

to my beloved ones

- Author** Anna Nurmi (née Peltoketo), M.Sc. (pharm.)
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
tel. +358407241315
e-mail: anna.nurmi@fimnet.fi
- Supervisors** Professor Raimo Hiltunen, Ph.D.
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
- Tarja Nurmi, Ph.D.
Research Institute of Public Health
School of Public Health and Clinical Nutrition
University of Kuopio
- Professor Matti J. Tikkanen, M.D.
Division of Cardiology
Department of Medicine
University of Helsinki
- Adjunct professor Sari Voutilainen, Ph.D., RD
Research Institute of Public Health
School of Public Health and Clinical Nutrition
University of Kuopio
- Reviewers** Professor Sari Mäkelä, M.D.
Food Development
Health Biosciences
Department of Biochemistry and Food Chemistry
University of Turku
- Adjunct professor Riitta Törrönen, Ph.D.
Food and Health Research Centre
School of Public Health and Clinical Nutrition
University of Kuopio
- Opponent** Adjunct professor Marina Heinonen, Ph.D.
Division of Food Chemistry
Department of Applied Chemistry and Microbiology
University of Helsinki

© Anna Nurmi 2008

ISBN 978-952-10-4554-7 (paperback)

ISBN 978-952-10-4555-4 (PDF, <http://ethesis.helsinki.fi/>)

ISSN 1795-7079

Cover picture: Oregano. Copyright: Kotimaiset Kasvikset ry / Sanna Peurakoski

Yliopistopaino, University print
Helsinki 2008, Finland

Health from herbs?

Antioxidant studies on selected Lamiaceae herbs *in vitro* and in humans

ANNA NURMI

Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki

and

Research Institute of Public Health
School of Public Health and Clinical Nutrition
University of Kuopio

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Pharmacy of the University of Helsinki,
for public examination in Auditorium 2 at Viikki Infocentre
(Viikinkaari 11) on March 29th, 2008, at 12 noon.

Helsinki 2008

CONTENTS

PREFACE	6
ABSTRACT	8
TIIVISTELMÄ	9
ABBREVIATIONS	10
LIST OF ORIGINAL PUBLICATIONS (I-V)	12
1. INTRODUCTION	13
2. REVIEW OF THE LITERATURE	16
2.1. Free radicals and lipid peroxidation	16
2.1.1. What are free radicals?	16
2.1.2. Sources of free radicals and other reactive species	17
2.1.3. Lipid peroxidation	20
<i>Chain reaction of lipid peroxidation</i>	21
<i>Diseases linked with oxidative stress</i>	22
<i>Cardiovascular diseases and the role of oxidised LDL in atherosclerosis</i>	22
2.2. Antioxidants	24
2.2.1. What are antioxidants?	24
2.2.2. Mechanisms of antioxidant defence in humans	24
2.2.3. Assessment of antioxidant capacity and lipid peroxidation	27
2.3. Phenolic compounds	30
2.3.1. What are phenolic compounds or polyphenols?	30
2.3.2. Phenolic compounds as antioxidants	33
2.3.3. Phenolic compounds and cardiovascular health	35
<i>Epidemiological evidence</i>	35
<i>Supplementation studies on phenolic compounds</i>	37
2.3.4. Dietary sources and intake of phenolic compounds	40
2.3.5. Bioavailability and metabolism of phenolic compounds	43
2.4. Lamiaceae herbs	48
2.4.1. What are Lamiaceae herbs?	48
2.4.2. Phenolic compounds in Lamiaceae herbs	48
2.4.3. Lamiaceae herbs as a source of dietary antioxidants	50
3. AIMS OF THIS STUDY	53
4. MATERIALS AND METHODS	54
4.1. Standard compounds and reagents	54
4.2. Plant material	55
4.3. Extraction techniques for the plant material (I)	55
4.4. Studies on the antioxidant properties of Lamiaceae extracts and phenolic constituents and metabolites of <i>O. onites</i> in vitro (I-IV)	56

4.5. Human studies (III, V)	59
4.5.1. Kinetic study (III)	59
<i>Subjects and study design</i>	59
<i>Analyses of O. onites extract and urine</i>	59
4.5.2. Supplementation study (V)	61
<i>Subjects, study design and supplement used</i>	61
<i>Measurement of oxidative stress and lipid peroxidation</i>	63
<i>Other laboratory measurements</i>	64
5. RESULTS	65
5.1. Extraction of phenolic antioxidants from sage (I)	65
5.2. Phenolic compounds in Lamiaceae extracts (II, III, IV) and study juices (V)	66
5.3. Antioxidant activity of Lamiaceae extracts, their phenolic constituents, human metabolites and study juices <i>in vitro</i> (II, IV, V)	67
5.4. Excretion of urinary phenolic metabolites of <i>O. onites</i> extract in humans (III)	69
5.5. Effects of <i>O. vulgare</i> extract supplementation on antioxidant biomarkers <i>in vivo</i> (V)	70
6. DISCUSSION	71
6.1. Evaluation of the methods used	71
6.1.1. Extraction of the herb material	71
6.1.2. Total phenolic assay and <i>in vitro</i> antioxidant methods	72
6.1.3. Kinetic study	76
<i>Subjects and study design</i>	76
<i>HPLC analyses of oregano extract and urine</i>	76
6.1.4. Supplementation study	77
<i>Subjects, study design and supplement used</i>	77
<i>Assessment of antioxidant capacity and lipid peroxidation</i>	78
6.2. Evaluation of the results	79
6.2.1. <i>In vitro</i> antioxidant activity and phenolic content of Lamiaceae herb extracts	79
6.2.2. <i>In vitro</i> antioxidant activity of phenolic constituents in <i>O. onites</i> extract and their urinary metabolites	80
6.2.3. Bioavailability and metabolism of <i>O. onites</i> extract in humans	81
6.2.4. Effects of <i>O. vulgare</i> extract supplementation on antioxidant biomarkers <i>in vivo</i>	82
7. SUMMARY AND CONCLUSIONS	84
8. REFERENCES	86
ORIGINAL PUBLICATIONS I-V	101

PREFACE

During the past eight years, the preparation of this thesis has introduced me into the captivating world of research on herb polyphenols and antioxidants. Without this work, most of you people mentioned in the following would have remained unknown to me. Today I can conclude that the price of sometimes so tangled-feeling labwork and writing was never too high for reaching this goal and getting to know you all. The expertise and facilities of three research units were employed along the way: In addition to my home laboratory at the Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki, I had the chance to work at the Division of Cardiology, Faculty of Medicine, University of Helsinki and at the Research Institute of Public Health, School of Public Health and Clinical Nutrition, University of Kuopio. My work was partially financed by projects of the National Technology Agency of Finland and the Ministry of Agriculture and Forestry as well as personal grants from the Finnish Cultural Foundation and the Juho Vainio Foundation. This support is gratefully acknowledged.

I am indebted for having had four excellent supervisors, all of whom are strong experts in their fields. My great appreciation and admiration is directed to my main supervisor, Raimo Hiltunen. In addition to providing up-to-date facilities to my work, he has believed in my work, tirelessly supported me and advised me on various matters. Even in some less exhilarating work-related matters he has always retained a twinkle in his eyes. The medical and especially cardiovascular expertise as well as supportive attitude of Matti J. Tikkanen is warmly acknowledged. The working atmosphere in his laboratory was enthusiastic and motivated. Tarja Nurmi has, as my co-author and supervisor, given an irreplaceable contribution to preparation of this thesis. Her ambitiousness and analytical expertise are something to really look up to, and I am truly thankful for all the effort. The collaboration with the University of Kuopio would not have been the same without my fourth supervisor, Sari Voutilainen. Along the way we have explored both the jungles of epidemiology and Southern Thailand. These experiences and memories are indelible! In addition to all the inspiring scientific contents and support during my co-operation with people from Kuopio, the stunning atmosphere both on duty and the fabulous parties at leisure can not be left unmentioned.

Sari Mäkelä and Riitta Törrönen are warmly acknowledged for their interest and commitment in reviewing this thesis. The positive and constructive comments were precious in the final processing of the text.

I am grateful to the personnel of the Division of Pharmaceutical Biology for a friendly and relaxed atmosphere. Damien Dorman introduced me into the world of antioxidants as well as tutored me in the mysteries of British English - I think my accent will never be the same. The support of Magali Laasonen in many scientific and personal ups and downs is heartily acknowledged. Tuija Niinimäki deserves my sincere thankyou for cosy and flexible assistance and teamwork at the lab.

The research team of Matti J. Tikkanen deserves my warm thoughts for all the help and support during the numerous LDL oxidation assays that were carried out in their laboratory. The warm-hearted and expertised guidance of Terhi Kuusisto and conversations with Maija Badeau were especially valuable for this work.

The personnel of the Research Institute of Public Health, University of Kuopio has always made me feel warmly welcome. Jaakko Mursu, my co-author and travel mate on congress trips, gets my warm thoughts for always keeping up the right responsible but laid-back attitude. Sirkku Karhunen is thanked for her expert help in the analyses and for accommodation during my visits to Kuopio.

All my co-authors not already mentioned are warmly acknowledged for their contributions. Teriaka Ltd. is thanked for pleasant co-operation as well as for providing the herb material used in these studies.

During this time, I was not always certain whether this project would ever reach its end. Fortunately, I am blessed with dear friends, who had all the faith for me and my capabilities. Each and every one of you is thanked for being there for me. The same applies for my dear mother, father, sister, brother and grandmother, whose belief and support never ran out. I would not be here without you.

Even though this academic merit warms my heart, the pride and joy of my life is without a doubt my family: my loving and encouraging husband Ville and our stunning daughter Aamu. Nothing ever compares to you. - I guess one PhD in a family would have been enough, but now our children will have to deal with two.

Tampere, 14.2.2008

ABSTRACT

High dietary intake of fruits and vegetables has been linked to protection against chronic diseases, of which cardiovascular diseases (CVD) are the major cause of death in Finland. One of the hypotheses underlying the mechanism behind this effect is the antioxidant action of several dietary factors, including vitamins C and E, carotenoids and polyphenolic compounds. Polyphenols are consumed in large quantities in plant rich diets and observations on their antioxidant potency *in vitro* have made them a hot research topic during the past twenty years. The present study deals with the antioxidant activity of selected members of the plant family Lamiaceae – sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.) and two oregano species (*Origanum vulgare* L., *Origanum onites* L.). Due to the abundance of various phenolic compounds in these herbs, they are considered as a potential source of health-beneficial natural components with usage as health-promoting dietary supplements.

The applicability of hydrodistillation, methanolic and ethanolic extraction, and pressurised hot-water extraction (PHWE) to extract phenolic antioxidants from sage was studied. Based on the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[•]) scavenging activity of the different extracts, PHWE was the most effective technique. The hydrodistillation method was chosen for the further production of study extracts due to sensory properties of the extracts suitable for human consumption. The technique was also easily applicable in a larger scale production. The *in vitro* antioxidant properties of water extracts from sage, rosemary, thyme and oregano (*O. vulgare*) were characterised using three radical scavenging assays, a reductive capacity assay and a human low-density lipoprotein (LDL) oxidation assay. The total phenolic content of these extracts was determined. Depending on the method used, the extracts showed varying degrees of antioxidant activity. The absorption and metabolism of oregano (*O. onites*) extract in humans was evaluated by analysing the urinary excretion of phenolic metabolites using liquid chromatographic methods. The excretion of phenolic metabolites in urine was markedly increased following a single ingestion of *O. onites* extract, and the pattern of urinary metabolites suggested effective metabolism to take place. The *in vitro* antioxidant activity of phenolic constituents and human metabolites of *O. onites* were studied in DPPH[•] and LDL oxidation assays. The phenolic acids from *O. onites* showed a range of antioxidant activity in the assays used, and some degree of activity was observed for many of the aglycone forms of their human metabolites. In a double-blinded, placebo-controlled clinical trial, the effects of oregano (*O. vulgare*) extract consumption on different biomarkers of antioxidant capacity and lipid peroxidation were studied in healthy men. No short- or long term effects on the antioxidant parameters followed were observed.

This work revealed several aspects on the problematic nature of studies on antioxidants. Even though these extracts and their constituents seem promising antioxidant candidates *in vitro* and are at least to some extent available in the body, they lack effects on an array of lipid peroxidation and antioxidant capacity parameters *in vivo*. The findings suggest that their potential health-beneficial effects on humans are not mediated via direct antioxidant action. However, the epidemiologically observed association between the high intake of polyphenol rich fruits and vegetables and the decreased risk of CVD, as well as accumulating *in vitro* evidence on several bioactivities of polyphenols support the theory that polyphenols may have favourable effects on human health mediated via some other mechanisms.

TIIVISTELMÄ

Epidemiologisten tutkimusten perusteella runsaasti kasviksia ja hedelmiä sisältävä ruokavalio vähentää riskiä sairastua sydän- ja verisuonisairauksiin. Yksi vahvimmista suojavaikutusta selittävistä teorioista pohjautuu ruokavalion hapettumista estävien eli antioksidatiivisten komponenttien, kuten C- ja E-vitamiinin, karotenoidien ja fenolisten yhdisteiden, toimintaan ihmiselimestössä. Tässä työssä tutkittiin Lamiaceae-heimon maustekasveja: salviaa (*Salvia officinalis* L.), timjamia (*Thymus vulgaris* L.), rosmariinia (*Rosmarinus officinalis* L.) ja kahta oreganolajiketta (*Origanum vulgare* L. ja *Origanum onites* L.), jotka sisältävät runsaasti antioksidatiivisia fenolisia yhdisteitä.

Työssä tutkittiin vesitislauksen, metanoli- ja etanoliuuton sekä paineistetun kuumavesiuuuton käyttöä fenolisten antioksidanttien uuttamiseen salviasta. Uutteiden fenoliprofiili selvitettiin nestekromatografisesti ja niiden antioksidanttitehoa 1,1-difenyyl-2-pikryylihydratsyyliiradikaalin (DPPH[•]) sieppaajina tutkittiin. Paineistettu kuumavesiuutto osoittautui menetelmistä tehokkaimmaksi. Osatöissä käytettävien uutteiden valmistamiseen valittiin vesitislauksella, jolla valmistettujen uutteiden maku- ja hajuominaisuudet olivat käyttötarkoitukseen soveltuvat, ja joka oli helposti siirrettävissä suurempaan tuotantomittakaavaan. Salvian, rosmariinin, timjamin ja oreganon (*O. vulgare*) vesiuutteiden kokonaisfenolipitoisuus määritettiin, ja niiden antioksidanttiominaisuuksia koeputkimalleissa (*in vitro*) tutkittiin radikaalinsieppauskykyä, rautaionien pelkistämiskykyä ja low density lipoprotein (LDL) -kolesterolin hapettumisenestokykyä mittaavilla menetelmillä. Kaikki uutteet toimivat antioksidanteina käytetyissä koeputkimalleissa, mutta aktiivisuusjärjestys vaihteli menetelmittäin. Oreganon (*O. onites*) fenolisten happojen imeytymistä ja metaboliaa ihmiselimestössä arvioitiin tutkimalla uutteen nauttimisen jälkeen virtsaan erittyviä fenolisia metaboliitteja nestekromatografisesti. Metaboliittien määrä lisääntyi merkittävästi osoittaen, että ainakin osa uutteen fenolisista komponenteista imeytyi elimistöön. Yhdisteprofiilin perusteella uutteen fenoliset hapot metaboloituivat tehokkaasti. Saman uutteen fenolisten komponenttien ja niiden metaboliittien antioksidanttitehoa tutkittiin DPPH[•]- ja LDL-malleissa. Uutteen fenolisilla yhdisteillä sekä niiden metaboliittien aglykonimuodoilla havaittiin antioksidanttiaktiivisuutta; metaboliittien aktiivisuudet olivat lähtöaineitaan alhaisempia. Kaksoissokkoutetussa, lumekontrolloidussa kliinisessä kokeessa tutkittiin, onko oreganouutteella (*O. vulgare*) vaikutuksia elimistön antioksidanttikapasiteettiin tai rasvojen hapettumista kuvastaviin mittareihin terveillä miehillä. Lyhyt- tai pitkäaikaisia vaikutuksia ei havaittu.

Tutkitut yrtit sisälsivät voimakkaita vesiliukoisia antioksidantteja, joista suurin osa oli fenolisia happoja. Ainakin osa oreganon fenolisista yhdisteistä imeytyi ihmiselimestöön ja metaboloitui tehokkaasti. Siitä huolimatta 45 koehenkilöllä tehdyssä syöttökokeessa ei havaittu antioksidanttivaikutuksia yleisesti käytetyillä, luotettavina pidetyillä mittareilla. Tulokset viittaavat siihen, että yrttien fenolisten yhdisteiden mahdolliset terveyshyödyt eivät välittyisi suoran antioksidantti-vaikutuksen kautta. Runsaasti fenolisia yhdisteitä sisältävän, kasvis- ja hedelmäpainotteisen ruokavalion epidemiologisesti havittu yhteys alentuneeseen sydän- ja verisuonisairauksien riskiin sekä fenolisten yhdisteiden lukuisat bioaktiiviset mekanismit koeputkimalleissa viittaavat kuitenkin siihen, että terveyshyötyä voisi välittyä jonkin muun mekanismin kautta.

ABBREVIATIONS

AAPH	2,2'-Azobis(2-aminopropane)
ABTS^{•+}	2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) anion radical
ALAT	alanine aminotransferase
CEAD	coulometric electrode array detector
CHD	coronary heart disease
CVD	cardiovascular disease
DPPH[•]	1,1-diphenyl-2-picrylhydrazyl radical
EDTA	ethylenediamine tetraacetic acid
FC	Folin-Ciocalteu
GAE	gallic acid equivalent
GC-MS	gas chromatography - mass spectrometry
GI	gastrointestinal
γ-GT	gamma-glutamyltransferase
HDL	high-density lipoprotein
H₂O₂	hydrogen peroxide
HP	high phenolic
HPLC	high performance liquid chromatography
IC₅₀	inhibitory concentration 50% (for example, sample conc. yielding 50% inhibition of DPPH [•] radical absorbance)
LDL	low-density lipoprotein
LOD	limit of detection
LP	low phenolic
MCT	monocarboxylic acid transfer
NADPH	nicotinamide adenine dinucleotide phosphate
O₂^{•-}	superoxide radical
¹O₂	singlet oxygen
OH[•]	hydroxyl radical
ORAC	oxygen radical absorbance capacity
ox-LDL	oxidised form of LDL
<i>O. vulgare</i>	<i>Origanum vulgare</i> L.
<i>O. onites</i>	<i>Origanum onites</i> L.
PBS	phosphate-buffered saline
PHWE	pressurised hot-water extraction
PI	percentage inhibition
PUFA	polyunsaturated fatty acids
RH	fatty acid side chain of PUFA
RO[•]	alkoxyl radical
ROO[•]	peroxyl radical
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
<i>R. officinalis</i>	<i>Rosmarinus officinalis</i> L.

SOD	superoxide dismutase
<i>S. officinalis</i>	<i>Salvia officinalis</i> L.
TBA	thiobarbituric acid
TEAC	Trolox [®] equivalent antioxidant capacity
TRAP	total radical-trapping antioxidant parameter
<i>T. vulgaris</i>	<i>Thymus vulgaris</i> L.
VLDL	very low-density lipoprotein
US	United States
UV	ultraviolet

LIST OF ORIGINAL PUBLICATIONS (I-V)

This thesis is based on the following original communications, referred to by their Roman numerals:

- I Ollanketo, M., **Peltoketo, A.**, Hartonen, K., Hiltunen, R. & Riekkola, M.-L. (2002). Extraction of sage (*Salvia officinalis* L.) by pressurized hot water and conventional methods: antioxidant activity of the extracts. *Eur Food Res Tech* 215:158-163.
- II Dorman, H.J.D., **Peltoketo, A.**, Hiltunen, R. & Tikkanen, M.J. (2003). Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chem* 83:255-262.
- III **Nurmi, A.**, Nurmi, T., Mursu, J., Hiltunen, R. & Voutilainen, S. (2006) Ingestion of oregano extract increases excretion of urinary phenolic metabolites in humans. *J Agric Food Chem* 54:6916-6923.
- IV **Nurmi, A.**, Nurmi, T., Hiltunen, R., Tikkanen M.J. & Voutilainen, S. Antioxidant properties of phenolic constituents of oregano and their human metabolites *in vitro*. Submitted.
- V **Nurmi, A.**, Mursu, J., Nurmi, T., Nyssönen, K., Alfthan, G., Hiltunen, R., Kaikkonen, J., Salonen, J.T. & Voutilainen, S. (2006) Consumption of juice fortified with oregano extract markedly increases excretion of phenolic acids but lacks short- and long-term effects on lipid peroxidation in healthy nonsmoking men. *J Agric Food Chem* 54:5790-5796.

In addition, some unpublished results are presented.

1. INTRODUCTION

Oxygen is essential for the life of all higher eukaryotic organisms. However, even in the very fundamental metabolic reactions occurring in the cells, reactive forms of oxygen – so called free radicals- capable of damaging various to biomolecules such as proteins, deoxyribonucleic acid (DNA) or polyunsaturated fatty acids are formed (Davies, 1995). In addition to the normal production of free radicals in cell respiration and metabolism, excessive amounts of reactive oxygen species (ROS) can be formed and elevated oxidative stress caused due to a number of reasons such as smoking, inflammation, strong physical activity, ischemia or trauma. The lack of necessary protective nutrients or other antioxidant substances in diet or certain hereditary pathological conditions may also affect negatively the pro-oxidant – antioxidant balance in the body. If the amount of free radicals and oxidative stress exceeds the capacity of body's antioxidant defence system, oxidative damage may result. The oxidation of lipids, carbohydrates, proteins and nucleic acids in the body has been linked e.g. with cardiovascular and neurological diseases, chronic inflammations and cancer (Kehrer, 1993; Gutteridge & Halliwell, 1994; Mariani *et al*, 2005; Valko *et al*, 2006; Halliwell, 2007). Even though the causality between oxidative stress and diseases is not clear (Gutteridge & Halliwell, 1994; Stocker & Keaney, 2004; Halliwell, 2007), free radicals are produced in greater amounts during several human diseases and types of tissue injury. Probably one of the most studied effects of oxidative stress on biomolecules is the oxidative modification of low-density lipoprotein (LDL) (Steinberg *et al*, 1989), role of which in the aetiology of atherosclerosis is well recognised. Today, antioxidants and their proposed beneficial effects on health have become an issue that interests, in addition to researchers of the field, conscious public worldwide.

In order to maintain the essential antioxidant - pro-oxidant balance, human body is protected against oxidative stress by a complex antioxidant system. Antioxidants delay or prevent oxidative events (Halliwell, 1994a), and do not initiate further oxidative processes during their action (Bors *et al*, 1990; Galati *et al*, 2002). Protective mechanisms in the human body suppress the production of free radicals and other reactive species, scavenge these species, chelate transition metals needed to convert poorly reactive species into more reactive ones, repair damage to the target molecules or destroy badly damaged molecules and replace them with new ones (Gutteridge & Halliwell, 1994). The antioxidant defence system consists of a

network of enzymatic and nonenzymatic as well as endogenous and exogenous factors acting synergistically (Masella *et al*, 2005). Of the enzymatic factors, e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase scavenge oxidising species, whereas proteins such as albumin, ceruloplasmin and transferrin participate in the antioxidant defence by binding transition metal ions iron and copper. Another type of enzymatic antioxidant action is to detoxify secondary products from reactions of ROS with macromolecules. Of the non-enzymatic antioxidants, α -tocopherol (often also called Vitamin E, even though Vitamin E comprises also other related substances) is of crucial importance in protection of lipophilic molecules (Gutteridge & Halliwell, 1994). Due to its effective radical scavenging capacity and molecular properties, it powerfully protects cell membranes from oxidation. Also lipophilic ubiquinol-Q10 and probably carotenoids have an antioxidative role in the body.

In human serum and other extracellular fluids, hydrophilic antioxidants participate in protection against oxidative damage. Ascorbic acid, also called Vitamin C, acts as a free radical scavenger and probably recycles α -tocopherol back to its active form. Also uric acid/urate and bilirubin contribute to the antioxidant capacity of plasma. Vitamins C and E and carotenoids cannot be synthesized in human body, and must thus be derived from diet. Another class of dietary antioxidants is the diverse group of phenolic compounds, synthesised as secondary metabolites in plants. During the recent years, the epidemiological evidence on the health-beneficial effects of diets rich in polyphenols (Bazzano *et al*, 2003; Hung *et al*, 2004; Mink *et al*, 2007), has triggered wide scientific interest to polyphenol research. Even though findings are still not conclusive (Scalbert *et al*, 2005), they suggest high intake of flavonoids to be linked with decreased risk of CVD (Huxley & Neil, 2003; Arts & Hollman, 2005). As flavonoids are present in same dietary sources with other polyphenols, this finding might concern other classes of polyphenols as well. Oxidative damage is associated with and may be involved in the pathogenesis of major age-related diseases, due to which the mechanism behind the health-protective action of phenolic compounds has been proposed to be linked to their antioxidant action (Halliwell *et al*, 2005; Scalbert *et al*, 2005); a number of *in vitro* and animal studies on the effects of phenolic compounds favour the suggested antioxidant hypothesis. Based on this antioxidant theory, various polyphenol supplementation studies have been carried out in humans in order to provide proof for the suggested mechanism of action. A part of these studies have showed antioxidant effects on some parameters used, whereas others have reported

the lack of effects (Halliwell *et al*, 2005; Scalbert *et al*, 2005; Williamson & Manach, 2005). The most studied sources of polyphenols have been tea, red wine and cocoa (Williamson & Manach, 2005). In addition to the antioxidant hypothesis, also other pertinent mechanisms behind the health beneficial effects of action have been proposed. These include effects on the cardiovascular system as well as complex regulation of cell functions (Halliwell, 2007); however, this thesis is based on and focuses on the antioxidant hypothesis.

