cm*s.

The assay for the MDA determination is was performed as follows:

	Final concentration	**Amount**
MPI	7.41 mM	650 µl
Sample of RB assay		200 µl
HCl	1.875 M	150 µl

The assay was measured spectrophotometrically after an incubation of 40 min at 45 °C at 586 nm.

The assay for the aldehyde determination was as follows:

	Final concentration	**Amount**
MPI	7.41 mM	650 µl
Sample of RB assay		200 µl
Methanesulfonic acid	2.7 M	150 µl

The assay was measured spectrophotometrically at 586 nm after an incubation of 40 min at 45 °C.

III.2.8. Fractionation of the buckwheat herb

To obtain more information about the qualitative and quantitative content of polyphenols, the buckwheat herb extract was fractionated with Sephadex LH-20.
100 g of the ground herb (pebble mill, 20 Hz, 5 min) was extracted for 20 min with 500 ml of cold methanol at 8 °C in an ultrasonic bath and subsequently centrifuged at 10000 rpm for 10 min at 8 °C. The resulting supernatant was pooled. The pooled pellet was extracted twice with 300 ml cold methanol as mentioned above. The pooled supernatants were reduced under vacuum to approximately 500 ml and the pellet discarded. The methanol extract was divided in three portions of 200 ml and each portion was extracted with 500 ml ice-cold petroleum ether in the elutriator (= removal of the lipophilic components in the extract). This step was repeated until the petroleum ether phase appeared to be colourless. The addition of *aqua bidest* to the methanol extract, as well as the cooling of the solutions, achieves a better separation of the phases. The aqueous methanol phases were pooled after the treatment with petroleum ether and extracted 8-9 times with 400 ml ethyl acetate as 300-ml aliquots. The ethyl acetate phases were pooled and dried under vacuum. The residue was dissolved in methanol/*aqua bidest* (MeOH ≤ 30 %) and lyophilised. 2.3 g of the lyophilised ethyl acetate phase were dissolved in *aqua bidest* and applied to a Sephadex LH-20 column. The elution starts with 1 l *aqua bidest*, followed by ethanol 99 % and is finished with acetone.

The eluate was collected in different fractions:

Eluting solvent *aqua bidest*: 2 x 500 ml, 1 x 150 ml;

Eluting solvent ethanol: 500 x 10 ml;

Eluting solvent acetone: 9 x 100 ml.

The single fractions were screened photometrically and via thin layer chromatography. Corresponding fractions were pooled, dried under vacuum, dissolved in *aqua bidest* and lyophilised. The lyophilised fractions were screened with HPLC (10 mg/ml in methanol). The fractionation resulted in 18 different fractions (A-R).

III.2.9. Thin layer chromatography

Single fractions of the buckwheat herb were screened using thin layer chromatography prior to pooling. With a glass capillary, 10-20 µl of each single fraction were applied to the TLC-plates. The chromatographic runs were done in appropriate running buffers for the TLC-plates (BAW for cellulose-plates, TAA for silica gel-plates). Under UV light, the bands of the dried plates are noted. After spraying the cellulose-plates with Naturstoffreagens A, the bands were noted again under UV light. Silica gel-plates were analyzed similarly, but sprayed with DMACA. The spraying with Naturstoffreagens A or DMACA effects a change in the UV spectrum of the bands and allows visualization of new bands respectively to identify single compounds and fractions. At the beginning of the fractionation, cellulose plates were used, because chlorogenic acid, hydroxyl cinnamic acid, their derivates as well as flavones and flavonols are detectable with them. Silica gel-plates were used later, because flavanols and their oligomers are detectable with them. DMACA as a specific indicator for flavanols forms a blue-green complex with them.

III.2.10. HSA Isolation

Two preparations of HSA were used in the experiments; a fatty acid-free preparation supplied by Sigma (called HSA1); and a preparation isolated from a plasma pool (HSA2).

The HSA2 was purified from plasma by affinity chromatography. Columns (l = 30 cm, ∅ = 1.5 cm) of 10 ml bed volume with Cibacron Blue 3GA matrix were prepared according to the protocol of Sigma. The columns were washed and equilibrated with 50 ml *aqua bidest* and 5 ml plasma (1:10 diluted) applied. After the plasma entered the bed, the column was washed with 80 ml *aqua bidest* to remove unbound proteins (e.g. glucoproteins). Bound HSA was released from the column with 0.01 M Tris-HCl with 1.5 M NaCl buffer at pH 7.5 (see III.1.3.7) and the column regenerated with 30 ml borate buffer. The resulting HSA was desalted with a DG-10 desalting column and lyophilised. To obtain uniform HSA material the lyophilised protein was dissolved in 12 ml 5 mM phosphate buffer pH 7.2 to obtain a stock solution (c = 467 µM) and stored at -20 °C until required.

III.2.11. Quenching of the tryptophan fluorescence for HSA binding studies

A common assay for investigating binding abilities is the fluorescence quenching of the single tryptophan due to the binding of the compounds in the hydrophobic pocket in subdomain IIA of HSA. Molecules binding close to or with Trp, are able to quench its fluorescence if they possess

a) specific groups that interact with Trp (contact quenching) or
b) light-absorbing groups that function as acceptors for the resonance energy transfer.

At an excitation wavelength > 295 nm only Trp is excited and the tyrosine residues do not contribute to the emission.

Fluorescence spectrophotometer settings:

Excitation:	298 nm
Emission:	290-500 nm
Slits:	ex 2.5 nm, em 5 nm
Scan rate:	200 nm/min
Temperature:	30 °C

The assay was performed as follows:

	Final concentration	**Amount**
Phosphate buffer pH 7,2	5 mM	add up to 1 ml
Sample	IV.7.	0.1 ml
HSA1 or HSA2	5 µM	0.1 ml or 11 µl

After mixing, the pre-warmed solutions were measured immediately.

III.2.12. Cell culture

The HepG2 cell line used was generated from hepatic carcinoma cells and was cultivated as a monolayer culture in culture dishes (∅ = 10 cm).

The cells were subcultured routinely once a week in a class II safety cabinet. The media was removed with a pipette, the cells washed twice with 10 ml PBS buffer each time. To detach the cells from the dish surface, 2 ml trypsin/EDTA solution was added; the cells were covered with a film of trypsin/EDTA by tilting the dish and excess trypsin/EDTA solution was removed. After an incubation of 9 min at 37 °C the trypsin was inactivated by adding of 10 ml media, the cells were transferred and pooled in an appropriate tube. A cell count was performed, the cells seeded in a fresh culture dish with a cell density of $2*10^4$ cells/cm^2 and cultivated in an incubator at 37 °C and 5 % CO_2.

Cell count and dilution for seeding:

A cell count was performed in a haemocytometer. Four squares composed of 16 smaller squares were counted and the mean of the count ($\equiv x*10^4$ cells/ml) calculated. The recommended cell density for seeding was $2*10^4$ cells/cm^2, this results in the following equation for the dilution:

$$(Z * A) / M = X \qquad \text{where:}$$

Z = Recommended cell density ($2*10^4$ cells/cm^2)
A = Area of the dish (cm^2)
M = Mean of the cell count ($\equiv x*10^4$ cells/ml)
X = Needed amount of cells (ml)

The following vessels were used:

Vessel	Area (cm^2)	Volume of media (ml)
Culture dishes (∅ 10 cm)	55	10
6 well plates (∅ 1.5 cm)	9.4	4

The amount of media per dish was calculated as follows:

$$V - X = D \qquad \text{where:}$$

V = Volume of media per dish (ml)
X = Needed amount of cells (ml)
D = Amount of added media (ml)

HepG2 cells at 80-90 % confluence were incubated at 37 °C, 80 % relative humidity and 5 % CO_2 with the samples (for concentrations see IV.8.) dissolved in fcs-free EMEM$^+$ medium. To a 1 ml aliquot of the medium, 1 ml methanol with 1 mM ascorbic acid was added to precipitate the proteins and to stabilize the flavonoids. The sample was centrifuged at 16250 g for 10 min at 4 °C and filtered (0.22 nm) for HPLC analysis. The HPLC analysis was performed at the Institute of Food Research.

III.2.13. Calculations and statistical evaluations of the results

All experiments were done in quadruplicates unless stated otherwise and the data found in the charts are the corresponding means with standard deviations σ_{n-1}.

The calculations were done with Microsoft Excel 7.0, the statistical evaluations and the graphs for the experiments IV.1. - IV.5. and IV.8. were done using Microcal Origin 6.1. The statistical evaluation and chart for the experiment IV.6. were done in Microsoft Excel 7.0.

For experiment IV.7. the program GraFit 5 was used for the calculations and charts. The statistical calculations of the k_q-values (Tab. 26) were done according to PRECHT and KRAFT (1993).

IV. RESULTS

IV.1. Antioxidant properties of human plasmas

IV.1.1. I-values of the plasma pool

Human plasma was tested in seven different assays in concentrations of 1-500 µl plasma per 2 ml assay to investigate its antioxidant potentials. The I-values of each single assay were determined, to compare the individual test systems, and are shown in Fig. 20.

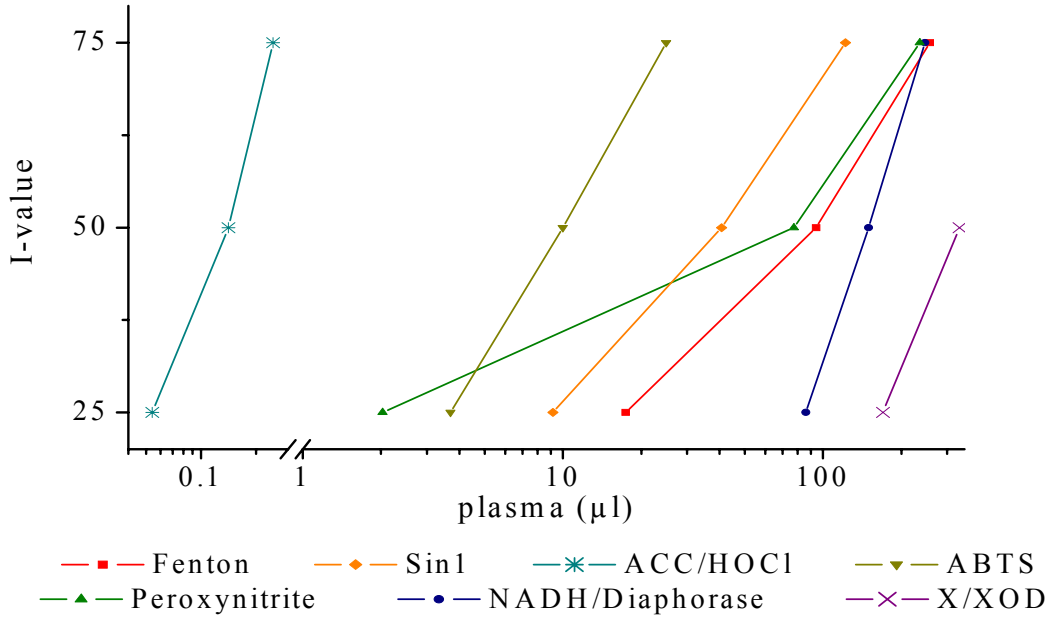


Fig. 20: I-values of the plasma pool in the individual test systems

The I-values represent the amounts of plasma, which inhibit the basic reaction (= 100 %) by 25 % (≡ I_{25}-value), 50 % (≡ I_{50}-value) and 75 % (≡ I_{75}-value), respectively. To inhibit the reaction in the ACC/HOCl assay, the lowest amount of plasma is needed (I_{25} = 0.054 µl, I_{50} = 0.14 µl, I_{75} = 0.25 µl), which indicates the most sensitive system. At the ABTS-system the I-values range from I_{25} = 3.7 µl, I_{50} = 10 µl to I_{75} = 25 µl still beyond an amount of 100 µl. The I-values for Sin-1 are I_{25} = 9.16 µl, I_{50} = 40.79 µl and I_{75} = 122.12 µl, at the Fenton assay they are I_{25} = 17.41 µl, I_{50} = 94.17 µl and I_{75} = 256.97 µl. The graph of the NADH/diaphorase-system shows a

steeper rise than the rest of the shown graphs with following I-values: I_{25} = 85.92 µl, I_{50} = 149.87 µl and I_{75} = 246.21 µl. The least sensitive test system is the xanthine/xanthine oxidase-system where no I_{75}-value can be measured, the other two I-values are I_{25} = 170.16 µl and I_{50} = 334.39 µl. For the peroxynitrite assay a low amount of plasma (= 2.03 µl) is needed to achieve 25 % inhibition, while the two other I-values (I_{50} = 77.38 µl and I_{75} = 235.74 µl) were reached after a larger step.

IV.1.2. Plasma pool versus BSA content

Two different plasma pools were compared to BSA concentrations corresponding to the total protein content in plasma. The systems used were ABTS, Fenton, ACC/HOCl and Sin-1 (Fig. 21-24).

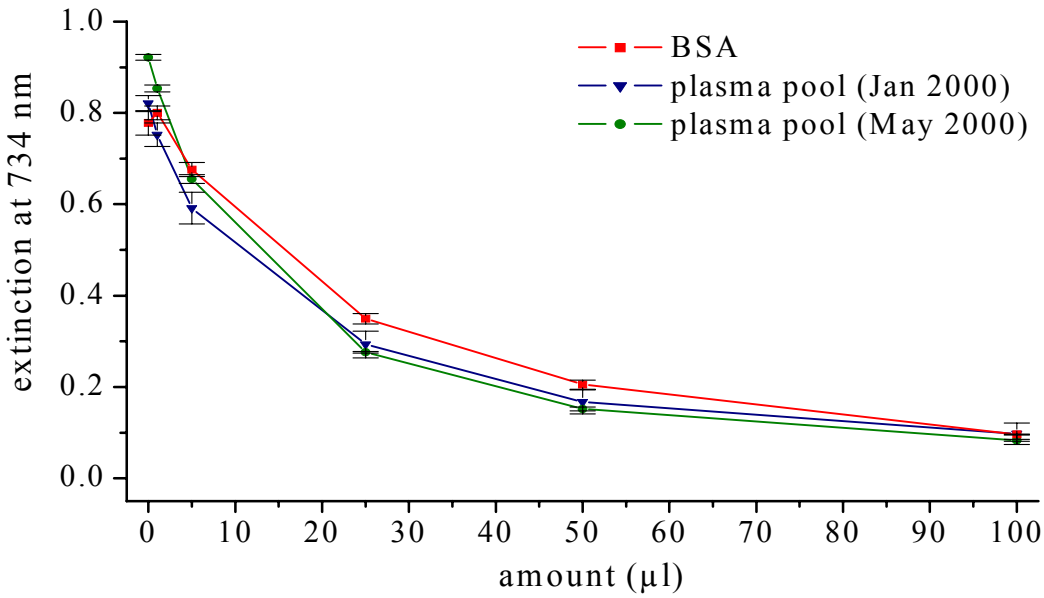


Fig. 21: ABTS assay, comparison of plasma pool vs. BSA, n = 4, BSA stock solution = 70 mg/ml

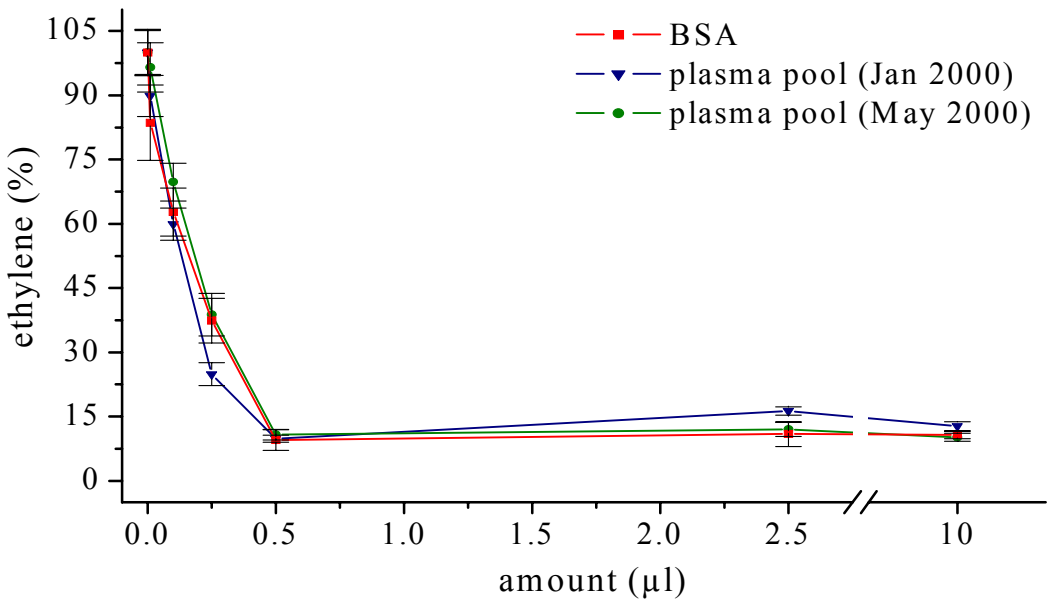


Fig. 22: ACC/HOCl assay, comparison of plasma pool vs. BSA, n = 4, BSA stock solution = 70 mg/ml

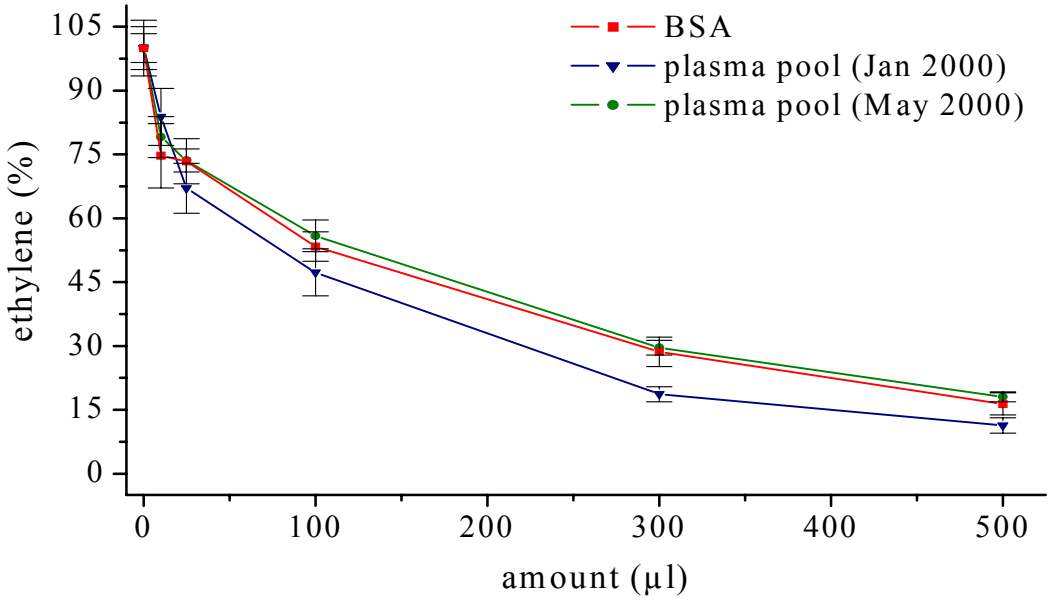


Fig. 23: Fenton assay, comparison of plasma pool vs. BSA, n = 4, BSA stock solution = 70 mg/ml

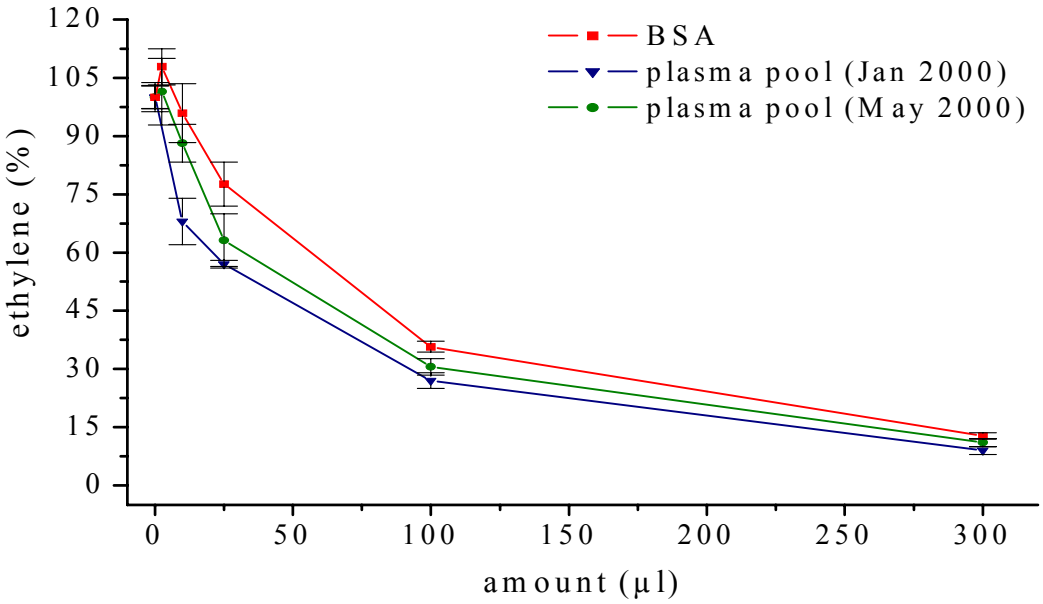


Fig. 24: Sin-1, comparison of plasma pool vs. BSA, n = 4, BSA stock solution = 70 mg/ml

The ABTS assay (Fig. 21; III.2.7.1) was chosen because it simulates lipid peroxidation in an aqueous milieu and is a commonly used assay to investigate antioxidant capacity. The ACC/HOCl assay (Fig. 22) is the most sensible assay to determine the antioxidant potentials of plasma (Fig. 20; IV.1.1.) and represents the processes in activated leukocytes (III.2.7.2.). The Fenton assay (Fig. 23) was chosen as an iron catalytic assay to generate $OH^{\bullet}$ radicals (III.2.7.3.), while Sin-1 (Fig. 24) was selected because of the peroxynitrite production (III.2.7.4.). As seen in all figures, the protein content in the plasma is mainly responsible for the antioxidant abilities.

IV.1.3. Single-plasma samples versus plasma pool

Single-plasma samples from 25 healthy volunteers were investigated in the four-mentioned (III.1.2.) assays to compare differences with the plasma pool (Fig. 25-28). The content of the lipophilic antioxidants in both the single-plasma samples and the plasma pool (Tab. 02) were determined via HPLC.