Lamiaceae herbs, such as oregano and thyme, are part of a typical Mediterranean kitchen and diet. Especially oregano is consumed world-wide in pizza seasonings, and as pot herbs are today available in shops around the year, the regular consumption of fresh herbs is increasing also in Finland. In addition to their culinary properties, Lamiaceae herbs are a rich source of potentially health-beneficial antioxidant polyphenols (Dragland *et al*, 2003). Even though their antioxidant properties *in vitro* are acknowledged, studies on their effects on humans are scarce. In the present work, selected Lamiaceae herbs with a common culinary usage were studied for their antioxidant properties *in vitro* and in humans. After assessing the *in vitro* antioxidant properties of sage, rosemary, thyme and two oregano species, the issues of bioavailability, metabolism and potential antioxidant action of oregano extracts in humans were studied using a variety of parameters reflecting antioxidant status as well as lipid peroxidation.

2. REVIEW OF THE LITERATURE

2.1. Free radicals and lipid peroxidation

2.1.1. What are free radicals?

Free radicals are atomic or molecular species capable of independent existence that contain one or more unpaired electrons in their outer orbital (Gutteridge & Halliwell, 1994). This chemical character makes free radicals highly reactive. The reactivity varies from one free radical to another and is dependent on the environment they are presented in. As most free radicals are derived from molecular oxygen, they are usually referred to as ROS. Also reactive nitrogen and chlorine species of clinical significance exist, and some species without real radical nature i.e. unpaired electrons are usually included in the concept of free radicals due to their reactive nature, for example hydrogen peroxide (H₂O₂). Selected radical and nonradical oxidants with significance *in vivo* are presented in Table 1. The species considered to be the major contributors to oxidative stress in the human body (Huang *et al*, 2005) are marked with an asterisk (*).

Table 1. Selected radical and nonradical oxidants with significance *in vivo*.

Name	Chemical formula
Superoxide radical*	O ₂ ^{•-}
Hydroxyl radical*	OH [•]
Peroxynitrite*	ONOO ⁻
Nitric oxide radical	NO [•]
Nitrogen dioxide radical	NO ₂ [•]
Alkoxy radical	RO [•]
Peroxy radical*	ROO [•]
Hydrogen peroxide*	H ₂ O ₂
Hypochlorous acid	HOCl
Singlet oxygen*	¹ O ₂

2.1.2. Sources of free radicals and other reactive species

The metabolism of every aerobic organism produces ROS. Endogenously, they are mainly formed as by-products of electron transport chains of cell perspiration in mitochondria (Halliwell & Gutteridge, 1990a). Formation of ROS also occurs in different enzymatic reactions of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and other oxidising enzymes in phagocytes, fibroblasts, smooth muscle cells and endothelial cells (Halliwell, 1994a; Stocker & Kearney, 2004). Phagocyte NADPH oxidase generating superoxide radicals is the most significant source of ROS production in the vascular system; xanthine oxidase, myeloperoxidase and nitric oxide synthases are examples of other enzymes that produce reactive oxygen and nitrogen species in human body. Autoxidation reactions of molecules such as catecholamines, tetrahydrofolates, quinones, thiols or flavins (Halliwell, 1994a) also produce free radicals capable of initiating oxidative events. Lipoxxygenases and cyclooxygenases are involved in the synthesis of leukotrienes and prostaglandins, which have functions e.g. in mediating inflammation and regulating vascular tone and platelet aggregation; some of these enzymes are capable of oxidizing LDL (Stocker & Kearney 2004). Transition metals iron and copper catalyse oxidative reactions in the human body, and even if the amounts of free transition metal ions in body are low, iron stored in heme, hemoglobin or myoglobin has potential to initiate oxidative reactions (Halliwell, 1994b; Rice-Evans, 1994).

Exposure to ischemia, excessive exercise and infections are also conditions that can result in increased production of ROS. Exogenous exposure for ROS results e.g. from ionising (ultraviolet [UV], visible, thermal) radiation, excessive availability of transition metals or oxygen, certain drugs (e.g. paracetamol), toxic chemicals (e.g. carbon tetrachloride), cigarette smoke, excessive use of alcohol, air pollutants, herbicides (Gutteridge & Halliwell, 1994; Rice-Evans, 1994) or oxidised foods or beverages (Shahidi, 1997). The dietary burden of ROS results from peroxidised fatty acids or oxysterols that are absorbed in chylomicrons and finally incorporated into endogenous lipoproteins, or the oxidized dietary lipids may initiate oxidative cascades in the intestinal mucosa, resulting in oxidation of chylomicron lipids (Botham *et al*, 2005). The endogenous burden of ROS is, however, considered to be of far greater significance as compared to ROS from outside the body. The reactive species occurring most commonly in human is superoxide radical, $O_2^{\cdot-}$.

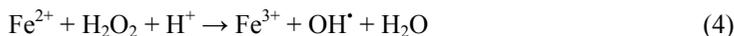
The primary source of ROS, in the form of superoxide, is the NADPH oxidase complex that catalyses the one-electron reduction of molecular oxygen (equation 1). The electrons required for production of $O_2^{\cdot-}$ are supplied by NADPH (eqn. 2):



$O_2^{\cdot-}$ itself has a rather low reactivity, but is capable of reacting further and dismutating to H_2O_2 (eqn. 3):



Even though H_2O_2 itself is not highly reactive, it is diffusible and in presence of transition metals copper or iron in tissues or body fluids can give rise to formation of hydroxyl radicals (OH^{\cdot}), according to the so-called Fenton reaction (eqn. 4):



The formation of OH^{\cdot} is thus dependent on the availability of Fe^{2+} ions. It is also possible that products from this reaction react further (eqn. 5), resulting in formation of reactive ferryl ion:



Another reaction resulting in formation of hydroxyl radical takes place between superoxide and hydrogen peroxide in the presence of metal catalysts iron or copper (the Haber-Weiss –reaction, eqn. 6):

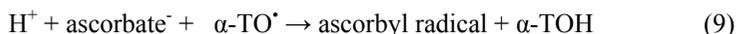


Oxidised transition metal ions (Fe^{3+} , Cu^{2+}) may also react with $\text{O}_2^{\cdot-}$ and be reduced (eqns. 7 and 8):



This capacity of $\text{O}_2^{\cdot-}$ to reduce iron and copper to forms that catalyse oxidative reactions is the main cause for its deleteriousness. OH^{\cdot} radical again is the most reactive oxygen species, and its capability to react with practically any biomolecule at high, diffusion-controlled reaction rate makes it extremely toxic (Halliwell, 1994a). It potently oxidises lipids e.g. in cell membranes affecting their permeability characters and in lipoproteins, resulting in changes in the particle surface structure and thus altering its metabolism in the body.

Radical action is often perceived only as oxidizing, i.e. a radical abstracts or accepts a single electron from a nonradical. Radicals can, however, also act as reducing agents thus donating a single electron to a nonradical (Stocker & Keaney, 2004). One of the main parameters governing the action of radicals is their reduction potential, which means the feasibility of one compound to chemically reduce another compound. Reduction potentials have been established for a number of biologically relevant compounds in order to predict the direction of reactions (Buettner, 1993). Difference in this thermodynamic parameter also offers an explanation for the recycling of α -tocopheryl radical ($\alpha\text{-TO}^{\cdot}$) back to its active form by ascorbate (eqn. 9):



Originally, free radicals and other reactive species were believed to have their key role in the microbe defence. However, it is now known that they are also produced in the body in other contexts related to normal physiological processes via cell

signalling (Seifried *et al*, 2007). To fulfil the criteria for a cell signalling factor, ROS must be produced in cells as they are stimulated to do so i.e. the production is purposeful, have an action either in the cell where it is produced or a cell nearby, and to be removed to quench or reverse the signal (Hancock *et al*, 2001). The role of ROS depends on the species; OH[•] radicals are extremely detrimental to biomolecules whereas at least H₂O₂ and O₂^{•-} act as cell signalling factors. NADPH oxidase was first found in neutrophils producing superoxide as a response to microbial attack, but more recently it has also been shown to be present in cells with no role in host defence (Jones & Hancock, 2000), such as endothelial cells (Jones *et al*, 1996), mesangial cells (Radeke *et al*, 1991) and osteoclasts (Steinbeck *et al*, 1994). These cells produce levels of ROS remarkably lower as compared to phagocytic cells, and the location and distribution of these cells in the body is unexpected in terms of host defence, suggesting that the proposed role in signalling is justified. In resting cells, no NADPH activity is detected, and the activity is tightly controlled by several mechanisms (Jones & Hancock, 2000). This strengthens the hypothesis of ROS having a key role in signalling cascades (Hancock *et al*, 2001).

2.1.3. Lipid peroxidation

If the amount of ROS exceeds the capacity of the antioxidant defence system in the body, oxidative stress results and severe cellular damage may occur (Halliwell & Cross, 1994). In unfavourable circumstances, free radicals are capable of reacting with various biomolecules and inducing oxidative damage e.g. to proteins, nucleic acids, carbohydrates and lipids. In this thesis, the discussion is focused on oxidation of lipids and diseases with pathogenesis linked to oxidative processes. To clarify the terminology in the thesis, terms “oxidation” and “peroxidation” will be used synonymously. This is despite the fact that sometimes the term “peroxidation” is limited to use in oxidative processes induced by a peroxy moiety (Kehrer & Smith, 1994). Lipid peroxidation has been defined as the oxidation of polyunsaturated fatty acids (PUFA) by a radical-induced chain reaction (Frei, 1996) to form a lipid hydroperoxide (ROOH) which then reacts further. Oxidative chain reactions may occur in biological membranes or different lipoprotein particles circulating in blood. Of all lipoproteins, LDL is of the main interest in this study.

Chain reaction of lipid peroxidation

As free radicals react with nonradicals, new radicals are generated. Hence, free radical reactions tend to proceed as chain reactions. The chain reaction –like process of lipid peroxidation is divided into three stages: initiation, propagation and termination. The reaction may be initiated by action of transition metals iron or copper, oxidative intracellular enzymes such as lipoxygenases or myeloperoxidases, or free radicals. In the following, the free radical –initiated cascade of lipid peroxidation is discussed more in detail.

The oxidation reaction initiates, as free radicals or ROS abstract hydrogen atoms from the fatty acid side chain (RH) of PUFA and carbon-centered radicals are formed (eqn. 10) (Halliwell, 1995):



PUFA molecules have more than one double bond in their carbon chain, which facilitates the abstraction of hydrogen by reactive species. The rate of oxidation of fatty acids increases in relation to their degree of unsaturation. The process of oxidation proceeds to propagation phase, as carbon-centered radicals formed in the initiation phase react with oxygen as follows (eqn. 11):



The peroxy radicals (ROO^{\bullet}) formed can attack adjacent fatty acid side chains, resulting in formation of ROOH (eqn. 12):



ROOH may be decomposed into lipid alkoxy (RO^{\bullet}) and ROO^{\bullet} radicals in the presence of catalysing transition metals, inducing further oxidative damage.

The on-going chain reaction of oxidation is terminated when either PUFAs feasible to oxidation or oxygen supply are extinguished, or when the radicals formed are present in such excess that they start quenching each other (eqn. 13):



Diseases linked with oxidative stress

In 1956, the process of ageing was for the first time suggested to be linked to the oxidation of biomolecules (Harman, 1956). Today, the evidence on the role of free radicals in ageing is strong (Rahman, 2007; Viña *et al*, 2007). Oxidative damage is connected with and probably has a role in the aetiology of several important age-related diseases such as Alzheimer's disease and Parkinson's disease, cataract and cardiovascular diseases (Davies, 1995; Mariani *et al*, 2005), which has led to the assumption that antioxidants such as vitamins C and E, and based on their epidemiologically observed health-protective action, also foods rich in phenolic compounds, could have a role in protection against these diseases (Halliwell *et al*, 2005; Scalbert *et al*, 2005). In this thesis, further discussion is limited to oxidation of lipids, which plays a crucial role in the aetiology of cardiovascular diseases (CVD). The emphasis is put on its potential role in the pathogenesis, and in the other hand, prevention of CVD.

Cardiovascular diseases and the role of oxidised LDL in atherosclerosis

CVD are the major cause for morbidity and mortality in the developed countries. Even though the cardiovascular health programme carried out in Finland since late 1970's has succeeded in decreasing the general risk factor level of the population (Vartiainen *et al*, 2000), in 2006, CVD still accounted for more than 40% of all deaths in Finland (Statistics Finland, 2006). Within the variety of pathological conditions under the classification of CVD, coronary heart disease (CHD) and stroke were of the major significance, with 25% and 9% death causality, respectively. As the lifetime expectancy increases, the cost of CVD in the health care increases and reduces the available resources for other health priorities. The major and independent risk factors for CVD are male gender, ageing, smoking, hypertension, high serum total and LDL cholesterol, low serum high-density

lipoprotein cholesterol and diabetes (Grundy *et al*, 1999). Also genetic factors make a strong contribution to the risk of CVD. The pathological processes linked to the development of CVD begin already in the childhood (Kuller, 2006). Low physical activity, obesity, high intake of saturated fatty acids, cholesterol and salt, low intake of ω -3 and ω -6 PUFA and low intake of fiber and vegetables increase the risk of CVD (Dietary Guidelines Advisory Committee, 2005). The choices, by which an individual may beneficially modify the risk of CVD, are linked to healthier life-style and dietary habits.

According to the widely accepted theory of “oxidative modification hypothesis”, the oxidation of PUFAs in LDL particles has an important role in the initiation and progression of atherosclerosis (Steinberg *et al*, 1989; Witztum, 1994; Berliner & Heinecke, 1996; Stocker & Keane, 2004). The level of LDL, or more specifically, the level of apolipoprotein B (apoB), and the number of LDL particles are important determinants of the development of atherosclerosis (Kuller, 2001). Human atherosclerosis is an arterial disease, which slowly progresses predominantly in the medium size arteries such as the coronary, carotid and vertebral arteries, but also in the aorta and arteries supplying the lower extremities (Clarke & Armitage, 2002). The main site for LDL oxidation has been assumed to be in the arterial intima, and only to a small extent in plasma (Witztum, 1994). In the early stage of LDL oxidation, *in vitro* evidence shows minimally oxidized LDL to induce production of monocyte chemotactic protein, resulting in recruitment of inflammatory cells and increased production of ROS (Stocker & Keane 2004). The oxidation is initiated by free radicals, and catalysed by transition metal ions (Pinchuk & Lichtenberg, 2002). At the early stage of oxidation, the surface structure of LDL particle remains intact; as the oxidation proceeds, apoB-100 on the LDL surface is modified. Most of LDL oxidation occurs following the entry of LDL particles into the arterial intima when these particles lose the protection of water-soluble antioxidants present in plasma. Oxidized LDL is recognized by scavenger receptors of macrophages residing in the arterial intima and avidly internalized into these cells. Unlike LDL receptors, scavenger receptors are not regulated by any negative feedback mechanism, and the macrophages continue to internalize oxidized LDL until they become lipid-laden foam cells, and finally burst resulting in accumulation of cholesterol in the artery wall. This initiates the formation of the atherosclerotic plaque. The plaques consisting of cholesterol, calcium, proteins and other components gradually obstruct the arteries. The fragility of these plaques may lead to ruptures and, consequently, to formation of a

blood clot obstructing the artery. As the thrombus propagates, it, combined with vasoconstriction of the artery, leads to total blockage of the blood flow resulting in damage of the heart or brain i.e., heart attack or stroke. The theory of oxidised LDL (ox-LDL) being of crucial significance in the pathogenesis of CVD is supported by the findings showing that ox-LDL is present in atherosclerotic lesions both in animals and humans (Stocker & Keaney, 2004). Also other mechanisms related to oxidative stress may contribute to the progression of atherosclerosis: for example, superoxide radicals inactivate nitrogen oxide and thus enhance proatherogenic mechanisms such as impaired vasodilatation, platelet aggregation and leukocyte adherence to endothelium (Landmesser & Harrison, 2001).

2.2. ANTIOXIDANTS

2.2.1. What are antioxidants?

From a biological point of view, antioxidants have been defined as substances that in concentrations lower compared to the substrate susceptible to oxidation are capable of delaying or inhibiting oxidative processes (Halliwell, 1994a). In this definition, “substrate” refers to any oxidisable molecule *in vivo*: lipids, proteins, carbohydrates and DNA. As antioxidants react to protect biological targets from oxidation, they are themselves oxidised. The stability of an oxidised antioxidant molecule is essential, as to prevent oxidation from progressing, oxidised antioxidants must retain low reactivity towards biomolecules (Bors *et al*, 1990). The use and meaning of term antioxidant depends on the scientific discipline: for example, in food sciences, antioxidants comprise both substances used to preventing fats in foods from becoming rancid as well as dietary antioxidants. In this thesis, a biological perspective on antioxidants is adopted.

2.2.2. Mechanisms of antioxidant defence in humans

Cells in the human body are throughout prone to attacks of ROS and resulting oxidative reactions. If the pro-oxidant-antioxidant balance of the human body is disturbed due to excessive oxidative stress or inadequate antioxidant defense, lipids, carbohydrates, proteins and nucleic acids are exposed to oxidative damage. Balance between oxidative stress and protection by body’s antioxidant defence system is a vital condition for the health of human tissues and organisms. Human body uses all of

the following antioxidant mechanisms: scavenging oxidizing species, either by enzymes or by direct chemical reactions; minimizing the formation of oxygen-derived and other reactive species, binding metal ions needed to convert poorly reactive species such as H_2O_2 or $\text{O}_2^{\cdot-}$ into highly reactive ones; repairing damage to the target and destroying badly damaged target molecules and replacing them with new ones (Gutteridge & Halliwell, 1994).

Radical scavenging enzymes SOD, catalase, glutathione peroxidase, thioredoxin reductase and peroxiredoxin provide the first-line defence against oxidizing species (Masella *et al*, 2005). The antioxidant function of SOD found both intra- and extracellularly is based on its ability to catalyse the dismutation of $\text{O}_2^{\cdot-}$ into oxygen and H_2O_2 . As $\text{O}_2^{\cdot-}$ is one of the major pro-oxidants in human body, SOD capable of reacting rapidly with ROS accounts for antioxidant function of great significance *in vivo*. Catalase functions via decomposing excessive H_2O_2 to oxygen and water by an extremely rapid turnover rate. Glutathione peroxidase, found practically in all human tissues, comprises a family of enzymes, which reduce ROOH to their corresponding alcohols and H_2O_2 to water. Glutathione peroxidase, as well as glutathione *S*-transferase, aldo-keto reductase and aldehyde dehydrogenase, also detoxify secondary products from reactions of ROS with macromolecules, whereas proteins albumin, ceruloplasmin and transferrin chelate transition metal ions. Imbalance of micronutrients such as copper or selenium in human body may lead to dysfunction of enzymatic antioxidant defense, as these substances have a role in antioxidant defense as antioxidant enzyme cofactors. Also substances that inhibit oxidative enzymes, for example cyclooxygenase, act as antioxidants in the body. Even though the harmful effects of a number of radicals are counteracted by enzymatic antioxidants, no enzymatic action is known to scavenge ROO^{\cdot} , OH^{\cdot} , $^1\text{O}_2$ or ONOO^{\cdot} , reactive species which are, together with $\text{O}_2^{\cdot-}$ and H_2O_2 , among the six major factors causing oxidative damage in human body (Huang *et al*, 2005). For this reason, effective radical scavenging by nonenzymatic antioxidants is fundamental to complement the defense system.

All human cell membranes consist of phospholipid bilayers with large number of PUFA molecules prone to oxidative attacks, damage of which leads to detrimental alterations in membrane fluidity and function. Thus, antioxidant protection of the lipid phase is of crucial significance in human body. The most important of lipophilic antioxidants in human body is vitamin E. It comprises multiple isomers of four tocopherols and four tocotrienols, of which α -tocopherol is of major

importance (Halliwell *et al*, 2007). Molecules of all isomers contain a chromanol ring with hydroxyl group, and a hydrophobic side chain, which allows the molecules to fuse into biological membranes. The hydroxyl group attached to chromanol ring is capable of donating a hydrogen atom or an electron and thus scavenging radicals. Vitamin E protects biomembranes from oxidation by an effective chain-breaking antioxidant action, maintaining normal permeability characters and cellular composition (Gutteridge & Halliwell, 1994; Morrissey & Sheehy, 1999). During the radical-scavenging action, α -tocopherol is converted to a radical, i.e. consumed. Interestingly, even though the antioxidant action and the epidemiological inverse association of vitamin E and CVD are well recognised, recent clinical trials have failed to show detectable effects of lipid peroxidation parameters or mortality (Clarke & Armitage, 2002).

Another compound capable of protecting the membranes of human body is ubiquinol, also called coenzyme Q-10. Ubiquinol has its key role in the mitochondrial electron transport chain, but may also act either by direct chain-breaking radical scavenging or by regenerating α -tocopherol radicals back to functional α -tocopherol (Gutteridge & Halliwell, 1994). Also lipophilic carotenoids, especially β -carotene, have been suggested to participate in the antioxidant protection of human body. Carotenoids are antioxidants *in vitro*, and high plasma β -carotene concentrations have been associated with a decreased risk of CVD; however, large scale clinical trials have failed to show antioxidant effects in human and in fact, even pro-oxidant effects at higher dosages have been reported (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994; Voutilainen *et al*, 2006).

Of the hydrophilic non-enzymatic antioxidants, vitamin C, or ascorbic acid, and glutathione are most probably the two with major significance. Ascorbic acid has several metabolic roles in the human body, but is also widely thought to be an important antioxidant *in vivo*, both directly and concurrently with vitamin E. It can reduce radicals from a variety of sources, and also appears to participate in recycling vitamin E radicals (see eqn. 9). It is, similarly to vitamin E, readily absorbed from the human gastrointestinal (GI) tract, and human body has evolved mechanisms for taking up ascorbate and transporting it into cells (Halliwell, 1999). Glutathione is a tripeptide (glutamyl-cysteinyl-glycine) with a reactive thiol group, which provides an abundant target for radical attacks. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving

glutathione reductase and the electron acceptor NADPH. Glutathione also is capable of regenerating α -tocopherol radicals back to their active form. Also uric acid/urate and bilirubin act as extracellular scavengers of different reactive species in the body fluids; uric acid may also chelate transition metal ions whereas bilirubin has the potential to be involved in recycling of α -tocopherol radical (Stocker & Keaney, 2004).

Of the abovementioned factors of human antioxidant defence, vitamins E and C or carotenoids cannot be synthesized in the human body and must thus be obtained from dietary sources. In dietary plants and plant-derived foods and beverages, these components as well as a wide variety of other phytochemicals - minerals, dietary fibre, phytosterols and phenolic compounds - are administered to humans. They have been suggested to exert health-beneficial properties and to be associated with decreased risk of various diseases (Meltzer & Malterud, 1997). At the moment, the antioxidant hypothesis suggests also dietary polyphenols to be involved in the network of biologically relevant antioxidants. In chapter 2.3., these phytochemicals and their significance to humans is discussed in more detail.

2.2.3. Assessment of antioxidant capacity and lipid peroxidation

The interest focused on antioxidants and their action has resulted in the development of an array of methods to assess antioxidant capacity and lipid peroxidation. Of the methods available today, none alone provides enough information to assess or characterize the whole process of oxidation, and can thus be referred to as the ultimate method of choice. Differences in methods and their modifications used, lack of adequate chemical characterization of samples tested, lack of usage of robust standards as well as differences in the interpretation of results obtained have lead to a situation, in which comparison and pooling of results from different published studies is very difficult, if not unfeasible. Stating that a certain sample tested is a good antioxidant is meaningless without separating out the methods used (Gutteridge & Halliwell, 1994). Only providing of the specific conditions such as temperature, possible coreactants, reaction media and reference points enables discussing on the antioxidant activity of a sample or a substance (Huang *et al*, 2005). For the abovementioned reasons, consensus on the necessity of standardizing the use of antioxidant methodologies is rising. In the following, selected methods for assessing antioxidant capacity and lipid peroxidation are briefly described with limitation to methods considered as potential reference

methods or otherwise relevant in the context of this study. The activities of antioxidant enzymes such as blood/plasma glutathione peroxidase, SOD or catalase have also been used to evaluate the effect of dietary antioxidants or antioxidant supplementation; however, this approach is excluded from this methodological discussion.