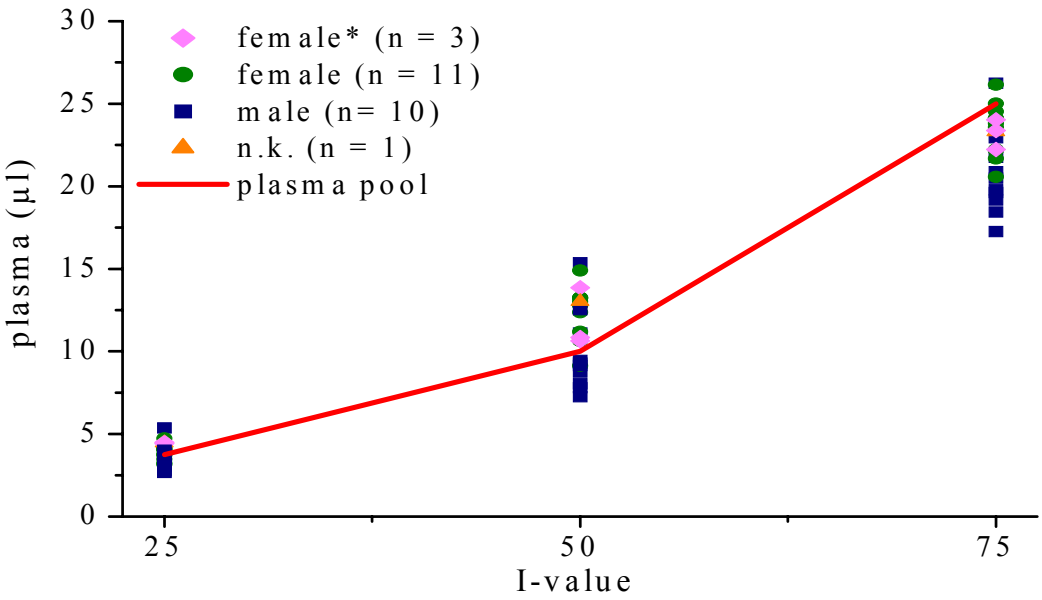


Fig. 25: ABTS assay, comparison of single-plasma samples (n = 25) vs. plasma pool, n.k. = gender not known, female* = menstruation

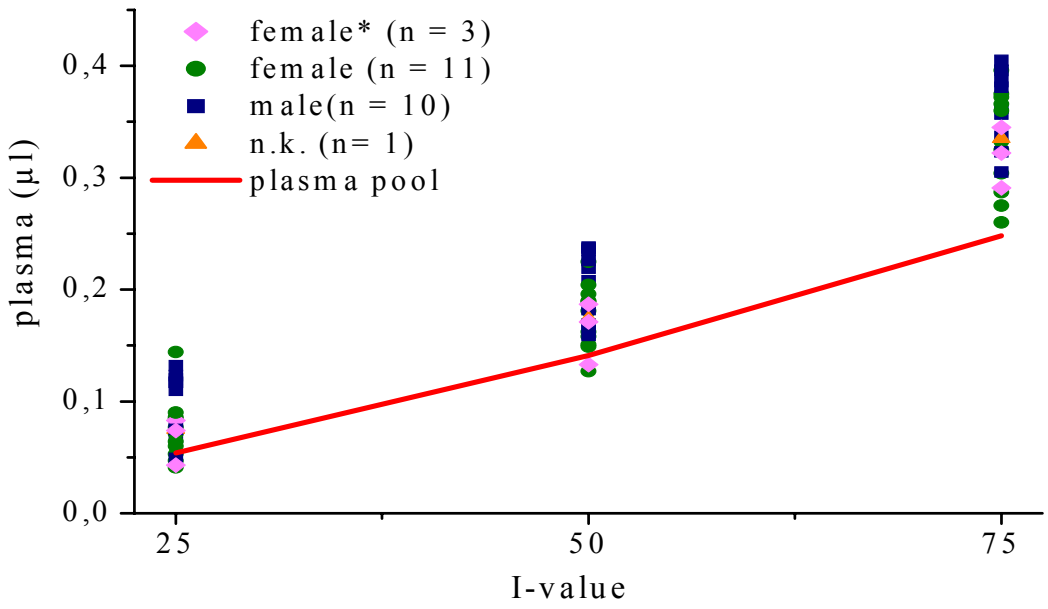


Fig. 26: ACC/HOCl assay, comparison of single-plasma samples (n = 25) vs. plasma pool, n.k. = gender not known, female* = menstruation

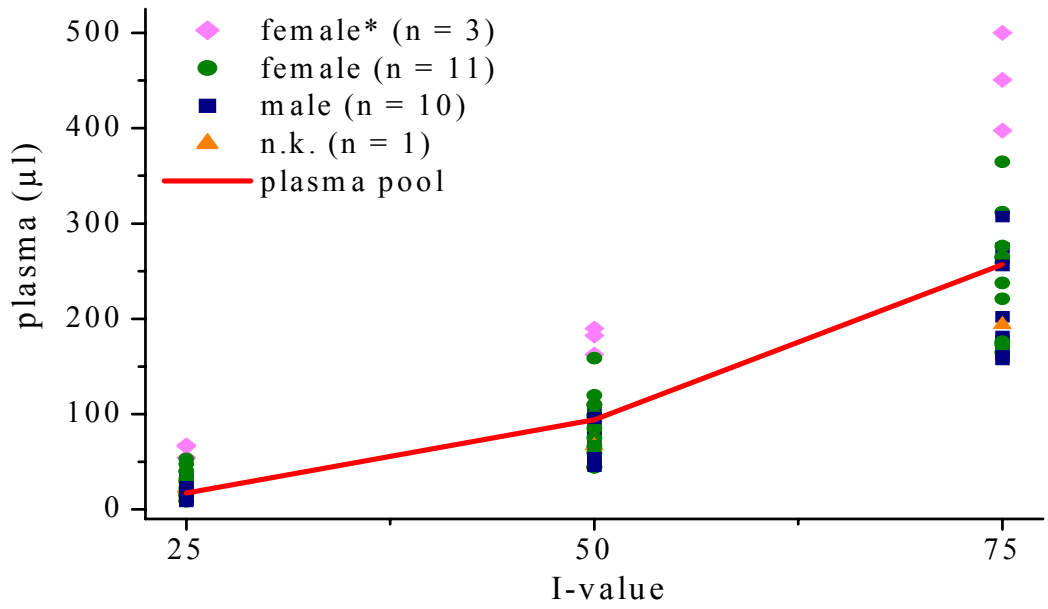


Fig. 27: Fenton assay, comparison of single-plasma samples (n = 25) vs. plasma pool, n.k. = gender not known, female* = menstruation

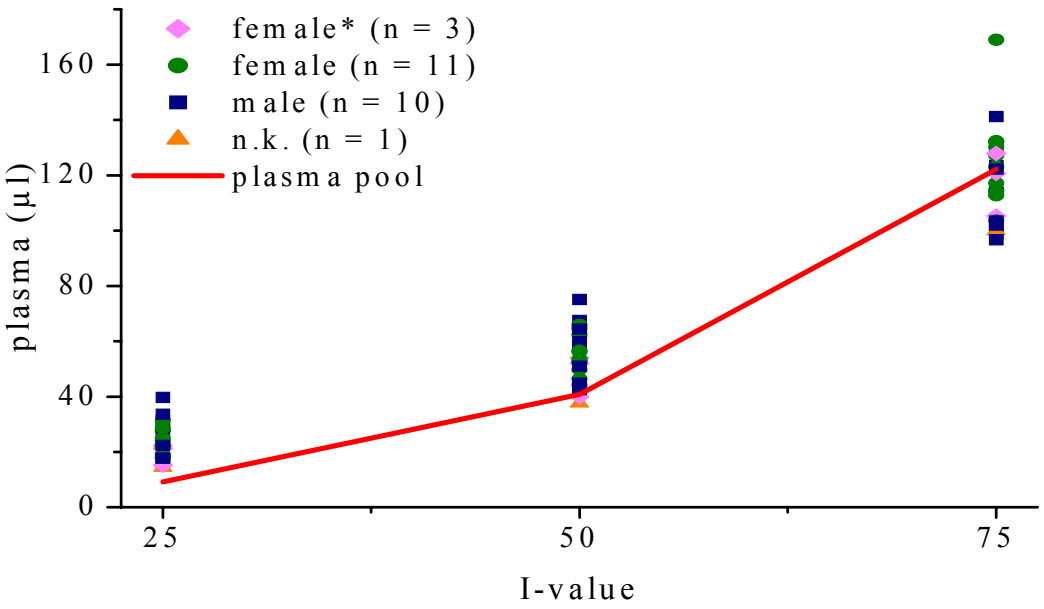


Fig. 28: Sin-1, comparison of single-plasma samples (n = 25) vs. plasma pool, n.k. = gender not known, female* = menstruation

The reasons to choose the above assays to compare the single plasma sample with the plasma pool were the same as mentioned in IV.1.2. As seen in all four figures (Fig. 25-28), the individuals exhibit a scatter around the plasma pool. This scatter reflected the different concentrations of lipophilic antioxidants in the individual volunteers (Tab. 02).

Tab. 02: Concentrations of lipophilic antioxidants in the plasma pool and single-plasma samples

Volunteer	α-Tocopherol (µM)	Lycopene (µM)	β-Carotene (µM)	Ubiquinon eox (µM)	Ubiquinone red (µM)
Pool/I	30.36	1.28	1.23	0.70	1.10
Pool/II	31.41	1.19	1.24	0.75	1.19
01/I	42.48	1.02	1.15	0.52	0.44
01/II	45.63	1.01	0.94	0.50	0.17
02/I	34.16	0.82	1.60	0.65	n.d.
02/II	35.75	0.90	1.73	0.72	n.d.
03/I*	23.33	0.85	2.08	0.36	1.23
03/II*	25.00	0.68	1.78	0.32	1.29
04/I	36.92	1.14	1.10	0.82	0.64
04/II	37.56	0.96	0.96	0.93	0.68
05/I	51.85	1.45	3.27	0.50	2.09
05/II	52.67	1.50	3.62	0.60	2.22
06/I	41.37	0.86	2.32	0.69	0.78
06/II	40.17	0.76	2.38	0.72	0.77
07/I*	34.03	0.81	0.66	0.76	0.63
07/II*	37.02	1.20	0.76	0.74	0.35
08/I	35.77	0.80	0.68	0.56	n.d.
08II	34.86	0.92	0.86	0.64	0.40
09/I	30.65	1.77	1.65	0.42	0.95
09/II	29.14	1.45	1.48	0.34	1.19
10/I	36.26	1.37	1.51	0.53	0.99
10/II	35.77	1.18	1.40	0.53	1.08
11/I	30.43	1.07	0.52	0.75	1.18
11/II	29.26	1.20	0.52	0.68	1.13
12/I	56.20	1.58	3.72	1.50	1.43
12/II	55.27	1.75	3.98	1.08	1.38
13/I*	33.24	1.60	0.27	0.88	0.87
13/II*	32.94	1.08	0.25	0.90	0.88
14/I	43.05	1.17	1.32	0.44	0.87
14/II	41.08	1.10	1.23	0.41	0.57
15/I	51.58	0.50	1.40	0.55	0.56
15/II	50.42	0.37	1.67	0.59	0.32
16/I	26.80	0.45	0.56	0.47	2.54
16/II	26.94	0.42	0.63	0.49	2.21
17/I	26.15	0.74	0.35	0.42	1.25
17/II	26.23	0.63	0.27	0.32	0.69
18/I	74.55	0.52	0.28	1.34	1.99
18/II	74.42	0.46	n.d.	0.72	0.32
19/I	74.39	0.37	2.23	0.71	12.06
19/II	78.55	0.48	2.59	0.76	13.99
20/I	29.67	1.19	0.86	0.88	1.00
20/II	27.54	0.39	0.33	0.95	0.77
21/I	50.50	1.00	0.91	1.30	0.21
21/II	55.78	1.44	0.85	0.77	0.28

22/I	50.85	1.26	1.45	1.90	1.26
22/II	49.54	1.53	1.42	1.30	1.47
23/I	42.45	1.51	0.90	2.01	2.13
23/II	41.70	1.73	1.03	2.15	3.34
24/I	34.04	0.71	0.55	1.39	0.63
24/II	38.21	1.04	0.40	0.90	0.40
25/I	29.89	1.66	1.61	1.08	3.47
25/II	29.51	2.03	2.11	1.17	3.94

n.d. = not detectable, female* = menstruation

The total protein content of all volunteers and the plasma pool was determined with the Coomassie Blue assay. There were no significant differences between the single-plasma samples (data not shown; mean values ~ 68.65 ± 5.09 mg/ml).

It is shown that gender has no influence on the antioxidant capacities of plasma when the ABTS (Fig. 22), ACC/HOCl (Fig. 23) and Sin-1 (Fig. 25) assays are used. Whereas in the Fenton assay (Fig. 24), female volunteers who had their menstruation at the time of the blood taking clearly differ from the other volunteers. The means and standard deviations of all single-plasma samples are shown in Tab. 03:

Tab. 03: Calculated means and standard deviations for the I-values of the single-plasma samples compared to the plasma pool in four assays

I-value	ABTS		ACC/HOCl		Sin-1		Fenton		
	pool (µl)	x±stdev (µl)	pool (µl)	x±stdev (µl)	pool (µl)	x±stdev (µl)	pool (µl)	x±stdev (µl)	x±stdev ∩F* (µl)
25	4	3.87±0.65	0.05	0.08±0.03	9	23.22±6.39	17	28.88±17.74	24.22±13.35
50	10	11.26±2.26	0.14	0.19±0.03	414	53.42±9.72	94	94.12±41.76	83.11±29.33
75	25	22.3±2.34	0.25	0.34±0.04	122	118.49±16.41	257	252.75±103.61	228.26±59.28

F* = female with menstruation at the time of blood taking; ∩ = excluding

The calculated means of the single-plasma samples are consistent with the values of the plasma pool for the ABTS, ACC/HOCl and Sin-1 assays (Tab. 03). At the Fenton assay the mean values scatter strongly when the data of the menstruating women are included. However, the means fit well with the plasma pool when the data of the menstruating women are excluded from the calculation.

IV.2. Antioxidant properties of different buckwheat extracts

The antioxidant capacities of different buckwheat extracts (see III.1.3.15.) were investigated using four different assays (Fenton, Sin-1, Rose Bengal and X/XOD) likewise with adding plasma (≡ I_{50}-value) to the assay. The flavonoid concentrations of the single extracts were determined via HPLC.

Tab. 04: Flavonoid concentrations and amounts, calculation based on 1 g extracting material

Ex-tract	Catechin		Epicatechin		Rutin		Quercitrin		Quercetin		Kaempferol	
	(µM)	(µg/g)	(µM)	(µg/g)	(µM)	(mg/g)	(µM)	(µg/g)	(µM)	(µg/g)	(µM)	(µg/g)
1 A	109.95	2216.6	12.85	259.05	1014.8	46.84	77.83	2425.7	0.23	5.4	0.63	12.52
1 B	1.99	4.01	10.79	217.52	3.43	0.158	0.07	2.18	n.d.	n.d.	n.d.	n.d.
1 C	3.12	6.29	15.59	314.29	3.24	0.149	0.19	5.92	n.d.	n.d.	n.d.	n.d.
2 A	315.65	1832.7	156.59	909.16	798.64	10.62	164.46	1474.9	0.61	4.13	2.52	14.43
2 B	9.79	56.84	2.5	14.52	1.87	0.045	0.66	5.92	0.89	6.02	0.14	0.8
2 C	23.29	135.22	22.88	132.84	2.89	0.038	4.38	39.28	0.32	2.17	n.d.	n.d.
3 A	158.34	689.49	99.06	431.36	1948.1	19.42	264.66	1780.1	49.01	248.68	0.25	1.07
3 B	n.d.	n.d.	7.9	34.4	11.63	0.116	0.64	4.31	n.d.	n.d.	n.d.	n.d.
3 C	16.89	73.55	11.67	50.82	6.58	0.066	0.27	1.82	n.d.	n.d.	n.d.	n.d.

n.d. = not detectable,

A = herb, B = whole kernels, C = kernels grounded

1 = aqueous extract, 2 = alkalic-ethanolic extract, 3 = ethanolic extract

The concentrations of the samples differ due to the extraction method used (Tab. 04). The alkalic-ethanolic extraction has the highest concentrations, with catechin and epicatechin, followed by the ethanolic and aqueous extraction. The ethanolic extraction reached the highest values for the flavonoids rutin and quercitrin, followed by the aqueous and alkalic-ethanolic extract. Amongst the samples, the herb possesses the highest concentrations of flavonoids. The sample 'kernels grounded' had higher values than the sample 'whole kernels'. This indicated that the compounds of the kernels are more accessible for extraction after the milling process.

IV.2.1. Fenton assay

The Fenton assay generates iron-catalytic OH-radicals with EDTA as an iron chelator to increase the ethylene production. The effect of the extracts in this assay is shown in the following figure.

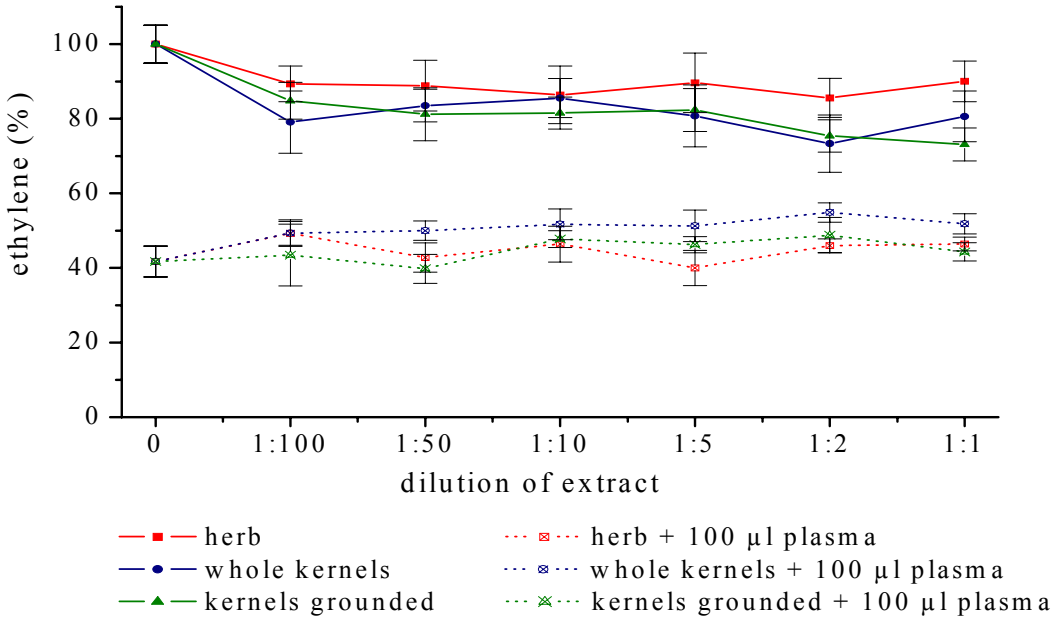


Fig. 29: Fenton assay, ethanolic extracts, n = 4

The ethanolic extract shows no influence in the Fenton assay as seen in Fig. 29. The addition of plasma (amount corresponds to the I_{50}-value, see IV.1.1.) causes an inhibition, which can be attributed to the antioxidant capacities of the plasma. The other extracts exhibited similar results in the Fenton assay, the graphs of these results are not shown.

IV.2.2. Sin-1

Sin-1 cleaves under physiological conditions in $O_2^{-\bullet}$ and NO to form ONOOH. The abilities of the extracts to scavenge $O_2^{-\bullet}$ or ONOOH are shown in the following figures. Additionally the supplementary effect of 40 µl plasma (≡ I_{50}-value) was investigated.

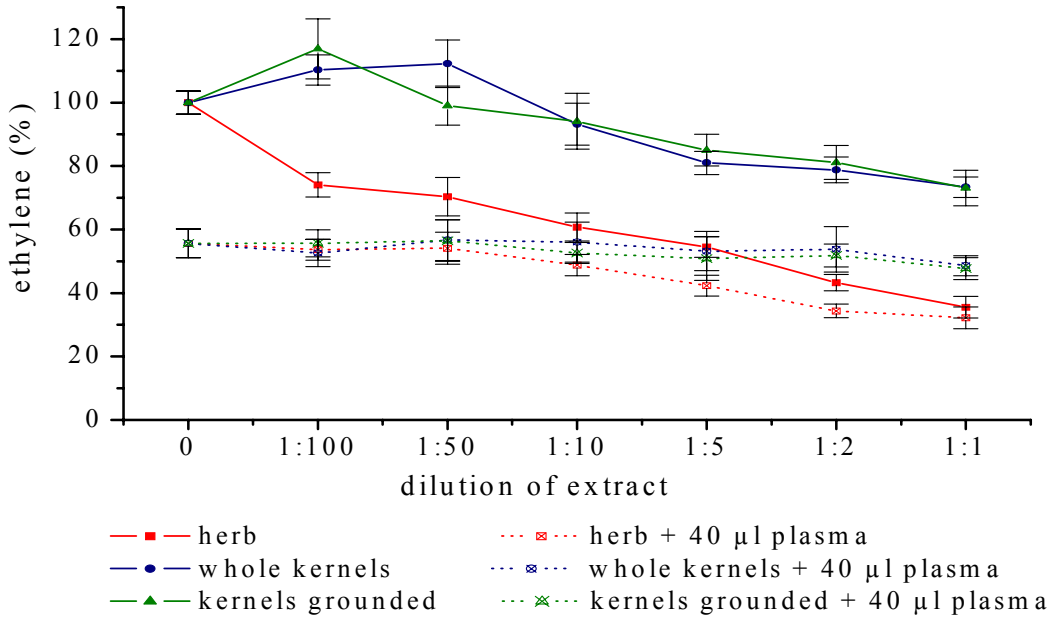


Fig. 30: Sin-1, aqueous extracts, n = 4

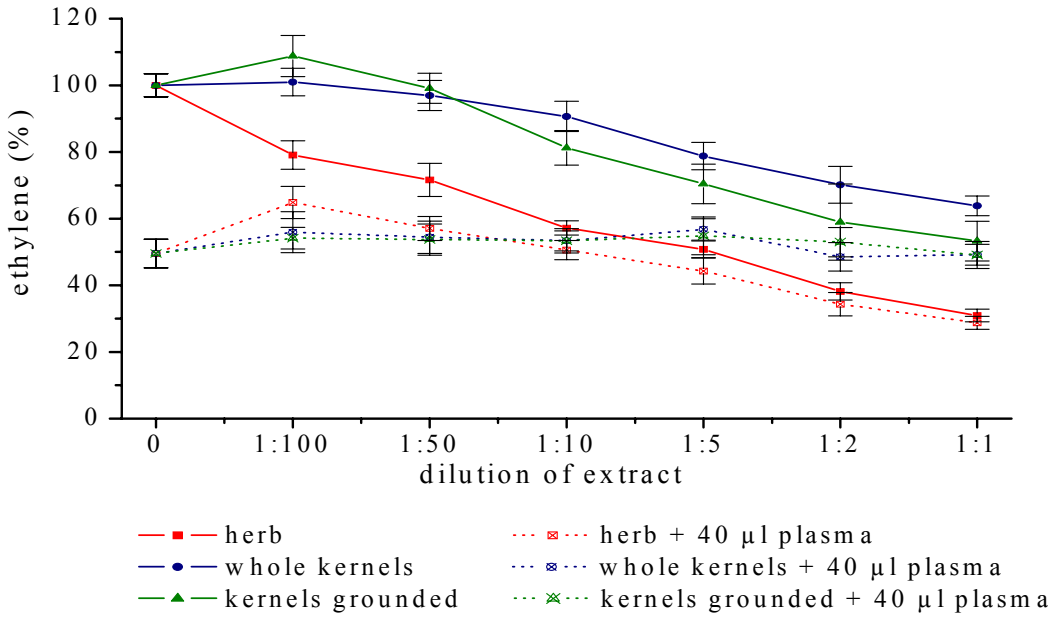


Fig. 31: Sin-1, alkalic-ethanolic extracts, n = 4

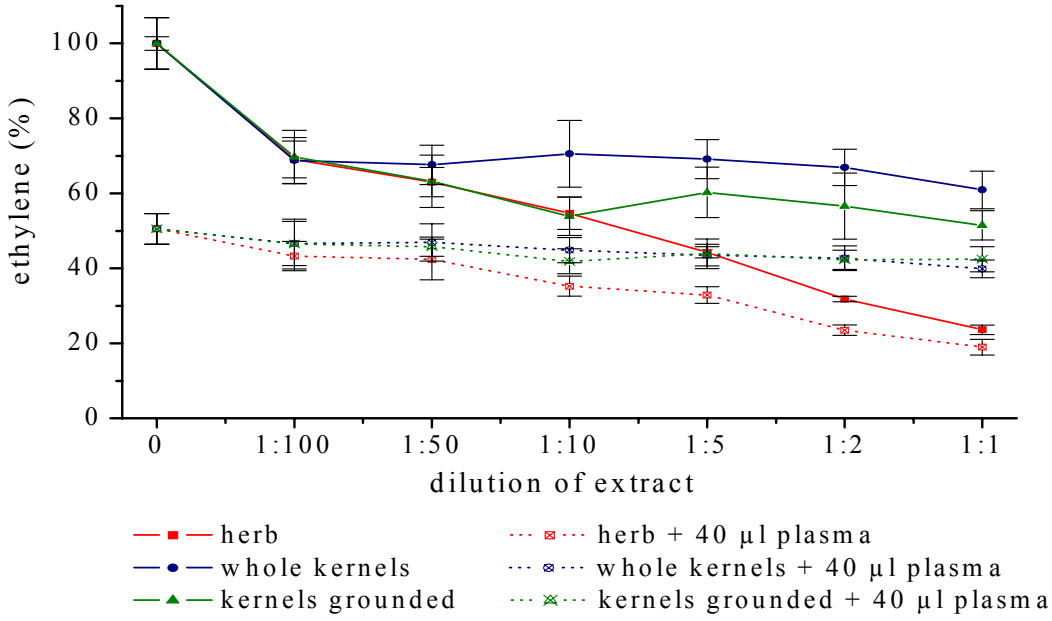


Fig. 32: Sin-1, ethanolic extracts, n = 4

Fig. 30-32 show that the 'herb' sample exhibited the best efficiency with all three extraction methods in the Sin-1 assay. Even the addition of plasma (corresponding to the I_{50}-value, see IV.1.1.) did minimize the positive effect of the 'herb' only slightly. The sample 'kernels grounded' was more effective than the sample 'whole kernels', but this effect was nearly cancelled with the addition of plasma. In the Sin-1 assay there was the following order of efficiency for the extraction methods: ethanolic, aqueous, and alkalic-ethanolic.