The antioxidant capacity of foods, botanicals, dietary supplements as well as human samples such as plasma or urine has been evaluated using different methods reflecting their free radical scavenging or reducing capacity. Based on evaluation of data presented in antioxidant-related congresses and journal articles, some of these methods have recently been proposed to be considered for standardization, or as reference methods, when assessing antioxidant capacity. In their expert review, Prior *et al* (2005) state that a standardized method for testing antioxidant capacity should fulfil the following requirements: measuring chemistry that actually occurs in potential applications, employing a biologically relevant source of radicals, simplicity, defined endpoint and known chemical mechanism, reasonable instrumentation, good reproducibility, adaptable for testing antioxidants of varying partition coefficient as well as adaptable for high-throughput approach analyses. Also, the methods suggested to be used as standards in measuring antioxidant activity need to have been used for a sufficient time and in a number of laboratories, establishing their strengths and weaknesses. Consequently, Prior *et al* suggest the following assays as methods of choice for standardizing assessment of antioxidant capacity: the oxygen radical absorbance capacity (ORAC) assay, the total radical trapping capacity parameter (TRAP) assay, the Folin-Ciocalteu (FC) method and possibly the Trolox equivalent antioxidant capacity (TEAC) assay. Of these assays, Huang *et al* (2005) suggest in their review FC method as the method of choice for the determination of reductive capacity, and ORAC assay as the standard method for determining the peroxy radical scavenging capacity of a sample.

ORAC and TRAP assays measure the inhibition of peroxy radical mediated oxidation thus reflecting the chain breaking action of an antioxidant (AH) by hydrogen atom transfer mechanism (eqn. 14):



The FC method measures the reductive capability of a sample by single electron transfer mechanism (eqn. 15):



TEAC assay reflects the radical scavenging capacity of a sample against 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) anion radical (ABTS^{•+}) by single electron transfer mechanism. In this assay, ABTS is oxidized resulting in formation of ABTS^{•+} radical, and the radical scavenging action of a sample is determined based on decrease in radical absorbance at 734nm. The TEAC value for the sample is calculated from a Trolox[®] calibration curve. A number of modifications of this method have been established, presented also on names other than TEAC, but they all are based on detecting the scavenging capacity against ABTS^{•+} radical.

In addition to TRAP, FC and TEAC methods described above, DPPH[•] radical scavenging assay, Fe³⁺-ethylenediamine tetraacetic acid (EDTA)/H₂O₂/ascorbate-catalysed deoxyribose oxidative degradation assay for determining the OH[•] scavenging activity, Fe³⁺ to Fe²⁺ reduction capacity assay by thiocyanate method, copper-induced LDL oxidation assay, copper-induced serum oxidation assay, LDL baseline conjugated dienes assay and determination of plasma F₂-isoprostane formation were employed in this study.

The DPPH[•] radical scavenging assay reflects the capacity of a sample to reduce this nitrogen centered radical mainly by single electron transfer mechanism; the hydrogen atom transfer mechanism has supposedly a marginal role (Huang *et al*, 2005). In the deoxyribose oxidative degradation assay, OH[•] radicals are produced in aqueous reaction solution containing FeCl₃ and H₂O₂ by Fenton chemistry (see chapter 2.1.2). Ascorbate and EDTA are included in the reaction mixture for their metal chelating properties. To assess the degree of deoxyribose degradation, the oxidative degradation product malonaldehyde is condensed with thiobarbituric acid, and quantified by spectrophotometrical measurements at 532 nm (Halliwell *et al*, 1987). Antioxidants capable of OH[•] radical scavenging act by hydrogen atom transfer mechanism. Fe³⁺ to Fe²⁺ reduction capacity assay (thiocyanate method) is based on determination of capacity of a sample to reduce Fe³⁺ to Fe²⁺ by single electron transfer mechanism.

The *in vitro* (also referred to as *ex vivo*) oxidation of LDL is most often initiated by copper ions (Esterbauer *et al*, 1989), but also iron or azo compounds producing hydroxyl radicals at a constant rate (Noguchi *et al*, 1993), or oxidative enzymes

may be used (Noguchi & Niki, 1998). The oxidation resistance of unsaturated fatty acids in human LDL particles either without addition of antioxidant or with addition of the antioxidant sample is determined by continuously measuring the formation of conjugated dienes at 234 nm, and most often, the antioxidant activity is determined as the lag-phase prior to the maximal rate of oxidation. Antioxidants in this assay may act by inhibiting the onset of oxidation and/or decreasing the maximal rate of oxidation (Pinchuk & Lichtenberg, 2002). The determination of human serum resistance against copper-induced oxidation reflects the *in vivo* antioxidant capacity of the whole serum, whereas the so-called LDL baseline conjugated diene method has been developed to indicate the state of non-induced LDL oxidation *in vivo* (Ahotupa & Vasankari, 1999).

F₂-isoprostanes, products of nonenzymatic oxidation of arachidonic acid, are chemically stable and are thus possible to determine in plasma or urine, in which they are finally excreted. Increased concentrations of these substances have been reported in association with risk factors for CVD: smoking, diabetes and hyperlipidemia (Patrono & FitzGerald, 1997). Most often they are analysed from human samples using gas chromatographic – mass spectrometric methods. Also F₃- and F₄-isoprostanes, arising from peroxidation of eicosapentaenoic acid residues and docosahexaenoic acid residues, respectively, can be measured to determine the peroxidation of different PUFA residues *in vivo* (Halliwell, 1999).

2.3. PHENOLIC COMPOUNDS

2.3.1. What are phenolic compounds or polyphenols?

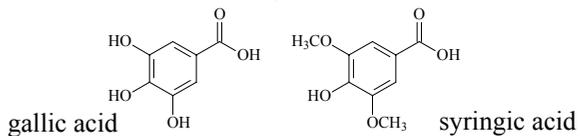
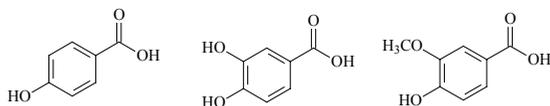
Phenolic compounds are plant secondary metabolites produced either from phenylalanine via the acetate pathway or from its precursor shikimic acid via the shikimate pathway (Harborne, 1989). Being widely distributed and synthesized throughout the plant kingdom, with exception of fungi and algae, phenolic compounds are one of the most numerous classes of substances. By 1993, they were known to comprise more than 8000 substances (Harborne, 1993). Even though the structural variety within this class of compounds is substantial, all phenolic compounds are characterised by the presence of a phenolic ring, i.e. a benzene ring substituted with a hydroxyl group. Although the literal reading of “polyphenolic” refers to a structure with more than one hydroxyl group, terms

“phenolic compounds” and “polyphenols” are most often used in parallel. For verbal flexibility, this practice also applies to this thesis.

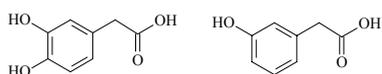
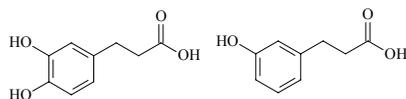
Phenolic compounds occur mainly in conjugated forms in plants, as conjugation of hydroxyl groups with sugars (most often glucose) increases the solubility of polyphenols and makes them suitable to be stored in plant vacuoles (Bravo, 1998). Polyphenols have several important physiological functions in plants: they are essential for the regulation of growth and participate in the reproduction by attracting pollinating insects, and also protect plants against UV radiation, harmful insects and infections (Harborne, 1989; Korkina, 2007). They can be divided into different subclasses as a function of the number of phenol rings and structural elements binding these rings to each other (Manach *et al*, 2004). Based on this classification, distinctions are made between the phenolic acids, flavonoids, stilbenes and lignans; the diversity is further expanded as polyphenols are associated with various carbohydrates and organic acids and with each other. The class of flavonoids, for example, may be further divided into 6 subclasses depending on the type of heterocycle in their molecular structure: flavonols, flavones, isoflavonoids, flavanones, flavanols (i.e. catechins) and anthocyanidins (Manach *et al*, 2004). Of the polyphenol subclasses, phenolic acids and flavonoids are the most common and have often been described to be the most important as regarding the intake and potential effects in humans (Bravo, 1998). As may be deduced by the large number of phenolic compounds and the discrepancy between the different subclasses, their chemical and biological properties vary greatly. Phenolic acids, the class of phenolic compounds with the main weight in this study, can be divided to those with a simple hydroxybenzoic acid structure (such as protocatechuic acid) and to those with hydroxycinnamic acid structure (such as caffeic acid or rosmarinic acid) (Manach *et al*, 2004). So far, the group of phenolic acids has been studied far less as compared to flavonoids. Especially hydroxybenzoic acids have not been considered to be of greater nutritional interest, as they are present in edible plants only in low amounts, with exception of some red fruits, berries, onions and cereals (Manach *et al*, 2004; Mattila *et al*, 2005; Mattila *et al*, 2006). Hydroxycinnamic acids are commonly found in plants, and representatives of this class, such as caffeic acid, chlorogenic acid and ferulic acid, have been studied more. This study and literature review focuses on phenolic acids with some discussion on flavonoids related to our work. As an example of different phenolic structures, the molecules of main interest in this study are shown in Figure 1. In addition to phenolic acids and their metabolite aglycones, flavonoids luteolin (a representative of flavone subclass) and eriodictyol (a

representative of flavanone subclass) identified from *O. onites* extract are presented as structural examples of the flavonoid class.

p-hydroxybenzoic acid protocatechuic acid vanillic acid

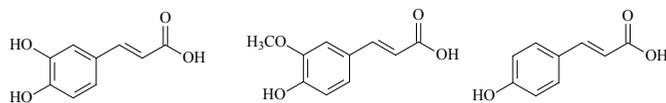
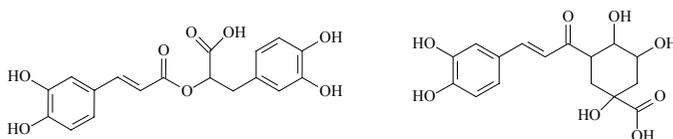


3,4-dihydroxyphenylpropionic acid *m*-hydroxyphenylpropionic acid



3,4-dihydroxyphenylacetic acid *m*-hydroxyphenylacetic acid

rosmarinic acid chlorogenic acid



caffeic acid ferulic acid *p*-coumaric acid

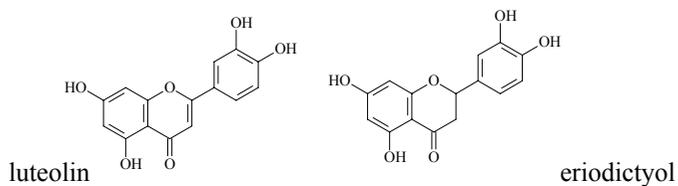


Figure 1. Phenolic compounds with the main interest in this study.

2.3.2. Phenolic compounds as antioxidants

The antioxidant potential of phenolic compounds has been shown in a number of *in vitro* studies. They are capable of direct chain-breaking antioxidant action by radical scavenging: in addition to being capable of scavenging of several non-physiological radicals such as DPPH• and ABTS^{•+} (Kosar *et al*, 2003; Payet *et al*, 2005; Cai *et al*, 2006), they are capable of scavenging a variety of reactive species such as superoxide, hydroxyl and peroxy radicals and hypochlorous acid with significance *in vivo* (Halliwell *et al*, 2005). They can also suppress the formation of reactive oxygen species by chelating transition metal ions capable of catalyzing oxidative reactions (Morel *et al*, 1994; Mira *et al*, 2002). It has been stated that without these catalysts the harmful event caused by free radicals would be much less (Halliwell *et al*, 1992); this points out the significance of antioxidants with transition metal chelating or reducing action. Furthermore, polyphenols inhibit oxidative enzymes such as lipoxygenases and cyclooxygenases involved in inflammatory reactions (Laughton *et al*, 1991; Sadik *et al*, 2003; O'Leary *et al*, 2004), thus suppressing the formation of free radicals. These mechanisms are classified as preventive antioxidant action. For example, the inhibitory or delaying action of polyphenols against oxidation in the LDL oxidation assay, seen as increased lag-time and/or decreased rate of LDL oxidation in the propagation phase relates to several mechanisms of antioxidant action. In addition to radical scavenging, metal chelation, reductive action and enhancing of hydroperoxide decomposition (Pinchuk & Lichtenberg, 2002), polyphenols have been suggested to inhibit the oxidation of LDL by binding certain proteins containing catalytic metal sites (Teissedre *et al*, 1996; Satué-Gracia *et al*, 1997).

In addition to having potential for independent antioxidant action, polyphenols have been suggested to spare essential antioxidants. Selected flavonoids have been shown to be able to reduce the ascorbyl radical, *i.e.* to protect vitamin C (Bors *et al*, 1995). The regeneration of α -tocopherol through reduction of the α -tocopherol radical has been suggested as one mode of action for polyphenols, for example caffeic acid (Rice-Evans *et al*, 1995, 1996; Nardini *et al*, 1995; Natella *et al*, 1999). In study of Nardini *et al* (1995), caffeic acid was also shown to protect β -carotene against oxidation. Also synergism between different antioxidants has been suggested; for example, in copper-induced oxidation of human LDL *in vitro*, combining phenolic compounds with each other resulted in synergistic protection against LDL lipid and protein oxidation (Yeomans *et al*, 2005; Cirico & Omaye,

2006), although also opposite result showing antagonism between phenolic compounds in the LDL assay have been reported (Meyer *et al*, 1998).

The direct antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical (equation 14 in 2.2.3.), and the stabilization on the phenoxyl radical by delocalization of unpaired electrons around the aromatic ring (Rice-Evans *et al*, 1996; Frankel & Meyer, 2000). The lower the bond-dissociation energy in the aromatic hydroxyl group, the faster will the reaction with a radical be (Wright *et al*, 2001). In addition to hydrogen donation, also single electron transfer mechanism (equation 15 in 2.2.3.) with ionisation potential as the crucial determinant for electron transfer capability is involved in the antioxidant action of polyphenols (Wright *et al*, 2001). However, hydrogen donation mechanism is considered to be more important for the radical scavenging action of most polyphenols (Wright *et al*, 2001). The radical scavenging capacity depends on the chemical structure of phenolic compound in question, affecting also its capacity to protect different biomolecules from oxidation. Even though details depend on the radical used, the following general structure-activity rules seem to apply for phenolic acids: 1) radical scavenging action requires a free hydroxyl group, 2) effective radical scavenging requires the presence of an aromatic *ortho*-dihydroxyl moiety, 3) radical scavenging activity is increased as the number of free hydroxyl groups increases, and 4) electron-donating substituent(s) such as methoxy group(s) next to aromatic hydroxyl group(s) enhance the hydrogen donation ability (Rice-Evans *et al*, 1996; Natella *et al*, 1999; Cai *et al*, 2006; Siquet *et al*, 2006). Of these requirements, the presence of an *ortho*-dihydroxyl moiety also applies to capability of transition metal chelation (Nardini *et al*, 1995; Natella *et al*, 1999). As a second hydroxyl group is introduced in the *ortho*-position in the phenol structure, the antioxidant effectiveness is increased due to the stabilisation of the phenoxyl radical through an intramolecular hydrogen bond (Rice-Evans *et al*, 1996). In radical scavenging, the effects of the alkyl spacer between the carboxylic acid group and the aromatic ring seem more complex. A double bond in the alkyl chain has been suggested to enhance the radical scavenging activity (Cai *et al*, 2006), whereas some other studies have reported the opposite (Silva *et al*, 2000; Siquet *et al*, 2006). In the work of Siquet *et al* (2006), the effect of alkyl chain length was also studied using DPPH[•] and ABTS^{•+} radicals, and the radical scavenging activity of benzoic acid derivatives was increased by addition of an alkyl chain. This is in accordance with earlier work of Rice-Evans *et al* (1996). An explanation for this

phenomenon is that the alkyl side chain stabilises the phenoxyl radical formed (Brand-Williams *et al*, 1995; Siquet *et al*, 2006).

In the case of flavonoids, the structural criteria for maximal radical scavenging are: 1) the *ortho*-dihydroxy structure in the B-ring, 2) the 2,3-double bond in conjugation with the 4-oxo function in the C-ring, and 3) the 3- and 5-hydroxy groups with the 4-oxo-function in the unsaturated C-ring. (Bors *et al*, 1990; Rice-Evans *et al*, 1996; Heijnen *et al*, 2002; Cai *et al*, 2006). If all these criteria are fulfilled, as in case of flavonols, a conjugated double bond system allowing electron delocalization in the molecule is formed, stabilising the radical and enhancing the antioxidant activity (Bors *et al*, 1990; Rice-Evans *et al*, 1996). In heterogeneous substrates, such as in foods or biological systems, the antioxidant action and structure-activity relationship become more complex, as the physico-chemical properties of the antioxidant, like lipophilicity, solubility and partition between lipid and aqueous phase become significant (Frankel, 1995). The structure-activity relationship is not only significantly affected by the test system used and the biological targets to be protected, but also by the modes of inducing oxidation and by the method used to determine oxidation. Also, most often a variety of antioxidant mechanisms are involved (Frankel & Meyer, 2000).

2.3.3. Phenolic compounds and cardiovascular health

Epidemiological evidence

During the past fifteen years, tens of epidemiological studies on the role of fruits, vegetables and polyphenols, especially flavonoids, have been published. The starting point for the renewed interest in phenolic compounds was the Zutphen Elderly Study, in which a relation between the high intake of flavonoids and the reduced risk of CVD was first established (Hertog *et al*, 1993a). Since then, several studies have detected an association between the consumption of polyphenol-rich foods or different polyphenols and the risk of cardiovascular diseases (Mennen *et al*, 2004; Andersen *et al*, 2006), myocardial infarction (Hirvonen *et al*, 2001; Geleijnse *et al*, 2002; Tavani *et al*, 2006), stroke (Keli *et al*, 1996; Knekt *et al*, 2002) or CHD and/or cardiovascular disease mortality (Hertog *et al*, 1995; Knekt *et al*, 1996; Yochum *et al*, 1999; Arts *et al*, 2001a; Mink *et al*, 2007). Also studies with no association have been reported (e.g. Arts *et al*, 2001b; Rimm *et al* 1996; Sesso *et al*, 2003; Lin *et al*, 2007), and for example in a study by Hertog *et al*

(1997), the intake of flavonols was associated with increased risk of all-cause mortality. Explanations for this discrepancy have been suggested to be found in possible confounding lifestyle factors. Some meta-analyses on cohort and case-control studies have also been carried out. In a meta-analysis of Peters *et al* 2001, including data from altogether 17 studies, moderate tea consumption decreased the risk of myocardial infarction by 11%. As Huxley & Neil (2003) made a meta-analysis of 7 studies, high intake of flavonols reduces the risk of CHD by 20%. So far, epidemiological studies assessing the cardiovascular protective effects of polyphenols have focused on flavonoids, especially flavonols and catechins, and to a lesser extent, catechin polymers i.e. procyanidins. The association between the intake of phenolic acids, compounds of the main interest in the context of this thesis, and cardiovascular diseases has not been studied. The source of phenolic acids most studied in epidemiological work is coffee. However, results from epidemiological studies on coffee have remained controversial. In a meta-analysis by Myers & Basinski (1992), including data from 11 studies, no association between coffee consumption and risk of CHD was found. A J-shaped association between coffee consumption and CVD has been reported (Panagiotakos *et al*, 2003; Happonen *et al*, 2004). Consumption of whole grain cereals has also been shown to protect against CVD (Flight & Clifton, 2006); however, the role of phenolic acids found in whole grains in the protection is currently not known.

Even though the data from epidemiological studies is not fully consistent, they seem to indicate that persons who consume more fruits and vegetables or other rich sources of polyphenols often have lower prevalence of important risk factors for CVD as well as decreased risk of developing CVD incidents such as CHD (Bazzano *et al*, 2003; Hung *et al*, 2004; Arts & Hollman, 2005; Manach *et al*, 2005). The biological mechanisms behind the protective effects of fruits, vegetables and other polyphenol rich foods are not entirely clear and are likely to be multiple. Many nutrients and phytochemicals in fruits and vegetables, including fiber, potassium, and folate, could be independently or jointly responsible for the apparent reduction in CVD risk. The low dietary glycemic load and energy density of fruits and vegetables may also play a significant role. However, support for the hypothesised antioxidant action and protective effects of phenolic compounds against cardiovascular diseases has been found in supplementation studies both in animals and in humans.

Supplementation studies on phenolic compounds

Supplements rich in flavonoids have been shown to have antioxidant effects in different animal models. For example, reduced progression of atherosclerosis in apolipoprotein E-deficient mice has been reported following consumption of red wine, quercetin or catechin, an effect associated with reduced susceptibility of LDL to oxidation (Hayek *et al*, 1997). Administration of green or black tea decreased the susceptibility of LDL to oxidation in rabbits (Tijburg *et al*, 1997). In study of Yamakoshi *et al* (1999), proanthocyanidin-rich extract from grape seeds decreased the levels of lipid peroxidation products in plasma and inhibited the development of aortic atherosclerosis in cholesterol-fed rabbits. Red wine polyphenol supplementation showed to offer protection against early atherosclerosis in hamsters, the mechanism suggested to have been linked e.g. with increased plasma antioxidant capacity and elevated levels of antioxidant enzymes (Auger *et al*, 2005). Dried grape powder inhibited development of atherosclerosis in apolipoprotein E deficient mice and reduced macrophage atherogenicity against LDL (Fuhrman *et al*, 2005). The effect could have been at least partly mediated by increased serum antioxidant capacity. In majority of animal studies reporting preventive effects against atherosclerosis or other pathological changes linked to cardiovascular diseases, levels of oxidative stress in animals were decreased. The polyphenol sources most often used have been tea, cocoa and red wine.

Similarly to animal studies, also human polyphenol interventions have focused on the effects of catechins, flavonols and procyanidins from tea, red wine and cocoa (Williamson & Manach, 2005). Also studies on other phenolic compounds, for example isoflavonoids have been carried out: in study of Tikkanen *et al* (1998), administration of soy-derived isoflavonoids decreased LDL oxidation susceptibility in humans. For the most studied classes of polyphenols, sufficient data from supplementation studies exists to evaluate the type and extent of their antioxidant effects in humans (Williamson & Manach, 2005). Catechins from green tea, black tea or tea polyphenol concentrates have been found e.g. to increase plasma antioxidant capacity (Sung *et al*, 2000; Young *et al*, 2002; Reddy *et al*, 2005), decrease levels of lipid peroxidation products in plasma (Nakagawa *et al*, 1999; Freese *et al*, 1999; Reddy *et al*, 2005), increase plasma vitamin C concentrations (Gomikawa & Ishikawa, 2002) and increase the resistance of LDL to oxidation (Ishikawa *et al*, 1997). Supplementation with red wine, red wine dealcoholised to

remove the confounding effects of ethanol, or red wine polyphenols (procyanidins, anthocyanidins and quercetin) have resulted in similar findings: increased antioxidant capacity (Carbonneau *et al*, 1997; Serafini *et al*, 2000), lowered levels of lipid oxidation products in plasma and increased resistance of LDL against oxidation (Fuhrman *et al*, 1995; Nigdikar *et al*, 1998; Tsang *et al*, 2005). In addition, decreased F₂-isoprostane formation has been reported (Caccetta *et al*, 2001; Pignatelli *et al*, 2006). Cocoa polyphenols, mainly procyanidins and catechins, have also exerted some antioxidant effects in humans (see review of Williamson & Manach, 2005); however, the proof of their effects on plasma antioxidant capacity or lipid peroxidation does not seem as strong as for tea and red wine polyphenols. Of the flavonol class, quercetin is the most studied representative. In intervention studies using supplements rich in quercetin, some effects suggesting cardiovascular protective antioxidant action *in vivo* have been shown (McAnlis *et al*, 1999; Chopra *et al*, 2000); however, the evidence is limited and many studies have failed to show antioxidant effects (Williamson & Manach, 2005). For polyphenols in aforementioned classes of compounds, also vasodilatory effects and improvement of endothelial function have been described, most probably due to increased production of nitric oxide (Duffy & Vita, 2003; Stoclet *et al*, 2004; Vita, 2005; Williamson & Manach, 2005). Furthermore, e.g. decreased blood pressure, inhibition of platelet aggregation, angiogenesis and cell migration and proliferation in the vascular system have been attributed to these compounds (Stoclet *et al*, 2004; Williamson & Manach, 2005). The epidemiologically observed cardiovascular protection by high polyphenol diets could be partly explained by these effects. Although beneficial changes in the aforementioned parameters have been observed, also lack of effects has been reported (Halliwell *et al*, 2005; Williamson & Manach, 2005). Taken all these observations together, it seems that polyphenols of these classes tend to transiently increase the plasma antioxidant capacity, the effect on lipid peroxidation being less announced.

Results from human intervention studies using phenolic acids as supplement are remarkably lower in number, and the show of their antioxidant effects *in vivo* is not as strong. Studies on coffee, containing e.g. high amounts of chlorogenic acid, have however suggested that phenolic acids might exert antioxidant action in humans. Coffee was shown to increase plasma antioxidant capacity by Natella *et al* (2002). In the recent work of the same group (Natella *et al*, 2007), coffee drinking was shown to increase the incorporation of conjugated forms of caffeic acid, *p*-coumaric acid and ferulic acid into LDL particles, and the oxidation resistance of LDL was

increased. Some support for the potential *in vivo* antioxidant properties of phenolic acids can be found in animal studies. Caffeic acid supplementation increased plasma antioxidant capacity in rats, and although caffeic acid was not incorporated into LDL particles, the resistance of LDL against oxidation was enhanced (Nardini *et al*, 1999). Furthermore, caffeic acid increased levels of Vitamin E in plasma and LDL particles. In a study of Balasubashini *et al* (2004), ferulic acid supplementation of rats with induced diabetes resulted in decreased lipid peroxidation and increase in antioxidant enzymes. In a recently published study of Luceri *et al* (2007), supplementation of rabbits with *p*-coumaric acid resulted in significantly increased plasma antioxidant capacity and decreased platelet aggregation. Even though this finding was made in an animal model, it is still interesting, as *p*-coumaric acid is one of the most commonly occurring phenolic acids. In another recent work, supplementation of high-cholesterol fed rats with *p*-hydroxybenzoic acid or *p*-hydroxyphenylpropionic acid, metabolites of citrus flavonoid naringenin, decreased levels of plasma and hepatic lipid peroxidation products and increased the levels of antioxidant enzymes. Also lipid-lowering effects e.g. in plasma total cholesterol and triglycerides were observed (Jeon *et al*, 2007). Yang *et al* (2006) demonstrated in high-cholesterol fed rats that plasma antioxidant capacity was correlated with atherosclerotic index.