IV.2.3. X/XOD assay

The effect of the extracts in the X/XOD assay, an *in vitro* assay mimicking ischaemia/reperfusion is shown in the following figures. Three distinct plasma pools had to be used as supplement (≡ I_{50}-value), which resulted in varying ethylene yields.

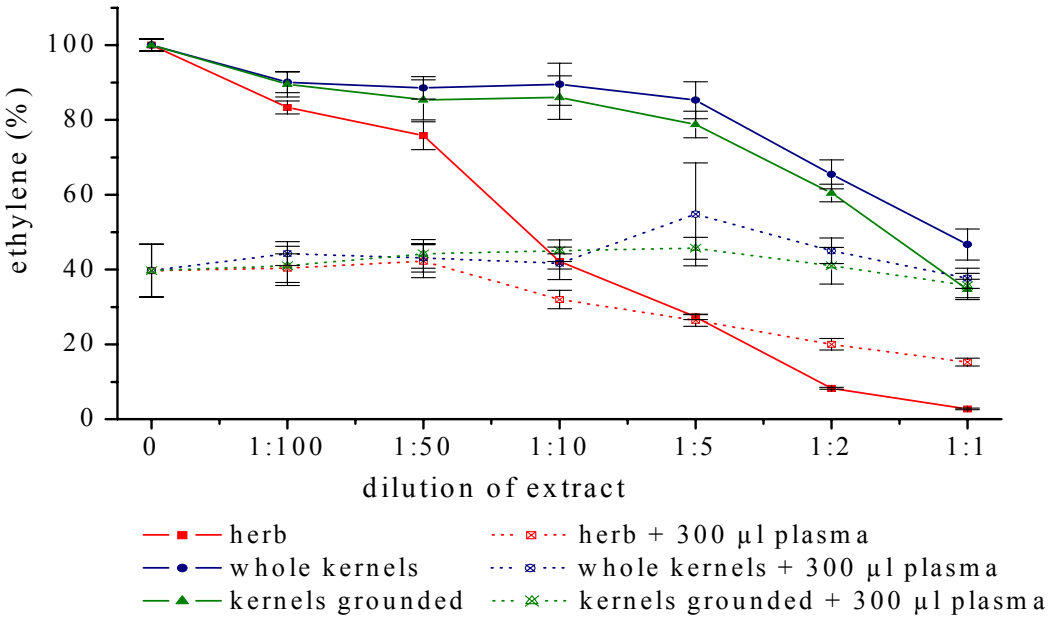


Fig. 33: X/XOD assay, aqueous extracts, n = 4

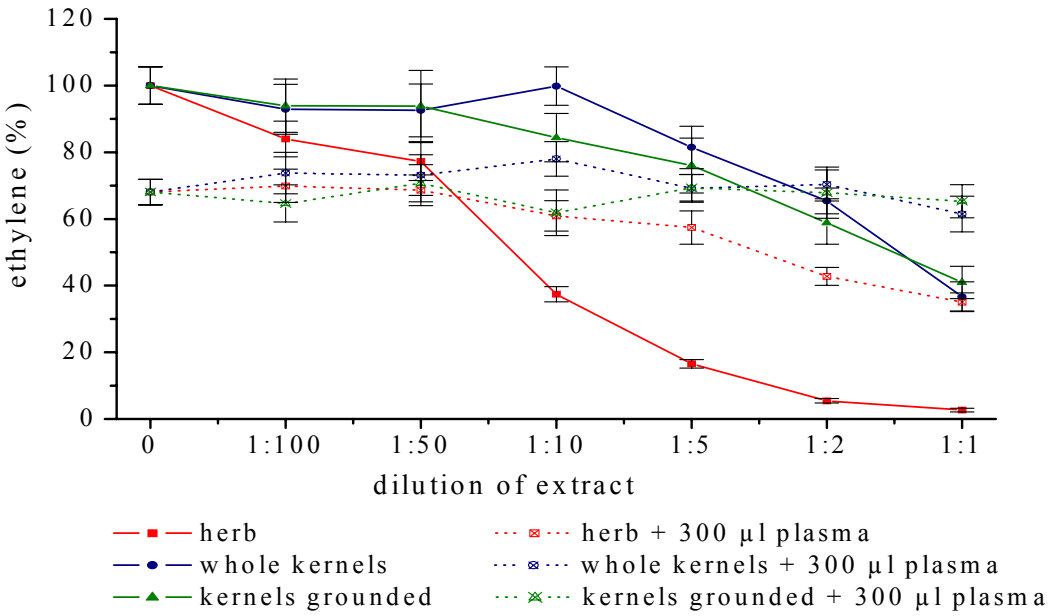


Fig. 34: X/XOD assay, alkalic-ethanolic extracts, n = 4

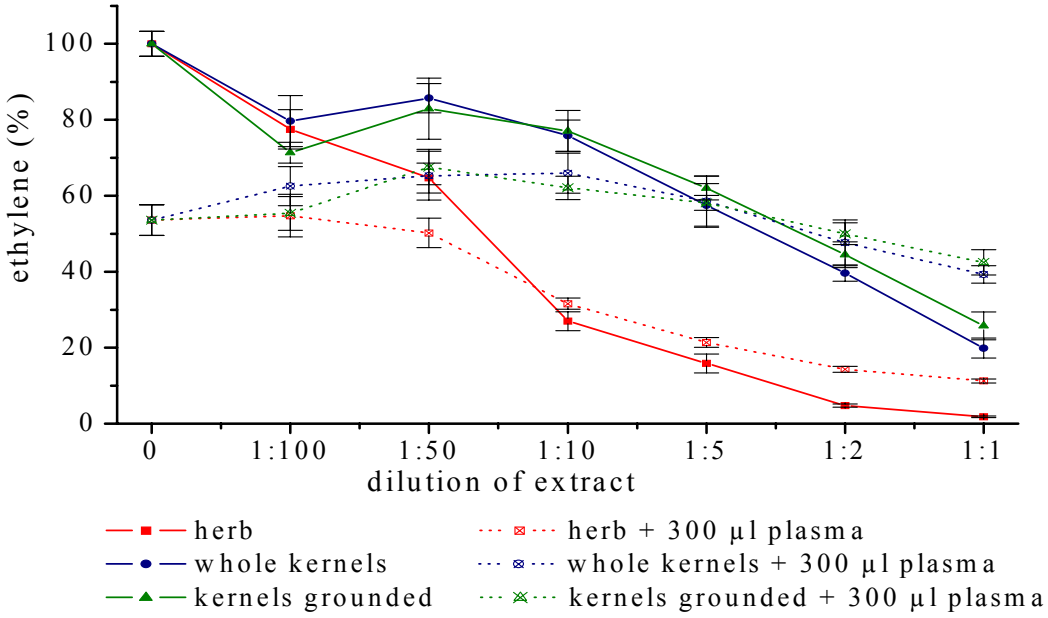


Fig. 35: X/XOD assay, ethanolic extract, n = 4

Fig. 33-35 show the 'herb' sample exhibiting the best efficiency in the X/XOD assay (as it did in the Sin-1 assay (IV.2.2.)). Nevertheless, adding plasma (corresponding to the I_{50}-value) minimized the positive effect of the aqueous and alkalic-ethanolic extract considerably. The ethanolic extract generally showed a better efficacy while there are nearly no differences between the aqueous and alkalic-ethanolic extract.

IV.2.4. Rose Bengal assay

The Rose Bengal assay was used to investigate the inhibitory properties of the extracts in the lipid peroxidation. The results are shown in the following figures.

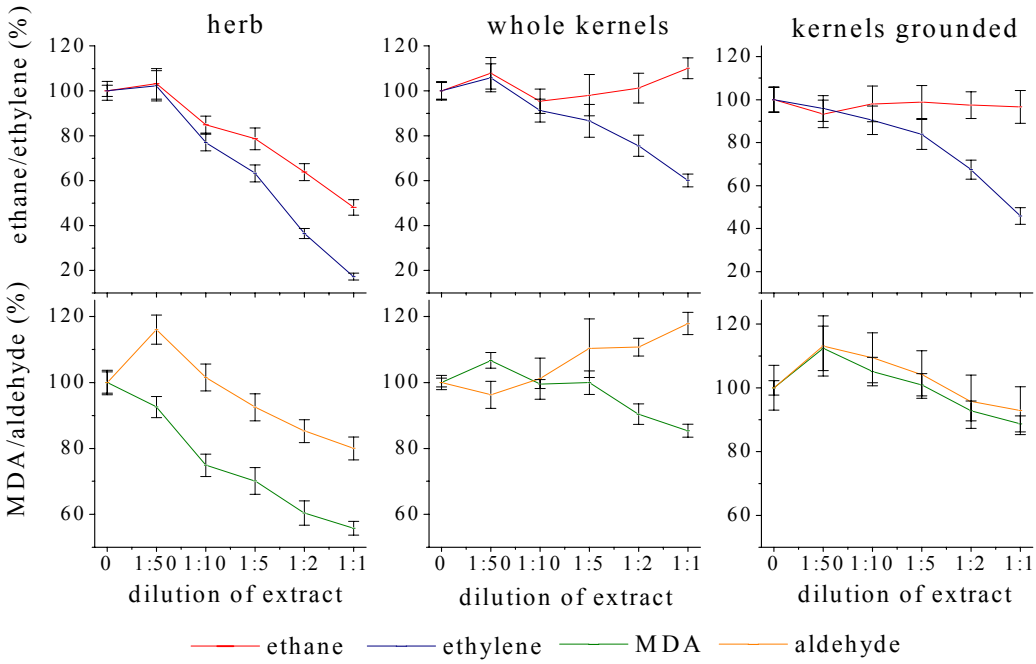


Fig. 36: Rose Bengal assay, aqueous extracts, n = 4

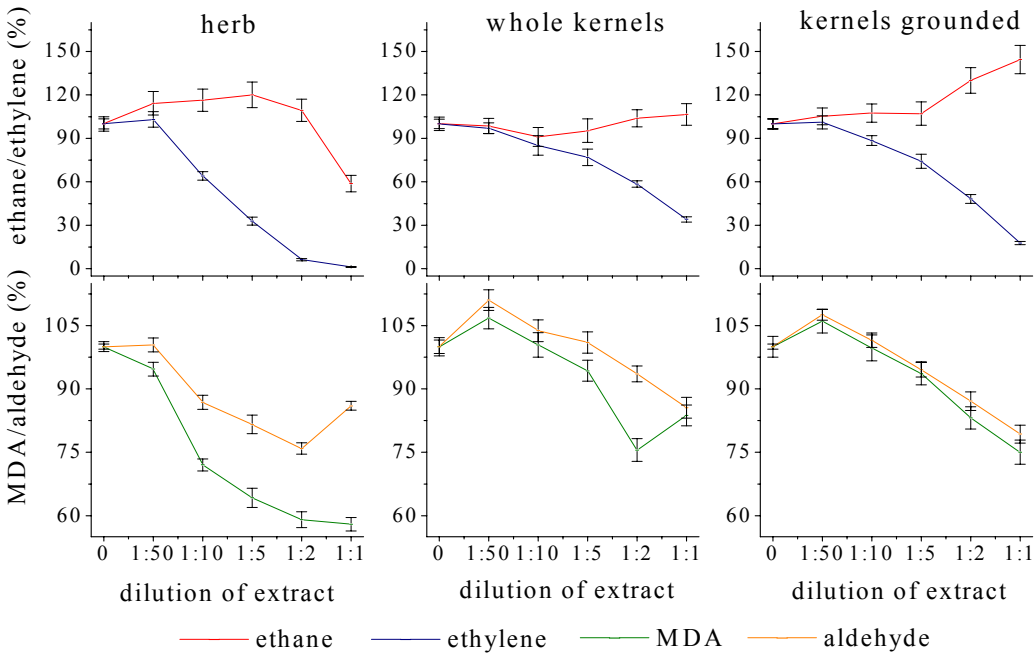


Fig. 37: Rose Bengal assay, alkalic-ethanolic extracts, n = 4

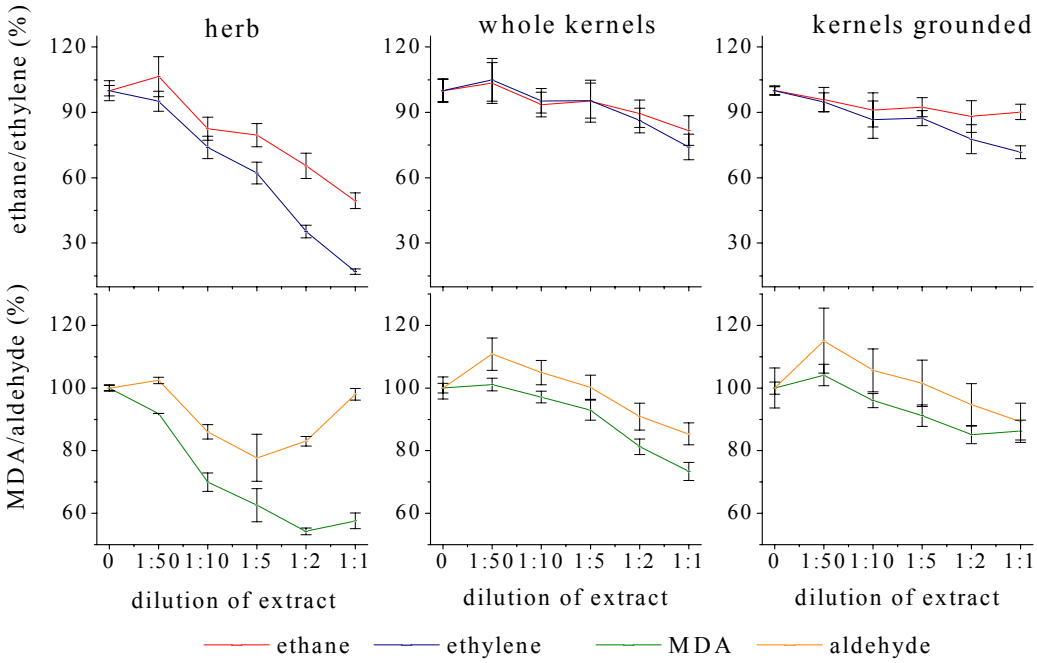


Fig. 38: Rose Bengal assay, ethanolic extracts, n = 4

Fig. 36-38 show the 'herb' extract having the best efficiency in the Rose Bengal assay. Apart from copper-chelating abilities, the herb also exhibited an antioxidant capacity. The 'kernel' samples show mainly copper-chelating abilities. Both the ethanolic extract and the aqueous extract had the same properties in this assay. The results of the 1:1 dilution at the MDA/aldehyde of the 'herb' sample as alkalic-ethanolic extract and ethanolic extract (Fig. 37 and 38) were due to the coloured extracts that caused a higher extinction.

IV.3. Fractionation of the buckwheat herb

From the buckwheat herb 7.27 g ethyl acetate extract was obtained as starting material. The fractionation resulted in 18 fractions (A-R), which were obtained from 2.3 g of the ethyl acetate extract.

Tab. 05: Amounts of the lyophilised starting material ethyl acetate extract (EtOAc extract) and the fractions (obtained from 2.3 g EtOAc extract)

Fraction	Amount (g)	Fraction	Amount (mg)	Fraction	Amount (mg)
EtOAc	7.27				
A	0.92	**G**	9.07	**M**	0.77
B	0.15	**H**	12.44	**N**	3.01
C	0.17	**I**	16.35	**O**	1.28
D	0.24	**J**	15.60	**P**	7.94
E	0.64	**K**	12.28	**Q**	19.22
F	0.032	**L**	18.24	**R**	6.70

The evaluation of the fractions was performed using HPLC at the Fachgebiet für Obstbau. The method was established by LUX-ENDRICH (1998) with detection at 280 nm and a derivatization with DMACA to detect flavan-3-ols at a wavelength of 720 nm. The retention times for the derivatization shift by about 2-3 min compared to the retention times at $\lambda = 280$ nm. This is considered in the given retention times, which were shown for the wavelength of 280 nm. For samples which are also detected at the wavelength of 720 nm the HPLC-areas for this wavelength were given in brackets. Peak areas under 5 mAU were not considered for tabulating and the calculations of the amounts because the error of the integration of these small areas was considered too high to give reproducible results. All phenolic acids were calculated with the chlorogenic acid standard. Unknown substances, detectable at $\lambda = 720$ nm, were calculated with the B2 standard. The unknown flavonol was calculated as rutin. The numbers (1-29) indicate the identical substances in different fractions. The identification of the substances was rendered more difficult as for many of the detected peaks no standards exist and due to technical problems with the HPLC, several of the spectra files were corrupted. A definite classification of some substances as flavan-3-ols was therefore not possible and the likewise possible reaction of DMACA with indols aggravates the problem.

Tab. 06: Results of the HPLC run of Fraction A, HPLC areas under 5 mAU are not considered for tabulating

Fraction A

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
6.50	7.33			Unknown
6.97	12.02			Unknown
9.97	5.31			Unknown
22.61	6.60			Unknown
119.67	363.18	256/353	121.72	**Rutin**
134.16	5.44	261/351	1.7	**Quercitrin**

Tab. 07: Results of the HPLC run of Fraction B, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction B

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
34.4	211.28	255/325	7.28	**Phenolic acid** (1)
115.89	184.59	256/353	10.09	**Rutin**

Tab. 08: Results of the HPLC run of Fraction C, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction C

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
7.88	44.18	265		Unknown (2)
9.89	22.08			Unknown
12.47	16.84	285/293		Unknown (3)
13.35	5.31			Unknown
14.68	287.32	324	11.22	**Phenolic acid** (4)
19.25	17.83	310	0.696	Phenolic acid
22.87	18.40	284		Unknown (5)
26.15	51.74	325	2.02	Phenolic acid
27.47	20.98	321	0.819	**Phenolic acid** (6)

33.38	59.84	255/324	2.34	**Phenolic acid** (1)
36.57	12.17			Unknown
48.78	12.78			Unknown
115.02	427.07	256/352	26.45	**Rutin**
136.49	6.13	246/343	0.380	**Flavonol** (7)

Tab. 09: Results of the HPLC run of Fraction D, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction D

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
7.88	43.03	265		Unknown (2)
11.80	112.96	258/293		Unknown (3)
15.55	7.23	324	0.398	**Phenolic acid** (4)
17.08	5.90			Unknown
22.13	20.22	284		Unknown (5)
27.47	22.33	321	1.23	**Phenolic acid** (6)
32.21	9.41			Unknown
33.79	188.37	255/324	10.38	**Phenolic acid** (1)
41.31	38.35	308		Unknown
59.51	12.40			Unknown
78.05	16.87			Unknown
91.11	7.78			Unknown (8)
112.33	17.91			Unknown (9)
115.60	658.49	256/352	57.57	**Rutin**
130.32	32.96	261/351	2.69	**Quercitrin**
137.07	24.42	246/343	2.14	**Flavonol** (7)
139.74	5.83			Unknown
151.21	8.29			Unknown
172.41	13.63	315		Unknown

Tab. 10: Results of the HPLC run of Fraction E, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction E

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
20.18	4.35 (10.29)	280	1.55	**Catechin ***
36.17	24.00 (72.21)	279	2.55	**Epicatechin ***
91.50	5.45			Unknown (8)
107.68	8.31			Unknown
112.51	26.95			Unknown (9)
115.43	1182.03	256/352	275.59	**Rutin**
129.70	639.33	261/351	138.94	**Quercitrin**
137.23	19.73	246/343	4.60	**Flavonol** (7)
146.82	7.56	256/369	1.15	**Quercetin**
149.30	8.14			Unknown

Tab. 11: Results of the HPLC run of Fraction F, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction F

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
18.67	11.60			Unknown
30.65	57.17 (67.37)	278	1.71	**B2** *
56.99	8.97			Unknown
71.53	5.44			Unknown
97.30	8.30			Unknown
113.75	24.94			Unknown (9)
115.86	1050.62	256/352	12.25	**Rutin**
130.79	25.58	261/351	0.278	**Quercitrin**
146.78	57.86	256/369	0.440	**Quercetin**

Tab. 12: Results of the HPLC run of Fraction G, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction G

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
21.30	4.80 (5.62)		40.41	Unknown *
23.33	11.13 (11.15)	278	80.18	Unknown (10) *
32.11	114.33 (129.00)	278	927.59	**B2** *
61.54	163.47 (105.52)	276	758.76	Unknown (11) *
122.92	15.98			Unknown
170.72	7.02			Unknown

Tab. 13: Results of the HPLC run of Fraction H, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction H

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
17.66	3.22 (5.20)		51.28	Unknown *
24.05	55.62 (64.15)	278	632.67	Unknown (10) *
32.97	2.48 (13.42)	278	132.35	**B2** *
33.84	11.30	279		Unknown
44.83	6.15		50.00	**C1**
48.13	5.99 (7.69)		75.84	Unknown *
61.29	57.20 (40.80)	276	402.38	Unknown (11) *
95.80	4.12 (7.17)		70.71	**B5** *
107.98	9.72			Unknown (12)
169.25	6.47			Unknown
170.17	11.77			Unknown (13)

Tab. 14: Results of the HPLC run of Fraction I, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction I

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
7.91	6.24			Unknown (14)
12.84	6.22			Unknown (15)
13.57	22.20			Unknown (16)
18.34	7.70			Unknown
22.16	13.22 (9.34)		121.07	Unknown *
29.91	19.74 (23.73)		307.59	Unknown *
31.20	10.29			Unknown
32.46	29.17 (26.63)	278	345.18	**B2** *
35.73	1.55 (11.00)		142.58	Unknown *
38.84	8.85			Unknown
39.42	12.73 (7.07)		91.64	Unknown *
41.11	32.11 (26.03)		337.41	Unknown (17) *
43.75	391.42 (363.95)		4853.26	**C1** *
45.53	9.03			Unknown
50.24	16.69 (6.25)		81.01	Unknown *
54.21	5.54			Unknown
56.67	13.25 (11.64)		150.88	Unknown *
70.05	5.48			Unknown
76.06	7.77			Unknown
86.16	5.60			Unknown
89.38	8.75 (5.39)		69.87	Unknown *
92.85	141.45 (248.76)		3224.47	Unknown *
106.17	26.20 (9.79)		126.90	Unknown (13) *
110.66	18.11 (37.81)		490.10	Unknown *
115.23	6.67			Unknown (18)
116.16	7.59			Unknown
155.71	5.77			Unknown
168.60	5.86			Unknown
170.14	6.41			Unknown (12)
172.26	27.82			Unknown
183.88	6.01			Unknown