It is important to notice that study setting has an ultimate key role in clinical studies, and cautiousness is needed in the interpretation of study results. For example, elevated levels of F₂-isoprostanes after fasting have been reported (Richelle *et al*, 1999; Lee *et al*, 2004). E.g. Lotito & Frei (2004) have shown that the increase observed in parameters reflecting plasma antioxidant status following consumption of apples was due to increase in urate, endogenous plasma antioxidant, caused by fructose present in apples, and not due to antioxidant activity of apple polyphenols *in vivo*.

To conclude, although the data supporting the antioxidant significance of polyphenols *in vivo* remains inconsistent, the evidence on the antioxidant potential of phenolic compounds *in vitro*, the epidemiological observations on the disease-preventing properties of diets rich in polyphenols, and the recognised role of oxidative damage in the aetiology of various diseases suggest the antioxidant hypothesis behind their health benefits to be reasonable. As mentioned above in the case of flavonoids, phenolic compounds have also several other potential mechanisms of action which may, independently or more probably concurrently be

involved in the observed protection against CVD. In addition to effects on the cardiovascular system, several effects may be mediated via complex regulation of cell signalling systems (Halliwell, 2007).

2.3.4. Dietary sources and intake of phenolic compounds

Due to the ubiquitous production of phenolic compounds in plants, practically all plant-derived foods and beverages act as sources of dietary phenolics making them important constituents of the nonenergetic part on human diet. The most commonly mentioned rich sources of dietary polyphenols probably are fruits and vegetables, berries, tea, red wine, cocoa and coffee. In addition to these, e.g. herbs, cereals, nuts, beer and cider provide a source for polyphenol intake. The content of phenolic compounds in different foods or beverages varies by several orders of magnitude (Bravo, 1998). Depending on the amounts and types of polyphenols present, foods exert different *in vitro* antioxidant capacities, most often determined as TEAC values. In Table 2., some dietary sources of different types of polyphenols are presented together with determined TEAC values for these products.

Table 2. Some dietary sources of different types of polyphenols and their TEAC values. PP = polyphenol.

Type of PP	Examples	Product	PP content	TEAC
Hydroxybenzoic acids	Gallic, vanillic, protocatechuic acid	Blackberry	80-270	3.990
		Raspberry	60-100	2.334
		Strawberry	20-90	2.15
		Rye grain	9 ¹	1.164 ²
Hydroxycinnamic acids	Caffeic, chlorogenic, ferulic, <i>p</i> -coumaric acid	Blueberry	2000-2200	2.154
		Oregano, dried	1440 ³	40.299
		Kiwi	600-1000	1.325
		Wheat bran	434 ¹	1.577 ⁴
		Rye grain	116 ¹	1.164 ²
		Apple	50-600	0.224-0.536
		Potato	100-190	0.168-0.417
		Coffee	350-1750	1.249
Flavonols	Quercetin, myricetin, kaempferol	Yellow onion	350-1200	0.236
		Broccoli	40-100	0.248
		Blueberry	30-160	2.154
		Apple	20-40	0.224-0.536
Flavones	Hesperetin, eriodictyol	Parsley	240-1850	7.430
Flavanones	Luteolin, apigenin	Orange juice	215-685	0.566
Anthocyanins	Cyanidin, malvidin, delphinidin	Black currant	1300-4000	0.644-1.313 ⁵
		Blueberry	250-5000	2.154
		Black grape	300-7500	0.316
		Red wine	200-350	2.135
Monomeric flavanols	Catechin, epicatechin	Chocolate	460-610	2.567-4.188
		Green tea	100-800	0.441-0.969 ⁵
		Black tea	60-500	0.388-0.770 ⁵
		Apple	20-120	0.224-0.536
		Red wine	80-300	2.135

If not otherwise indexed, polyphenol contents (mg/100g or mg/100mL) refer to Manach *et al*, 2004 (see for references).

If not otherwise indexed, TEAC values (mmol/100g) refer to Halvorsen *et al*, 2006 (see for references).

¹ Mattila *et al*, 2005

² Pellegrini *et al*, 2006

³ Kosar *et al*, 2003

⁴ Martínez-Tomé *et al*, 2004

⁵ Schlesier *et al*, 2002

When examining these TEAC values, it needs to be considered that the values for all the berry and vegetable samples were determined from raw samples, and they are usually largely increased by cooking procedures (Halvorsen *et al*, 2006). Furthermore, TEAC values between studies cannot be fully compared with each other. For example, in paper of Pellegrini *et al*, the TEAC value for fresh *O. vulgare* leaves was 3.065 mmol/100g, and in the paper of Shan *et al* (2005), dried *O. vulgare* was given a TEAC value of 100.7 mmol/100g. The values reported for different food products by Halvorsen *et al* (2006) can, however, within the study be used to rank the samples in order of antioxidant activity (Van den Berg *et al*, 1999).

Even though the analytical techniques have developed side by side with the growing knowledge of polyphenols, no ultimate information on dietary intake of polyphenols is available. Challenges lay in the chemical complexity of this group of compounds; the range of polyphenolic compounds is not yet fully covered. Another difficulty is related to the fact that no official agreement exists on the analytical methodologies, resulting in pieces of data that are hard to compare and combine. About 30 years ago, the estimate for the average daily intake of flavonoids in United States (US) was around 1g (Kühnau, 1976). Later on, the proposed figures for the intake of some individual flavonoids or flavonoid groups in Europe or in the US have been of the magnitude of tens of milligrams (Hertog *et al*, 1993b; Hertog *et al*, 1997; Leth & Justesen, 1998; Yochum *et al*, 1999; Arts *et al*, 2001a; Knekt *et al*, 2002; Sesso *et al*, 2003). In the 1997 Dietary Survey of Finnish Adults (National Public Health Institute, 1998) the average total intake of flavonoids in Finland was estimated to be 55 mg/d. The main contributing sources were fruits (67%), followed by tea, wine and other beverages (altogether 25%), vegetables (5%) and berries (3%). In a recent population based study of middle-aged eastern Finnish men, Mursu *et al* (2007) reported the mean flavonoid intake to be 129 mg/d; of this figure, 84% were catechins, which were not included in the earlier intake calculations. The intakes of other flavonoids were significantly lower: flavonols, anthocyanidins and flavanones had a 7%, 6% and 3% contribution to the total intake, respectively. In Finnish diet, especially berries are a significant source of anthocyanins (Heinonen, 2007). Recent estimates for the intake of anthocyanins and proanthocyanidins are around few hundred milligrams (Gu *et al*, 2004; Manach *et al*, 2004). The lower intake figures, for example in Finnish diet, are most probably due to a limited number of compounds included in the calculations. In national FINDIET 2002 study, the consumption of fruits, vegetables and berries was found to be higher among Finnish women than in men (National Public Health

Institute, 2003). In all parts of Finland, oranges or orange juice were the most frequently consumed fruits / fruit products. Also apples were among the three most frequently used fruits / fruit products throughout Finland.

Another remarkable source of polyphenols in Finnish diet is coffee, average consumption of which is several cups per day. The intake of phenolic acids from coffee may rise up to several hundreds of milligrams, the main compound being chlorogenic acid (Clifford, 1999). In addition to the most commonly occurring chlorogenic acid, high amounts of phenolic acids such as ferulic acid, caffeic acid and *p*-coumaric acids are also present e.g. in cereals, vegetables, fruits and berries (Rice-Evans *et al*, 1996; Macheix & Fleuriet, 1998; Clifford, 1999; Kaur & Kapoor, 2001; Manach *et al*, 2004). The intake of phenolic acids has been studied much less as compared to flavonoids. Based on their wide distribution in commonly consumed dietary plants and plant products, they must make a significant contribution to the daily intake of phenolic compounds; according to one estimation, they might account for on third of the daily polyphenol intake (Schwedhelm *et al*, 2003).

2.3.5. Bioavailability and metabolism of phenolic compounds

In order to exert systemic effects in the human body, polyphenols need to be absorbed from the GI tract and distributed in blood circulation and target tissues. Thus, knowledge on the bioavailability and metabolism of these compounds is essential as their potential bioactivities are evaluated. Due to the structural diversity of phenolic compounds, this area has not been easily covered. Until 1990's, the absorption of phenolic compounds from the GI tract was believed to be marginal. After Hollman *et al* (1995) published their findings on the absorption of quercetin, this field has been intensively studied. However, more knowledge is still needed on these topics in humans. This review mainly focuses on those compounds most relevant in the context of this study.

Several mechanisms are involved in the absorption of dietary phenolic compounds, the main mechanism in the permeation being passive diffusion (Scalbert & Williamson, 2000). Once ingested, polyphenols are exposed to acidic and digestive enzyme rich conditions of stomach. A small portion of aglyconic phenolic compounds may be absorbed from the stomach, but due to lack of glucosidases in gastric fluid, glycosides usually proceed to the small intestine. As an exception,

anthocyanins have been shown to be absorbed as intact glycosides from stomach (McGhie & Walton, 2007). Absorption from rat stomach has been shown for ferulic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid and gallic acid (Konishi *et al*, 2006). 5 minutes after the administration of these compounds by gastric intubation, plasma concentrations were increased in the following order: gallic acid = chlorogenic acid < caffeic acid < *p*-coumaric acid = ferulic acid. This matched the earlier results from Caco-2 cell work of Konishi *et al* (2003), Konishi & Shimizu (2003) and Konishi & Kobayashi (2004a), according to which the absorption of ferulic acid, *p*-coumaric acid and, to a lesser extent, caffeic acid, involved active transportation by monocarboxylic acid transfer mechanism, MCT. The affinity of gallic acid, which has a slow gastric absorption rate, for MCT is low, and esterified phenolic acids, for example chlorogenic acid, have no affinity to MCT at all. The main mechanism in the absorption of caffeic acid and rosmarinic acid in Caco-2 cells was paracellular diffusion, (Konishi & Kobayashi, 2004b; Konishi & Kobayashi, 2005), and also passive diffusion was involved. Two plasma peaks for ferulic acid were detected, first for the intact molecule at 5 minutes and another for conjugated ferulic acid at 15 minutes, which suggest two parallel mechanisms of gastric absorption for ferulic acid (Konishi *et al*, 2006). The first would be rapid permeation in the intact form and the other a slower permeation process for conjugated forms. Rosmarinic acid was not included in the list of analytes in this study, but as it is, similarly to chlorogenic acid, an ester of caffeic acid, one might expect it to have absorption properties close to those of chlorogenic acid. In another work of Konishi *et al* (2005), rosmarinic acid peaked in plasma 10 minutes after administration by gastric intubation to rats.

In the small intestine, enzymes capable of breaking down glycosidic bonds in phenolic compounds are present. Many polyphenols occur in glycosylated forms, and the removal of the glycoside residues in the gut is expected to increase the intestinal absorption by diffusion (Scalbert & Williamson, 2000). Some polyphenols such as free phenolic acids, flavonoid aglycones and some flavonoid glucosides, for example quercetin glucosides, are absorbed in the small intestine by passive diffusion, but the majority is not (Manach *et al*, 2004). The MCT absorption is active also in the small intestine, and in the colon. When polyphenols enter the colon, the microbial metabolism becomes a key factor in the absorption and metabolism. The colonic microflora is, for example, responsible for the cleavage of esterified phenolic acids (Schwedhelm *et al*, 2003). After enzymatic microbial deconjugation of any phenolic conjugate reaching the colon, two possible

routes exist: absorption of intact polyphenol through epithelium of colon and subsequent transfer to blood circulation, either in the free aglycone form or in conjugated form, or microbial degradation of original polyphenol into lower molecular mass metabolites, which then are absorbed. After absorption, polyphenols are mainly bound to plasma albumin and methylated, sulphated or glucuronised in the liver (Manach *et al*, 2004). The conjugation may also occur in the intestinal mucosa. In human colon, caffeic acid and chlorogenic acid are metabolised into lower molecular weight compounds, namely *m*-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and *m*-coumaric acid (Rechner *et al*, 2004). The absorption of these metabolites has been shown in Caco-2 cells to involve the MCT mechanism as well as paracellular diffusion (Konishi & Kobayashi, 2004b). In study of Rechner *et al* (2002a), the majority of *in vivo* phenolic compounds were reported to be derived from the colon metabolism, subsequently metabolised in the liver into glucuronides. The levels on intact conjugated polyphenols were considerably lower. Thus, bioavailability studies determining only the intact original compounds or their conjugates ignore the contribution of these colonic products. The discrepancy between very low detected absorption of procyanidins and their observed effects in intervention studies could possibly be explained by underestimation of their absorption by detection of the intact molecules or their conjugates in urine, leaving potentially bioactive microbial metabolites undetected. The mechanism(s) of action, extent and significance of the various metabolites need to be better established in the future.

Usually, less than 1-26% of ingested flavonoids are found in human urine (0.4-1.4% for flavonols, 0.5-6% for catechins, 2.5-26% for isoflavones and 1-7% for anthocyanins) (Scalbert & Williamson, 2000). This suggests that their intestinal absorption is limited, or that they are effectively metabolised either in tissues or by the colonic microflora (Bravo, 1998). In colon, flavonoids are metabolised into a variety of low-molecular weight phenolic compounds, for example hydroxyphenylpropionic acids, hydroxyphenylacetic acids and hydroxybenzoic acids (Winter *et al*, 1989; Manach *et al*, 2004). In addition to being excreted in urine, polyphenols may also be excreted in the bile in their original or metabolised form and be subject to further metabolism in the colon (Manach *et al*, 2005).

Of the phenolic acid class, the bioavailability and metabolism of chlorogenic acid and caffeic acid have been studied most often (Manach *et al*, 2005). Of 1000 mg of caffeic acid ingested, 27% was reported to be excreted in urine (Jacobson *et al*,

1983). In another study, the percentages of absorbed caffeic acid and chlorogenic acid from small intestine of ileostomy subjects were 95% and 33%, respectively (Olthof *et al*, 2001). This suggests that part of chlorogenic acid is absorbed from the small intestine. Contrary to this observation, Nardini *et al* (2002) suggested chlorogenic acid from coffee to be metabolised into caffeic acid in the colon, and subsequently absorbed. However, the plasma maximum concentration of caffeic acid was observed 30 minutes after ingestion of coffee, which rather refers to absorption from the small intestine than from the colon. Furthermore, esterase activity capable of hydrolysing esters of hydroxycinnamic acids, such as chlorogenic acid or rosmarinic acid, has been found in mucosa-cell free extract of all areas of human small intestine (Andreasen *et al*, 2001), as well as in the colon, but not in tissues such as intestinal mucosa or liver, or biological fluids such as gastric fluid, duodenal fluid or plasma (Plumb *et al*, 1999; Olthof *et al*, 2001; Rechner *et al*, 2001). Despite the partial absorption of chlorogenic acid in the upper part of the GI tract, the major part of chlorogenic acid probably reaches the colon, where colonic microflora metabolises it into lower molecular weight phenolic compounds, such as *m*-coumaric acid and *m*-hydroxyphenyl-propionic acid, which are actively absorbed by MCT (Konishi & Kobayashi, 2004b).

Dietary ferulic acid from tomatoes (Bourne & Rice-Evans, 1998) and beer (Bourne *et al*, 2000) were reported to be absorbed intact, with 11-25% of ferulic acid from tomatoes being excreted in urine. The maximum urinary excretion was observed 7-8 hours after the ingestion. The bioavailability of rosmarinic acid, characteristic phenolic acid in Lamiaceae herbs, has been evaluated in two human studies. In study of Nakazawa & Ohsawa (2000), only glucuronide conjugates of apigenin and 2,4,5-trimethoxycinnamic were found in human urine after ingestion of *Perilla frutescens* extract, whereas rats excreted e.g. *m*-hydroxyphenylpropionic acid and conjugates of caffeic acid, *p*- and *m*-coumaric acid, ferulic acid, luteolin and apigenin. After ingestion of 200 mg of rosmarinic acid in *Perilla* extract, about 6% of rosmarinic acid and its metabolites (methylated rosmarinic acid, caffeic acid, ferulic acid and *m*-coumaric acid) were excreted in urine of human volunteers (Baba *et al*, 2005). Among other compounds, intact rosmarinic acid was detected in plasma, with peak concentration at 30 minutes after the ingestion. Based on Caco-2 cell work (Konishi & Kobayashi, 2005) and rat studies (Konishi *et al*, 2005), rosmarinic acid has been suggested to be absorbed mainly by paracellular diffusion. Also, as rosmarinic acid seemed to be resistant for mucosa esterases in Caco-2 cells, majority of it was suggested to be degraded into lower molecular weight

compounds, such as *m*-coumaric acid and hydroxylated phenylpropionic acids, in the colon, followed by efficient absorption e.g. by active MCT mechanism.

To conclude, the molecular properties of phenolic substances greatly affect their rate and extent of intestinal absorption as well as the range of metabolites in plasma and urine (Bravo, 1998; Scalbert & Williamson, 2000; Manach *et al*, 2004). As dietary polyphenols enter human body, they are effectively metabolised to antioxidatively less active molecules (Rechner *et al*, 2002a; Masella *et al*, 2005). Even though polyphenols seem to be low in toxicity, they still are xenobiotics to human body and are treated as ones. The bioavailability of most polyphenols is rather low (Manach *et al*, 2005). Even though most phenolic acids and flavonoids are at least to some extent absorbed through the GI tract, the plasma concentrations of individual compounds are usually less than 1 $\mu\text{mol/L}$; this is partially due to intensive metabolism by tissues and colonic microflora (Scalbert & Williamson, 2000; Rechner *et al*, 2002b; Manach *et al*, 2005). Concentrations of polyphenols and their metabolites *in vivo* are lower than those of other antioxidants such as ascorbic acid and α -tocopherol (Schwedhelm *et al*, 2003; Manach *et al*, 2005; Williamson *et al*, 2005). For example, ascorbic acid is present in plasma or serum at level of 50-70 $\mu\text{mol/L}$ and α -tocopherol between 20-30 $\mu\text{mol/L}$ (Schwedhelm *et al*, 2003). As the plasma antioxidant capacity has been shown to be around 1 mmol/L or more, it seems rather unlikely that a 1 $\mu\text{mol/L}$ addition of a polyphenol would have remarkable effects (Halliwell, 2007). However, epidemiological observations on the cardiovascular protection as well as antioxidant and cardiovascular protective properties in intervention studies suggest that polyphenols are health beneficial, via antioxidant or some other mechanism.

In order to maintain high concentrations of phenolic compounds in plasma, several doses should be ingested daily (Van het Hof *et al*, 1999). This is especially true for compounds with rapid absorption, metabolism and excretion pattern; metabolism and uptake in the colon results in prolonged half-lives (Schwedhelm *et al*, 2003). The absorption also depends on the matrix the compounds are present in; for example, ferulic acid is absorbed slower from cereals than from beer (Andreasen *et al*, 1999; Bourne *et al*, 2000). Furthermore, foods and their phytochemicals present in the GI tract may slow or inhibit the absorption (Manach *et al*, 2004).

Another perspective for polyphenols not being fully absorbed is that they have the potential for antioxidant action within the GI tract. Present in high concentrations in the stomach, they might exert local antioxidant effects by scavenging various types of

reactive species or chelating transition metal ions (Halliwell, 2007). This could also occur in the small intestine, and even more, in the colon, as ascorbic acid and α -tocopherol are mainly absorbed in the small intestine, polyphenols remaining the main group of dietary antioxidants present (Halliwell, 2007). Especially smaller molecular size metabolites of polyphenols are present in high concentrations in the colon (Jenner *et al*, 2005). In addition to their potential radical scavenging or metal chelating action, their inhibitory effects on e.g. oxidizing enzymes and matrix metalloproteinases might be beneficial for the local health of colon.

2.4. LAMIACEAE HERBS

2.4.1. What are Lamiaceae herbs?

Lamiaceae (syn. Labiatae) herb family consists of more than 200 genus and 3500 species (Watson & Dallwitz, 2007). These herbs have been used also in traditional medicine, but are mainly known for their culinary properties. For example oregano, rosemary, sage and thyme used in this study are typical seasonings in the Mediterranean region, and especially oregano is consumed in larger quantities all around the world as part of pizza seasoning mix. Oregano, rosemary, sage and thyme have a GRAS status given by the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2006), meaning that they are generally recognised as safe for human consumption without limitations on intake. This fact, combined with promising data on the antioxidant properties of phenolic compounds, has triggered both scientific and commercial interest towards this herb family.

2.4.2. Phenolic compounds in Lamiaceae herbs

Lamiaceae herbs are rich in polyphenols: compounds from several classes: di- and triterpenes, simple phenolics, phenolic acids and flavonoids are found in representatives of this family of herbs. From sage, for example, more than 160 different phenolic constituents have been identified (Lu & Foo, 2002). This review focuses on partial characterisation of the phenolic profiles of Lamiaceae herbs used in this study: *Rosmarinus officinalis* L. (*R. officinalis*), *Salvia officinalis* L. (*S. officinalis*), *Thymus vulgaris* L. (*T. vulgaris*), *Origanum vulgare* L. (*O. vulgare*) and *Origanum onites* L. (*O. onites*). Some of the identified phenolic compounds in these herbs are presented in Table 3.

Table 3. Some phenolic constituents found in Lamiaceae herbs.

Herb	Identified phenolic(s)	Reference
<i>R. officinalis</i>	luteolin, hesperidin	Okamura <i>et al</i> , 1994
	carnosic acid, carnosol	Bicchi <i>et al</i> , 2000
	<i>p</i> -hydroxybenzoic, syringic, vanillic, protocatechuic and caffeic acid	Zgórka & Głowniak, 2001
	rosmarinic acid	Cuvelier <i>et al</i> , 1996
<i>S. officinalis</i>	gallic, <i>p</i> -coumaric, ferulic, chlorogenic and dihydroxycaffeic acid	Kivilompolo <i>et al</i> , 2007
	carnosol, carnosic acid, rosmanol, epirosmanol, rosmadial, methyl carnosate	Cuvelier <i>et al</i> , 1994
	<i>p</i> -hydroxybenzoic and caffeic acid	Wang <i>et al</i> , 2000
	protocatechuic acid	Zgórka & Głowniak, 2001
	vanillic, ferulic and rosmarinic acid	Cuvelier <i>et al</i> , 1996
	salvianolic acids	Lu & Foo, 1999
	(rosmarinic acid dimers)	
	<i>p</i> -coumaric acid	Lu & Foo, 2000
	gallic, vanillic, chlorogenic and dihydroxycaffeic acid	Kivilompolo <i>et al</i> , 2007
	apigenin, luteolin, hesperetin	Cuvelier <i>et al</i> , 1996
<i>T. vulgaris</i>	kaempferol	Shan <i>et al</i> , 2005
	<i>p</i> -hydroxybenzoic and vanillic acid	Zgórka & Głowniak, 2001
	protocatechuic, syringic, caffeic, <i>p</i> -coumaric, ferulic, rosmarinic and dihydroxycaffeic acid	Kivilompolo <i>et al</i> , 2007
	chlorogenic acid	Kivilompolo & Hyötyläinen, 2007
<i>O. vulgare</i>	apigenin, luteolin	Justesen & Knuthsen, 2001
	<i>p</i> -hydroxybenzoic, vanillic, caffeic, <i>o</i> -coumaric and ferulic acid	Gerothanassis <i>et al</i> , 1998
	gallic, vanillic, caffeic, <i>p</i> -coumaric, ferulic, rosmarinic, chlorogenic and dihydroxycaffeic acid	Kivilompolo <i>et al</i> , 2007
	apigenin, luteolin	Justesen & Knuthsen, 2001
	quercetin	Exarchou <i>et al</i> , 2002
	kaempferol	Shan <i>et al</i> , 2005
	eriodictyol, naringenin	Exarchou <i>et al</i> , 2003
<i>O. onites</i>	<i>p</i> -hydroxybenzoic, vanillic, caffeic and ferulic acid	Gerothanassis <i>et al</i> , 1998
	rosmarinic acid	Pizzale <i>et al</i> , 2002
	apigenin, luteolin	Kosar <i>et al</i> , 2003

In polar extracts of Lamiaceae herbs, phenolic acids are the major class of phenolic compounds. Phenolic acids (caffeic acid, gallic acid, *p*-coumaric acid and rosmarinic acid, depending on the herb in question) were reported to be the major type of phenolic compounds in methanolic extracts of *O. vulgare*, thyme, rosemary and sage in study of Shan *et al* (2005). The major part of aqueous sage extract phenolics consists of phenolic acids, mainly caffeic acid derivatives (Lu & Foo, 2002). The amounts of phenolic acids in these herbs varies, depending on the herbs in question, from tens of milligrams per 100 grams of herb to even tens of milligrams per 1 gram of herb; flavonoids are present at the level of few milligrams to tens of milligrams per 100 g (Justesen & Knuthsen, 2001; Shan *et al*, 2005; Kivilompolo *et al*, 2007). The total phenolic values of methanolic Lamiaceae extracts, determined as gallic acid equivalents, are in the range of 5-10 g gallic acid equivalent (GAE)/100g dry weight (Shan *et al*, 2005). Rosmarinic acid, the major phenolic component of most Lamiaceae extracts, may even reach concentrations in the range of 1-2.5 g/100g dry weight (Shan *et al*, 2005). The concentrations determined show wide variation between studies. In addition to phenolic acids, phenolic diterpenes such as carnosic acid and carnosol were reported especially in rosemary and sage, and to a lesser extent, in thyme. Also some amounts of volatile compounds, for example carvacrol and thymol, as well as flavonoids were found in all four extracts. Rosmarinic acid has been identified as the predominant phenolic compounds in aqueous extracts of sage and thyme (Zheng & Wang, 2001) and in 60% ethanol extracts of oregano, sage, rosemary and thyme (Kivilompolo & Hyötyläinen, 2007). The phenolic content of these herbs depends e.g. on the growing location and harvesting time, as was seen for rosmarinic acid (Yesil-Celiktas *et al*, 2007).