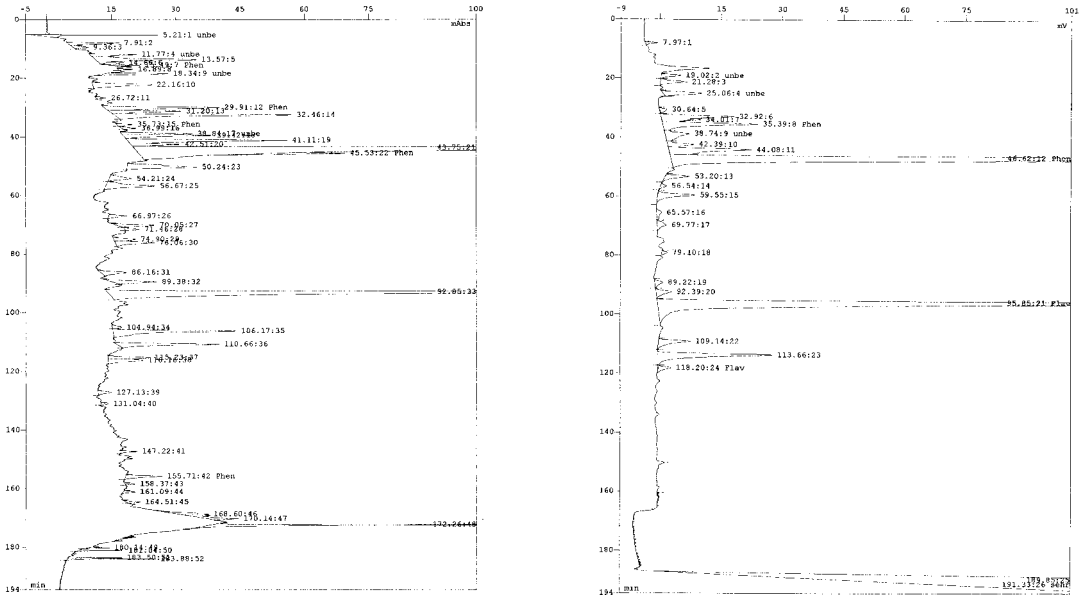


Fig. 39: Chromatograms of fraction I at λ = 280 nm (left) and with the derivatization of DMACA at λ = 720 nm (right)

Tab. 15: Results of the HPLC run of fraction J, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction J

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
7.96	5.20			Unknown (14)
11.88	5.28			Unknown (15)
13.38	5.86 (5.57)		68.89	Unknown (16) *
15.51	5.31			Unknown (19)
40.95	92.25 (46.04)		569.40	Unknown (17) *
48.44	14.02			Unknown
115.79	5.73 (11.32)		140.00	Unknown (18) *
178.06	9.52			Unknown
180.63	32.27			Unknown

Tab. 16: Results of the HPLC run of fraction K, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction K

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
8.02	5.02			Unknown (14)
11.94	5.23			Unknown (15)
13.40	18.43 (14.89)		144.96	Unknown (16) *
15.51	10.33			Unknown (19)
17.16	5.52			Unknown
20.29	6.59			Unknown
21.75	11.13			Unknown
26.42	31.00 (31.80)		309.59	Unknown *
40.48	5.83			Unknown (19)
47.44	45.58 (30.34)		295.38	Unknown *
55.84	6.63 (8.03)		78.18	Unknown *
76.81	30.89 (11.00)		107.09	Unknown *
122.41	9.41 (8.90)		86.65	Unknown *
136.43	8.54 (7.60)		73.99	Unknown *
169.30	17.48			Unknown
171.70	9.81			Unknown

Tab. 17: Results of the HPLC run of Fraction L, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction L

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
11.90	5.29			Unknown (15)
50.56	6.93			Unknown
73.66	5.33			Unknown

Tab. 18: Results of the HPLC run of Fraction M, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction M

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
8.02	8.16			Unknown (20)
11.95	7.50			Unknown (15)
15.59	5.74			Unknown (21)
30.40	17.06 (20.23)		12.35	Unknown (22) *
31.96	9.79			Unknown (23)
36.11	13.81 (10.49)		6.40	Unknown (224) *
53.19	3.36 (7.63)		4.66	Unknown *
176.54	5.78			Unknown (25)

Tab. 19: Results of the HPLC run of Fraction N, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction N

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
8.05	7.17			Unknown (20)
12.04	7.07			Unknown (15)
15.67	7.84			Unknown (21)
30.53	8.84 (7.90)		18.85	Unknown (22) *
32.13	6.43 (5.87)		14.01	Unknown (23) *
36.38	9.83 (7.19)		17.16	Unknown (24) *
50.96	13.34			Unknown (26)
126.61	12.14 (4.57)			Unknown (27) *
176.52	6.54			Unknown (25)

Tab. 20: Results of the HPLC run of Fraction O, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Fraction O

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
30.31	9.54 (9.22)		9.36	Unknown (22) *
31.89	6.29 (6.75)		6.85	Unknown (23) *
36.07	12.39 (8.00)		8.12	Unknown (24) *
50.57	10.38			Unknown (26)
123.42	5.48			Unknown
126.13	30.92 (12.45)		12.63	Unknown (27) *
176.53	9.30			Unknown (25)
183.93	5.94			Unknown

Tab. 21: Results of the HPLC run of Fraction P, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction P

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
125.68	9.47			Unknown (27)

Tab. 22: Results of the HPLC run of Fraction Q, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction Q

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Concentration (mM)	Substance
11.95	5.06			Unknown
15.56	5.84			Unknown (28)
17.25	5.38			Unknown
50.32	10.92			Unknown
180.02	5.05			Unknown
183.81	11,92			Unknown (29)

Tab. 23: Results of the HPLC run of Fraction R, HPLC areas under 5 mAU are not considered for tabulating, numbers in brackets indicate identical substances in different fractions

Fraction R

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (µg)	Substance
15.55	5.82			Unknown (28)
182.78	24.29			Unknown (29)

Tab. 24: Results of the HPLC run of ethyl acetat extract, numbers in the brackets indicate identical substances as in the fractions, samples detected at λ = 720 nm are marked with * and the corresponding areas are given in brackets

Ethyl acetate extract

Retention time (min)	HPLC-areas (mAU)	λ_{max} (nm)	Amount (mg)	Substance
6.70	10.08			Unknown
8.05	9.23			Unknown
12.03	9.97			Unknown
14.86	48.99	324	25.87	**Phenolic acid** (4)
21.77	5.21 (1.04)	280	0.563	**Catechin** *
27.20	13.26		7.00	**Phenolic acid** (6)
31.53	3.21 (4.54)	278	8.28	**B2** *
34.77	47.59	255/325	25.13	**Phenolic acid** (1)
37.69	4.08 (14.99)	280	5.07	**Epicatechin** *
46.36	1.47 (1.27)		2.38	**C1** *
118.95	13.98			Unknown (9)
122.13	1356.70	256/353	1136.76	**Rutin**
136.47	185.82	255/346	145.17	**Quercitrin**
142.68	21.43	246/343	17.96	**Flavonol** (7)
152.17	6.56			Unknown

IV.4. Antioxidant properties of the fractions

Due to the low amounts of the fractions M-R (see IV.3., Tab. 05) only the fractions A-L and the ethyl acetate extract (EtOAc-extract) were tested in four test systems (Fenton, Sin-1, Rose Bengal and X/XOD). For a better chart layout, the results for the Sin-1 and X/XOD assays are shown as I_{50}-values. The I_{50}-value corresponds to the amount of sample that inhibits the basic reaction (= 100 %) about 50 %.

IV.4.1. Fenton assay

The fractions A-L as well as the EtOAc-extract exhibit the same effects in the Fenton assay as seen in Fig. 29 (IV.2.1.). Neither the EtOAc-extract nor the fractions had antioxidant capacities in this assay, therefore no graphics are shown of the results.

IV.4.2. Sin-1

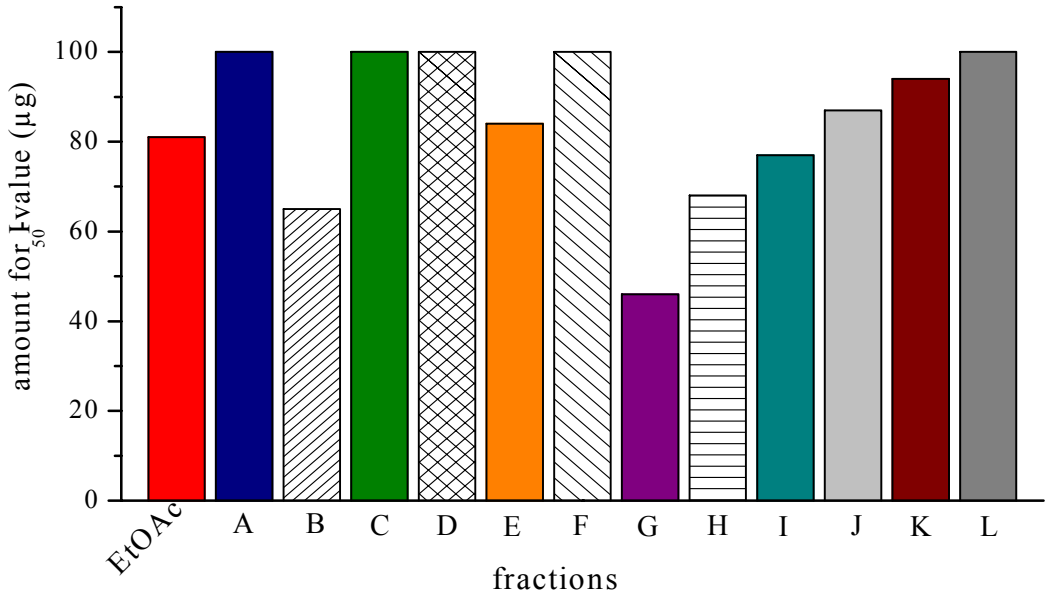


Fig. 40: Sin-1, I_{50}-values of the EtOAc-extract and the fractions A-L

The following order of efficiency of the fractions can be established in the Sin-1 assay (Fig. 40): G > B > H > I > E > J > K > A = C = D = F = L, the EtOAc-extract with a value of ~ 80 µg ranges between fraction I and E.

IV.4.3. X/XOD assay

The X/XOD assay mimics ischaemia/reperfusion *in vitro*. The properties of the EtOAc-extract and the fractions A-L in the X/XOD assay are shown in Fig. 41.

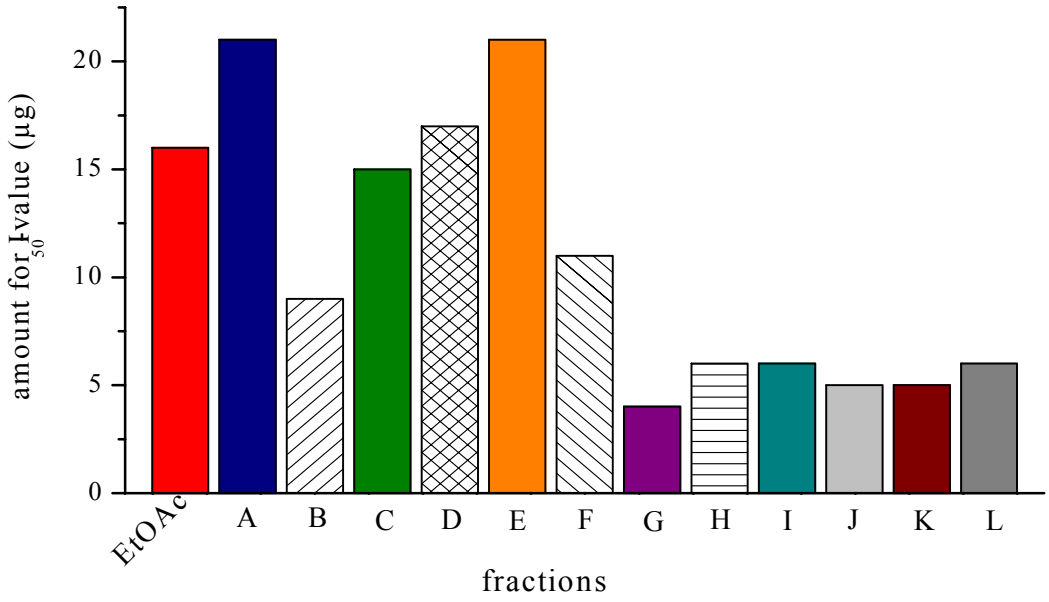


Fig. 41: X/XOD assay, I_{50}-value of the EtOAc-extract and the fractions A-L

In the X/XOD assay the fractions G-L exhibited the best efficacy (Fig. 41). The order of efficacy of all fractions was as follows: G > J = K > H = I = K > B > F > C > D > A = E, the EtOAc-extract ranges with ~ 16 µg for the I_{50}-value between the fractions C and D.

IV.4.4. Rose Bengal assay

The inhibitory properties of the EtOAc-extract and the fractions A-L in the lipidperoxidation were investigated in the Rose Bengal assay. The results are shown in the following figures.

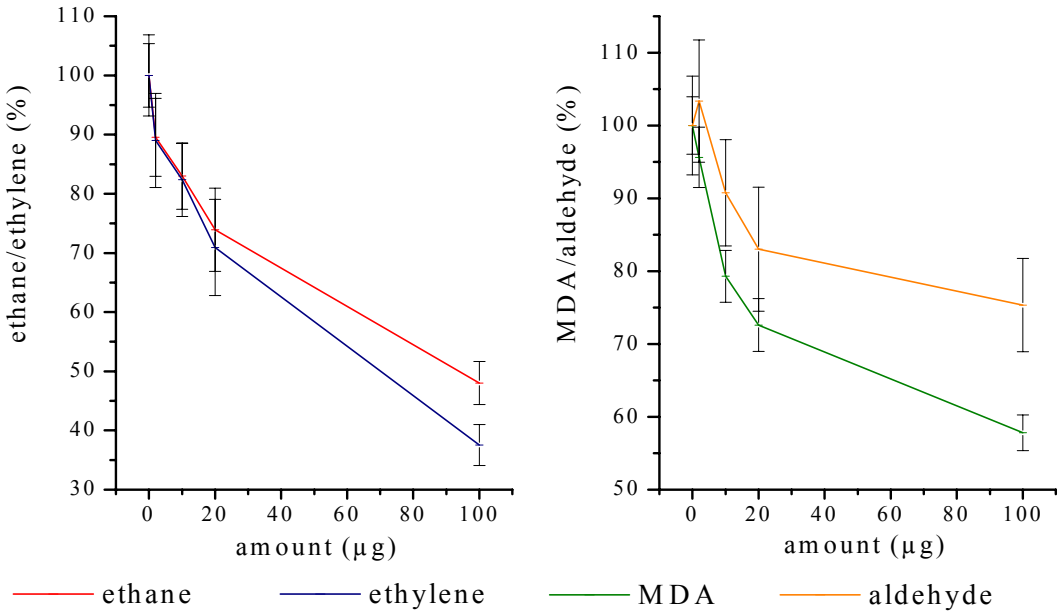


Fig. 42: Rose Bengal assay, EtOAc-extract, n = 4

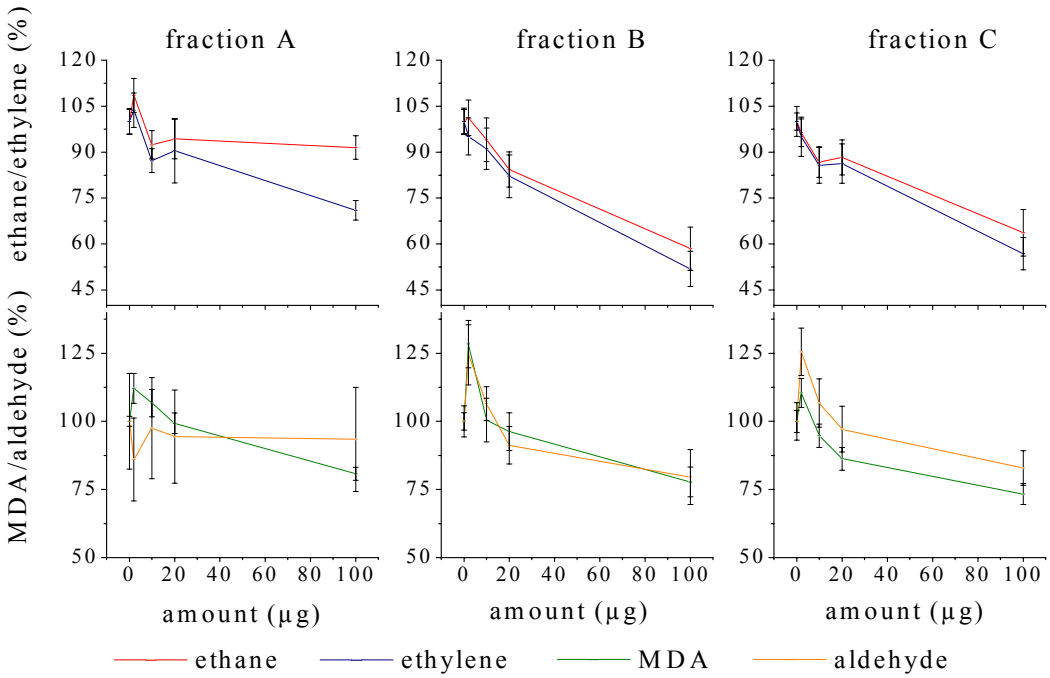


Fig. 43: Rose Bengal assay, fractions A-C, n = 4

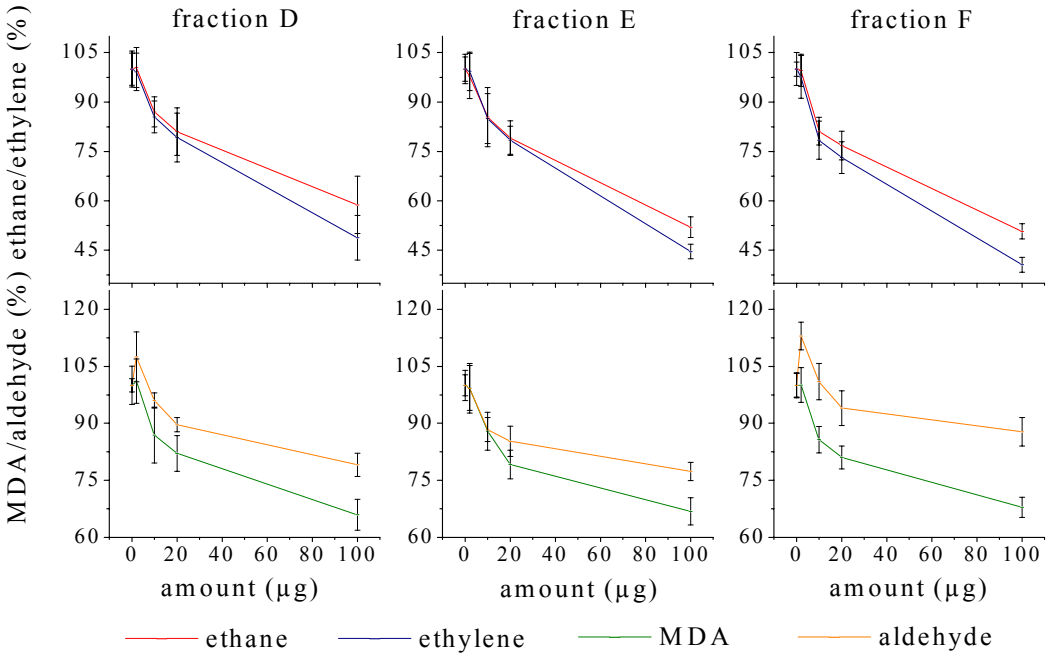


Fig. 44: Rose Bengal assay, fractions D-F, n = 4

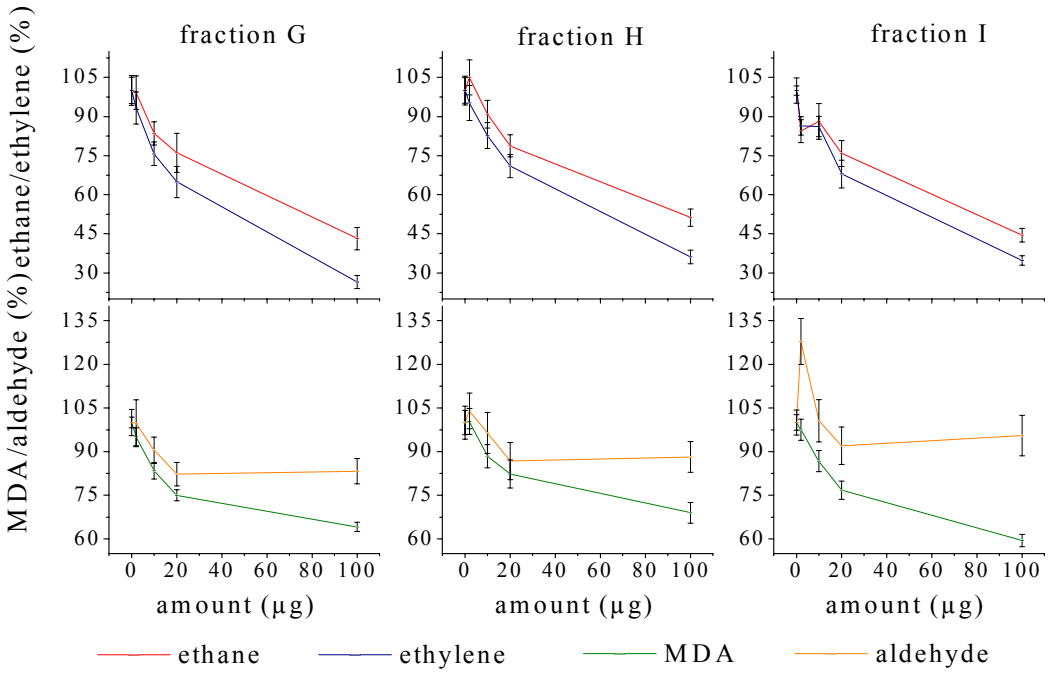


Fig. 45: Rose Bengal assay, fractions G-I, n = 4

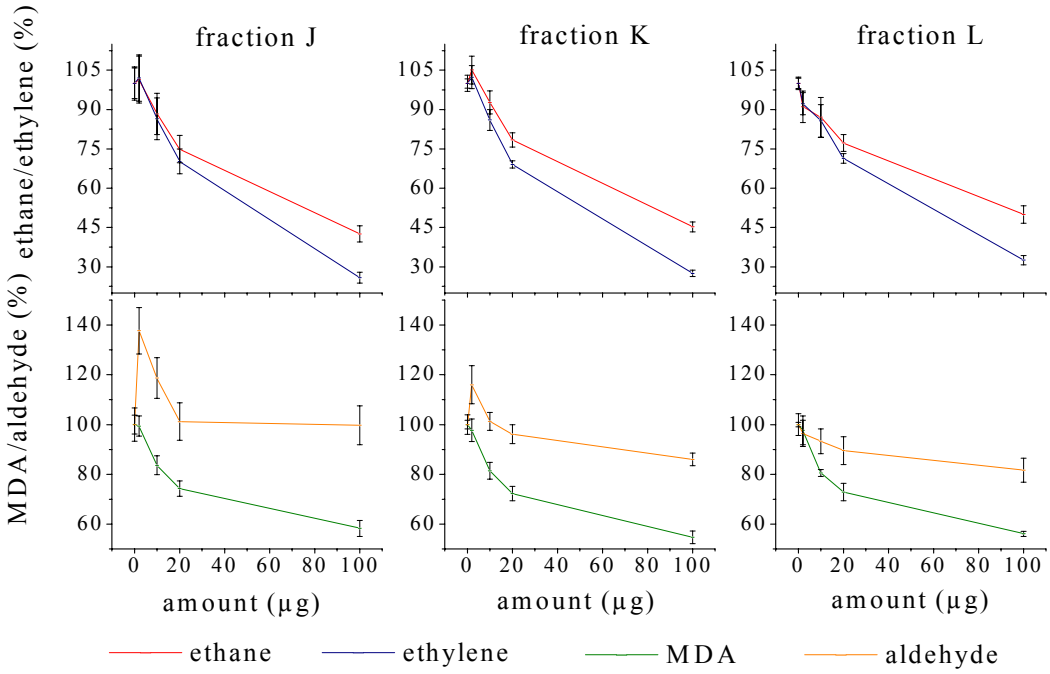


Fig. 46: Rose Bengal assay, fractions J-K, n = 4

Fig. 42-46 show, the EtOAc-extract, as well as the fractions A-L, had an effect in the Rose Bengal assay. The efficiency of the fractions increases starting from fraction A. Due to the decreasing ethane and ethylene production the fractions possessed either an antioxidant capacity and/or copper-chelating ability.