2.4.3. Lamiaceae herbs as a source of dietary antioxidants

Lamiaceae herbs have been reported to contain high levels of dietary antioxidants (Zheng & Wang, 2001; Dragland *et al*, 2003; Shan *et al*, 2005; Halvorsen *et al*, 2006), and the *in vitro* antioxidant potency of these herbs has been revealed in numerous studies (Exarchou *et al*, 2002; Pizzale *et al*, 2002; Dorman *et al*, 2004). In Norwegian-Japanese study of Dragland *et al*, an intake of 1 gram of these herbs has been suggested to make a relevant contribution (>1mmol) to the total intake of plant antioxidants, and even to be a better source of dietary antioxidants than many other food groups. Among the 50 food products with the greatest TEAC values, 13

spices were found, oregano having the second highest antioxidant value after cloves (Halvorsen *et al*, 2006).

The total use of herbs as well as type of herbs used varies largely in different countries. Lamiaceae herbs are typical seasoning ingredients in the Mediterranean countries, in which they are regularly consumed in amounts higher than in Finland. The incidence of CVD in countries of southern Europe, such as Spain, Italy, France and Greece, is lower than in countries in northern Europe or The United States (Serra-Majem *et al*, 2006). The explanation for the observed difference has most often been the diet traditionally consumed in the countries of the Mediterranean region. The diet is characterised by the following eight components: high monounsaturated to saturated fat ratio, high consumption of legumes, cereals (including bread), fruit, vegetables and red wine, moderate consumption of milk and other dairy products, and low consumption of meat and meat products (Trichopoulou *et al*, 1999; Lairon, 2007). It is rich in fibre, unsaturated fats and polyphenols, and has been described as an optimal diet for cardiovascular protection. In addition to an established positive association between Mediterranean diet and CVD, observational studies have found positive association between the consumption of the aforementioned diet and longevity. Furthermore, a negative association between the diet and certain cancers and other nutrition-related diseases has been established (Serra-Majem *et al*, 2006). The credibility of these studies has, however, suffered from low numbers of study participants as well as differences in the methodology used to define the intervention. As reviewed by Estruch *et al* (2006) and Lairon (2007), the results from intervention studies are conflicting. In 2006, preliminary results from the first large-scale, long-term (about 5 years) clinical trial on the effects of Mediterranean diet enriched with extra virgin olive oil or nuts in 9000 high-risk patients for CVD in Spain were published (Estruch *et al*, 2006). At three-month time point from the beginning of the study, the two groups with oil/nuts enriched Mediterranean type diet showed, compared to the low-fat diet group, beneficial changes in the following intermediate markers of cardiovascular disease: plasma glucose levels, systolic blood pressure and total cholesterol/HDL cholesterol ratio. In the group consuming olive oil enriched Mediterranean diet also plasma levels of C-reactive protein, an inflammatory marker, were decreased. Even though this short-term substudy of the long-term study did not detect the clinical outcomes, it showed that consumption of enriched Mediterranean diet favourably affects intermediate outcomes of CVD. However,

the contribution of polyphenols to observed cardiovascular protection could not be distinguished from that of PUFAs or dietary fiber.

The consumption of fresh herbs in Finland has increased and diversified during the recent years. One of the strongest factors contributing to the increased consumption is that pot-grown herbs are today available throughout the year. Furthermore, the international trends in cooking have introduced new herbs into Finnish kitchens. Instead of using herbs as seasoning just in cooked dishes, the polyphenol and antioxidant content, as well as flavour, of different salad dishes might be markedly enriched by additions of fresh herbs (Ninfali *et al*, 2005). Considering their high content of polyphenols with a significant contribution to the dietary antioxidant intake, the regular use of fresh and dried herbs from the Lamiaceae family may help one to assemble a healthy, balanced diet rich in health-beneficial phytochemicals.

3. AIMS OF THIS STUDY

During the past twenty years, intensive research has been focused on the oxidation of biomolecules and the potential health promoting or disease preventing effects of dietary antioxidant polyphenols abundant in fruits and vegetables. Lamiaceae herbs with a traditional culinary usage especially in the Mediterranean region are rich in antioxidant phenolic compounds, but a very little is known about their potential health effects in humans. This study on the antioxidant properties of Lamiaceae herbs represents a progression starting from finding an extraction technique applicable for producing extracts suitable for use as nutritional supplements, proceeding to evaluation of the antioxidant properties of these extracts and their phenolic constituents in laboratory test models, and finally ending up in elucidating their bioavailability, metabolism and effects on lipid peroxidation biomarkers in humans.

The aims of the present work were:

- 1) To study the use of different techniques to extract Lamiaceae herbs with emphasis on finding a technique for producing antioxidant-rich extracts with properties suitable for human consumption,
- 2) to study the antioxidant properties of these extracts and phenolic constituents and metabolites thereof in different *in vitro* models,
- 3) to examine the bioavailability and metabolism of phenolic acids from oregano extract in humans, and
- 4) to study the short and long term effects of oregano extract on biomarkers of antioxidant capacity and lipid peroxidation in humans.

4. MATERIALS AND METHODS

4.1. Standard compounds and reagents

The standard compounds used and their origins of purchase are presented in Table 4. The purity of all the standards used was 98% or higher, and all other reagents (origins presented in original publications) were of analytical or HPLC grade. Water used was purified using a Millipore RO 12 plus system (Millipore Corp., Bedford, MA, US).

Table 4. Standard compounds used and their origins of purchase.

Compound	Origin of purchase
1,3-dihydroxybenzene (resorcinol)	Riedel-de-Hagen
<i>p</i> -hydroxybenzoic acid	Extrasynthese
2,3-dihydroxybenzoic acid	Fluka
2,4-dihydroxybenzoic acid	Fluka
2,5-dihydroxybenzoic acid	Fluka
2,6-dihydroxybenzoic acid	Fluka
3,4-dihydroxybenzoic acid (protocatechuic acid)	Sigma
3,5-dihydroxybenzoic acid	Fluka
3,4,5-trihydroxybenzoic acid (gallic acid)	Sigma
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	Fluka
4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid)	Sigma
<i>m</i> -hydroxyphenylacetic acid	Sigma
3,4-dihydroxyphenylacetic acid	Sigma
<i>m</i> -hydroxyphenylpropionic acid	Apin chemicals
3,4-dihydroxyphenylpropionic acid	Apin chemicals
<i>o</i> -coumaric acid	Sigma
<i>m</i> -coumaric acid	Fluka
<i>p</i> -coumaric acid	Sigma
caffeic acid	Sigma
4-hydroxy-3-methoxycinnamic acid (ferulic acid)	Sigma
sinapic acid	Sigma
rosmarinic acid	Extrasynthese
chlorogenic acid	Sigma
catechin	Sigma
epicatechin	Sigma
catechin gallate	Sigma
epicatechin gallate	Sigma
epigallocatechin	Sigma
epigallocatechingallate	Sigma
gallocatechin	Sigma
apigenin	Fluka

apigenin-7- <i>O</i> - β -glucoside	Extrasynthese
eriodictyol	Apin chemicals
fisetin	Sigma
hesperetin	Sigma
isorhamnetin	Apin chemicals
kaempferol	Sigma
luteolin	Sigma
luteolin-7- <i>O</i> -glucoside	Extrasynthese
myricetin	Sigma
naringenin	Apin chemicals
naringin	Sigma
quercetin	Sigma
rhamnetin	Apin chemicals
rutin	Sigma

4.2. Plant material

Representatives of four genus of Lamiaceae herb family were used in this study: *S. officinalis* (sage, study I), *R. officinalis* (rosemary, study II), *T. vulgaris* (thyme, study II), *O. vulgare* (oregano, studies II and V) and *O. onites* (oregano, studies III and IV). *O. vulgare* and *O. onites* are two oregano species of the major culinary importance. Their nomenclature and synonyms are rather confusing, as both are often referred to as pot marjoram. *O. vulgare* is grown all over the Europe and has thus been called European oregano, whereas *O. onites* is grown especially in Greece and Crete and has thus been called Greek or Cretan oregano. All the herbal material, consisting of aerial parts of these herbs was received air-dried and cut in air-tight plastic containers from Teriaka Ltd, Helsinki, Finland and stored at room temperature. As an exception to other herbal samples, *O. onites* material was pre-treated by a steam-distillation procedure by Türer Ltd, Izmir, Turkey.

4.3. Extraction techniques for the plant material (I)

In study I, the extraction of phenolic antioxidants from sage was studied using four different extraction methods: pressurised hot-water extraction (PHWE), extraction with methanol, 70% ethanol and hydrodistillation. The details on extraction techniques other than the hydrodistillation used in the production of the study extracts are presented in study I. For the hydrodistillation, 50 g of dry herb material was boiled with 500 ml of water in a round-bottom flask connected to a Ph Eur hydrodistillation apparatus for two hours. The water was removed and 300 ml of pure water added, following which the herb material was further boiled for one

hour. During the two-step boiling the volatile components were removed from the plant material. The plant material was removed by filtering and the water fractions were combined, freeze-dried and stored at 4°C until used.

4.4. Studies on the antioxidant properties of Lamiaceae extracts and phenolic constituents and metabolites of *O. onites* in vitro (I-IV)

Analysis of total phenols in the study extracts. The total phenolic compounds in the study extracts were determined as GAE (Singleton *et al*, 1999). A weighed amount of dry extract was dissolved into accurate volume of water, and the solution was transferred to a volumetric flask. Water and undiluted Folin-Ciocalteu reagent were added and after 1 min, 20% (w/v) Na₂CO₃ was added and the flask was filled up to volume with H₂O. In the reaction, reducing substances in the sample react with Folin-Ciocalteu reagent, resulting in formation of chromogens which can be spectrophotometrically measured at 760 nm. After 1 h incubation at 25 °C, the absorbance of the solution was measured at 760 nm and the GAE value calculated using a calibration curve prepared previously with gallic acid. The GAE values are the mean of duplicate analyses.

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) scavenging assays. The ability of the samples to scavenge free radical DPPH• was determined spectrophotometrically by measuring a decrease in the absorbance caused by the radical at 517 nm. As an antioxidant donates an electron to a DPPH• radical molecule, the purple colour and corresponding absorbance of the radical at 517 nm disappears. Two DPPH• assays were used: In study II, assay of Gyamfi *et al*, 1999 was used and in study IV, the assay of Goupy *et al*, 1999 was employed. The principle of these two assays is the same, the main difference being that in the former, the reaction solution is buffered. Briefly, for the assay in study II the extracts were diluted in Tris-HCl (2-amino-2-[hydroxymethyl]-1,3-propanediol –hydrochloric acid) buffer to a concentration series and methanolic DPPH• solution was added. Control consisted of buffer and DPPH• in methanol. After a 30 minute reaction period, the absorbance of the solutions was recorded at 517 nm. The percentage inhibition (PI) of the radical absorbance was calculated as follows: $PI = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$. The IC₅₀ value (sample concentration yielding a 50% inhibition of the radical absorbance) was then estimated using Sigma-Plot 2001 (version 7.0, SPSS Inc., Chicago, IL, US). The results were expressed as IC_{50(mg/mL)}. In study IV, samples were diluted to a concentration series in methanol, and methanolic DPPH• solution

was added. After 30 minutes, the absorbance of control and samples at 517 nm was determined. The (PI) of the radical absorption was calculated as described above. The IC_{50} values were calculated using the equation $IC_{50} = C_1 - \Delta C$, where $\Delta C = [(C_1 - C_2) \times (PI_1 - 50)] / (PI_1 - PI_2)$ and PI_1 is the antioxidant activity value superior to 50% inhibition and PI_2 is inferior to 50% inhibition; C_1 and C_2 are the sample concentrations corresponding to PI_1 and PI_2 , respectively. The results were expressed as $1/IC_{50(mg/mL)}$ values.

Fe³⁺ to Fe²⁺ reduction capacity assay. The reductive capacities of the extracts were assessed using ferric to ferrous reductive activity as determined spectrophotometrically from the formation of Perl's Prussian blue coloured - complex (Yildirim *et al*, 2000). Extracts were diluted in distilled water, phosphate buffer (pH 7.0) and 1% (w/v) potassium ferricyanide [$K_3Fe(CN)_6$] solution were added. After a 30-minute incubation at 50°C, trichloroacetic acid (10%, w/v) was added and the mixture was centrifuged for 10 minutes. Finally, a volume of the upper layer was mixed with water and $FeCl_3$ solution (0.1%, w/v), and the absorbance was recorded at 700 nm. The results were expressed as relative reduction efficiency as compared to ascorbic acid.

ABTS^{•+} decolourisation assay. The determination of ABTS^{•+} radical scavenging was carried out as described by Re *et al*, 1998. The ABTS^{•+} radical was produced by reacting aqueous ABTS solution with $K_2S_2O_8$ solution in the dark for 12–16 h at ambient temperature, and adjusting the UV absorbance at 734 nm to 0.700 (± 0.020) with water at 30°C. The extracts were diluted into a concentration, in which sample added to ABTS^{•+} resulted in a 20% - 80% inhibition of the blank absorbance. After ABTS^{•+} was added to extract samples and Trolox™ standards, the absorbance at 734 nm was recorded at 1 min intervals for 10 minutes. The percentage inhibition of the blank absorbance was plotted as a function of concentration and the TEAC value was calculated using Trolox™ calibration curve.

Fe³⁺-EDTA/H₂O₂/ascorbate-catalysed deoxyribose oxidative degradation assay. The determination of OH[•] scavenging was carried out as described by Halliwell *et al*, 1987. A reaction mixture containing deoxyribose, phosphate buffer, extract, $FeCl_3$, EDTA (ethylenediamine tetraacetic acid), H_2O_2 and ascorbic acid was incubated at 37°C for 60 min. Butylhydroxytoluene, trichloroacetic acid and thiobarbituric acid (TBA) were added and the incubation was continued for 20 min at 80°C. The reaction was stopped by ice bath. Butanol was added, the tubes were vortexed and centrifuged for 10 minutes. The degree of deoxyribose degradation

was finally determined by measuring the absorbance of the organic layer at 532 nm. If the extract was capable of inhibiting the oxidative degradation of deoxyribose, the absorbance was lower as compared to that of the control. The PI was calculated as follows: $PI = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$.

Copper-induced LDL oxidation assay. The ability of the herbal extracts or single phenolic compounds to inhibit or delay the $CuSO_4$ -induced oxidation of LDL unsaturated fatty acids *in vitro* was assessed using a microwell-plate modification of the method by Esterbauer *et al*, 1989. A detailed presentation of the method is presented in study II. EDTA-plasma was separated from blood of healthy volunteers by centrifugation, following which the very low density lipoprotein (VLDL) and LDL fractions were isolated in a density gradient (cut-off densities $d < 1.006 \text{ g ml}^{-1}$ and $1.006 < d < 1.063 \text{ g ml}^{-1}$, respectively) by sequential ultracentrifugation (Havel *et al*, 1955). The LDL fraction was carefully collected, packed into cryo-tubes and stored at $-80 \text{ }^\circ\text{C}$ until used (maximum 2 months). Prior to the oxidation experiment, the EDTA used as a metal chelator was removed from the LDL fraction in Sephadex G-25 columns (Pharmacia Biotech, Uppsala, Sweden) using phosphate-buffered saline (PBS) as the eluent. After the removal of EDTA, the protein content of LDL fraction was determined by a method modified from that of Lowry *et al*, 1951 using BioRad Protein Assay Reagents (BioRad Laboratories, Hercules, CA, US) on a Perkin Elmer HTS7000+ BioAssay Reader (Buckinghamshire, United Kingdom) at 750 nm. Finally, the protein concentration was adjusted to $105 \text{ }\mu\text{g/mL}$ with PBS. For the oxidation, diluted LDL was pipetted on UV microtiter plate. The extracts diluted in water or single compounds diluted in ethanol were added to wells (except for the control), and the oxidation was initiated by aqueous $CuSO_4$ solution. The oxidation of LDL was assessed by measuring the formation of PUFA oxidation products, conjugated dienes, at 235 nm with the Perkin Elmer BioAssay Reader. The state of oxidation was followed at three minute intervals for 10 hours or 16 hours, depending on the samples in question. The duration (min) of the lag phase, i.e. the time preceding the propagation phase of the oxidation, was calculated from the intercepts of the tangent lines in the oxidation curves.

4.5. Human studies (III, V)

The human studies and analyses presented in the original publications III and V were carried out in the Research Institute of Public Health, University of Kuopio.

4.5.1. Kinetic study (III)

Subjects and study design

Six volunteers (four women and two men) aged 28-38 years with normal health (no chronic diseases or regular use of antioxidant supplements or medication, body mass index (BMI) <32 kg/m²) were recruited for this study among the staff of Research Institute of Public Health, Kuopio. For 7 days before the experiment, the subjects consumed a diet poor in phenolic compounds. This low-phenolic diet consisted mainly of dairy products, meat, fish, chicken, potato, pasta, rice and white bread, and was continued for the duration of the follow-up time. To determine the baseline urinary excretion of phenolic acids, 24-hour urine was collected before the experiment. At 0 hours the participants ingested a single dose (3.75 g) of *O. onites* extract packed in hard-gelatine capsules. This dose corresponds to 13.9 g of dried herb. All aliquots of urine production were collected at 2, 4, 6, 8, 12, 24, 30, 36 and 48 hour intervals after the ingestion of the extract. The volume of the collected urine samples was determined and aliquots of 2x10 mL were stored at -70 °C until analysed.

Analyses of O. onites extract and urine

HPLC apparatus and analytical conditions. Analyses were carried out with high performance liquid chromatography (HPLC) using coulometric electrode array detector (CEAD) (ESA Inc. Chelmsford, MA, US). HPLC system consisted of two pumps, autosampler with cooled sample tray and a thermostat chamber (37 °C) for columns and the detector. The detector consisted of two detector cells each containing four electrode pairs (channels) and one reference electrode to measure the background signals. A different potential was set on each channel to oxidize the analytes of interest. The potentials of channels 1-8 selected according the electrochemical properties of the analytes to achieve the highest sensitivity possible were 20, 100, 320, 390, 570, 600, 630 and 680 mV, respectively.

The mobile phase consisted of two eluents A: 50 mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ buffer pH 2.3:methanol (90:10, v/v) and B: 50 mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ buffer pH 2.3:methanol:acetonitrile (40:40:20, v/v/v). The flow rate was 0.3 mL/min and different gradient elution for the extract and urine samples was used. The injection volume used was 10 μl . The following columns were employed: guard column Quick Release C18 (Upchurch Scientific Inc., WA) 10 x 3 mm packed with 5 μm particles and analytical column Inertsil ODS-3 (GL Sciences Inc., Japan) 150 x 3 mm packed with 3 μm end-capped particles. Limits of detection (LOD) and linear ranges for signals were determined to evaluate the suitability of the detection conditions for phenolic acids in urine and in *O. onites* extract. The oregano extract was screened for the presence of the 45 compounds presented in Table 4. Based on the phenolic acid composition of the extract, the following phenolic acids were analysed from urine samples: *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, syringic acid, vanillic acid, *m*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, rosmarinic acid and chlorogenic acid.

Sample pre-treatment for oregano extract. An amount of dry *O. onites* extract was weighed and water was added, followed by ascorbic acid and hydrolysis reagent containing 2 500 U/mL of β -glucuronidase from *Helix pomatia* in sodium acetate buffer pH 4.1. Sample was incubated at 60 °C for 2 hours. Into cool sample, methanol was added, sample was shaken vigorously and centrifuged 10 min at 2500 rpm. The supernatant was transferred into volumetric flask, which was filled up to volume with methanol. The resultant solution was further hydrolysed with sodium hydroxide for 1 h at 90 °C. Into cool sample, methanol was added and sample was centrifuged 10 min at 2500 rpm. Supernatant was transferred into volumetric flask, which was filled up to volume with methanol. Both fractions were diluted with mobile phase prior to the HPLC run. Sodium hydroxide treatment was applied to quantify the total amount of ferulic acid present in the extract. Rosmarinic, protocatechuic, gallic, chlorogenic, caffeic, sinapic, *p*-, *m*-, and *o*-coumaric acids were quantified after the enzyme hydrolysis. Also *m*-hydroxy- and 3,4-dihydroxyphenylacetic acids were measured from the extract to confirm their presence merely in the urine samples, thus being pure *in vivo* metabolites. A weighed amount of *O. onites* extract was also dissolved in 80 % methanol and phenolic acids were measured after appropriate dilution to confirm the stability of the ester bond in rosmarinic acid molecules during the enzyme hydrolysis.

Sample pre-treatment for urine. Urine samples were thawed and 0.5 mL of urine was taken into analysis. Ascorbic acid was added, followed by 0.5 mL of enzyme hydrolysis reagent containing 2 500 U/mL of β -glucuronidase from *Helix pomatia* (Burgundy snail) in sodium acetate buffer pH 5. Sample was mixed and incubated overnight at 37 °C. Into cool sample, HCl was added and sample was extracted twice with diethyl ether in a multitube shaker (2 min at 2000-2500 rpm). The diethyl ether phase was separated after freezing the water phase. The extracts were combined, evaporated under nitrogen flow and dissolved in methanol. Prior to the HPLC run, the sample was diluted with eluent A. In each assay, a reagent blank containing water instead of urine was prepared and treated similarly with the samples. *Helix pomatia* enzyme mixture contained small amounts of some phenolic acids, thus the blank was subtracted from the samples. The recoveries of the phenolic acids were evaluated by adding standards into water and treating them similarly with the sample. Control sample was used to check the overall performance of the sample pre-treatment procedure.

4.5.2. Supplementation study (V)

Subjects, study design and supplement used

45 non-smoking male volunteers from the Kuopio area were recruited for the 4-week double-blinded supplementation trial using the following inclusion criteria: no severe obesity (BMI < 32 kg/m²), no regular use of medicines or nutritional supplements with antioxidant or lipid lowering properties, no chronic diseases like diabetes, CHD or other major illness, and willingness to consume 375 mL of the study beverage daily for 4 weeks. The subjects were randomly assigned to consume daily 375 mL of the study beverage: mango-orange juice (placebo group, n = 15), mango-orange juice enriched with oregano extract containing 300 mg GAE total phenolic compounds (low phenolic [LP] group, n = 15) or mango-orange juice enriched with oregano extract containing 600 mg GAE total phenolic compounds (high phenolic [HP] group, n = 15).

The placebo juice was prepared from an orange-mango juice concentrate (Tekno-Food, Finland). The concentrate was chosen to be used due to its very low antioxidant capacity and phenolic content (unpublished observations). To prepare the LP and HP juices, 5.35 g/L and 10.7 g/L of oregano extract were added in the placebo juice, respectively. The daily dose of study beverages consisted of three

125 mL brick packages, the daily dose of the oregano extract being 2 g in the LP group and 4 g in the HP group. These doses correspond to 5.55 g and 11.1 g of dried oregano, respectively. The subjects were instructed to consume beverages with meals, one package in the morning, one in the afternoon and one in the evening. The subjects were advised to refrain from consuming tea, red wine, cocoa and chocolate one week prior to and during the study and to avoid the use of alcohol and analgesics three days before and vigorous physical activity one day before the study visits. They were also given instructions for the four-day food recording that was demanded at the baseline and during the last week of the intervention period to control possible confounding factors and to check the compliance to given instructions. The regular consumption of study beverages was checked by a questionnaire designed for the study.

In this work, both short- and long term effects of juice consumption were studied. The short-term study was conducted at the study baseline (day 0) before the long-term supplementation study using the same subjects and study groups as in long-term study. Blood samples were drawn at the baseline after an overnight (10 h) fast and 1.5 hours after the consumption of 250 mL (two tetra brick packages) of study juices. The same selection of analyses was carried out both in the short- and long term studies, *i.e.* at the baseline, 1.5 hours after ingestion of the first dose of study beverages and after the 4-week supplementation period. The subjects collected 24-h urine samples on the day preceding to the baseline study visit, and during the last 24 hours of the supplementation period. Volume of the urine was determined and aliquots were stored at -70 °C until urinary phenolic acids were determined.

Determination of the total phenolic content and phenolic compounds in the O. vulgare extract and in the study beverages. The total phenolic content of the oregano extract was determined as described earlier in 4.4. The analyses of individual phenolic compounds in the extract and in the study juices were carried out as described in 4.5.1.

Measurement of DPPH• radical scavenging capacity of the study beverages. The measurement of the DPPH• radical scavenging capacity of the study beverages was carried out as described in 4.4. (Goupy *et al.*, 1999). The IC₅₀ value means the dilution of each juice resulting in 50% inhibition of the radical absorbance, and the results are expressed as 1/IC₅₀.

Measurements of oxidative stress and lipid peroxidation

Plasma TRAP. Plasma TRAP was determined from plasma with a method modified from (Metsä-Ketelä, 1991) as previously described by Nyysönen *et al*, 1997. Briefly, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was added into luminol containing buffer and the formation of peroxy radicals in the thermal decomposition of AAPH was followed with a luminometer (Model 1251, Bio Orbit Oy, Turku, Finland) at 32°C. After 15 minutes, plasma sample extinguishing the chemiluminescence was added, and the TRAP value was calculated from the duration of the absence of chemiluminescence.

Resistance of serum lipids to oxidation. The resistance of serum lipids to oxidation was measured as described earlier (Nyysönen *et al*, 1997). In brief, serum was diluted in phosphate buffered saline, pH 7.4. Oxidation was initiated by an addition of CuCl₂ into diluted, pre-warmed (30 °C) serum. The formation of conjugated dienes was followed by monitoring the change in the UV-absorbance at 234 nm on a Beckman DU-6401 spectrophotometer (Fullerton, California, US) equipped with a six-cuvette automatic sample changer. The absorbance was recorded every 5 min for 4 h. The lag-time was determined as the time period before the propagation phase (maximum rate of oxidation).

Baseline conjugated dienes. The oxidation of LDL *in vivo* was assessed as the amount of conjugated dienes as described previously (Ahotupa *et al*, 1998). In brief, serum LDL was isolated by precipitation with buffered heparin. The precipitate was re-suspended in phosphate buffered saline. The cholesterol concentration was determined and the rest of the suspension was used for the measurement of conjugated dienes. Lipids were extracted from the LDL by a mixture of chloroform and methanol (3:1), dried under nitrogen, re-dissolved in cyclohexane, and the amount of conjugated dienes was measured spectrophotometrically at 234 nm and 300 nm. The absorbance at 300 nm was subtracted from that at 234 nm to reduce background interference, and the concentration of conjugated dienes in LDL was calculated per concentration of cholesterol in LDL.

Plasma F₂-isoprostanes. The plasma free F_{2α} -isoprostane concentrations were determined at Oy Jurilab Ltd., Kuopio, Finland. A deuterated prostaglandin F_{2α} internal standard was added to plasma, and F_{2α} -isoprostanes were extracted with C₁₈ and silica minicolumns. Compounds were converted to pentafluorobenzyl ester

trimethylsilylether derivatives and analyzed by a gas chromatographic-mass spectrometric assay (Morrow & Roberts, 1994).

Other laboratory measurements

Safety measurements. Serum alanine aminotransferase (ALAT, ALT/GPT Cat No 981110, Thermo Electron, Vantaa, Finland) and creatinine (Creatinine, Cat No 981811, Thermo Electron, Vantaa, Finland) were measured with a Kone Specific clinical chemistry analyzer (Thermo Electron, Vantaa, Finland). The catalytic concentration of gamma-glutamyltransferase (γ -GT, Cat no 981093, Thermo Electron, Vantaa, Finland) was measured using the International Federation of Clinical Chemistry method (Shaw *et al*, 1983) with Kone Specific clinical chemistry analyzer.

Serum lipids and lipoproteins. Serum cholesterol (Cholesterol, Cat No 981261, Thermo Electron, Vantaa, Finland) and triglycerides (Triglycerides GPO-PAP, Cat No 701904, Roche Diagnostics, Mannheim, Germany) were determined with enzymatic colorimetric tests using Kone Specific clinical chemistry analyzer. The serum concentration of high-density lipoprotein (HDL) cholesterol was measured from supernatant after magnesium chloride dextran sulphate precipitation. The serum concentration of LDL cholesterol was determined by a direct cholesterol measurement (Cat No 981656, Thermo Electron, Vantaa, Finland).

Serum fatty acids. Serum fatty acids (arachidonic, linoleic and linolenic acid) were extracted from serum with chloroform-methanol (2:1) and water was added. The two phases were separated after centrifugation and the upper phase was discarded. To the chloroform phase, methanol-water (1:1) was added and the extraction was repeated. The chloroform phase was evaporated under nitrogen. For methylation, the residue was treated with sulphuric acid-methanol (1:50) at + 85 °C for 2 h. The mixture was extracted with petroleum ether. The fatty acids from the ether phase were determined by a model 5890 gas chromatograph (Hewlett Packard, Avondale, Pennsylvania, US) equipped with a flame ionization detector and NB-351 capillary column (HNU-Nordion, Helsinki, Finland).

Serum folate and plasma homocysteine. Serum folate concentrations were measured by Quantaphase II radioimmunoassay (Bio-Rad, Hercules, California, US). The serum total homocysteine concentrations were analyzed by HPLC at the National Public Health Institute, Helsinki, Finland (Schwab *et al*, 2002).

5. RESULTS

5.1. Extraction of phenolic antioxidants from sage (I)

In this work, the radical scavenging activity towards DPPH[•] radical was used to evaluate the applicability and efficiency of different methods to extract antioxidants from sage. The highest radical scavenging activity (1/IC₅₀ value 25) was attributed to the total extract from PHWE without solid phase trapping. Radical scavenging activity of the extracts obtained by hydrodistillation and ultrasonication-assisted methanol extraction were similar (1/IC₅₀ values 14.3 and 12.0, respectively) and slightly weaker than the activity of the extract obtained by maceration with 70% ethanol (1/IC₅₀ value 19.3).

PHWE was tested at three temperatures: 70, 100 and 150 °C. The extraction at 100 °C yielded the highest radical scavenging activity in the extract. This was despite the higher recovery of identified phenolic analytes (rosmarinic acid, carnosol, carnosic acid and methyl carnosate) obtained by extraction at 150 °C. The HPLC profile of PHWE extract without solid phase trap was similar to the one obtained by ultrasonication-assisted methanol extraction. Of the tested solid phase traps, cyclohexyl trapping material resulted in consistently higher radical scavenging activity as compared to C18 trapping material, despite the fact that rosmarinic acid was more strongly attached to C18 trap material.

The hydrodistillation method with the most polar solvent, water, extracted only the polar compounds from sage. The second most polar solvent, 70% ethanol, also extracted some of the less polar compounds. Methanol with a less polar nature easily extracted less polar compounds. However, among conventional extraction techniques the highest antioxidant activity was achieved in the maceration with 70% ethanol, and also the highest recovery of rosmarinic acid was achieved with this solvent.

As the hydrodistillation method was used to prepare extracts for usage in further studies, the mean yields of dry extracts (% weight of the original material) for different herbs were: *S. officinalis* 25%, *R. officinalis* 24%, *T. vulgaris* 29%, *O. vulgare* 36% and *O. onites* 27%.

5.2. Phenolic compounds in Lamiaceae extracts (II, III, IV) and study juices (V)

In study II, the amounts of total phenolic compounds determined as GAE in the studied extracts were: oregano 149mg/g, rosemary 185mg/g, sage 166mg/g and thyme 96mg/g. In this work, no HPLC analyses for the individual phenolic compounds were carried out. The total phenolic content of *O. onites* extract used in study III and study IV was determined to be 134mg GAE/g. In the single dose of 3.75 g oregano extract ingested in study III, the calculated amount of total phenolic compounds was about 500 mg GAE.

To identify as many phenolic constituents of *O. onites* as possible in study III, altogether 45 compounds were included in the list of analytes to be screened. As a result, the following phenolic acids were quantified: rosmarinic acid (12.7 mg/g), protocatechuic acid (2.1 mg/g), *p*-coumaric acid (1.0 mg/g), ferulic acid (0.50mg/g), chlorogenic acid (0.31mg/g) and gallic acid (0.15mg/g). In total, the dose ingested contained 63 mg (223 μ mol) of quantified phenolic acids. Also luteolin (3.55 mg/g) and eriodictyol (0.19 mg/g) were found in the extract; however, they were not included in the list of analytes in urine analyses.

In study V, *O. vulgare* extract was used as antioxidant polyphenol supplement. The total phenolic content of the study extract, determined as GAE, was 156 mg/g. In the HPLC analyses, the same selection of analytes as in study III were used, and the following phenolic acids were quantified: rosmarinic acid (16.03 mg/g), protocatechuic acid (1.30 mg/g), ferulic acid (0.37 mg/g), gallic acid (0.22 mg/g), caffeic acid (0.18 mg/g), chlorogenic acid (0,16 mg/g) and *p*-coumaric acid (0,06 mg/g). Of the flavonoids, luteolin (1.99mg/g) and eriodictyol (0.11 mg/g) were found. In the publication V, the described phenolic composition of *O. vulgare* extract was incorrect, i.e. the extract was reported to have a phenolic profile different from the one reported here. The explanation for this discrepancy is that in the published article, analysis results from a different batch of *O. vulgare* extract were erroneously reported. The phenolic composition of *O. vulgare* extract presented above is the correct phenolic content of the supplement used. The reported total phenolic contents i.e. 300 mg GAE and 600 mg GAE per day for LP and HP juices, respectively, were correct. The correct daily doses of quantified phenolic compounds in the study juices were 41 mg in the LP group and 82 mg in the HP group, corresponding to 131 μ mol and 262 μ mol of compounds, respectively.

In the HPLC analyses of study juices, the following phenolic compounds were identified (Table 5.):

Table 5. Identified phenolic acids and flavonoids (mg/L) in study (V) juices. LP = low phenolic, HP = high phenolic.

Compound	Placebo juice	LP juice	HP juice
rosmarinic acid	-	74.64	155.9
protocatechuic acid	-	8.03	17.57
ferulic acid	1.05	7.08	14.65
caffeic acid	-	1.83	3.39
<i>p</i> -coumaric acid	0.04	6.40	12.45
luteolin	-	23.08	42.63
eriodictyol	-	1.08	1.97
naringenin	0.31	*	*
hesperetin	1.13	0.75	0.69

* = identified but not quantified due to partial overlapping of the signal.

5.3. Antioxidant activity of Lamiaceae extracts, their phenolic constituents, human metabolites and study juices *in vitro* (II, IV, V)

In study II, the *in vitro* antioxidant activity of four *Lamiaceae* extracts: oregano, rosemary, sage and thyme was studied using five different assays. In Table 6., the ranking order of the extracts in these five assays is given.

Table 6. The ranking order of Lamiaceae extracts in study II using five different in antioxidant methods. * Sum of rankings = the combined ranking from the five assays.

Method	Sage	Oregano	Rosemary	Thyme
Fe ³⁺ to Fe ²⁺ reduction	1	3	2	4
DPPH [•] scavenging	2	3	1	4
ABTS ^{•+} scavenging	1	2	2	3
Deoxyribose assay (OH [•])	1	2	3	3
LDL oxidation	2	3	1	4
* Sum of rankings	7	13	9	18

Based on all the five assays used, sage extract was the one with the highest antioxidant activity, closely followed by rosemary extract. Oregano extract exhibited antioxidant activity inferior to these two extracts, but clearly superior to that of thyme extract. In the iron reductive capacity assay, the reductive power of all the extracts was lower as compared to that of ascorbic acid, a strong reductive agent used as a standard in this assay. The relative reductive efficiency varied from 0.099 (thyme) to 0.201 (sage), compared to ascorbic acid having a value of 1.000. In the radical scavenging assays, sage showed the strongest antioxidant activity, followed by rosemary and oregano. Of the tested samples, thyme exhibited clearly the weakest radical scavenging capacity. In ABTS^{•+} assay, the determined TEAC values (mmol/L) were in the range of 4.9-15.5., depending on the sample and the time point of determination. In the LDL oxidation assay, the extracts were capable of delaying the onset of LDL oxidation. None of the extracts were capable of slowing down the rate of LDL oxidation in the propagation phase, as judged by the slopes of oxidation curves.

In study IV, the antioxidant activity of phenolic acids identified from *O. onites* as well as their urinary human metabolites (from study III) was evaluated using DPPH[•] scavenging and LDL oxidation assays. Based on their radical scavenging activity, compounds could be classified into inactive, intermediately active and highly active ones. The following compounds were practically inactive against DPPH[•] radical (activity too low for determination of IC₅₀ value): *p*-hydroxybenzoic acid, vanillic acid, *m*-hydroxyphenylacetic acid, *m*-hydroxyphenylpropionic acid and *p*-coumaric acid. Intermediate activity was observed for the following compounds, in decreasing order of activity: syringic acid > eriodictyol > caffeic acid > chlorogenic acid > protocatechuic acid > ferulic acid. The highest radical scavenging capacity was observed, in decreasing order of activity, for gallic acid > 3,4-dihydroxyphenylacetic acid > luteolin = rosmarinic acid > 3,4-dihydroxyphenylpropionic acid. Based on their molar lag-phase prolonging effect in the LDL oxidation assay, compounds were classified into inactive, intermediately active and highly active ones. The inactive compounds included ferulic acid, syringic acid, vanillic acid, *p*-coumaric acid, *m*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and *m*-hydroxyphenylpropionic acid. Gallic acid, 3,4-dihydroxyphenylpropionic acid and protocatechuic acid had an intermediate antioxidant effect in this assay. The following compounds, in decreasing order of activity, showed a strong increasing effect on the lag-time: luteolin > eriodictyol > rosmarinic acid > caffeic acid > 3,4-dihydroxyphenylacetic acid > chlorogenic acid.

The juices used in study V were tested for their DPPH^{*} scavenging activities. The 1/IC₅₀ values for the juices were 16, 491 and 861 for placebo juice, LP juice and HP juice, respectively.

5.4. Excretion of urinary phenolic metabolites of *O. onites* extract in humans (III)

In study III, six volunteers ingested 3.75g of *O. onites* extract packed in hard gelatine capsules and the urinary excretion of phenolic metabolites was followed for 48 hours. The dose corresponded to 500 mg GAE of total phenolic compounds and 63 mg (223 μ mol) of identified phenolic acids. A marked increase was observed in the urinary excretion of phenolic metabolites. During 0-24 hours of the follow-up time, the excretion was about four-fold (403 ± 69 μ mol/d) as compared to baseline excretion, 95 ± 11 μ mol/d. During 24-48 hours of the follow-up, the excretion was about double (172 ± 21 μ mol/d) as compared to the baseline.

At the baseline, the major compound excreted in urine was vanillic acid, followed by *p*-hydroxybenzoic acid, *m*-hydroxyphenylacetic acid, ferulic acid and 3,4-dihydroxyphenylacetic acid, respectively. Low amounts of protocatechuic acid, syringic acid, *p*-coumaric acid and caffeic acid were also detected. During the first day of the follow-up, the main compound excreted was *p*-hydroxybenzoic acid, followed by, in decreasing order of quantity, vanillic acid, *m*-hydroxyphenylacetic acid, protocatechuic acid, ferulic acid and 3,4-dihydroxyphenylacetic acid. Minor amounts of syringic acid, caffeic acid and *p*-coumaric acid were also detected. During the second day, *m*-hydroxyphenylacetic acid was the main compound excreted in urine, followed by, in decreasing order of quantity, vanillic acid, *p*-hydroxybenzoic acid, ferulic acid and 3,4-dihydroxyphenylacetic acid. Low amounts of protocatechuic acid, caffeic acid, *p*-coumaric acid and syringic acid were also excreted. Rosmarinic acid, gallic acid or chlorogenic acid were not detected in any urine samples at the baseline or during the 48-hour follow-up time.

During the total 48-hour follow-up time, the mean increasing effect of the supplement on the excretion of identified phenolic compounds (baseline extraction subtracted) was altogether 385 ± 114 μ mol. The main metabolite excreted was *p*-hydroxybenzoic acid, representing 45% of the identified urinary phenolics.

5.5. Effects of *O. vulgare* extract supplementation on antioxidant biomarkers *in vivo* (V)

In the *O. vulgare* extract supplementation study, the compliance of the participants was good. None of the participants dropped out during the 4-week study. No adverse effects were reported. No significant changes were detected in serum ALAT or γ -GT between study groups during the study period. Serum creatinine concentration decreased by 2% ($P=0.123$) in LP group and increased by 5% ($P=0.015$) in HP group. The significant increase in HP group was mostly due to $>10 \mu\text{mol/L}$ increase in three study subjects. In the post hoc analysis, the increase of serum creatinine concentration was significantly greater in the HP group as compared with LP group ($P=0.002$), but no significant difference was seen between HP and placebo groups ($P=0.144$). Pearson's correlation coefficient (R) between changes in excretion of total phenolics and changes in serum creatinine was 0.25 ($P=0.107$) in the whole study population. In a linear regression model, the variables with strongest associations with increase in serum creatinine concentration were changes in serum TRAP concentration (standardised coefficient 0.44, $P=0.001$), urinary excretion of vanillic acid (0.36, 0.009), serum lagtime (-0.38, 0.004), plasma total homocysteine (0.30, 0.023) and serum γ -GT (0.34, 0.010), adjusted R^2 for model was 0.44, $P<0.001$.

At the baseline, the study groups did not differ from each other by physical characteristics or age. However, a significant difference was observed in the baseline urinary excretion of phenolic compounds ($P=0.013$): the excretion in the placebo and LP group were 26% and 72% higher than in HP group, respectively.

The consumption of placebo juice or juices enriched with oregano extract did not have effects on the concentrations of serum lipoproteins (triglycerides, total-, LDL- or HDL cholesterol), or plasma tHcy concentrations. Changes in the 24-hour excretion of phenolic compounds were different between the study groups: the excretion decreased 10% in the placebo group, while excretion increased 2% in the LP and 85% in the HP groups ($P < 0.001$).

A favourable effect was seen in plasma TRAP within HP group, as TRAP increased significantly in short-term measurements ($P=0.017$). Conversely, an unfavourable effect was noticed in long-term measurement within LP group, as the level of LDL baseline conjugated dienes increased ($P=0.023$). No significant changes between the study groups in either short-or long-term measurements of antioxidant capacity or lipid peroxidation were found.

6. DISCUSSION

6.1. Evaluation of the methods used

6.1.1. Extraction of the herb material

Of the two water-based methods, PHWE exhibited higher extraction efficiency than hydrodistillation at similar temperature. The high pressure in PHWE possibly eases the penetration of water into the plant matrix or otherwise physically alters the matrix, resulting in extraction of also the less polar compounds such as carnosic acid. PHWE showed to offer a promising alternative to conventional solid-liquid techniques in the extraction from plant matrixes of natural compounds with a variety of polarities. The hydrodistillation method was, however, chosen for the production of the study extracts due to several reasons. As shown for sage in study I, the hydrodistillation method extracted only components of polar nature as compared to the other techniques studied; the same finding was confirmed in a recent study of Kivilompolo & Hyötyläinen (2007). The more lipophilic constituents of Lamiaceae herbs, for example diterpenes, in human extract supplements were avoided. The major reason for choosing this technique was that the sensory properties of the produced extracts were acceptable, as the characteristic strong aroma and taste of Lamiaceae herbs was weakened due to removal of volatile oils during the hydrodistillation. As purified water was used as the extraction solvent, risk of organic solvent residues remaining in the extracts was avoided.

The hydrodistillation method was, contrary to pressurised hot water extraction, easily transformable to a larger scale production, as was seen in the preparation of *O. vulgare* extract for study V. As can be seen from the yields of dry extracts by the hydrodistillation method, a large portion of plant material was extractable with boiling water. In a study of Parejo *et al* (2002), steam distillation (volatile oil removing process) was shown to increase the antioxidant capacity, determined as radical scavenging capacity, of Lamiaceae herb extracts as compared to non-distilled extracts. Further support for our choice of extraction technique for the preparation of study extract from *O. vulgare* was presented by Kivilompolo & Hyötyläinen (2007), who reported that the rosmarinic acid content of *O. vulgare* extract prepared similarly to our hydrodistillation method yielded a higher recovery

of rosmarinic acid from herb material as compared to extraction with 60% ethanol, and similar to 80% methanol.

6.1.2. Total phenolic assay and *in vitro* antioxidant methods

In this work, the rough evaluation of the total phenolic content of the study extracts was carried out by using the FC reagent. The more accurate analysis of the phenolic content was carried out using chromatographic techniques. The FC method has been widely used and has many advantages: it is simple, sensitive and precise, and if the original procedure is accurately followed, comparable results can be produced (Prior *et al*, 2005). There are, however, problems concerning the use of this method: depending on the sample in question, several other constituents than phenolic compounds are capable of reducing action and may thus co-react in the assay. The most common interfering substances are ascorbic acid, sugars, aromatic amines, organic acids and Fe^{2+} (Prior *et al*, 2005). When interpreting the results, the possible confounding effects of co-reactants have to be kept in mind. Also the use of varying modifications of the original procedure of Singleton & Rossi (1965) has resulted in problems when comparing results between studies. A properly validated version of this assay traditionally used for determination of the total phenolic content of the sample has been suggested as an antioxidant method of choice for determining the total reductive capacity of samples (Huang *et al*, 2005).

The reductive capacity of the extracts was studied using the Fe^{3+} to Fe^{2+} reduction assay. This method, similarly to FC assay and DPPH[•] assay is based on the determination of the the electron donating ability of the sample, and as they should reflect the same type of antioxidant action, it is interesting to compare the results from these assays. The major drawback of the Fe^{3+} to Fe^{2+} reduction assay is that it cannot detect antioxidants acting by hydrogen transfer mediated radical scavenging mechanism (Prior *et al*, 2005). However, the reductive power of a sample still reflects the ability to maintain and modulate redox tone in plasma and tissues. As the Fe^{3+} to Fe^{2+} reduction assay is totally based on the electron transfer process, it can, combined with other antioxidant assays, be used to characterize the antioxidant properties of a sample in question.

The DPPH[•] radical scavenging assay used to study the radical scavenging properties of the study extracts and phenolic compounds by electron donation, and possibly to a lesser extent by hydrogen atom transfer, was the first antioxidant

method set up in our laboratory in Helsinki. The method has often been utilised in antioxidant research due to its simplicity, rapidity and minor instrumentation requirements (Prior *et al*, 2005). In contrast to the chemically generated radicals such as $\text{ABTS}^{\bullet+}$, DPPH^{\bullet} may be utilized in aqueous and nonpolar organic solvents such as benzene and can be used to examine both hydrophilic and lipophilic antioxidants. However, it has been hard to compare the DPPH^{\bullet} scavenging capacity data between different laboratories or from the same group at different times because the results are highly dependent e.g. on the reaction time, the concentration of DPPH^{\bullet} used and the antioxidant(s) in the assay mixture. Another drawback is that this radical is, as compared to biologically relevant radicals, very long-lived and the rating of radical-scavenging activity based on just DPPH^{\bullet} assay may lead to misinterpretations. Also, due to molecular structure of DPPH^{\bullet} radical (Figure 2.), the radical scavenging action of an antioxidant is largely dependent on its molecular size: in case of larger molecules such as flavonoid glycosides, the steric hindrance of DPPH^{\bullet} radical affects the capability of antioxidant action (Prior *et al*, 2005), and may result in too low activities reported.

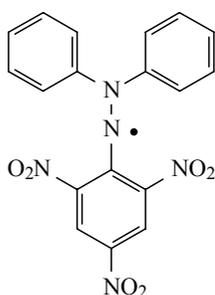


Figure 2. Structure of the DPPH^{\bullet} radical molecule.

The radical scavenging properties if the study extracts were also studied using $\text{ABTS}^{\bullet+}$ radical. Advantages of this TEAC method are its operational simplicity and rapidity, usability for both hydrophilic and lipophilic antioxidants as well as possibility of automation (Prior *et al*, 2005). On the other hand, $\text{ABTS}^{\bullet+}$ radical is non-physiological, and use of a short time-point detection of absorbance may result in low TEAC values. Also variations in the assay conditions pose challenges for comparison of results between studies. It has been stated that the use of TEAC assay is not ideal to quantitatively evaluate the antioxidant properties of a sample,

but it can be used to provide a ranking order of antioxidant samples (Van den Berg *et al*, 1999).

The antioxidant properties of the study extracts were also studied in the deoxyribose oxidative degradation assay. This assay has been designed for testing the capability of antioxidants to scavenge OH[•] radicals generated in reactions between hydrogen peroxide and iron ions. The approach of generating and testing a radical with real biological relevance is ideal; however, the use of metal ions in radical generation can lead to misinterpretations, as metal chelating action cannot be distinguished from radical scavenging action (Huang *et al*, 2005). In the assay modification we used, the excess of iron ions was chelated with EDTA. The real relevance of *in vitro* scavenging activity of OH[•] radicals by a sample has also been criticised: detrimental OH[•] radicals are short-lived and react readily with practically any molecule nearby, compared to which dietary antioxidants in human body are present in insignificant amounts. Thus, the preventive action against OH[•] radical formation by metal chelation or decomposition of H₂O₂ into harmless products *in vivo* by polyphenols is more feasible, and the approach of measuring the capacity of food phytochemicals to prevent the *in vitro* formation of OH[•] radicals instead of measuring the OH[•] radical scavenging capacity has been suggested (Huang *et al*, 2005).