IV.5. *In vivo* supplementation with buckwheat tea and Pycnogenol

This experiment shows that supplementation of a volunteer with buckwheat tea or Pycnogenol enhances the antioxidant properties of the plasma. A 28 year-old healthy female volunteer was supplemented with buckwheat tea for a period of 14 d. After a reasonable washing-out period, the same volunteer was supplemented with Pycnogenol for a period of 14 d. Almased® powder used as diet during the supplementation period is a protein drink, which supplies its user with all essential minerals and vitamins. The experimental data are shown in Tab. 25.

Tab. 25: Experimental data of the *in vivo* supplementation

Supplement	Pharmaceutical form	Dose	Period	Diet
Buckwheat herb	Tea	3 x daily 1.8 g herb brewed in 125 ml water; brewing time 10 min	14 d	Almased®
Pycnogenol	Capsule	2 x daily 49.4 mg ± 2.74 mg	14 d	Almased®
Blood was taken before and after the supplementation				

The plasma samples were analyzed in the following assays: X/XOD, NADH/Diaphorase, Sin-1, Fenton and peroxynitrite (Fig. 47).

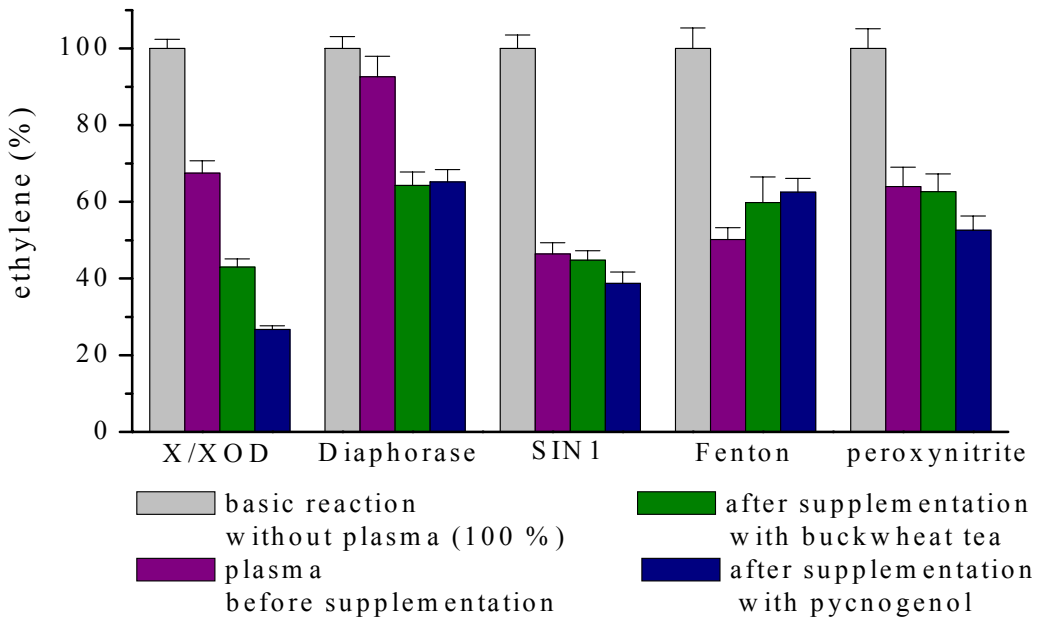


Fig. 47: Plasma samples of the *in vivo* supplementation, n = 4

Fig. 47 shows, the *in vivo* supplementation enhanced the plasma in both cases when investigated in the X/XOD and NADH/Diaphorase assay, whereas in the Sin-1 and peroxynitrite only the Pycnogenol supplementation exhibits an effect. In the Fenton assay, an increase in the ethylene production was registered due to the supplementation. The plasma was tested in amounts corresponding to the I_{50}-value of the plasma pool in these assays (X/XOD: 300 µl; NADH/Diaphorase: 100 µl; Sin-1: 40 µl; Fenton: 100 µl; peroxynitrite: 100 µl).

IV.6. Copper-induced LDL oxidation with quercetin metabolites

Five quercetin metabolites, which are detectable in human plasma after a quercetin rich meal as well as quercetin, isoquercitrin and catechin were tested in the copper-induced LDL oxidation (see III.2.4.) to investigate their protective abilities.

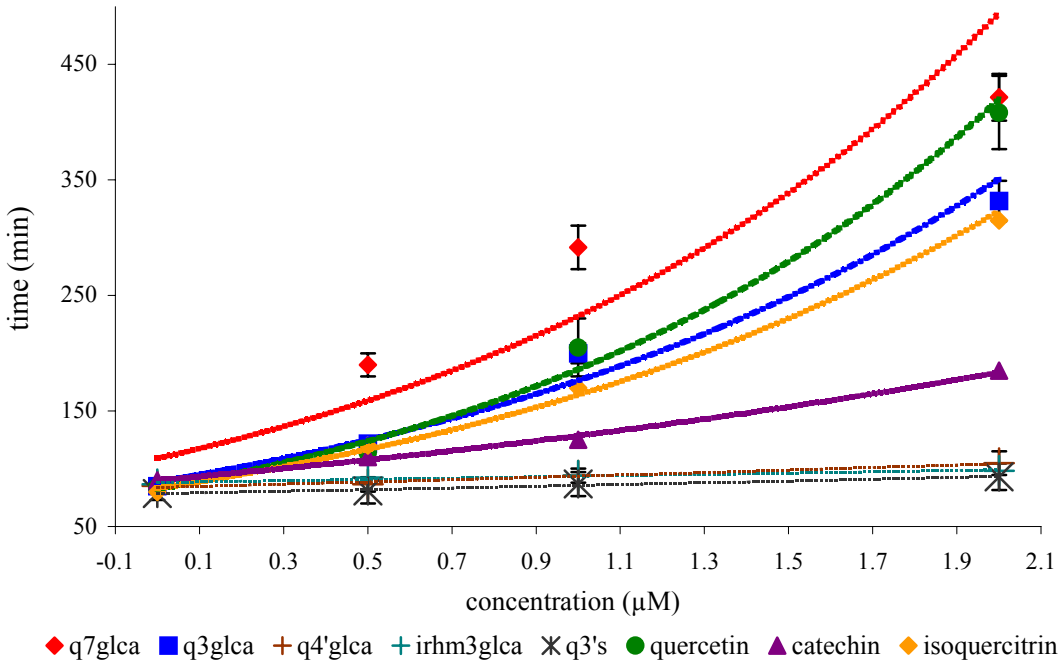


Fig. 48: Lag times of catechin, isoquercitrin, quercetin and metabolites in the copper-induced LDL oxidation, n = 3

In Fig. 48, the lag times versus the concentration of the samples are shown and all samples exhibit an exponential rise in the lag time on increase of concentration. The chosen concentrations were in the physiological range of the metabolites. The following order can be estimated for the protective abilities of the samples:

q7glca > quercetin > q3glca ≈ isoquercitrin > catechin > q4'glca > irhm3glca > q3's

The following equations and r-squared values were calculated for the best fit lines of the data:

sample	equation	r-squared
quercetin:	$y = 45.11e^{0.53x}$	$R^2 = 0.97$
isoquercitrin:	$y = 83.17e^{0.68x}$	$R^2 = 0.996$
catechin:	$y = 90.17e^{0.36x}$	$R^2 = 0.995$
q7glca:	$y = 108.93e^{0.76x}$	$R^2 = 0.879$
q3glca:	$y = 51.48e^{0.46x}$	$R^2 = 0.994$
q4'glca:	$y = 83.83e^{0.11x}$	$R^2 = 0.969$
irhm3glca:	$y = 88.19e^{0.059x}$	$R^2 = 0.908$
q3's:	$y = 78.44e^{0.087x}$	$R^2 = 0.975$

With r-squared values of 0.879 to 0.996, it is evident that the best fit lines match well with the data and together with the calculated equations the exponential rise of the results is confirmed.

IV.7. HSA binding studies with quercetin metabolites

A common assay for the investigation of binding abilities is the fluorescence quenching of the single tryptophan in the hydrophobic pocket of subdomain IIA. Two HSA preparations, a fatty acid-free preparation (HSA1) and an isolated fraction (HSA2) were compared to determine possible differences in the binding capacity of both preparations.

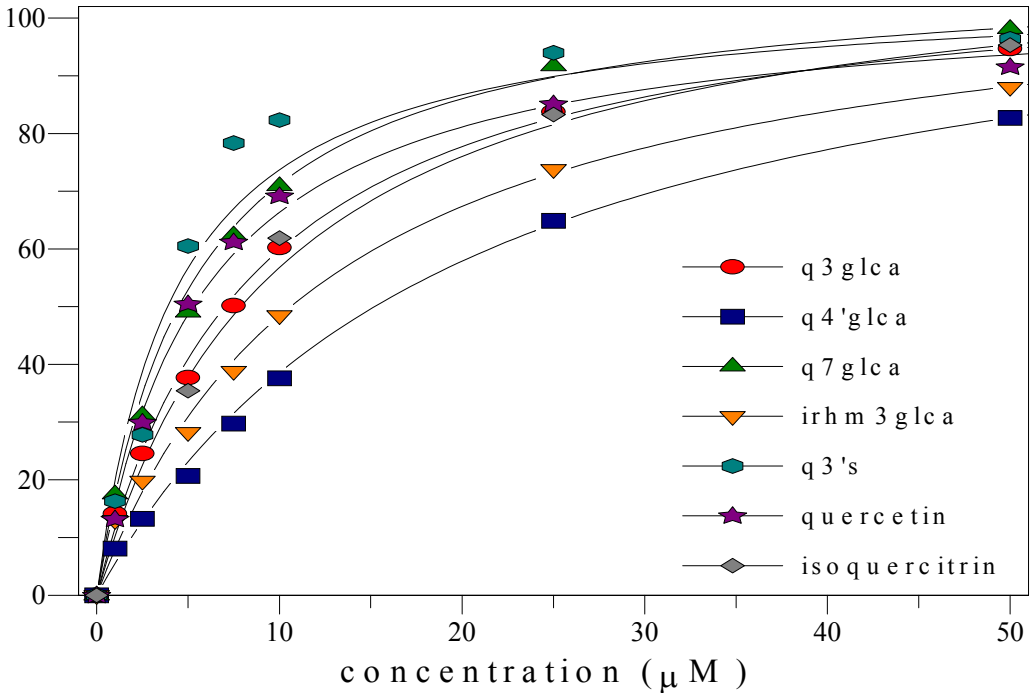


Fig. 49: Fluorescence quenching of HSA1, n = 3, stdev ≤ symbols

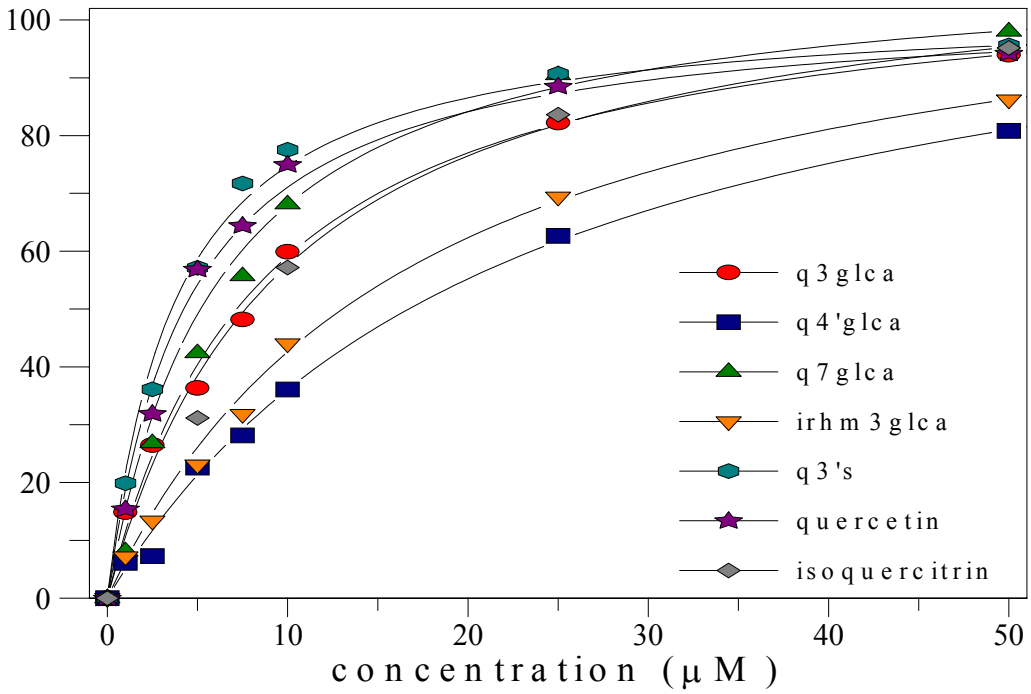


Fig. 50: Fluorescence quenching of HSA2, n = 3, stdev ≤ symbols

Fig. 49 and 50 demonstrate the percentage quenching of the two HSA preparations versus the concentration of the samples. It is evident that the differences in the tryptophan fluorescence for both preparations were small. The following equation for 'single-site binding' describes the graphs:

$$100*(1-F[C]/F_{max}) = \frac{[C]*Q_{cap}}{k_q+[C]}$$ where:

[C] = concentration of the sample

Q_{cap} = maximal quenching capacity

k_q = concentration of C to reach ½ quenching

F = fluorescence

The calculated k_q-values of the tested samples are shown in Tab. 26:

Tab. 26: k_q-values (µM) for all tested samples

Sample	HSA1		HSA2	
	k_q-value	stdev	k_q-value	stdev
quercetin	$5.73^{a.\#}$	0.46	$4.49^{e.\#}$	0.16
isoquercitrin	10.24^{b}	0.96	9.81^{f}	0.88
q7glca	5.26^{a}	0.24	6.24^{g}	0.51
q3glca	8.54^{b}	0.31	8.64^{f}	0.33
q4'glca	20.05^{c}	1.08	22.62^{h}	1.49
irhm3glca	$12.92^{d.+}$	0.45	$17.21^{i.+}$	1.01
q3's	4.28^{a}	0.65	3.76^{e}	0.30

(a-i): different letters indicate significant difference, $P < 0.025$ (t-test)

#,+: indicate significant difference between the two HSA preparations, $P < 0.05$ (t-test)

Tab. 26 shows the k_q-values range 4.28-20.05 µM for the HSA1 preparation and 3.76-22.62 µM for the HSA2 preparation. For both preparations there were no significant differences between the samples except for quercetin and irhm3glca. There was no significant difference between quercetin, q7glca and q3's with HSA1, the same applies to isoquercitrin and q3glca. With HSA2 there is no significant difference between quercetin and q3's and between isoquercitrin and q3glca. The following order based on the k_q-values (Tab. 26) of the samples was determined:

HSA1: q3's ≈ quercetin ≈ q7glca > q3glca ≈ isoquercitrin > irhm3glca > q4'glca

HSA2: q3's ≈ quercetin > q7glca > q3glca ≈ isoquercitrin > irhm3glca > q4'glca

IV.8. HepG2 cell culture

The stability of quercetin and metabolites in the cell media was investigated. The ability of four different additives to inhibit the autoxidation of quercetin and to stabilize it were tested. The same additives were used with the stability experiments of the metabolites.

IV.8.1. Influence of additives on the stability of quercetin

The influence of ascorbic acid, EDTA and desferal (c = 0.5 mM) to prevent the autoxidation of quercetin in the media was investigated in the presence and absence of HepG2 cells.

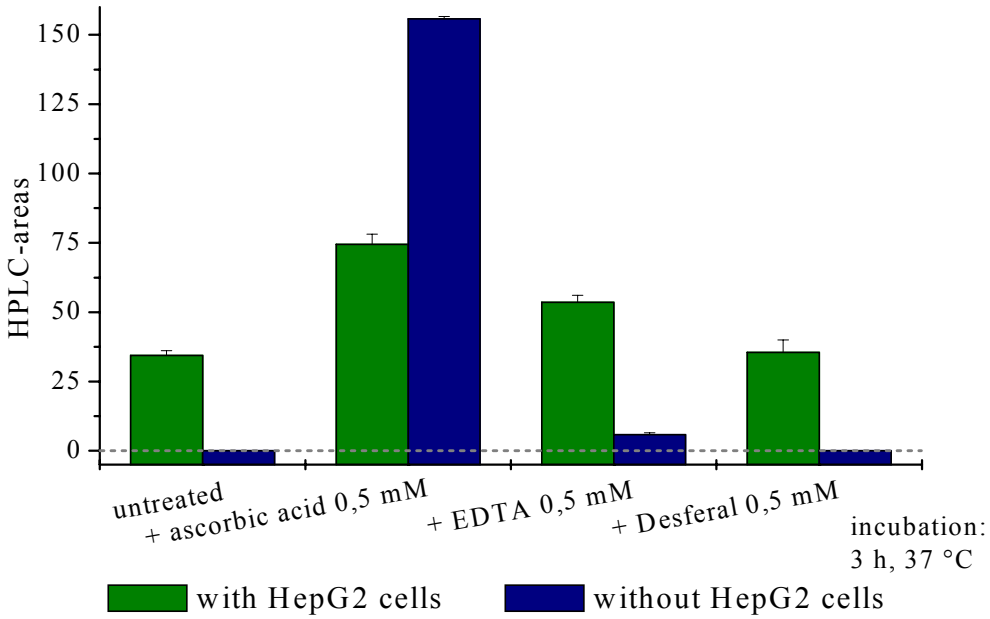


Fig. 51: Influence of ascorbic acid, EDTA and Desferal (c = 0,5 mM) on the stability of quercetin, n = 3
HPLC-areas quercetin 10 µM = 192.61 ± 1.39 (≡ 100 %)

The addition of ascorbic acid (Fig. 51) showed the highest positive effect on the stability of quercetin. In the presence of cells, about 39 % of quercetin can be detected whereas in the absence of cells 81 % are detectable. EDTA and desferal had no or a slight effect in the tests without cells (quercetin recovery: EDTA ≈ 3 %; desferal ≈ 0 %). Cells had a stabilising effect on quercetin (quercetin recovery ≈ 18 %). The combination with EDTA had an additive effect (quercetin recovery: EDTA ≈ 28 %; desferal ≈ 18 %).

IV.8.2. Influence of HSA on the stability of quercetin

HSA can bind quercetin (see IV.7.) and may protect quercetin due to this binding. The effectiveness of the protection of HSA was compared to the results of the stability studies with ascorbic acid (see IV.8.1).

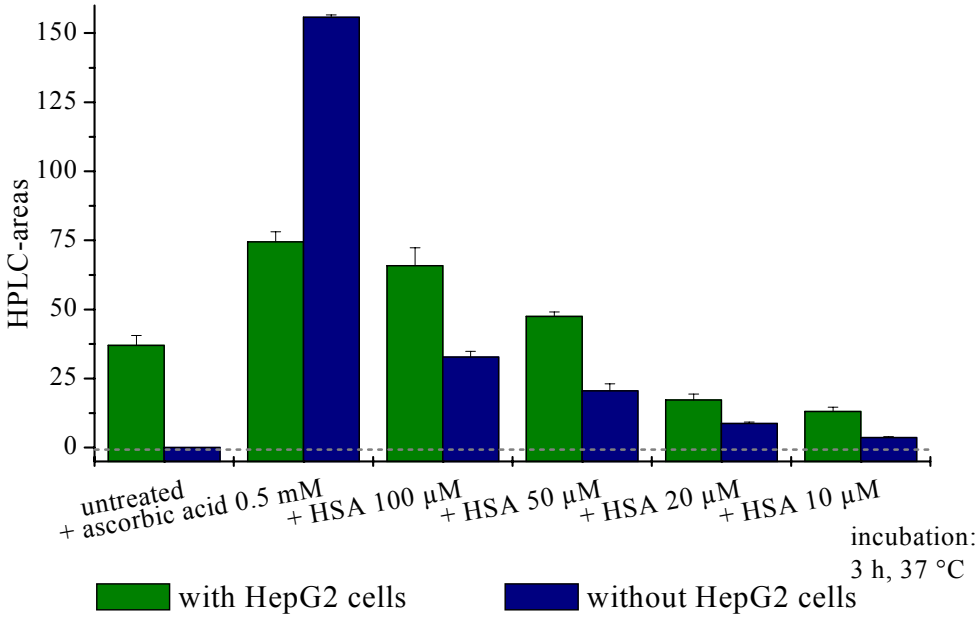


Fig. 52: Influence of HSA and ascorbic acid on the stability of quercetin, n = 3
HPLC-areas quercetin 10 µM = 192.61 ± 1.39 (≡ 100 %)

The highest positive effect (Fig. 52) was exhibited by ascorbic acid in the media without cells (quercetin recovery ≈ 81 %). The addition of HSA also increased the detectable quercetin in the absence of cells (quercetin recovery: HSA 100 µM ≈ 17 %, HSA 50 µM ≈ 11 %, HSA 20 µM ≈ 5 %, HSA 10 µM ≈ 2 %). There is little difference between the ascorbic acid and 100 µM HSA treatment (quercetin recovery: ascorbic acid ≈ 39 %, HSA 100 µM ≈ 34 %). Even the addition of 50 µM HSA increased the amount of quercetin treatment (quercetin recovery ≈ 11 %). Whereas an addition of 20 µM or 10 µM HSA resulted in no increase of quercetin (quercetin recovery: HSA 20 µM ≈ 9 %, HSA 10 µM ≈ 7 %) compared to untreated cells. However, the combination of HSA and cells had a positive effect on the amount of quercetin.

IV.8.3. Investigations on the stability of quercetin metabolites

Quercetin, and the metabolites quercetin-3- (q3glca), quercetin-7- (q7glca), quercetin-4'-glucuronic acid (q4'glca) and quercetin-3'-sulphate (q3's) were tested for their stability, adding either ascorbic acid (c = 0.5 mM) or HSA (c: 100 - 10 µM) to the media.

IV.8.3.1. Quercetin-3-glucuronic acid

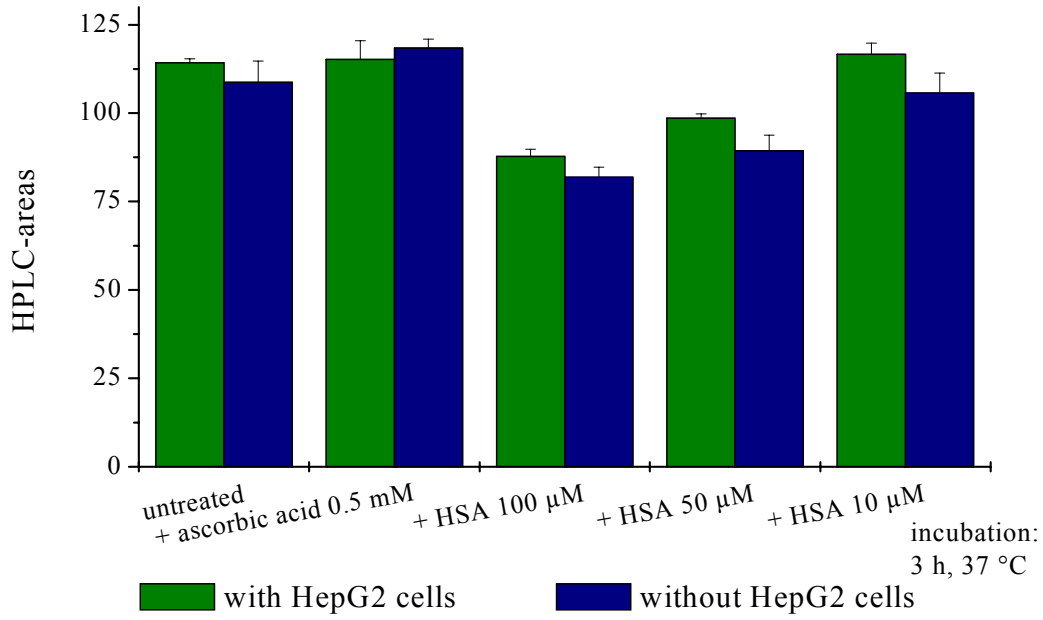


Fig. 53: Influence of ascorbic acid and HSA on the stability of q3glca, n = 3
HPLC-areas q3glca 10 µM = 189.24 ± 0.51 (≡ 100 %)

There is little difference in the stability of q3glca in the untreated sample in the presence and absence of cells (q3glca recovery: with cells ≈ 62 %, without cells ≈ 58 %). Even the addition of ascorbic acid does not significantly increase the amount of q3glca (q3glca recovery: with cells ≈ 61 %, without cells ≈ 63 %). However, the addition of HSA in concentrations of 100 µM and 50 µM decreased the q3glca amount and the 10 µM treatment did not significantly differ from the untreated sample. Q3glca recovery: HSA 100 µM with cells ≈ 46 %, without cells ≈ 43 %; HSA 50 µM with cells ≈ 52 %, without cells ≈ 47 %; HSA 10 µM with cells ≈ 62 %, without cells ≈ 56 %. Q3glca was taken up and metabolized by the cells.

IV.8.3.2. Quercetin-7-glucuronic acid

The stability of q7glca in the cell media was tested in the presence and absence of cells and with the addition of ascorbic acid and HSA. The result is shown in Fig. 54.

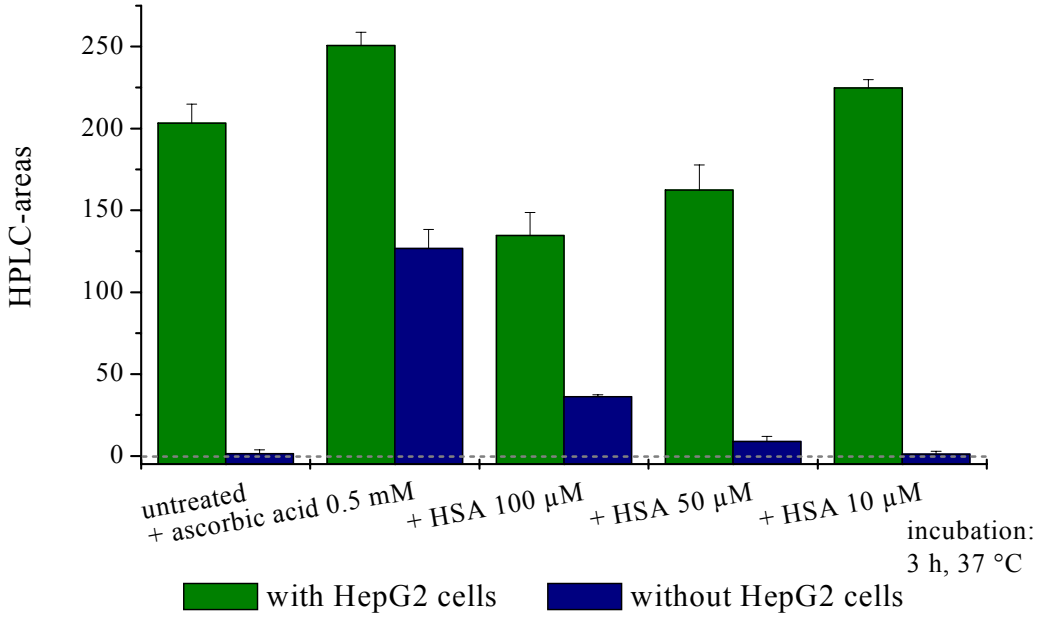


Fig. 54: Influence of ascorbic acid and HSA on the stability of q7glca, n = 3
HPLC-areas q7glca 10 µM = 236.96 ± 4.54 (≡ 100 %)

Fig. 54 shows the degradation of q7glca in the untreated sample in the absence of cells (q7glca recovery ≈ 0.57 %). This degradation can be prevented by the addition of ascorbic acid to give a recovery of q7glca of 53 %. The addition of 100 µM HSA stabilized q7glca in the absence of cells (q7glca recovery ≈ 15 %); though to a lesser extent than ascorbic acid. The recoveries of q7glca for the 100 µM and 50 µM HSA treatment in the absence of cells are 4 % and 0.44 %, respectively. The test in the presence of cells showed that they have a stabilising effect on q7glca (q7glca recovery ≈ 86 %) and an addition of ascorbic acid resulted in 100 % recovery of the metabolite. The addition of HSA (100 and 50 µM) decreased the amount of q7glca to give a recovery of 57 % and 69 %, respectively. At a protein concentration of 10 µM a slight increase was detected (q7glca recovery ≈ 95 %); compared to q7glca in the presence of cells. Q7glca was taken up and metabolized by the cells.

IV.8.3.3. Quercetin-4'-glucuronic acid

The stability of q4'glca in the cell media was tested in the presence and absence of cells and with the addition of ascorbic acid and HSA. The result is shown in Fig. 55.

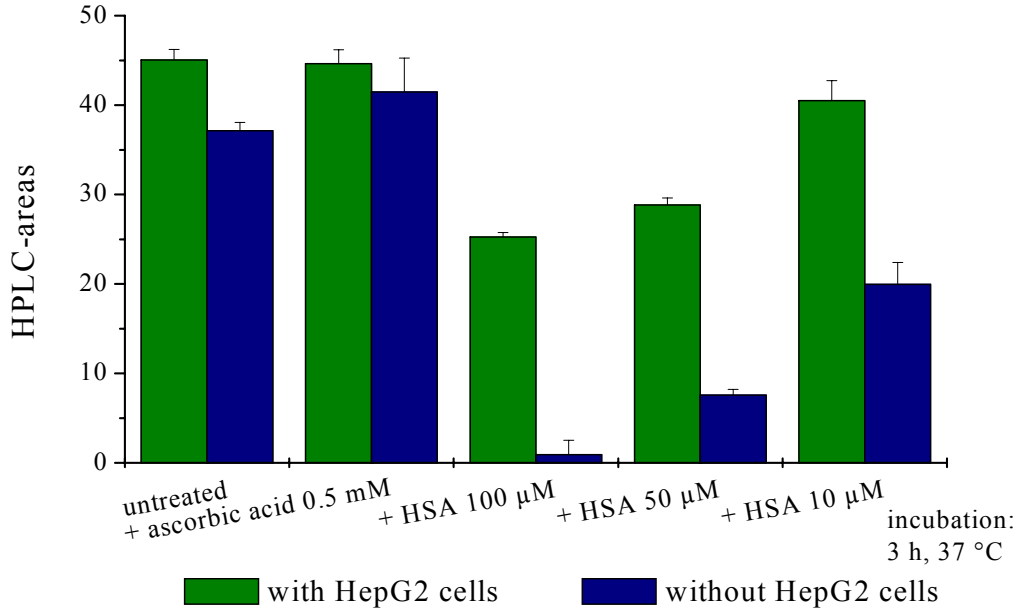


Fig. 55: Influence of ascorbic acid and HSA on the stability of q4'glca, n = 3
HPLC-areas q4'glca 10 µM = 87.78 ± 1.65 (≡ 100 %)

Q4'glca showed a degradation (Fig. 55) in the untreated sample in the absence of cells (q4'glca recovery ≈ 42 %). An addition of ascorbic acid increased its recovery to 47 %. The addition of HSA decreased the concentration of the metabolite in the absence of cells, which was due to the binding of the metabolite to the protein and was proven with the concentration-dependent increase of q4'glca with decreasing HSA concentrations. Q4'glca recovery: HSA 100 µM ≈ 1 %; HSA 50 µM ≈ 9 %; HSA 10 µM ≈ 23 %. The presence of cells increased the recovery in the untreated sample to 51 %. There was no difference between the untreated sample and the addition of ascorbic acid (recovery ≈ 51 % both) in the recovery of q4'glca in the presence of cells. The HSA treatment decreased the recovery of q4'glca in the presence of cells in a concentration-dependent manner due to the binding: HSA 100 µM ≈ 29 %; HSA 50 µM ≈ 33 %; HSA 10 µM ≈ 46 %.

IV.8.3.4. Quercetin-3'-sulphate

The stability of q3's in the cell media was tested in the presence and absence of cells and with the addition of ascorbic acid and HSA. The result is shown in Fig. 56.

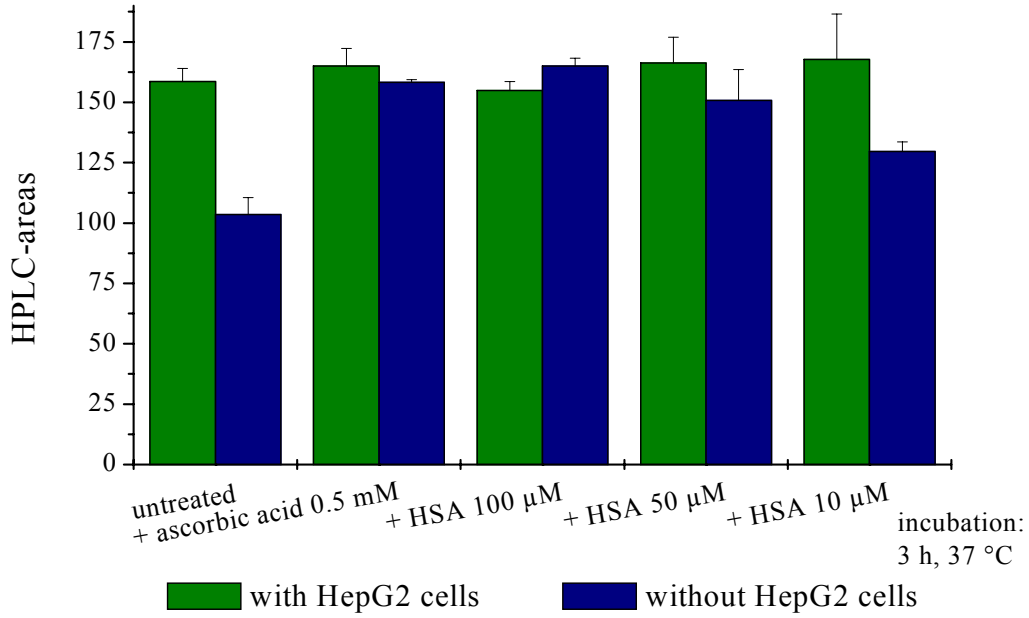


Fig. 56: Influence of ascorbic acid and HSA on the stability of q3's, n = 3
HPLC-areas q3's 10 µM = 348.52 ± 6.91 (≡ 100 %)

Q3s was degraded (Fig. 56) in the untreated sample both in the absence and presence of cells (q3s recovery: with cells ≈ 46 %; without cells ≈ 30 %). Addition of ascorbic acid had no effect on its recovery in the presence of cells (q3s recovery ≈ 47 %), whereas in the absence of cells an increased recovery was seen (q3s recovery ≈ 45 %) compared to the untreated sample. The addition of HSA increased the concentration of the metabolite in the absence of cells (q3s recovery: HSA 100 µM ≈ 47 %; HSA 50 µM ≈ 43 %; HSA 10 µM ≈ 37 %). The presence of cells had no effect on the recovery in the HSA treatment compared to the untreated sample. Q3s recovery: HSA 100 µM ≈ 44 %; HSA 50 µM ≈ 48 %; HSA 10 µM ≈ 48 %.

V. DISCUSSION

This thesis is divided in three parts which are inter-linked. Firstly, there are the investigations on the antioxidant abilities of plasma in different biochemical test systems. Secondly, there are the *in vitro* analyses on different buckwheat extracts, likewise with the combination of plasma, on buckwheat fractions and a small *in vivo* study to compare the supplementation of buckwheat with Pycnogenol. Finally, there are the studies with the flavonoid metabolites to examine their antioxidant properties and stability in cell culture media.

V.1. Antioxidant properties of human plasma

V.1.1. Plasma pool

Plasma contains an abundance of compounds with antioxidant functions. The main water-soluble antioxidants are proteins, uric acid and ascorbic acid, whereas the main lipid-soluble antioxidant in plasma is α-tocopherol. All these molecules contribute to the defence against oxidative stress in human plasma (STOCKER and FREI, 1991; DUTHIE, 1999; POLIDORI et al., 2000). The question addressed by the experiments with plasma was to investigate its behaviour in our test assays. Enzymatic antioxidants such as SOD, catalases or peroxidases are found in small concentrations in plasma and their functions are still unclear (STOCKER and FREI, 1991), their influence in the experiments is therefore assumed negligible. The antioxidant properties of plasma samples are due to non-enzymatic proteins and low-molecular weight molecules.

HOCl is a strong oxidant and aggressive bactericide, it is also known to inactivate proteins and oxidize thiols and ascorbic acid (ELSTNER, 1992; ELSTNER, 1993; HALLIWELL and GUTTERIDGE, 1999). Albumin is the main antioxidant for HOCl in plasma according to STOCKER and FREI (1991); the result in Fig. 22 is in accordance with this. A solution with a BSA-concentration corresponding to the total protein content of the plasma pool exhibited the same antioxidant properties compared to the two plasma pools used. This explains the good results in the ACC/HOCl assay (Fig. 20) being the assay with the lowest amounts of plasma needed to reach the I-values (I_{25} = 0.054 µl, I_{50} = 0.14 µl, I_{75} = 0.25 µl).

In the ABTS assay with I-values ranging from I_{25} = 3.7 µl, I_{50} = 10 µl to I_{75} = 25 µl (Fig. 20) the protein in the plasma is not the only antioxidant responsible for the result (Fig. 21). Low-molecular antioxidants, like uric acid or vitamin C, contributed to the antioxidant properties of the plasma. In this assay, the mechanism of the antioxidant effect remains unclear. A sample tested in this test system can either scavenge the ABTS-radical, react with the added H_2O_2 to prevent the production of the radical or inhibit the 'pseudo-peroxidase', which is formed by myoglobin and H_2O_2. The sample can be likewise a competitive substrate to ABTS for the myoglobin-H_2O_2 complex (personal communications: SCHEMPP, 2001; JANISCH et al., 2002). A single compound is already difficult to discuss due to these diverse mechanisms and the reaction of the complex mixture of plasma with different compounds can exhibit all three possible mechanisms mentioned to obtain the measured antioxidant effect.

The I-values of the Sin-1 assay are in the middle range with the following values: I_{25} = 9.16 µl, I_{50} = 40.79 µl and I_{75} = 122.12 µl (Fig. 20). The proteins of the plasma contribute to the antioxidant abilities in the Sin-1 assay (Fig. 24), but the other compounds of plasma also play a role in the antioxidant effect. There is no pure production of peroxynitrite from Sin-1. Sin-1 cleaves under physiological conditions in $O_2^{-\bullet}$ and NO thus being able to form ONOOH (BOHN and SCHÖNAFINGER, 1989; FEELISCH et al., 1989). Therefore, the measured effect can be due to the scavenging of the $O_2^{-\bullet}$ or the ONOOH, as NO does not react rapidly enough with molecules. Peroxynitrite oxidizes a number of biological molecules like protein thiols, iron/sulphur centres and causes nitration in proteins - especially on tyrosine residues (BECKMAN and KOPPENOL, 1996). The resulting superoxide radical reacts further with the iron present in the buffer and hydroxyl radicals result via Fenton chemistry (yield ~ 30 %; personal communications: SCHEMPP, 2003). An experiment with a pure peroxynitrite solution with plasma and BSA solution could give more information about the role of proteins on the antioxidant effects. The I-values from the peroxynitrite assay are not directly comparable with the Sin-1 assay. To achieve 25 % inhibition only a small amount of plasma is needed (I_{25} = 2.03 µl). However to reach 50 % and 75 % inhibition (I_{50} = 77.38 µl and I_{75} = 235.74 µl, respectively) more plasma is needed than in the Sin-1 assay. The difference of these two assays is due to the mixed effect in the Sin-1 assay, which makes a direct comparison impossible.

The I-values in the Fenton assay range from I_{25} = 17.41 µl, I_{50} = 94.17 µl to I_{75} = 256.97 µl (Fig. 20). The proteins play a main role in the antioxidant effect in this

assay. Nevertheless, it is not only albumin but also the metal-binding proteins are important as they chelate transition metals and therefore prevent the development of hydroxyl radicals (STOCKER and FREI, 1991). Fig. 23 shows the differences of the two plasma pools. The pool of January 2000 has a better antioxidant property than that from May 2000, which indicates that low-molecular compounds in plasma take part in the antioxidant effect of the pool. This result is explained by different volunteers giving blood for the two plasma pools.

The remaining two assays are enzymatic assays. The graph of the NADH/diaphorase assay is striking as it shows a steeper rise than the graphs of the other systems (Fig. 20), with I-values laying close together: I_{25} = 85.92 µl, I_{50} = 149.87 µl and I_{75} = 246.21 µl (Fig. 20). An inhibition of the enzyme by certain plasma compounds can be a possible explanation for this steeper rise (JANISCH et al., 2002).

The least sensitive system is the X/XOD assay with an I_{25}-value of 170.16 µl and an I_{50}-value of 334.39 µl (Fig. 20), an inhibition of 75 % could not be established with the tested plasma amounts. This result can be due to compounds found in the plasma itself like hypoxanthine or xanthine oxidase, which then increase the basic reaction.

V.1.2. Single-plasma samples

Single-plasma samples of 25 healthy volunteers were tested in four different assays and the resulting I-values compared to the plasma pool. For all single-plasma samples, the lipophilic antioxidants in the plasma were determined via HPLC (Tab. 02). Except for the volunteers #18 and #19, all concentrations for α-tocopherol, lycopene, β-carotene, $ubichinone_{ox}$ and $ubichinone_{red}$ are in the normal range. The higher concentrations for α-tocopherol in these two volunteers were due to their supplementation with vitamin E supplementation products. These two volunteers did not show any different behaviour in the four assays than the other volunteers.

The I-values of the single individuals scattered around the I-values of the plasma pool in three test systems (Fig. 25, 26, 28) forming a rather larger cluster with no results outside the norm. The formation of the cluster is due to the individual variation of antioxidants in the samples. After calculating the means and standard deviations of the single-plasma samples for all assays, it is clear that the means for the ABTS, ACC/HOCl and Sin-1 assays fit well with the established I-values of the plasma pool (Tab. 03). The standard

deviations are reasonable and represent the scatter of the individual samples around the pool. It is possible that diverse pools made from different volunteers move in-between this scatter. In the Fenton assay, three individuals were outside this cluster (Fig. 27) showing a decreased antioxidant property than the others. These three volunteers were found to be female and having their menstruation at the time of blood taking. The means for this assay had large standard deviations, which were due to the three out-laying volunteers. When the data of these three female volunteers were not considered for the calculation, the means and standard deviations fit nicely with the I-values of the plasma pool. The poor antioxidant property of the plasma of menstruating women was only be detected in the Fenton assay. Therefore, it can not be due to the loss of antioxidants due to the bleed, as this should then be detectable in the other systems as well. It is known that women are more prone to iron deficiency than men. The monthly loss of blood easily leads to a microcytic, hypochrome anaemia. About one third of the total iron in the body is bound to storage proteins like ferritin or haemosiderin, to myoglobin and other iron-containing proteins, two thirds of the iron is bound to haemoglobin. With a blood loss of about 30 ml arterial blood during menstruating women can easily become anaemic (HALLBERG and ROSSANDER-HULTÉN, 1991; HALLBERG et al., 1995; SCHMIDT and THEWS, 1997). Thus, with a decreasing ferritin concentration and transferrin saturation (HALLBERG and ROSSANDER-HULTÉN, 1991; HALLBERG et al., 1994), the iron from the Fenton assay should be taken up by transferrin and be removed from the Fenton reaction leading to a decrease in the radical production. The poor antioxidant property of the plasma of menstruation women must be explained otherwise. A possible change in the hormone status might be an explanation as the concentrations of both oestradiol and progesterone are very low at the time of the bleed (SCHMIDT, 1999).

V.2. Antioxidant properties of buckwheat

V.2.1. Buckwheat extracts

Buckwheat herb is used pharmaceutically in Germany to treat leg oedema and chronic venous insufficiency (IHME et al., 1996; QUETTIER-DELEU et al., 2000). There are also studies on the clinical effect of buckwheat herb on retinopathy in diabetes, where the authors recommend, due to their results, buckwheat herb for prevention and as a treatment (ARCHIMOWICY-CYRYLOWSKA et al., 1996). WÓJCICKI et al. (1995) investigated the effect of buckwheat extract (Fagorutin) on rabbits fed a high fat diet. The concentration of MDA decreased significantly to the control group as well as the total cholesterol and triglyceride content, when supplemented with buckwheat herb. The seeds of buckwheat are a part of the diet in Asia and Central Europe as substitutes of rice and cereal (DIETRYCH-SZOSTAK and OLESZEK, 1999; IKEDA et al., 2001; YAMASHITA and MURAKAMI, 2001). In this work, three different methods were used to extract the herb, whole kernels and grounded kernels. The aqueous extraction (SCHNEIDER-LEUKEL et al., 1992) is according to the use of the herb as an infusion of tea. The other two methods can be found in the literature for the analysis of the compounds of buckwheat seeds (alkalic-ethanolic extract: KREFT et al., 1999; ethanolic extract: WATANABE, 1998). For all three methods, the same amount of material was used (see III.1.3.15.) and the volume of the extracting media adapted accordingly. The extracts were not standardized on a single compound when tested in the assays. It was the aim of this work to compare the different extracts in four test systems. Obviously, an extract contains more than just flavonoids, namely glucose, amino acids, different compounds of the metabolism and, especially in the case of the kernels, soluble (depending on the extracting media) proteins. There were no methods to determine the presence of all these compounds. It is clear that all these compounds will contribute to a beneficial antioxidant effect. However, the secondary metabolites, here flavonoids, are the ‘active’ ingredients why plant extracts are used for medication. Therefore, the focus is on the flavonoids, which are listed in Tab. 04 for the different extracts. The ‘herb’ contains the most flavonoids, followed by the ‘kernels grounded’ and then the ‘whole kernels’. It can be said that compounds of the seeds are more easily accessible after a milling process. The concentrations vary between the different

extracting methods. This variation was due to the varying volumes of extracting media in each method (see III.1.3.15.). Calculated as the amounts per gram dry weight, the 'herb' appears to contain the most amounts of rutin, quercitrin and catechin when extracted with water. The other two extraction methods do not seem to extract exhaustively, or in the case of the alkalic-ethanolic method rutin, quercitrin and quercetin probably degrade (MAKRIS and ROSSITER, 2000). The authors (MAKRIS and ROSSITER, 2000) found that quercetin and rutin degrade rapidly under oxygen conditions in alkaline aqueous media, even at low temperature (t = 20 ± 1 °C). They could determine protocatechuic acid and two unidentified products as degradation products, this led to the assumption that the cleavage of the flavonol skeleton is involved in the degradation (NORDSTRÖM, 1968; BOULTON et al., 1999; MAKRIS and ROSSITER, 2000). The highest amount of epicatechin can be extracted with the alkalic-ethanolic method and the 'herb' sample; this can be due to the alkalic conditions, which might facilitate the isomerisation from catechin to epicatechin. A better extractability of epicatechin in alkalic conditions might also explain the higher amounts in this extract. This fact has to be investigated further do give clarity. When the catechin and epicatechin amount are added together as flavanols, the aqueous and alkalic-ethanolic extracts exhibit nearly the same values of these flavanols (1 A = 2.48 mg/g and 2 A = 2.74 mg/g, respectively). The ethanolic extract yields half of the flavanol amount (3 A = 1,12 mg/g) compared to the other two extraction methods.

The rutin content of the herb is about 4.7 % calculated with the rutin amounts of the aqueous extract, this is in accordance to the literature (KÖTTER, 1998; QUETTIER-DELEU et al., 2000; HAGELS, WAGENBRETH and SCHILCHER, 2001). Quercetin is only detected in higher amounts in the ethanolic extract of the 'herb'. This might be due to the extraction method as there are no significant findings of quercetin reported in the literature (KÖTTER, 1998; HAGELS, WAGENBRETH and SCHILCHER, 2001). All flavonols added together result in half the amount in the ethanolic extract, compared to the aqueous extract (1 A = 49.29 mg/g and 3 A = 21.45 mg/g, respectively). The alkalic-ethanolic extract contains only a fourth of the flavonols compared to the aqueous extract (1 A = 49.29 mg/g and 2 A = 12.16 mg/g, respectively). The low amount of flavonols in the alkalic-ethanolic extract must be due to the degradation of rutin, quercitrin and quercetin mentioned above. Even with a lesser volume of the extraction medium than in the alkalic-ethanolic extract, the ethanolic extracting method achieves higher flavonol amounts, but it can be either not-exhaustive or degradation takes place

during this extraction as well. It can be said that an aqueous extracting method achieves good results and therefore an infusion of tea provides its consumer with a high amount of flavanols and flavonols.