As the oxidation of LDL particles has been proposed as one of the major events in the aetiology of atherosclerosis, we chose to use the *in vitro* human LDL oxidation assay to characterise the antioxidant properties of our study extracts and some phenolic components. In copper-induced LDL oxidation model, lipid peroxidation requires continuous formation of radicals at the lipoprotein particle surface by the Cu⁺/Cu²⁺ redox cycle, in which hydroperoxides and LDL-associated reducing agents have an important role. Following the production of free radicals, peroxidation propagates via their interactions with PUFA. Even though the pathophysiological role of both copper and radical induced oxidation of LDL have been questioned, copper catalysed LDL oxidation model may be regarded as a reasonable model for oxidation of LDL *in vivo*, as it plays a catalyzing role and also induces production of free radicals when reacting with LDL-associated antioxidants or hydroperoxides (Pinchuk & Lichtenberg, 2002). This method is advantageous for providing information on the kinetics of oxidation and the spectroscopic determination of products of oxidation at 234 nm has little background interference (Pinchuk & Lichtenberg, 2002). However, there are some shortcomings: both conjugated diene hydroperoxides and 5-ketocholesterol absorb at the same

wavelength. As the method requires isolated LDL from different volunteers to be prepared on a regular basis, it is not possible to get analogous LDL preparations to be used. This limits the use of LDL oxidation assay as a consistent, high-throughput approach assay method. Pinchuk & Lichtenberg (2002) have stated that the key factor governing the lag-time in copper-induced oxidation model is the amount of pre-formed ROOH in the sample to be oxidized. Thus, an antioxidant may act by enhancing the rate of ROOH decomposition during the lag-phase. In addition, several other antioxidant mechanisms, independently or concurrently, can be involved: chelation of transition metal ions, inhibition of binding of metal ions to apolipoprotein surface, inhibition of inter- or inraparticle transfer of reactive intermediates, scavenging of free radicals or reducing of transition metal ions (Pinchuk & Lichtenberg, 2002). Due to several possible mechanisms of action, an observed increase in the lag-phase by addition of antioxidant cannot be simply attributed to a certain antioxidant mechanism. Additional information on the mechanisms of action involved might have been found by also using AAPH radical induction in the assay.

In Table 7., evaluation of characteristics of each method is presented.

Table 7. Evaluation of selected characteristics of *in vitro* antioxidant methods used in this study: simplicity of use, instrumentation required, biological relevance, and the principal antioxidant mechanism(s) involved in the assay. DPPH• = 1,1-diphenyl-2-picrylhydrazyl radical, TEAC = Trolox® equivalent antioxidant capacity, Fe red. = Fe³⁺ to Fe²⁺ reduction assay, OH• = deoxyribose degradation assay, FC = Folin-Ciocalteu assay, LDL ox. = copper-induced LDL oxidation assay. +, ++, +++ = desirable to highly desired characteristic; -, --, --- = less desirable to highly undesirable based on this characteristic. (Partially modified from Huang *et al*, 2005 and Prior *et al*, 2005).

Method	Simplicity	Instruments	Clinical relevance	Mechanism
DPPH•	+++	++	---	single electron transfer
TEAC	+	++	-	single electron transfer
Fe red.	+++	++	--	single electron transfer
OH•	-	++	+	hydrogen atom transfer
FC	++	++	--	single electron transfer
LDL ox.	-	-	++	mixed

To conclude, this study employed a range of assays to characterise the *in vitro* antioxidant properties of different Lamiaceae extracts as well as phenolic constituents and human metabolites of *O. onites*. The results obtained from different assays clearly demonstrate the inadequacy of using a single method to evaluate the antioxidant properties of a sample. Both single electron transfer and hydrogen atom transfer based radical scavenging assays were used. Assays determining the peroxy radical scavenging activity, such as ORAC or TRAP, would have been of greater physiological relevance as compared to DPPH[•] or ABTS^{•+} scavenging based assays. The TRAP method was, however, employed to study the antioxidant capacity of human plasma in study V.

6.1.3. Kinetic study

Subjects and study design

In the kinetic study, which followed the urinary excretion of phenolic metabolites of *O. onites*, data was obtained from six study participants. Although this is a rather low number of study subjects, it can still be considered as adequate for this type of experiment, especially as the interindividual variation remained reasonable. One week prior to and during the experiment, the participants consumed a polyphenol-restricted diet to avoid confounding effects from the diet. In many kinetic studies, the follow-up time has been restricted to few hours after the ingestion of the supplement. In our study, the urinary excretion of phenolic metabolites from oregano was followed for two days, which allowed us to detect both the rapid metabolites and the colonic metabolites with a prolonged elimination time.

HPLC analyses of O. onites extract and urine

The HPLC-CEAD combination in the analysis of phenolic compounds is sensitive and precise, but it has been criticised for the requirement of high expertise from its users. The methods used in this study to identify and quantify the separate phenolic compounds in the extract and urine samples, have been earlier used for similar purposes, and the analyses were carried out by expertised users of the system. Some limitations occur in the analysis of metabolites of phenolic compounds when using the CEAD detector: compounds without a free hydroxyl group, for example hippuric acid, cannot be detected. The analysis of phenolic compounds after acidic

or enzymatic hydrolysis simplifies the identification, but at a same time, the information is limited to aglycone forms of compounds. The presence of 45 phenolic compounds was screened in the oregano extract, and based on these analyses, the urine samples were screened for a selection of 13 phenolic compounds. Even though a large number of standards were used, the characterisation of the phenolic composition of hydrodistilled extract of *O. onites* remained partial. This is a general problem when using supplements of natural origin; the variety of compounds is huge, and as all the potential parent compounds are not identified, complete conclusion on the origins of metabolites cannot be drawn. On the other hand, the other approach in bioavailability studies is to use a supplement consisting of a single compound. In this case, the parent compound and thus the origin of metabolites is known, but the biological relevance of such a supplement remains negligible.

6.1.4. Supplementation study

Subjects, study design and supplement used

In the oregano supplementation study, a randomised, double-blinded, placebo-controlled study design was used. The recruited subjects were randomised into three groups, one group received placebo juice, one group received LP juice and one group HP juice, and neither the study subjects nor the research personnel were aware of their type of supplement before the analysis of the results. By the use of placebo juice, the possible effects of the juice base were controlled. The effects of supplementation on the antioxidant parameters were determined both short-term (1.5 hours after ingestion of 250 mL of the study juices) and long-term (after 4 weeks of regular juice consumption, 3x125 mL of study juices every day). One week prior to and during the experiment, the participants consumed a polyphenol-restricted diet, and a four-day food recording was collected from all the study participants at the baseline and in the end of the study. The participants were also advised to report any skipping of juice doses. The purpose of these steps was to diminish the effects of confounding dietary factors and to check the compliance towards given instructions.

All the recruited men were healthy non-smokers, and most probably had a normal, balanced redox status in their body. Thus, a question remained, whether participants with elevated oxidative stress, for example smokers, might have had a different response on the oregano antioxidant supplementation.

Assessment of antioxidant capacity and lipid peroxidation

Several methods were used to assess the antioxidant capacity and lipid peroxidation from blood samples obtained: plasma TRAP, resistance of serum lipids to copper-induced oxidation, levels of baseline (without induced oxidation) conjugated dienes in LDL, and the concentration of plasma F₂-isoprostanes. TRAP was used to measure the capacity of a sample to scavenge peroxy radicals, which are physiologically relevant reactive species formed in the process on lipid peroxidation *in vivo*. Often, TRAP is the antioxidant parameter on which supplementation with polyphenols has been reported to have an increasing effect. It detects all antioxidants capable of chain breaking antioxidant action and is not interfered by enzymatic antioxidants present in human samples (Huang *et al*, 2005). However, the assay is relatively complex and time-consuming, and requires high expertise and routine from its users.

The copper-induced oxidation of serum lipids measures the capacity of serum to resist the oxidative stress, whereas the baseline LDL conjugated dienes assay reflects the *in vivo* oxidation status of LDL particles. Both of these methods can be considered to have a reasonable clinical relevance.

The *in vivo* biomarker of lipid peroxidation considered as the most reliable and of the highest clinical value are the F₂-isoprostanes, highly specific products of nonenzymatic peroxidation of arachidonic acid (Roberts & Morrow, 1997; Halliwell, 1999; Basu, 2004; Kadiiska *et al*, 2005; Milne *et al*, 2007; Montuschi *et al*, 2007). The GC-MS method for determining F₂-isoprostanes from human samples is laborious and thus costly. The analysis requires expertise, and their use is still rather limited as compared to many other *in vivo* biomarkers of lipid peroxidation.

6.2. Evaluation of the results

6.2.1. *In vitro* antioxidant activity and phenolic content of Lamiaceae herb extracts

In study I, all the tested sage extracts exhibited varying degrees of radical scavenging activity, the efficacy not being straightly correlated with the rosmarinic acid content of extract in question. When the two solid-phase traps were used in the PHWE procedure, higher radical scavenging activity was addressed to extract obtained by using cyclohexyl trapping material. As the retention of rosmarinic acids to cyclohexyl phase is far less than to C18 phase, this observation suggests that the radical scavenging properties of PHWE extracts were strongly contributed by phenolic compounds other than rosmarinic acid, for example diterpenes present in the extract. The phenolic profile of total PHWE extract was very similar to that of methanol extract, suggesting that the solvent properties of water in PHWE resembled those of methanol. In methanolic sage extract, diterpenes were present in higher amount as compared to 70% ethanol extract and hydrodistillation extract. In hydrodistillation extract, no traces of diterpenes were found, but instead, more compounds of very polar nature were present in the extract. Of the traditional extraction techniques, maceration with 70% ethanol yielded the highest radical scavenging activity, together with highest concentration of rosmarinic acid. In this extract, some diterpenes were also found. Hydrodistilled extract exhibited radical scavenging activity slightly higher than the methanolic extract, even though the rosmarinic acid content in these extracts was similar, and the methanolic extract also contained some amounts of diterpene compounds. This suggests that the highly polar constituents of sage, only extracted by the hydrodistillation method, had a contribution to the observed radical scavenging action.

In study II, the total phenolic contents of aqueous Lamiaceae extracts varied from 99 mg GAE/g for thyme to 185 mg GAE/g for rosemary. For example, the total phenolic content of *O. vulgare* extract obtained by a hydrodistillation procedure was close to that of *O. vulgare* extract obtained by subcritical hot water extraction at 100°C, 168mg GAE/g (Rodríguez-Meizoso *et al*, 2006). All the four aqueous Lamiaceae extracts showed to be antioxidative in all our assays used. As the total phenolic assay is based on the determination of reductive capacity, i.e. electron transfer capacity, of a sample, it is not a surprise that the total phenolic contents of samples were well correlated with the results of Fe³⁺ to Fe²⁺ reduction assay and DPPH• radical assay:

rosemary and sage extracts with the highest total phenolic content showed the highest activity in these assays, followed by oregano. Thyme extract, with the lowest total phenolic content, exhibited the weakest activity in all four assays. In the methods assessing ABTS^{•+} or OH[•] radical scavenging capacity, the total phenolic content of the samples was not as well correlated with the antioxidant power. For example, in the deoxyribose assay i.e. the OH[•] radical scavenging assay, rosemary and thyme exhibited similar activity inferior to sage and oregano. In the LDL oxidation model, the order of antioxidant activity was in accordance with the total phenolic contents. The antioxidant mechanisms involved cannot be fully elucidated, as all types of antioxidant action in this assay result in an increased lag-time, and in polyphenol mixtures, like these extracts, several mechanisms of action are involved. However, as the rate of oxidation in the propagation phase was not affected, the mechanism(s) involved probably is not the chelation of copper ions, but rather the enhancing of the non-radical decomposition of ROOH, or scavenging or stabilisation of free radicals (Pinchuk & Lichtenberg, 2002).

6.2.2. *In vitro* antioxidant activity of phenolic constituents in *O. onites* extract and their urinary metabolites

The structure-activity relationship of the tested phenolic compounds in DPPH[•] radical scavenging assay and copper-induced LDL oxidation assay was not similar; however, mono-hydroxylated compounds exhibited antioxidant activity in neither assay. This is in agreement with earlier observations. Especially in the case of benzoic acid derivatives, an explanation for this could be the carboxylic acid moiety, which is electron withdrawing and hampers the hydrogen donation ability from aromatic hydroxyl group (Soobrattee *et al*, 2005). The most active compounds in both assays possessed an *ortho*-dihydroxyl moiety in their structure, suggesting this structural feature to have a key role in effective antioxidant action. *Ortho*-dihydroxylated phenolic compounds are capable of antioxidant action by several mechanisms, for example by chelating Cu²⁺ ions and scavenging free radicals (Nardini *et al*, 1995; Natella *et al*, 1999). An exception to this was gallic acid, trihydroxybenzoic acid, which was the most active against DPPH[•] radical. This superior radical scavenging activity of trihydroxylated phenolics compared to corresponding dihydroxylated compounds has been reported e.g. by Siquet *et al*, 2006. In the LDL oxidation model, the antioxidant activity of gallic acid was weaker than that of protocatechuic acid, which has been explained by Siquet *et al*

(2006) by the higher hydrophilicity as compared to corresponding dihydroxyl derivative.

Most of the urinary metabolites were low in antioxidant activity, but the *ortho*-dihydroxylated metabolites still possessed antioxidant capacity. Of these metabolites, 3,4-dihydroxyphenylacetic acid showed higher activity in both assays as compared to the corresponding propionic acid derivative. The ethanoic side chain length seems thus favourable as compared to propionic side chain length, which is in agreement with work of Siquet *et al*, 2006.

Taking the concentrations of different compounds in the extract into consideration, the major role in the antioxidant activity of *O. onites* extract can be attributed to rosmarinic acid, followed by luteolin and protocatechuic acid. Similar findings have been reported e.g. for *O. vulgare* extracts by Exarchou *et al* (2002). Even though some of the urinary metabolites exhibited high antioxidant activity in the *in vitro* assays used, they mainly occur as conjugates in the urine, which usually decreases their antioxidant potential *in vivo*.

6.2.3. Bioavailability and metabolism of *O. onites* extract in humans

The phenolic compounds from *O. onites* were shown to be at least partially absorbed, metabolised and excreted in urine in study III. The study subjects consumed a phenolic-restricted diet prior to and during the study, which minimised the role of diet as a source of phenolic metabolites. Low concentration of the main metabolite, *p*-hydroxybenzoic acid, was present in 24-hour baseline urine, but after ingestion of the extract, the excretion multiplied. The maximum urinary concentration of this compound was observed in urine within 4 hours from the ingestion of the extract, which indicates that the parent compound(s) was rapidly absorbed, metabolised and excreted in urine. *p*-Hydroxybenzoic acid has not been described as a human metabolite of polyphenol metabolism, although low amounts have been reported e.g. as *in vitro* fermentation products of quercitrin with human intestinal microflora (Kim *et al*, 1999) and as a metabolite of anthocyanin pelargonidin in rat urine (El Mohsen *et al*, 2006). Hydroxyphenylpropionic acids are metabolites of the colonic degradation of hydroxycinnamates as well as flavonoids (Rechner *et al*, 2002a), and *p*-hydroxybenzoic acid was suggested to be a potential metabolite from the human colonic metabolism of these compounds. In most studies, *p*-hydroxybenzoic acid has not been on the list of included analytes,

possibly because metabolism of aromatic acids in diet also produces *p*-hydroxybenzoic acid. Even though low amounts of *p*-hydroxybenzoic acid has been found in extracts of Lamiaceae herbs, in *O. onites* extract used in this study, no traces of it were detected.

In one of the few human studies on Lamiaceae herbs, rosmarinic acid from Perilla extract reached its maximum plasma concentration at 30 minutes after ingestion of the extract (Baba *et al*, 2005). This suggests that rosmarinic acid was, at least partially, absorbed from the stomach. If the rosmarinic acid in *O. onites* extract was absorbed in the stomach or in the upper part of the small intestine, it could have acted as a parent compound for *p*-hydroxybenzoic acid. Due to the partial characterisation of the study extract, the origin of the metabolites cannot be quantitatively elucidated. However, the total amount of oregano extract derived phenolic metabolites detected in the urine exceeded the amount of identified phenolic components in the extract.

Due to the 48-hour follow-up time, we also detected the excretion of colonic metabolites of the extract, the phenylacetic acids. In further analyses, we identified propionic acid derivatives from the colonic metabolism in the urine samples, and although they were not quantified, they further increased the amount of excreted urinary phenolic metabolites.

6.2.4. Effects of *O. vulgare* extract supplementation on antioxidant biomarkers *in vivo*

The lack of short- or long-time effects of oregano supplementation on parameters reflecting antioxidant status or lipid peroxidation was despite the use of several biomarkers with clinical relevance *in vivo*. *O. vulgare* extract exhibited antioxidant potential in different *in vitro* assays, and as it had a comparable content of identified phenolic compounds to that of *O. onites* extract used in study III, we were expecting to see some antioxidant effects *in vivo*. The lack of effects reawakened the questions that were also considered when designing the study setting: were the type or dose of phenolic compounds administered right? Were oregano polyphenols weakly absorbed or metabolically inactivated so rapidly that concentrations of polyphenols present *in vivo* were not high enough to have a role in antioxidant defence? Was the choice of recruiting healthy, nonsmoking study participants correct, or would the results have been different in subjects with an elevated level of oxidative stress due to some disease or exogenic exposing factor

such as tobacco smoking? Could some other biomarkers of lipid peroxidation or antioxidant status have shown effects?

During the past two decades, the research on polyphenols has mainly been targeted at their antioxidant properties. Today, accumulating data from a variety of polyphenol studies and the inconsistent findings on the antioxidant action of polyphenols in human body suggest that the classical direct antioxidant action is a simplistic way to conceive the bioactivity of polyphenols in humans (Noguchi, 2002; Williams *et al*, 2004). The concentrations of polyphenols or their metabolites in the human body may be inadequate as compared to other antioxidants to have any detectable effects on antioxidant status or lipid peroxidation, but the biological effects of polyphenols could be mediated via cell signalling, in which lower *in vivo* concentrations are needed. For example, oxidised LDL induces a number of genes that have a role in the initiation and progression of atherosclerosis. These genes regulating cell growth, survival, adhesion and inflammatory responses are up-regulated via nuclear receptor-dependent pathways by ox-LDL. The health-beneficial actions, protection of the cardiovascular and neurological system and prevention of cancer, may be mediated via modulatory actions of polyphenols on the regulation of gene expression involved in protein degradation and transcriptional pathways (Noguchi, 2002; Williams *et al*, 2004). In addition to the observed antioxidant action *in vitro*, polyphenols have been shown to have a huge variety of biological activities (reviewed by Halliwell, 2007): they are capable of inhibiting telomerase, glutamate dehydrogenase, cyclooxygenase, lipoxygenase, xanthine oxidase, matrix metalloproteinases, angiotensin-converting enzyme, proteasome, cytochrome p-450 and sulphotransferase enzyme activities, affecting signal transduction pathways and interacting with sirtuins, histone deacetylase enzymes with a role in the ageing process. They may also have interactions with cellular drug transport systems or glucose transmembrane transport, affect cell cycle regulation, protein glycation, modulate paraoxonase, myeloperoxidase and thyroid peroxidase activity, increase endothelial nitric oxide production and affect the platelet function. All of these effects have been shown *in vitro*, and it is still uncertain whether these potential modulatory actions have a role in human body. However, this diversity of potential mechanisms of health-beneficial actions suggest that the lack of effects in parameters reflecting direct antioxidant action does not equal lack of beneficial effects *in vivo*.

7. SUMMARY AND CONCLUSIONS

These studies represent a progression starting from studying of extraction techniques for Lamiaceae polyphenols, proceeding to *in vitro* antioxidant studies of these extracts and their phenolic constituents, and ending up in elucidating their actions in humans. Even though Lamiaceae extracts and their phenolic constituents were effective antioxidants in different *in vitro* models, and phenolic components of oregano were absorbed in humans, no antioxidant effects were seen in humans after oregano polyphenol supplementation. The promising antioxidant results of polyphenols from *in vitro* studies are thus not enough to predict their action in human body. In humans, issues of absorption, metabolism, distribution and concentrations in the blood and tissues are of crucial importance for effects of any substance. The fact that polyphenol concentrations in blood and tissues are rather low even after supplementation with polyphenols suggests that they may not be present at a concentration adequate to contribute to body's antioxidant defence at least at a detectable level.

Even though the most popular hypothesis behind the epidemiologically observed protection of polyphenol rich diets against CVD has been related to their antioxidant action, also other mechanisms than direct antioxidant activity may be involved. This might at least partially explain the lack of consistent evidence on the effects of clinical polyphenol supplementations. Diets rich in fruits, vegetables, berries and plant based beverages also contain a number of other phytochemicals that might contribute to the observed protection, including fiber, immunostimulatory agents, inducers of various enzymes such as oxygenases, vitamins, potassium, and folate, which could be independently or concurrently responsible for the apparent reduction in CVD risk. The low dietary glycemic load and energy density of diets rich in plant-derived foods and drinks may also play a significant role. It is also possible that high intake of these nutritional factors is linked to a generally healthy lifestyle, which comprises various health beneficial choices: non-smoking, regular physical activity, and diet in accordance with the dietary recommendations.

Today, there is a vogue for functional foods and nutritional supplements of natural origin, and their market is expanding worldwide. This work illustrates the problemacy of studying and interpreting the properties of a potential health-beneficial ingredient. Most of the antioxidant products on the market lack studies on their effects in humans, and they are referred to as antioxidants based on literature or in the most advanced case, based on incidental *in vitro* studies lacking reliable standardisation. Furthermore, no kinetic data of these substances in humans are available, and thus the extent of absorption, for example, remains unknown. The evidence of an ingredient or a mixture of ingredients exhibiting antioxidant activity *in vitro* is far from proven effects in humans. Until the situation changes so that the effects need to be substantiated scientifically in well-controlled intervention studies with human volunteers, my opinion is that the best dietary way to maintain a healthy redox balance is to consume a balanced diet rich in fruits, berries, herbs and vegetables, as well as beverages rich in phenolic compounds. This kind of diet, as a part of a whole healthy lifestyle, is most probably beneficial for maintaining a good cardiovascular health.

8. REFERENCES

- Ahotupa, M., Marniemi, J., Lehtimäki, T. *et al* (1998) Baseline diene conjugation in LDL as a direct measure of in vivo LDL oxidation. *Clin Biochem* 31:257-261.
- Ahotupa M. & Vasankari, T.J. (1999) Baseline diene conjugation in LDL lipids: an indicator of circulating oxidized LDL. *Free Radic Biol Med* 27:1141-1150.
- Andersen, L.F., Jacobs, D.R.J., Carlsen, M.H. & Blomhoff, R. (2006). Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. *Am J Clin Nutr* 83:1039-1046.
- Andreasen, M.F., Christensen, L.P., Meyer, A.S. & Hansen, Å. (1999) Release of hydroxycinnamic and hydroxybenzoic acids in rye by commercial plant cell wall degrading enzyme preparations. *J Sci Food Agric* 79:411-413.
- Andreasen, M.F., Kroon, P.A., Williamson, G. & Garcia-Conesa, M.-T. (2001) Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. *J Agric Food Chem* 49:5679-5684.
- Arts, I.C., Hollman, P.C., Feskens, E.J., Bueno de Mesquita, H.B. & Kromhout, D. (2001a) Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am J Clin Nutr* 74:227-232.
- Arts, I.C., Jacobs, D.R.J., Harnack, L.J., Gross, M. & Folsom, A.R. (2001b) Dietary catechins in relation to coronary heart disease death among post-menopausal women. *Epidemiology* 12:668-675.
- Arts, I.C. & Hollman, P.C. (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 81:317S-325S.
- Auger, C., Rouanet, J.-M., Vanderlinde, R. *et al* (2005) Polyphenols-enriched Chardonnay white wine and sparkling Pinot Noir red wine identically prevent early atherosclerosis in hamsters. *J Agric Food Chem* 53:9823-9829.
- Baba, S., Osakabe, N., Natsume, M. *et al* (2005) Absorption, metabolism, degradation and urinary excretion of rosmarinic acid after the intake of *Perilla frutescens* extract in humans. *Eur J Nutr* 44:1-9.
- Balasubashini, M.S., Rukkumani, R., Viswanathan, P. & Menon, V.P. (2004) Ferulic acid alleviates lipid peroxidation on diabetic rats. *Phytother Res* 18:310-314.
- Basu, S. (2004) Isoprostanes: novel bioactive products of lipid peroxidation. *Free Radic Res* 38:105-122.
- Bazzano, L.A., Serdula, M.K. & Liu, S. (2003) Dietary intake of fruits and vegetables and risk of cardiovascular disease. *Curr Atheroscler Rep* 5:492-499.
- Berliner, J.A. & Heinecke, J.W. (1996) The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 20:707-727.
- Bicchi, C., Binello, A. & Rubiolo, B. (2000) Determination of phenolic diterpene antioxidants in rosemary (*Rosmarinus officinalis* L.) with different methods of extraction and analysis. *Phytochem Anal* 11:236-242.