All extracts were tested in the following four test assays: Fenton, Sin-1, X/XOD and Rose Bengal assay. The Fenton assay was chosen as it produces iron-catalytic OH-radicals (see II.1. and III.2.7.3.). The Sin-1 assay was selected because of its production of peroxynitrite despite the probable mixing effect with the superoxide radical (see above V.1.). The X/XOD reaction is an enzymatic reaction mimicking ischaemia/reperfusion and it is known that quercetin is an inhibitor of the xanthine oxidase (ELSTNER, 1990). The photosensitizer Rose Bengal produces physically singlet oxygen and about 20 % superoxide radicals. In this assay, the influence of samples on the lipid peroxidation can be investigated.

None of the extracts, neither from the 'herb' nor from the 'kernels', exhibited any antioxidant property in the Fenton assay (Fig. 29). Therefore, the compounds of the extracts are not able to scavenge hydroxyl radicals. There is also no effect when plasma, at the amount equivalent to of the I_{50}-value, was added; the lesser ethylene production (Fig. 29) is due to the antioxidant ability of the plasma. A possible inhibition mechanism for the compounds is the chelating of the iron; this has to be investigated with a Fenton assay without EDTA as iron chelator. The formed iron-EDTA complex is too stable to be effected by the flavonoids.

The antioxidant effect at the Sin-1 assay is based on the scavenging of the ONOOH and the OH-radicals (see V.1.1.) or additionally the scavenging of the primarily produced superoxide. The 'herb' showed for all extracting methods the best efficiency (Fig. 30-32). It exhibited I_{50}-values with a dilution close to 1:5 for the aqueous and alkalic-ethanolic extract and with a dilution between 1:5 and 1:10 for the ethanolic extract. The I_{25}-values for the three 'herb' extracts were obtained with a dilution higher than 1:100. For neither the 'whole kernels' nor the 'kernels grounded' of all three extracting methods, the I_{50}-values were reached. However, the I_{25}-values are achieved with all three extracting methods for the samples of seeds. They lie between the stock solution and dilution of 1:2 for the aqueous extracting method for both kernel preparations; between 1:2 and 1:5 for the 'whole kernels' and between 1:5 and 1:10 for the 'kernels grounded' for the alkalic-ethanolic extract, respectively. The I_{25}-value for the kernel samples of the ethanolic extract was beyond the 1:100 dilution.

When plasma (amount ≡ I_{50}-value) was added to the assay, the antioxidant properties of

all samples were reduced (Fig. 30-32). This reduction of the antioxidant abilities of the extracts was due to the binding of the compounds to plasma proteins (BOULTON et al., 1998; see also V.3.). With plasma and the 'herb', an additional antioxidant effect was observed up to the dilution of 1:10 for the aqueous and alkalic-ethanolic extracts. The ethanolic extract of the 'herb' showed an additional antioxidant effect with plasma up to the 1:100 dilution is concerned. The seed samples had no additional antioxidant effect when extracted with water or alkalic-ethanolic. The ethanolic extraction of the 'kernels' with plasma achieved a slight additional antioxidant effect up to a dilution of 1:100 for both preparations.

In summary, the ethanolic extract possessed the best efficacy for all three samples, even when plasma was added, as it had the highest concentrations of flavonols (Tab. 04). The aqueous and alkalic-ethanolic extract exhibited the same properties even if they differed in the concentrations of compounds (Tab. 04). The effect in this assay must be mainly due to the flavonols as they are the main compounds in the ethanolic extract. At the two extracts (aqueous and alkalic-ethanolic) the flavonols and flavanols both contributed to the effect, as the concentrations of the compounds varied considerably between these two extracts.

In the X/XOD assay the compounds of all extracts either scavenged the resulting radicals (superoxide and hydroxyl radicals), inhibited the enzyme xanthine oxidase or chelated transition metals, but also mixed mechanisms occur in this assay. Inhibition of the xanthine oxidase has to be confirmed with an uric acid assay but is assumed here as this mechanism is known from quercetin and rutin (ELSTNER, 1990). As already seen in the Sin-1 assay, the 'herb' sample exhibited the best efficacy in this assay (Fig. 33-35). The I_{50}-values were below the 1:10 dilution for the aqueous and alkalic-ethanolic extract and nearly the 1:50 dilution for the ethanolic extract. The seed samples of the aqueous extraction reached the I_{50}-values with the stock solution for the 'whole kernels' and between the stock solution and a dilution of 1:2 for the 'kernels grounded', respectively. With the alkalic-ethanolic extraction, both kernel preparations obtained the I_{50}-values between the stock solution and a dilution of 1:2. Extracted with ethanol the two seed preparations were close together at the I_{50}-values, both laying between a dilution of 1:2 and 1:5.

Adding plasma (amount ≡ I_{50}-value) to this system, reduces the positive effects as in the Sin-1 assay. The yield of the ethylene of the plasma pools varied (Fig. 35-33) due to the use of three distinct plasma pools. The varying yields of ethylene were considered in the

estimating of the supplementary positive effects of the extracts. The additional positive effect was reached with the stock solution of both seed preparations and up to the 1:10 dilution for the 'herb' with the aqueous extraction. For the alkalic-ethanolic extraction, the additional effect was achieved with the stock solution of both kernel preparations and up to the 1:50 dilution for the 'herb'. The ethanolic extracted 'herb' showed an additional effect with plasma nearly up to the 1:100 dilution, both seed preparations nearly to the 1:5 dilution. As already mentioned for the Sin-1 assay, the ethanolic extraction of all samples possessed the best efficacy for all three samples, even when plasma was added. The flavonoids seemed to be the main active compounds, as their concentration was the highest within all extraction methods (Tab. 04). Assuming inhibition of the XOD the high concentration of quercetin was possibly promoting. The concentrations are even high enough to effect a positive result of the ethanolic extracts in addition of plasma. The proteins of the plasma cannot bind all compounds, and this explains the good result of these extracts. The aqueous and alkalic-ethanolic extracts again exhibited the same properties when tested without plasma. On addition of plasma, the alkalic-ethanolic extract of the 'herb' was better than the aqueous extracts. This result suggests that the flavanols and quercitrin play an important role here as their concentration is higher in the alkalic-ethanolic extract (Tab. 04). Due to their higher concentration, there was probably still a high enough concentration available to cause an effect, despite their binding to the plasma proteins.

The Rose Bengal assay is a complex system with different sites for test substances to interfere with the lipid peroxidation (see schemes 01 and 02). Chelating agents can chelate the transition metals; this leads to a decrease in ethylene production and to a possible decrease in the ethane production. The content of MDA declined while the aldehyde content remained the same or increased compared to the control. Scavengers terminate the radical chain, which is indicated with a decreased ethane/ethylene production and a declined MDA/aldehyde content or an unchanged aldehyde content. Quenchers are able to react with the excited molecules such as Rose Bengal or singlet oxygen, which results in a lower production of ethane/ethylene and a decrease in the MDA/aldehyde content. Due to these different mechanisms for a test sample, it was hard to judge the true effect of a sample, when evaluating the mode of action of the test substances. A complex mixture of different compounds, like an extract, is even harder to discuss, as it can exhibit a mixture of different mechanisms.

The 'herb' sample of all three extracting methods decreased both the ethylene and the

ethane production; this suggests a transition metal chelating and antioxidant property. The aqueous and ethanolic extract had the same properties (Fig. 36 and 38) and were slightly less efficient than the alkalic-ethanolic extract (Fig. 37). Flavonoids are able to chelate transition metals (BROWN et al., 1998; HIDER et al., 2001, see also V.3.) and to scavenge radicals (BORS et al., 1990; RICE-EVANS et al., 1995, see also V.3.). This result is therefore in agreement with the literature; a better prediction of the mechanism can be investigated with the Rose Bengal assay without copper. All kernel preparations exhibited a transition metal chelating ability with a decreased ethylene but a stable ethane production. The sample 'kernels grounded' had a better efficiency than the 'whole kernels' for all three extracting methods (Fig. 36-38). Therefore, it can be said that the chelating compounds of the seeds are more accessible for the extraction when they are milled. The range of efficiency of the extracts of the seed preparations was: alkalic-ethanolic ≥ aqueous > ethanolic. This leads to the suggestion that the compounds responsible for the result are more soluble in an alkalic milieu or aqueous solution than in ethanol. A possible explanation for this suggestion can be that the proteins of the seed exhibit metal chelating properties. Further investigations have to be made on this finding to clear any uncertainty.

V.2.2. Buckwheat fractions

The buckwheat herb was extracted and fractionated to investigate its polyphenol compounds. There are several studies on the contents of seeds of buckwheat, determining the polyphenol content, which is lower than in the leaves. In buckwheat phenylpropanoids such as coumaric acid, gallic acid, salicylic acid, cinnamic acid etc are found (POMERANZ, 1985; KÖTTER, 1998; HAGELS et al., 2001). Besides the flavons vitexin, isovitexin, orientin and isoorientin (KÖTTER, 1998; DIETRYCH-SZOSTAK and OLESZEK, 1999) buckwheat also contains flavans like flavan-3,4-diols and catechins as well as the flavonols quercitrin, hyperin and as the main compound rutin (POMERANZ, 1985; KÖTTER, 1998; HAGELS et al., 2001). The dianthron fagopyrin is only found in the buckwheat herb but is not, according to KÖTTER (1998), detected in the dried drug and the infusion of tea. The results of the fractionation are listed in tables 05-24. The extraction resulted in 7.27 g ethyl acetate extract (EtOAc extract) which corresponded to the flavonoid content of the herb. From this ethyl acetate

extract, 18 fractions could be produced with a Sephadex L-20 column. The amounts of each fraction are shown in Tab. 05. All fractions and the ethyl acetate extract were analysed via HPLC at the Fachgebiet für Obstbau. Due to technical problems with the HPLC, several spectra files of the runs were corrupted. This explains the missing spectra for some of the compounds. The missing spectra were problematic for the classification of the single peaks; especially as more of the polypropanoid compounds could then have been identified. Another reason why so many of the compounds remain unidentified is due to the lack of standards. Commercially available standards are often very expensive and for many compounds, there are no existing standards. Nevertheless, several peaks were identified and their findings are in accordance with the literature (e.g. proanthocyanidins, catechin, epicatechin and the flavonols; POMERANZ, 1985; KÖTTER, 1998). The fractionation also showed that some compounds appeared in only a few fractions (e.g. B2: found in fractions F-I; Tab. 11-14). While others were only be detected in one single fraction (e.g. catechin and epicatechin: found only in fraction E; Tab. 10). Rutin, which appeared from fraction A-F (Tab. 5-10), due to the overload of the Sephadex L-20 column with it. Therefore, not all of the rutin interacted with the Sephadex matrix and the excess of rutin was washed out during the first fractions. With less material loaded (< 2.3 g) this problem does not occur but then other compounds are in such small amounts in the fractions that they are not detectable with the HPLC. The whole extraction gave 1.14 g rutin (Tab. 24) for 100 g of buckwheat herb. All amounts of the fractions A-F (Tab. 5-10) give a rutin level of 1.6 g/100 g. The lower amount of rutin in the EtOAc extract was due to the error of the integration as the whole peak is out of the range of the HPLC with 1356.70 mAU. The EtOAc extract had to be diluted to give a better result with regard to rutin. The amount of rutin for the buckwheat herb did not correspond to the findings of the aqueous extract (Tab. 04), but agreed with the ethanolic extract of table 04. This discrepancy is explained by the different extraction methods. Calculated for 1 g herb, the methanolic extraction for the fractionation used 11 ml of MeOH, the ethanolic extraction 15 ml; whereas the aqueous extraction used 69 ml media. This explains the agreement between the ethanolic and methanolic extraction and the discrepancy with the aqueous extracting method. The content of 4.7 % rutin, as calculated for the aqueous extract, corresponds with the literature values for buckwheat herb used for tea infusion (KÖTTER, 1998; QUETTIER-DELEU et al., 2000; HAGELS et al., 2001). It can therefore be said, that neither the methanolic nor the ethanolic extracting method was exhaustive.

Due to the low amounts of the lyophilized fractions M-R (Tab. 05) only the fractions A-L and the ethyl extract (EtOAc extract) were tested in the following four assays: Fenton, Sin-1, X/XOD and Rose Bengal. The same assumptions as mentioned above for the buckwheat extracts hold for the test systems.

Neither the EtOAc extract nor the fractions A-L exhibited any effects in the Fenton assay (data not shown). These findings can be explained by a probable transition metal-chelating action in this assay, which can only be detected when the Fenton assay is done in the absence of EDTA. As mentioned above for the buckwheat extracts the complex of the EDTA with the iron was too stable to be affected by the polyphenols. It can also be said that the flavonoids of the buckwheat herb were poor scavengers of the hydroxyl radical, generated in this assay. This did not agree with the results of BORS and MICHEL (1999) who determined a scavenging reaction of flavans with OH-radicals. The calculated rate constants lie in the range of 1.0-7.1 x 10^9 $M^{-1}s^{-1}$ and were in the same range as for flavons or flavonols. Therefore, the single fractions should show an effect in this assay according to the literature. A possible explanation for this result might lie in the reactivity of the hydroxyl radicals. The reaction with KMB is possibly more favoured than with flavonoids, as its concentration in the assay is, at 1 mM, much higher than the flavonoid's.

There is the following order of efficiency at the Sin-1 assay: G > B > H > I > EtOAc >E > J > K > A = C = D = F = L (Fig. 40). The fraction G was the best one. The concentration of the procyanidin B2 was the highest in this fraction (Tab. 12). Obviously, a good efficiency in the Sin-1 corresponded with a high concentration of B2. It is unclear if it is the scavenging of the superoxide radical or the ONOOH, which resulted in this good effect. According to BORS et al. (2001), the rate constant of flavonoids with $O_2^{-\bullet}$ is in the intermediary range of 10^4 $M^{-1}s^{-1}$. Whereas JOVANOVIC et al. (1998) counted flavonoids among the most efficient superoxide radical scavengers with rate constants of 10^2 - 10^6 $M^{-1}s^{-1}$. The reaction is slower as with hydroxyl radicals (see above), but there was an effect seen in the Sin-1 assay. Therefore, a reaction with the ONOOH must have taken place. A mechanism of both scavenging properties could be an explanation. Considering the results of the Fenton assays (IV.2.1. Fig. 29 and IV.4.1.), the generated hydroxyl radicals were not scavenged by the fractions. The second best fraction was fraction B containing water-eluting compounds like phenolic acids and the washed-out excess of rutin. The result of this fraction must have been due to the additional effect of the phenolic acids. Rutin was just a minor part

in this fraction and even fractions with a higher concentration of rutin (E or F, Tab. 10 and 11) did not possess such a good property (Fig. 40). Fractions H and I are the next best fractions. They both contained mainly flavans. The concentration of the procyanidin B2 decreased in these two fractions and the other flavans and proanthocyanidins did not have such an impact as B2. The result of the EtOAc extract can be explained by the content of flavans. The percentage of the flavans in the EtOAc extract was lower than compared to the single fractions where they are concentrated. The result of the EtOAc extract lied in the addition of all compounds in it. Good results were still achieved for fractions E, J and K. Fractions J and K containing flavans (Tab. 15 and 16), whereas fraction E contained the flavans catechin, epicatechin and the flavonols quercetin, quercitrin and rutin (Tab. 10). The fractions with the least effect either contained the flavonols quercetin, quercitrin, rutin and the unknown flavonol (fraction A, C, D and F; Tab. 06, 08, 09 and 11), or unidentified compounds (fraction L, Tab. 17). In summary, it can be said that fractions containing flavans (starting with fraction E up to fraction K, Tab. 10-16) possessed good scavenging properties in the Sin-1 assay, procyanidin B2 showing the most impact of all flavans. To confirm if the active principle of the fractions in the X/XOD system was the inhibition of the enzyme, the scavenging of the radicals ($O_2^{-\bullet}$, H_2O_2, $^{\bullet}OH$) or an additive mechanism, has to be checked with an uric acid assay. As it is known that flavonoids inhibit the enzyme (ELSTNER, 1990), this might be the main principle besides the scavenging of the radicals. As already outlined in the Sin-1 assay, fraction G was the best in the X/XOD assay as well (Fig. 41). The high concentration of the procyanidin B2 also had a positive effect in the X/XOD assay. The second best fractions were fraction J and K, followed by the fractions H, I and K. In this test system all fractions with high concentrations of flavans showed good antioxidant properties. Good results were achieved by the fraction B and F. Fraction B, with the water-eluting phenolic acids and the washed-out excess of rutin (Tab. 07), and fraction F with a mixture of the flavonols and B2 (Tab. 11). The less active samples either contained a mixture of phenolic acids and flavonols (fraction C and D), the unidentified water-eluting compounds and rutin (fraction A) or a mixture of flavonols and the flavans epicatechin and catechin (fraction E). The EtOAc extract ranged between fraction C and D. The main compounds within are flavonols and the flavans, which can be found at a lower concentration. In conclusion, it can be said that a higher concentration of flavans and proanthocyanidins are more effective in the X/XOD assay than flavonols. This may be a

result of inhibition of the enzyme xanthine oxidase and/or in scavenging the radicals, which has to be investigated further. According to BORS and MICHEL (1999), both flavonols and flavans possess the same rate constants for scavenging superoxide radicals or hydroxyl radicals. Therefore, all fractions should react in the same way, unless the inhibition of the enzyme is more effective with flavans or procyanidins than with flavonols. On the other hand, BORS and MICHEL (2001) showed that the flavan-rich mixture Pycnogenol has a rate constant of 0.52×10^9 $M^{-1}s^{-1}$ for the reaction with hydroxyl radicals. This indicates that a mixture of these compounds has an even higher impact than fractions or single compounds.

The range of efficacy for the Rose Bengal assay was as follows: G = J = K > F = H = I = L > EtOAc > B = C = D = E > A (Fig. 42-45). The effects of the fractions and the EtOAc extract resulted mainly in the chelating of copper but antioxidant ability could also be seen. The differences between the single fractions were minor and, except for fraction A, negligible. From fraction F to L (Fig. 44-46) the copper chelating properties predominate. The stable aldehyde, with decreased MDA production and decreased ethane and ethylene formation, formulated this conclusion, especially as the graphs for ethane and ethylene are gaped more significantly than at the other fractions. These fractions were the ones containing mainly flavans and proanthocyanidins (Tab. 11-17). In fractions B to E (Fig. 43-44), the copper chelating properties and antioxidant properties were in balance. These fractions contained phenolic acids, flavonols and catechin/epicatechin (Tab. 07-10). Ethane and ethylene decreased at the same rate and even the aldehydes decreased. Fraction A is the least good, showing mainly copper chelating activity, as the ethane formation remained stable and only the ethylene decreased. The EtOAc extract lied in the middle in its efficiency, showing antioxidant and copper chelating properties. Its efficiency was due to the mixture of all compounds and, with higher amounts of the extract in the assay, the concentration of the flavans increases. This could explain the gap in the ethane and ethylene graphs (Fig. 42). Flavonoids possess transition metal chelating abilities (BROWN et al., 1998; YAMAMOTO et al., 1999; BORS et al., 2001; HIDER et al., 2001), with the possible binding sites at the catechol group in the B-ring, between the 3-OH and 4-oxo group or the 4-oxo and 5-OH group. MIRA et al. (2002) showed that flavonoids chelate preferably copper than iron and possess copper reducing abilities. Therefore, all compounds in the different fractions were able to chelate and/or reduce copper. The slight differences between the single fractions must be due to the different

concentrations of the compounds in each fraction. The antioxidant properties of the fractions were either due to the scavenging of the radicals produced by the lipid peroxidation or the quenching of the excited molecules. According to JOVANOVIC et al. (1998), flavonoids are very fast and efficient in quenching of singlet oxygen. The determined rate constants are 10^4 - 10^9 $M^{-1}s^{-1}$ for different flavonoids. The part of the copper chelating abilities has to be cleared with the Rose Bengal assay without copper.

V.3. *In vivo* supplementation with buckwheat tea and Pycnogenol

Buckwheat tea is given clinically to treat leg oedemas and fragility of the capillaries. In one study, supplementation with buckwheat tea resulted in decreasing volumes of leg oedema and is therefore considered a good medication for prevention and treatment of this (KIESEWETTER, 1998; SCHIMMEL, 1998).

A healthy volunteer was supplemented for 14 d with buckwheat tea and, as comparison, with Pycnogenol after a reasonable washing-out period. The diet of the volunteer was Almased® powder and water for the duration of the study. Almased® powder is the basis of a protein drink, which is used as nutrition to help to reduce weight, supplying the user with all essential minerals and vitamins. During the time of the study, the uptake of flavonoids was reduced to the supplements alone. Details of the supplementation can be found in Tab. 25. Plasma was taken at the start of each study and after 14 d. The plasma was tested in five test assays: X/XOD, diaphorase, Sin-1, Fenton and peroxynitrite assay (Fig. 47). There was an improvement of the plasma in two assays for both supplements (X/XOD and diaphorase assay) with Pycnogenol performing better than buckwheat tea. In two assays only the Pycnogenol supplementation showed an effect (Sin-1 and peroxynitrite), whereas in the Fenton assay deterioration can be seen (Fig. 47) for both supplements. The active principle in the plasma after a supplementation is not represented, according to the literature (MANACH et al., 1998; DONOVAN et al., 1999; MOON et al., 2000; DAY et al., 2001b; WITTIG et al., 2001), by the flavonoids itself. Metabolites, such as glucuronides, sulphates and methylates, are the circulating form of flavonoids in plasma. The results of this study therefore show that there is an uptake of compounds due to supplementation and these compounds enhanced the plasma even after their metabolization. The exact determination of the metabolites was not possible due to the

lack of methods at our institute. The results will be discussed as the flavonoids being the active principle but it must be considered that they are metabolised in the body. The deterioration measured in the Fenton assay must not be connected with the supplementation; it can also be a deterioration of the plasma itself due to the diet. As already seen with the buckwheat extracts and fractions (Fig. 29; V.2.), the flavonoids had no effect in this system. A test without EDTA in the Fenton assay could give a clearer picture about their abilities. In the X/XOD and diaphorase assay, both supplements exhibited an effect, with Pycnogenol being better in the X/XOD assay than buckwheat tea. The flavonoids can inhibit the enzymes of both assays and scavenge the produced radicals. Pycnogenol was better in the X/XOD assay, which is consistent with the results of the fractions (V.2.2.) where fractions with a high content of flavans and procyanidins achieved better results than fractions containing flavonols (Fig. 41). This result also agrees with JOVANOVIC et al. (1998) who counted the flavonoids among the most efficient superoxide radical scavengers. The difference in the result of the diaphorase assay must be due to a divert mechanism. The inhibition of the enzyme is probably not the main effect but the scavenging of the produced radicals. Flavans and flavonols have, according to BORS and MICHEL (1999), the same rate constants for superoxide radicals and hydroxyl radicals.

The buckwheat supplementation had no effect in the Sin-1 and peroxynitrite assays (Fig. 47) contrary to the Pycnogenol supplementation. This result corresponded to the result of the buckwheat fractions in the Sin-1 assay, where flavan- and procyanidin-rich fractions exhibited better properties than the others (Fig. 40; V.2.2.). The flavan-rich mixture Pycnogenol was therefore able to scavenge the superoxide radicals and ONOOH better than buckwheat tea. The concentrations of flavans and proanthocyanidins in the buckwheat tea were too low to achieve an effect. In the peroxynitrite assay, the supplemented plasma had to react with a pure peroxynitrite solution. When Pycnogenol was the supplement, it showed an effect in this assay. It can be said that flavans react with ONOOH, whereas flavonols did not have this ability. This also explains the good result with the Sin-1, as ONOOH seemed to be the main product.

In summary, it can be said that a supplement of buckwheat tea or Pycnogenol resulted in the uptake of antioxidant active compounds, which had an effect even after being metabolised. The effects varied when tested in different test systems, but flavans and proanthocyanidins generally obtained a better result compared to flavonols.

V.4. Effect of quercetin metabolites on LDL oxidation and HSA binding

V.4.1. LDL oxidation

Oxidized LDL is involved in the process of atherogenesis leading to heart disease and stroke, and therefore it is important to prevent its oxidation (ESTERBAUER et al., 1992). It is known that flavonoids inhibit lipid peroxidation and LDL oxidation and there are some studies, which investigated the protective effects of flavonoids (mainly quercetin) (RICE-EVANS et al., 1995; BROWN et al., 1998; MORAND et al., 1998; ZHU et al., 2000). The aim of these experiments was to investigate the protective effects of five different quercetin metabolites compared to quercetin, catechin and isoquercitrin.

All compounds under study prolonged the lag time of the copper-induced LDL oxidation in an increasing order (q3's < irhm3glca < q4'glca < catechin < isoquercitrin ≈ q3glca < quercetin < q7glca, see Fig. 48), which is consistent with the work of YAMAMOTO et al. (1999) who used quercetin glucosides in an equivalent study. The calculated best fits (p. 84) with r^2-values from 0.879 to 0.996 show, that the data points of the lag times fit very well with the best fit lines. All equations (p. 84) are exponential equations, therefore the increase of the concentration of a sample prolonged the lag time exponentially.

The protective capacities of flavonoids in the LDL oxidation was derived either from
a) binding to the Cu-receptor of the apoprotein B of the LDL particle,
b) regeneration of the endogenous antioxidants of the LDL particle (ZHU et al., 2000),
c) scavenging lipid peroxyl radicals (BORS et al., 1990; RICE-EVANS et al., 1995) or
d) chelating the copper(II)ions (BROWN et al., 1998, YAMAMOTO et al., 1999; BORS et al., 2001) with three possible metal-binding sites varying in binding affinities. The B ring with the catechol moiety occupies the binding site with the highest affinity compared to the sites between the 3-OH and 4-oxo group or the 4-oxo and 5-OH group (HIDER et al., 2001). These structures are also said to exhibit maximal radical-scavenging potential (BORS et al., 1990; 1995; 2001; RICE-EVANS et al., 1995; HEIJNEN et al., 2002).

The poor protective abilities of irhm3glca can be explained by the methylation at the 3'-hydroxyl group and glucuronidation at the 3-hydroxyl group. HIDER et al. (2001)

demonstrated that irhm3glca possesses only one potential metal-binding site between the 4-oxo and the 5-hydroxyl group. Having the 3'-and 3-position blocked, the radical-scavenging property of irhm3glca was also decreased (BORS et al., 1995). Q4'glca, glucuronated at the 4'-position, and q3's, with a sulphate at 3'-position in the B ring, both still possessed two possible binding sites for copper ions between the 3-OH and the 4-oxo group or between the 4-oxo and the 5-OH group. The slightly better protective abilities of q4'glca compared to irhm3glca and q3's could be due to the glucuronic acid which may provide a weak copper binding site at the B ring between the 3'-OH and the carboxyl group of the glucuronic acid. The sulphate at 3'-position did not seem to contribute to the copper binding at all. Hence, catechin should be in the same range as irhm3glca, with only one metal binding site between the 3'- and 4'-position. However, there may be a binding site between the hydroxyl groups at the 3- and 5-positions. The result was consistent with SOLEAS et al. (1997) who concluded that these positions are important for antioxidant activity in various lipid systems. As a flavan-3-ol, catechin possessed a good radical-scavenging property, which was due to the catechol group in the B ring and the saturated 2,3-bond (BORS et al., 1990). Isoquercitrin and q3glca, possessing a substitution at the 3-position, exhibited similar protective potential, both having two metal-binding sites between the 3'- and 4'-position and the 4-oxo and 5-OH group (HIDER et al., 2001) and the same radical-scavenging abilities: the catechol group in the B ring, the 2,3-double bond with the linked 4-oxo group and the free 5-OH group (BORS et al., 1990). The slightly better protection of q3glca may be due to the glucuronic acid providing another oxo- and hydroxyl-group, which might increase the metal-binding properties of the position 4 and 5. The protective ability of quercetin in this study is consistent with the results of BROWN et al. (1998). Quercetin can bind metal cations at three possible sites (HIDER et al., 2001) and possesses the ideal structure for maximum radical scavenging (BORS et al., 1990; 1995; RICE-EVANS et al., 1995). The best protective capacity in our study was exhibited by q7glca. According to YAMAMOTO et al. (1999) q7glca should prolong the lag time to the same extent as quercetin, possessing the same possible three metal-binding sites and radical-scavenging features. The result of q7glca could be explained by the substitution of glucuronic acid providing another possible binding site between the carboxyl group of the glucuronic acid and the 5-hydroxyl group of the A ring.

V.4.2. HSA binding

HSA contributes both to the colloid osmotic pressure and the binding of a wide variety of biological compounds (PETERS, 1985; HE and CARTER, 1992; TRYNDA-LEMIESZ et al., 2000). A common assay for the investigation of binding abilities is the fluorescence quenching of the single tryptophan in the hydrophobic pocket of subdomain IIA (PULLA REDDY et al., 1999; TRYNDA-LEMIESZ et al., 2000; DANGLES et al., 2001). In all analyses, defatted HSA was used as the binding can be influenced by bound fatty acids (CHEN, 1967; LEE and MCMENAMY, 1980). However, LEE and MCMENAMY (1980) showed that long chain fatty acids bind to subdomain IIIA, whereas subdomain IIA had one crevice binding small organic compounds and another crevice for medium chain fatty acids situated adjacent. The high affinity binding site found in the works of SYTNIK and LITVINYUK (1996) and PULLA REDDY et al. (1999) corresponds to the subdomain IIIA, usually occupied by long chain fatty acids in native HSA and is, according to BOULTON et al. (1998), not a major binding site for quercetin or small molecules in humans. In the study two different HSA preparations were used for comparison, a commercially available fatty acid free one (referred to as HSA1) and an isolated native one (referred to as HSA2). Both preparations exhibited the same nature in fluorescence, varying only in the relative intensity, with a higher yield for the HSA2 preparation (about 750 units) compared to the HSA1 (about 680 units) (data not shown).

The results of this study indicate that HSA is able to bind the tested compounds with an HSA:compound ratio up to 1:10, which is consistent with other studies (PULLA REDDY et al., 1999; TRYNDA-LEMIESZ et al., 2000). All metabolites of the flavonoids are bound to HSA and there are no free metabolites circulating in the plasma. This result is consistent with the study of BOULTON et al. (1998) who demonstrated the binding of quercetin to HSA was about 99.4 %.

The fluorescence abilities of both preparations varied to a certain degree, the k_q-values being slightly higher for HSA2 binding to all compounds except for q3's, q3glca, isoquercitrin and quercetin (Table 08). Nevertheless, the difference was only significant for quercetin and irhm3glca ($P < 0.05$). The binding abilities of the two HSA preparations can therefore be considered the same.

The quenching of the fluorescence involved the binding of a molecule in a short

distance from the Trp, the molecule possessing either specific groups to interact with the excited Trp or containing structures to absorb the energy from the excited Trp (SYTNIK and LITVINYUK, 1996; personal communications: E. BURSTEIN, 2002). The differences in the quenching behaviour of all compounds under study can be explained by their structural features. BORS et al. (1990; 2001) reported that the catechol group in the B ring and the 2,3-double bond linked with the 4-oxo group in the C ring contribute to electron delocalisation over the ring system. Therefore glucuronidation at the 4'-position effects the quenching as well as the binding to the highest extent as seen in Table 08 for q4'glca. DANGLES et al. (2001) assumed that flavonoids bind to BSA in a bidentate-mode. If this binding mode can be assumed for HSA as well, and the catechol group contributes to the mode, substitution at the B ring would influence the binding. The effectiveness of absorbing energy from the excited Trp was also decreased by glucuronidation at this position. If this was true, irhm3glca should be in the same range as q4'glca or less effective (Table 08) (DANGLES et al., 2001). The result of the quenching might be explained by the presence of the methyl group facilitating a closer contact to the excited Trp and could therefore absorb the energy better than q4'glca. Q3glca and isoquercitrin having only the 3-hydroxyl group substituted, the catechol structure in the B ring is still free for binding (DANGLES et al., 2001) and the scavenging features are only reduced for the 3-OH group. The difference in quenching behaviour is not significant (Table 08), but may be due to the different substitutions for both compounds; the glucuronic acid may be able to accept excitation energy more easily than glucose. Q3's exhibits the same quenching properties as quercetin (Table 08). Referring to the bidentate-mode of binding (DANGLES et al., 2001) the substitution with a sulphate at the 3'-position would appear to work as well as the catechol group. The electron delocalisation over the whole ring system should be the same as with quercetin. The difference between q7glca and quercetin (Table 08) with the HSA2 preparation could be explained with the sterical difference, as the binding and the electron delocalisation properties can be considered the same for both compounds. With medium chain fatty acids sharing this subdomain (LEE and MCMENAMY, 1980), the glucuronic acid as a substitute might obstruct the q7glca molecules entering the pocket.

V.5. HepG2 cell culture

Autoxidation of flavonoids occurs not only in alkaline media but also in neutral aqueous solutions. Protocatechuic acid, phloroglucinol, 2,4,6-trihydroxybenzoic acid and carbon monoxide were identified beside other unidentified, probable polymeric, products as products of the autoxidation of quercetin, kaempferol and rutin (NORDSTRÖM, 1968; JØRGENSEN et al., 1998; BOULTON et al., 1999; MAKRIS and ROSSITER, 2000). This autoxidation represents a problem for studies with cell culture. The degradation of flavonoids occurring in the cell media (oxygenated, tempered, transition metals) resulted in a decrease of the concentration under study. Additives with stabilizing effects, which did not interfere with the cells or tested flavonoids are desirable. In these experiments different additives (ascorbic acid, desferal, EDTA, HSA) were tested to examine their abilities to stabilize quercetin and its metabolites.

Fig. 51 shows the result of the stability of quercetin in cell media with and without cells. In the untreated sample in the absence of cells, quercetin was degraded after an incubation of 3 h, whereas the cells had a stabilizing effect on quercetin. This might be due to the uptake and accumulation of quercetin by the cells (BOULTON et al., 1999), but it is possible that the surface (especially proteins) of the cells also protects quercetin. When ascorbic acid was added, there was the recovery rate of quercetin increases both in presence and in absence of cells. The best result was achieved in the absence of cells, but the lower content in the sample with cells is due to the increased uptake of quercetin. The effect of ascorbic acid on the degradation of quercetin is attributed to the fact that ascorbic acid not only possesses strong reducing ability but also chelates transition metals (STOCKER and FREI, 1991; DUTHIE, 1999). Therefore, ascorbic acid can reduce formed phenoxyl radicals and chelate transition metals to prevent Fenton chemistry.

EDTA resulted in a small increase when added in the absence of cells (Fig. 51), the increase in the presence of cells corresponded to an additive effect of the cells and EDTA. The effect was not as good as an addition of ascorbic acid. EDTA is a chelator of transition metals and this explains this result. However, chelated iron is more active in this complex and stimulates Fenton chemistry, which would then lead to a degradation of quercetin. The iron chelator desferal showed no effect when added to the media, neither in presence nor in absence of cells. It can therefore be concluded, that the

transition metal iron does not play a major part in the degradation of quercetin in this media. Studies with a copper chelator can give clarity.

When HSA was added (Fig. 52), a concentration-dependent increase in the quercetin content both in the absence and presence of cells can be seen. With decreasing concentrations of HSA, a decrease of quercetin was measured. In the presence of cells, there was less quercetin found at HSA-concentrations of 10 and 20 µM than in the two higher concentrations. In a concentration of 100 µM HSA was still better than EDTA in the absence of cells. The effect of HSA can be either due to the binding of quercetin to HSA (see V.4.2.) or the binding of iron and copper to HSA. HSA possesses a binding site for copper and according to PETERS (1995) about 2 µM copper are bound to albumin in plasma. The antioxidant activity of HSA may also play a role in the protection against the autoxidation of quercetin.

After quercetin, the metabolites q3glca, q7glca, q4'glca and q3's were tested for their stability with ascorbic acid (c = 5 mM) and HSA (c = 100, 50 and 10 µM) as additives. A substitution at different positions of the quercetin skeleton has different effects on its degradation as the opening of the C-ring is the starting point for the degradation (NORDSTRÖM, 1968; JØRGENSEN et al., 1998; BOULTON et al., 1999; MAKRIS and ROSSITER, 2000).

There was no significant difference for the degradation of q3glca between the absence and presence of cells (Fig. 53). As there was no uptake of the metabolite by the cells, there was also no accumulation of q3glca in the cells. This is due to the substitution of the 3-OH group in the C-ring. According to JØRGENSEN et al. (1998), the acidity of this group is the breaking-up point of the flavonoid skeleton and a protection, or as in their studies, the lack of this group retards the degradation. The addition neither of ascorbic acid nor of 10 µM HSA had any positive effect on the content of q3glca in the media. On the other hand, at HSA-concentrations of 100 and 50 µM a decrease of q3glca was seen. This must be due to the binding of the compound to the protein. With a high recovery rate in the untreated sample, the binding to proteins can be more evident.

Q7glca degraded in the same degree as quercetin in the absence of cells (Fig. 54) whereas in the presence of cells a recovery of about 86 % was estimated. The cells had a stabilizing effect on q7glca, which was due to the uptake and a possible accumulation by the cells. With no substitutions at the catechol-group and the 3-OH group, q7glca was as liable to degradation as quercetin (JØRGENSEN et al.; 1998). In the absence of

cells, both ascorbic acid and HSA had a positive effect on the recovery rate of q7glca. Ascorbic acid to a higher extent than HSA: recovery rate ascorbic acid ≈ 53%; 100 µM HSA ≈ 15 %. With HSA, a concentration-dependent decrease of q7glca was seen in the media without cells. A decrease of the HSA concentration led to a decrease of q7glca in the media, obviously due to the stabilising effect of albumin. In the presence of cells, ascorbic acid shows an increasing effect on the recovery rate of q7glca, but with the HSA addition, a concentration-dependent decrease was seen. The addition of HSA (100 and 50 µM) decreases the amount of q7glca to a recovery of 57 % and 69 %, respectively. At a protein concentration of 10 µM, a slight increase can be detected (q7glca recovery ≈ 95 %) compared to q7glca in the presence of cells. This was the same effect as seen with q3glca (Fig. 53), the high recovery rate of q7glca with cells probably makes the binding of the metabolite to albumin evident. In the absence of cells, the binding might not be so apparent due to the high recovery rate compared to the untreated sample (recovery rate: untreated sample ≈ 0.57 %; 100 µM HSA ≈ 15 %).

In the experiment with q4'glca, the addition of ascorbic acid had just a slight effect on the recovery rate of the metabolite (Fig. 55) in the absence of cells. In the presence of cells, no difference between the untreated sample and the addition of ascorbic acid was seen. With the addition of HSA, a concentration-dependent decrease was exhibited in both presence and absence of cells. With HSA concentrations of 100 µM, the recovery of the metabolite decreased and increased with decreasing HSA concentrations. This result shows the binding of the metabolite to albumin.

Fig. 56 shows that q3's has a recovery rate of about 30 % in the untreated sample without cells. The addition of ascorbic acid and HSA had a positive effect on the recovery rate in the absence of cells (recovery rate: ascorbic acid ≈ 45 %; 100 µM HSA ≈ 47 %; 50 µM HSA ≈ 43 %; 10 µM HSA ≈ 37 %). Nevertheless, there was no significant difference between the samples with an addition of ascorbic acid and HSA and the untreated sample with cells (recovery rate: untreated sample ≈ 46 %; ascorbic acid ≈ 47 %; 100 µM HSA ≈ 44 %; 50 µM HSA ≈ 48 %; 10 µM HSA ≈ 48 %). The decreased degradation of q4'glca and q3's compared to quercetin must be due to the substituted catechol-group in the B-ring, where the degradation starts with the formation of a phenoxyl radical (JØRGENSEN et al., 1998).

Further work on this topic, especially with different chelators, will bring more clarity.

VI. SUMMARY/ZUSAMMENFASSUNG

Human plasma tested in biochemical assays (ACC/HOCl, ABTS, Sin-1, X/XOD, diaphorase, peroxynitrite, Fenton) achieves different I-values in each assay, indicating different antioxidant properties. When examined closer in four out of these seven assays (ACC/HOCl, ABTS, Sin-1, Fenton), it became obvious that the plasma proteins are the main responsible antioxidants. Only in the Sin-1 and Fenton assay, is the influence of the low-molecular antioxidants visible.

The comparison of 25 single-plasma samples with the plasma pool showed that they scatter closely around the pool. The means and standard deviations of these 25 samples fit well with the estimated I-values of the pool, except for the Fenton assay. In the Fenton assay, plasma of women with their menstruation at the time of the blood taking can be determined by the bad antioxidant value of their plasma. When the values of these volunteers are not considered for the means and standard deviations, there is no difference between the averaged samples and the pool.

The investigations on the extracts of the buckwheat herb and kernels determined different contents of flavonoids in the sample, which is due to the extracting method. In general, the extract of the herb showed the best antioxidant properties in three of the used test assays (Sin-1, X/XOD, Rose Bengal). None of the extracts had any effect in the Fenton assay that was done with EDTA as iron chelator. The aqueous extract of the herb, which corresponds to the infusion of tea, achieved nearly as good a result as the ethanolic extract; whereas an alkalic-ethanolic extraction can not be recommended.

The fractionation of the buckwheat herb resulted in the detection of a variety of peaks and compounds. Due to the lack of standards and problems with the HPLC, several peaks could not be identified. The identified peaks correspond with findings in the literature for buckwheat kernels. The herb contains mainly rutin, but it also contains catechin, epicatechin, the procyanidins B2, B5 and C1, quercitrin and different kinds of phenolic acids. When tested in the Fenton assay none of the fractions exhibits an effect. For the other assays (Sin-1, X/XOD, Rose Bengal), fractions containing high concentrations of flavans and procyanidins exerted the best properties.

An *in vivo* supplementation study with either buckwheat tea or Pycnogenol enhanced the antioxidant properties of the plasma of one volunteer. This corresponded with the results of the flavan-rich fractions of the buckwheat herb. The flavan-rich mixture Pycnogenol has a better impact on enhancing the plasma than buckwheat tea, which also shows effects. The active compounds must be flavonoids, which exhibit even after absorption and metabolization antioxidant properties.

Metabolites of quercetin, as they occur in human plasma after a quercetin-rich meal, protect LDL in the copper-induced LDL oxidation. The estimated best-fit lines of the lag times have exponential equations and they fit very well to the data points with their calculated r-squared values. The binding studies with the metabolites and two HSA preparations gave rise to the conclusion that there are no free metabolites circulating in human plasma. There were no significant differences between the two HSA preparations. The k_q-values of quercetin, q7glca and q3's are not significantly different; the same is for q3glca and isoquercitrin.

The degradation of quercetin and its metabolites in cell culture media is probably due to Fenton chemistry with copper ions. Ascorbic acid and HSA had a stabilizing effect on quercetin and q7glca, which degrades as quercetin. Q3glca is the most stable metabolite and neither addition of ascorbic acid or of HSA had any increasing effect. The recovery of q4'glca is affected by the binding of the metabolite to the HSA. Only with q3's are no effects of stabilization found neither with ascorbic acid nor with HSA. Further studies have to be done on this subject to give more clarity.

Humanes Plasma zeigt je nach angewandtem biochemischen Testsystem (ACC/HOCl, ABTS, Sin-1, X/XOD, Diaphorase, Peroxynitrit und Fenton) verschiedene antioxidative Eigenschaften, die sich in unterschiedlichen I_{50}-Werte widerspiegeln. Bei näherer Betrachtung in vier Systemen (ACC/HOCl, ABTS, Sin-1 und Fenton) wird deutlich, dass die Plasmaproteine hauptverantwortlich für die antioxidative Wirkung sind.

Der Vergleich von 25 Einzelplasmaproben mit dem Plasmapool zeigt, dass diese eng um den Pool verteilt sind. Die Mittelwerte und Standardabweichungen dieser 25 Einzelplasmaproben stimmen sehr gut mit den ermittelten I-Werten des Pools, mit Ausnahme des Fenton Systems, überein. Im Fenton System können Frauen, die zum Zeitpunkt der Blutabnahme ihre Menstruation haben, anhand der schlechteren antioxidativen Eigenschaften ihres Plasmas bestimmt werden. Wenn Daten dieser Probanden nicht in die Berechnung der Mittelwerte und Standardabweichungen mit einbezogen werden, zeigen die gemittelten Werte des Fenton Systems und des Pools keine Unterschiede.

Die Untersuchungen zu der Buchweizenteedroge und den Buchweizenkörnern ergab unterschiedliche Flavonoidgehalte in den Proben, aufgrund der unterschiedlichen Extraktionsmethoden. Der Extrakt der Buchweizenteedroge hatte generell die besten antioxidativen Eigenschaften in drei der verwendeten Testsysteme (Sin-1, X/XOD, Rose Bengal). Keiner der Extrakte zeigte eine Wirkung im Fenton System, welcher mit EDTA als Eisenchelator durchgeführt wurde. Der wässrige Extrakt, der einem Teeaufguss entspricht, erzielte fast so gute Ergebnisse wie der ethanolische Extrakt; wohingegen eine alkalisch-ethanolische Extraktion nicht empfohlen werden kann.

Die Fraktionierung der Buchweizenteedroge ergab verschiedene Peaks und Inhaltsstoffe. Aufgrund des Mangels an Standards und der Probleme mit der HPLC konnten einige Peaks nicht identifiziert werden. Die identifizierten Peaks stimmen mit den Angaben für Buchweizenkörner in der Literatur überein. Die Teedroge enthält hauptsächlich Rutin, aber ebenso Quercitrin, Catechin, Epicatechin, die Procyanidine B2, B5 und C1 sowie verschiedene Phenolcarbonsäuren. Im Fenton System zeigte keine der Fraktionen eine Wirkung. In den Systemen Sin-1, X/XOD, Rose Bengal besitzen Fraktionen mit einem hohen Anteil an Flavanen und Procyanidinen die besten Eigenschaften.

Eine *in vivo* Supplementierungsstudie mit Buchweizentee einerseits oder Pycnogenol andererseits, verbesserte die antioxidative Eigenschaft des Plasmas. Dies stimmt mit den Ergebnissen der flavanreichen Buchweizenfraktionen überein. Die flavanreiche Mischung Pycnogenol hat eine stärkere Auswirkung auf die Verbesserung des Plasmas als Buchweizentee, welcher ebenfalls Wirkung zeigt. Die aktiven Bestandteile müssen aufgenommene Flavonoide sein, die trotz ihrer Metabolisierung antioxidative Eigenschaften besitzen.

Quercetinmetaboliten, die im menschlichem Plasma nach einer quercetinreichen Mahlzeit detektiert werden, schützen LDL in der kupferinduzierten Dienkonjugation. Die ermittelten Ausgleichsgeraden der lag-Zeiten haben exponentielle Gleichungen und mit den berechneten Bestimmtheitsmassen fügen sie sich sehr gut an die Datenpunkte. Die Bindungsstudien mit den Metaboliten und den zwei HSA-Präparaten ermöglichen die Annahme, dass im menschlichen Plasma keine freien Metaboliten zirkulieren. Es gab keine signifikanten Unterschiede zwischen den beiden HSA-Präparaten. Die k_q-Werte von Quercetin, Q7glca und Q3‘s zeigen keine signifikanten Unterschiede, dasselbe gilt für Q3glca und Isoquercitrin.

Quercetin und seine Metaboliten degradieren im Zellkulturmedium möglicherweise aufgrund von Fenton Chemie mit Kupferionen. Ascorbinsäure und HSA haben eine stabilisierende Wirkung auf Quercetin und Q7glca, das wie Quercetin autoxidiert. Q3glca ist der stabilste Metabolit, weder die Zugabe von Ascorbinsäure noch von HSA erhöht seine Wiederfindungsrate. Die Wiederfindungsrate von Q4‘glca wird durch die Bindung des Metaboliten an HSA beeinträchtigt. Nur bei Q3‘s konnte weder durch die Zugabe von Ascorbinsäure oder von HSA eine stabilisierende Wirkung gefunden werden. Weitere Untersuchungen zu dieser Thematik können mehr Klarheit verschaffen.

VII. LITERATURE

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VIII. CURRICULUM VITAE

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