- Bors, W., Heller, W., Michel, C. & Saran, M. (1990): Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol* 186:343-355.
- Bors, W., Michel, C. & Schikora, S. (1995) Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: a pulse radiolysis study. *Free Radic Biol Med* 19:45-52.
- Botham, K.M., Bravo, E., Elliott, J. & Wheeler-Jones, C.P.D (2005) Direct interaction of dietary lipids carried in chylomicron remnants with cells on the artery wall: implications for atherosclerosis development. *Curr Pharm Des* 11:3681-3695.
- Bourne, L.C., Paganga, G., Baxter, D., Hughes, P. & Rice-Evans, C. (2000) Absorption of ferulic acid from low-alcohol beer. *Free Radic Res* 32:273-280.
- Bourne, L.C. & Rice-Evans, C. (1998) Bioavailability of ferulic acid. *Biochem Biophys Res Commun* 53:222-227.
- Brand-Williams, W., Cuvelier, M.E. & Berset, C. (1995): Use of free radical method to evaluate antioxidant activity. *Food Sci Technol - LEB* 28:25-30.
- Bravo, L. (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* 56:317-333.
- Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300:535-543.
- Caccetta, R.A.-A., Burke, V., Mori, T.A., Beilin, L.J., Puddey, I.B. & Croft, K.D. (2001) Red wine polyphenols, in the absence of alcohol, reduce lipid peroxidative stress in smoking subjects. *Free Radic Biol Med* 30:636-642.
- Cai, Y.Z., Sun, M., Xing, J., Luo, Q. & Corke, H. (2006) Structure–radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sci* 78:2872-2888.
- Carbonneau, M.-A., Leger, C.L., Monnier, L. *et al* (1997) Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu²⁺ -oxidizability: Possible explanation by phenolic location. *Eur J Clin Nutr* 51:682-690.
- Chalas, J., Claise, C., Edeas, M. *et al* (2001) Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed Pharmacother* 55:54-60.
- Cherubini, A., Vigna, G.B., Zuliani, G., Ruggiero, C., Senin, U. & Fellin, R. (2005) Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Des* 11:2017-2032.
- Chopra, M., Fitzsimons, P.E.E., Strain, J.J., Thurnham, D.I. & Howard, A.N. (2000) Nonalcoholic red wine extract and quercetin inhibit LDL oxidation without affecting plasma antioxidant vitamin and carotenoid concentrations. *Clin Chem* 46:1162-1170.
- Cirico, T.L. & Omaye, S.T. (2006) Additive or synergistic effects of phenolic compounds on human low density lipoprotein oxidation. *Food Chem Toxic* 44:510-516.
- Clarke, R. & Armitage, J. (2002) Antioxidant vitamins and risk of cardiovascular disease. Review of large-scale randomised trials. *Cardiovasc Drug Ther* 16:411-415.
- Clifford, M.N. (1999) Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *J Sci Food Agric* 79:362-372.

- Coskun, O., Kanter, M., Korkmaz, A. & Oter, S. (2005) Quercetin, a flavanoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and b-cell damage in rat pancreas. *Pharmacol Res* 51:117–123.
- Cuvelier, M.E., Berset, C. & Richard, H. (1994) Antioxidant constituents in sage (*Salvia officinalis*). *J Agric Food Chem* 42:665-669.
- Cuvelier, M.E., Richard, H. & Berset, C. (1996) Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J Am Oil Chem Soc* 73:645-652.
- Davies, K.J.A. Oxidative stress: the paradox of aerobic life. In: *Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives*. Rice-Evans, C., Halliwell, B. (eds.). Portland Press, London, UK, 1995.
- Dietary Guidelines Advisory Committee (2005) The Report of the Dietary Guidelines Advisory Committee on Dietary Guidelines for Americans, 2005. Electronic reference, retrieved 6.11.2007 from: <http://www.health.gov/dietaryguidelines/dga2005/report/>
- Dorman, H.J.D., Bachmayer, O., Kosar, M. & Hiltunen, R. (2004) Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. *J Agric Food Chem* 52:762-770.
- Dragland, S., Sanoo, H., Wake, K., Holte, K. & Blomhoff, R. (2003) Several culinary and medicinal herbs are important sources of dietary antioxidants. *J Nutr* 133:1286-1290.
- Duffy, S.J. & Vita, J.A. (2003) Effects of phenolics on vascular endothelial function. *Curr Opin Lipidol* 14:21-27.
- El Mohsen, M.A., Marks, J., Kuhnle, G. *et al* (2006) Absorption, tissue distribution and excretion of pelargonidin and its metabolites following oral administration to rats. *Brit J Nutr* 95:51-58.
- Esterbauer, H., Striegl, G., Puhl, H. & Rotheneder, M. (1989) Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Rad Res Commun* 6:67-75.
- Estruch, R., Martínez-González, M. A., Corella, D. *et al* (2006) Effects of a Mediterranean-style diet on cardiovascular risk factors. *Ann Intern Med* 145:1-11.
- Exarchou, V., Godejohann, M., van Beek, T. A., Gerothanassis, I.P. & Vervoort, J. (2003) LC-UV-solid-phase extraction-NMR-MS combined with a cryogenic flow probe and its application to the identification of compounds present in Greek oregano. *Anal Chem* 75:6288-6294.
- Exarchou, V., Nenadis, N., Tsimidou, M., Gerothanassis, I.P., Troganis, A. & Boskou, D. (2002) Antioxidant Activities and Phenolic Composition of Extracts from Greek Oregano, Greek Sage, and Summer Savory. *J Agric J Agric Food Chem* 50:5294-5299.
- Flight, I. & Clifton, P. (2006) Cereal grains and legumes in the prevention of coronary heart disease and stroke: A review of the literature. *Eur J Clin Nutr* 60:1145-1159.
- Frankel, E.N. (1995) Natural and biological antioxidants in foods and biological systems. Their mechanism of action, applications and implications. *Lipid Technol* 7:77-80.
- Frankel, E.N. & Meyer, A.S. (2000) The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J Sci Food Agric* 80:1925-1941.

- Freese, R., Basu, S., Hietanen, E. *et al* (1999) Green tea extract decreases plasma malondialdehyde concentration but does not affect other indicators of oxidative stress, nitric oxide production, or hemstatic factors during a high-linoleic acid diet in healthy females. *Eur J Clin Nutr* 38:149-157.
- Frei, B. (1996) Reactive oxygen species and antioxidant vitamins: Mechanism in action. *Am J Med* 97:5S-13S.
- Fuhrman, B., Lavy, A. & Aviram, M. (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 61:549-554.
- Fuhrman, B., Volkova, N., Coleman, R. & Aviram, M. (2005) Grape powder polyphenols attenuate atherosclerosis development in apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity. *J Nutr* 135:722-728.
- Galati, G., Sabzevari, O., Wilson, J.X. & O'Brien, P.J. (2002) Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* 177:91-104.
- Garrat, G., Jarrige, J.-F., Blanquet, S., Beyssac, E., Cardot, J.-M. & Alric, M. (2006) Gastrointestinal absorption and urinary excretion of *trans*-cinnamic and *p*-coumaric acids in rats. *J Agric Food Chem* 54:2944-2950.
- Geleijnse, J.M., Launer, L.J., Van der Kuip, D.A., Hofman, A. & Witteman, J.C. (2002) Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am J Clin Nutr* 75:880-886.
- Gerothanassis, I.P., Exarchou, V., Lagouri, V., Troganis, A., Tsimidou, M. & Boskou, D. (1998) Methodology for identification of phenolic acids in complex phenolic mixtures by high-resolution two-dimensional nuclear magnetic resonance. Application to methanolic extracts of two oregano species. *J Agric Food Chem* 46:4185-4192.
- Gomikawa, S. & Ishikawa, Y. (2002) Effects of catechins and ground green tea drinking on the susceptibility of plasma and LDL to oxidation *in vitro* and *ex vivo*. *J Clin Biochem Nutr* 32:55-68.
- Goupy, P., Hughes, M., Boivin, P. & Amiot, M.J. (1999) Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts of isolated phenolic compounds. *J Sci Food Agric* 79:1625-1634.
- de Groot, H. (1994) Reactive oxygen species in tissue injury. *Hepato-Gastroenterol* 41:328-332.
- Grundy, S.M., Pasternak, R., Greenland, P., Smith, S. Jr. & Fuster, V. (1999) AHA/ACC scientific statement: Assessment of cardiovascular risk by use of multiple-risk-factor assessment equations: a statement for healthcare professionals from the American Heart Association and the American College of Cardiology. *J Am Coll Cardiol* 34:1348-1359.
- Gu, L., Kelm, M., Hammerstone, J.F. *et al* (2004) Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J Nutr* 134:613-617.
- Gupta, S., Hussain, T. & Mukhtar, H. (2002) Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. *Arch Biochem Biophys* 410:177-185.
- Gutteridge, J.M.C. & Halliwell, B. *Antioxidants in nutrition, health, and disease*. Oxford University Press, Oxford, UK, 1994.

- Halliwell, B., Gutteridge, J.M.C. & Aruoma, O.I. (1987) The deoxyribose method: A sample test tube assay for determination of rate constants for reaction of hydroxyl radical. *Anal Biochem* 165:215-219.
- Halliwell, B. (1994a) Free radicals and antioxidants: a personal view. *Nutr Rev* 52:253-265.
- Halliwell, B. (1994b) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* 344:721-724.
- Halliwell, B. (1995) Oxidation of low-density lipoproteins: question of initiation, propagation and the effect of antioxidants. *Am J Clin Nutr* 61:670S-677S.
- Halliwell, B. (1999) Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutr Rev* 57:104-113.
- Halliwell, B. (2007) Dietary polyphenols: Good, bad, or indifferent for your health? *Cardiovasc Res* 73:341-347.
- Halliwell, B. & Cross, C.E. (1994) Oxygen-derived species: their relation to human disease and environmental stress. *Environ Health Perspect* 10:5S-12S.
- Halliwell, B. & Gutteridge, J.M.C. (1990a): Role of free radicals and catalytic metal ions in human disease: An Overview. *Methods Enzymol* 186:1-79.
- Halliwell, B., Gutteridge, J.M.C. & Cross, C.E. (1992) Free radicals, antioxidants, and human disease: Where are we now? *J Lab Clin Med* 119:598-620.
- Halliwell, B., Rafter, J. & Jenner, A. (2005) Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am J Clin Nutr* 81:268S-276S.
- Halvorsen, B.L., Carlsen, M.H., Phillips, K.M. *et al* (2006) Content of redox-active compounds (ie, antioxidants) in foods consumed in United States. *Am J Clin Nutr* 84:95-135.
- Hancock, J T., Desikan, R. & Neill, S J. (2001) Role of reactive oxygen species in cell signalling pathways. *Biochem Soc Transactions* 29:345-350.
- Happonen, P., Voutilainen, S. & Salonen, J.T. (2004) Coffee drinking is dose-dependently related to the risk of acute coronary events in middle-aged men. *J Nutr* 134:2381-2386.
- Harborne, J.B. *Methods in plant biochemistry, I: plant phenolics*. Academic Press, London, UK, 1989.
- Harborne, J.B. *The flavonoids: advances in research since 1986*. Chapman and Hall, London, UK, 1993.
- Harman, D. (1956) "Aging: a theory based on free radical and radiation chemistry". *J Gerontol* 11:298-300.
- Hayek, T., Fuhrman, B., Vaya, J. *et al* (1997) Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* 17:2744-2752.
- Heijnen, C.G.M., Haenen, G.R.M.M., Oostveen, R.M., Stalpers, E.M. & Bast, A. (2002) Protection of flavonoids against lipid peroxidation: structure activity relationship revisited. *Free Radic Res* 36:575-581.
- Heinonen, M. (2007) Antioxidant activity and antimicrobial effect of berry phenolics – A Finnish perspective. *Mol Nutr Food Res* 51:684-691.
- Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B. & Kromhout, D. (1993a) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 342:1007-1011.

- Hertog, M.G.L., Hollman, P.C.H., Katan, M.B. & Kromhout D. (1993b) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 20:21-29.
- Hertog, M.G.L., Kromhout, D., Aravanis C. *et al* (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries study. *Arch Int Med* 155:381-386.
- Hertog, M., Sweetnam, P., Fehily, A.M., Elwood, P.C. & Kromhout, D. (1997) Antioxidant flavonols and ischemic heart disease in Welsh population of men: the Caerphilly study. *Am J Clin Nutr* 65:1489-1494.
- Hirvonen, T., Pietinen, P., Virtanen, M. *et al* (2001) Intake of flavonols and flavones and risk of coronary heart disease in male smokers. *Epidemiology* 12:62-67.
- Hollman, P.C.H., de Vries, J.H.M., Vanleeuwen, S.D., Mengelers, M.J.B. & Katan, M.B. (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* 62:1276-1282.
- Huang, D., Ou, D. & Prior, D. (2005) The chemistry behind antioxidant assays. *J Agric Food Chem* 53:1841-1856.
- Hung, H.C., Josphipura, K.J., Jiang, R. *et al* (2004) Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst* 96:1577-1584.
- Huxley, R.R. & Neil, H.A. (2003) The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. *Eur J Clin Nutr* 57:904-908.
- Ishikawa, T., Suzukawa, M., Ito, T. *et al* (1997) Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. *Am J Clin Nutr* 66:261-266.
- Jacobson, E.A., Newmark, H., Baptista, J. & Bruce, W.R. (1983) A preliminary investigation on the metabolism of dietary phenolics in humans. *Nutr Rep Int* 28:1409-1417.
- Jenner, A.M., Rafter, J. & Halliwell, B. (2005) Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. *Free Radic Biol Med* 38:763-772.
- Jeon, S.M., Kim, H.K., Kim, H.J., Do, G.-M., Jeong, T.-S., Park, Y.B. & Choi, M.-S. (2007) Hypocholesterolemic and antioxidative effects of naringenin and its two metabolites in high-cholesterol fed rats. *Transl Res* 149:15-21.
- Jones, O.T.G. & Hancock, J.T. The NADPH oxidase of neutrophils and other cells. In: *Free radicals and inflammation* (Winyard, P.G., Blake, D.R. & Evans, C.H., eds). Birkhäuser Verlag AG, Basel, Switzerland, 2000.
- Jones, S.A., O'Donnell, V.B., Wood, J.D., Broughton, J.P., Hughes, E.J. & Jones, O.T. (1996) Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol Heart Circ Physiol* 271:1626-1634.
- Justesen, U. & Knuthsen, P. (2001) Composition of flavonoids in fresh herbs and calculation of flavonoid intake by use of herbs in traditional Danish dishes. *Food Chem* 73:245-250.
- Kadiiska, M.B., Gladen, B.C., Baird, D.D. *et al* (2005) Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl₄ poisoning? *Free Radic Biol Med* 38:698-710.
- Kaur, C. & Kapoor, H.C. (2001) Antioxidants in fruits and vegetables – the millennium's health. *Int J Food Sci Tech* 36:703-725.
- Kehrer, J. (1993) Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol* 23:21-48.

- Kehrer, J.P. & Smith, C.V. Free radicals in biology: sources, reactivities, and roles in the etiology of human diseases. In: *Natural Antioxidants in Human Health and Disease*. Frei, B. (ed.). Academic Press, San Diego, US, 1994.
- Keli, S.O., Hertog, M.G.L., Feskens, E.J.M. & Kromhout, D. (1996) Dietary flavonoids, antioxidant vitamin and incidence of stroke. The Zutphen Study. *Arch Intern Med* 156:637-642.
- Kim, D. H., Kim, S. Y., Park, S. Y. & Han, M. J. (1999) Metabolism of quercitrin by human intestinal bacteria and its relation to some biological activities. *Biol Pharm Bull* 22:749-751.
- Kivilompolo, M. & Hyötyläinen, T. (2007) Comprehensive two-dimensional liquid chromatography in analysis of Lamiaceae herbs: characterisation and quantification of phenolic acids. *J Chromatogr A* 1145:155-164.
- Kivilompolo, M., Obürka, V. & Hyötyläinen, T. (2007) Comparison of GC-MS and LC-MS methods for the analysis of antioxidant phenolic acids in herbs. *Anal Bioanal Chem* 388:881-887.
- Knekt, P., Järvinen, R., Reunanen, A. & Maatela, J. (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *BMJ* 312:478-481.
- Knekt, P., Kumpulainen, J., Järvinen, R. *et al* (2002) Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 76:560-568.
- Konishi, Y. & Kobayashi, S. (2004a) Microbial metabolites of ingested caffeic acid are absorbed by the monocarboxylic acid transport (MCT) in intestinal Caco-2 cell monolayers. *J Agric Food Chem* 52:6418-6424.
- Konishi, Y. & Kobayashi, S. (2004b) Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal Caco-2 cell monolayers. *J Agric Food Chem* 52:2518-2526.
- Konishi, Y. & Kobayashi, S. (2005) Transepithelial transport of rosmarinic acid in intestinal Caco-2 cell monolayers. *Biosci Biotechnol Biochem* 69:583-591.
- Konishi, Y. & Kobayashi, S. & Shimizu, M. (2003) Transepithelial transport of *p*-coumaric acid and gallic acid in intestinal Caco-2 cell monolayers. *Biosci Biotechnol Biochem* 67:2317-2324.
- Konishi, Y. & Shimizu, M. (2003) Transepithelial transport of ferulic acid in intestinal Caco-2 cell monolayers. *Biosci Biotechnol Biochem* 67:856-862.
- Konishi, Y., Zhao, Z. & Shimizu, M. (2006) Phenolic acids are absorbed from the rat stomach with different absorption rates. *J Agric Food Chem* 54:7539-7543.
- Korkina, L.G. (2007) Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cell Mol Biol* 53:15-25.
- Kosar, M., Dorman, H.J.D., Bachmayer, O., Baser, K.H.C. & Hiltunen, R. (2003) An improved online HPLC-DPPH[•] method for the screening of radical scavenging compounds in water extracts of Lamiaceae plants. *Chem Nat Comp* 39:161-166.
- Kühnau, J. (1976) The Flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* 24:117-191.
- Kuller, L.H. (2001) Prevention of cardiovascular disease and the future of cardiovascular disease epidemiology. *Int J Epidemiol* 30:66S-72S.
- Kuller, L.H. (2006) Nutrition, lipids and cardiovascular disease. *Nutr Rev* 64:15S-26S.
- Lairon, D. (2007) Intervention studies on Mediterranean diet and cardiovascular risk. *Mol Nutr Food Res* 51:1209-1214.

- Landmesser, U. & Harrison, D.G. (2001) Oxidant stress as a marker for cardiovascular events: Ox marks the spot. *Circulation* 104:2638–2640.
- Laughton, M.J., Evans, P.J., Moroney, M.A., Hoult, J.R.S & Halliwell, B. (1991): Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. *Biochem Pharmacol* 42:1673-1681.
- Lee, C.Y., Jenner, A.M. & Halliwell, B. (2004) Rapid preparation of human urine and plasma samples for analysis of F₂-isoprostanes by gas chromatography – mass spectrometry. *Biochem Biophys Res Commun* 320:696-702.
- Leth, T. & Justesen, U. Analysis of flavonoids in fruits, vegetables and beverages by HPLC-UV and LC-MS and estimation of the total daily flavonoid intake in Denmark. In: *Polyphenols in food*. Amadó, R., Andersson, H., Bardócz, S. & Serra, F. (eds.). Office for Official Publications of the European Communities, Luxembourg, Belgium, 1998.
- Lin, J., Rexrode, K.M., Hu, F. *et al* (2007) Dietary intakes on flavonols and flavones and coronary heart disease in US women. *Am J Epidemiol* 165:1305-1313.
- Lotito, S.B. & Frei, B. (2004) The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids. *Free Radic Biol Med* 37:251-258.
- Lu, Y. & Foo, L.Y. (1999) Rosmarinic acid derivatives from *Salvia officinalis*. *Phytochemistry* 51:91-94.
- Lu, Y. & Foo, L.Y. (2000) Flavonoid and phenolic glycosides from *Salvia officinalis*. *Phytochemistry* 55:263-267.
- Lu, Y. & Foo, L.Y. (2002) Polyphenolics of *Salvia* – A review. *Phytochemistry* 59:117-140.
- Luceri, C., Giannini, L., Lodovici, M. *et al* (2007) *p*-Coumaric acid, a common dietary phenol, inhibits platelet activity *in vitro* and *in vivo*. *Brit J Nutr* 97:458-463.
- Macheix, J.-J. & Fleuriet, A. Phenolic Acids in Fruits. In: *Flavonoids in Health and Disease*. Rice-Evans, C. & Packer, L. (eds.). Marcel Dekker Inc., NJ, US, 1998.
- Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79:727-747.
- Manach, C., Williamson, G., Morand, C., Scalbert, A. & Rémésy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 81:230S-242S.
- Mariani, H., Polidori, M.C., Cherubini, A. & Mecocci, P. (2005) Oxidative stress in brain aging, neurodegenerative and vascular diseases: An overview. *J Chromatogr B* 827:65-75.
- Martínez-Tomé, M., Murcia, M.A., Frega, N., Ruggieri, S., Jiménez, A.M., Roses, F. & Parras, P. (2004) Evaluation of antioxidant capacity of cereal brans. *J Agric Food Chem* 52:4690-4699.
- Masella, R., Di Benedetto, R., Vari, R., Filesi, C. & Giovannini, C. (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J Nutr Biochem* 16:577-586.
- Mattila, P., Hellström, J. & Törrönen, R. (2006) Phenolic acids in fruits, berries and beverages. *J Agric Food Chem* 54:7193-7199.

- Mattila, P., Pihlava, J.-M. & Hellström, J. (2005) Content of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J Agric Food Chem* 53:8290-8295.
- McAnlis, G.T., Mceneny, J., Pearce, J. & Young, I.S. (1999) Absorption and antioxidant effects of quercetin from onions, in man. *Eur J Clin Nutr* 53:92-96.
- McGhie, T.K. & Walton, M.C. (2007) The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol Nutr Food Res* 51:702-713.
- Meltzer, H.M. & Malterud, K.E. (1997) Can dietary flavonoids influence the development of coronary heart disease? *Scand J Nutr* 41:50-57.
- Mennen, L.I., Sapinho, D., de Bree A. *et al* (2004) Consumption of foods rich in flavonoids is related to a decreased cardiovascular risk in apparently healthy French women. *J Nutr* 134:923-926.
- Metsä-Ketelä, T. Luminescent assay for total peroxy radical-trapping capability of plasma. In: *Bioluminescence and chemiluminescence: current status*. Stanley, P.E. & Kricka, L.J. (eds.). John Wiley & Sons Inc, Chichester, UK, 1991.
- Meyer, A.S., Heinonen, M. & Frankel, E.N. (1998): Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chem* 61:71-75.
- Milne, G.L., Sanchez, S.C., Musiek, E.S. & Morrow, J.D. (2007) Quantification of F₂-isoprostanes as a biomarker of oxidative stress. *Nature Protocols* 2:221-226.
- Mink, P.J., Scrafford, C.G., Barraj, L.M. *et al* (2007) Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr* 85:895-909.
- Mira, L., Fernandez, M.T., Santos, M., Rocha, R., Florencio, M.H. & Jennings, K.R. (2002) Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radic Res* 36:1199-1208.
- Montuschi, P., Barnes, P. & Roberts, L.J. 2nd. (2007) Insights into oxidative stress: the isoprostanes. *Curr Medic Chem* 14:703-717.
- Morel, I., Lescoat, G., Cillard, P. & Cillard, J. (1994) Role of flavonoids and iron chelation in antioxidant action. *Methods Enzymol* 234:410-443.
- Morrissey, P.A. & Sheehy, P.J.A. (1999) Optimal nutrition: vitamin E. *Proc Nutr Soc* 58:459-468.
- Morrow, J.D. & Roberts, L.J. (1994) Mass spectrometry of prostanoids: F₂-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods Enzymol* 233:163-174.
- Mursu, J., Nurmi, T., Tuomainen, T.-P., Ruusunen, A., Salonen, J.T. & Voutilainen, S. (2007) The intake on flavonoids and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease risk factor study. *Brit J Nutr* 98:814-818.
- Myers, M.G. & Basinski, A. (1992) Coffee and coronary heart disease. *Arch Intern Med* 152:1767-1772.
- Nakagawa, K., Ninomiya, M., Okubo, T. *et al* (1999) Tea catechin supplementation increases antioxidant capacity and prevents phospholipid hydroperoxidation in plasma of humans. *J Agric Food Chem* 47:3967-3973.
- Nakazawa, T. & Ohsawa, K. (2000) Metabolites of orally administered *Perilla frutescens* extract in rats and humans. *Biol Pharm Bull* 23:122-127.

- Nardini, M., Cirillo, E., Natella, F. & Scaccini, C. (2002) Absorption of phenolic acids in humans after coffee consumption. *J Agric Food Chem* 50:5735-5741.
- Nardini, M., D'Aquino, M., Tomassi, G., Gentili, V., Di Felice, M. & Scaccini, C. (1995) Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radic Biol Med* 19:541-552.
- Nardini, M., Natella, F., Gentili, V., Di Felice, M. & Scaccini, C. (1999) Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: an *in vivo* study. *Arch Biochem Biophys* 342:157-160.
- Nardini, M., Natella, F., Scaccini, C. & Ghiselli, A. (2006) Phenolic acids from beer are absorbed and extensively metabolized in humans. *J Nutr Biochem* 17:14-22.
- Natella, F., Nardini, I., Di Felice, M. & Scaccini, C. (1999) Benzoic and cinnamic acid derivatives as antioxidants: structure-activity relation. *J Agric Food Chem* 47:1453-1459.
- Natella, F., Nardini, M., Giannetti, I., Dattilo, C. & Scaccini, C. (2002) Coffee drinking influences plasma antioxidant capacity in humans. *J Agric Food Chem* 50:6211-6216.
- Natella, F., Nardini, M., Belevi, F. & Scaccini, C. (2007) Coffee drinking induces incorporation of phenolic acids into LDL and increases the resistance of LDL to *ex vivo* oxidation in humans. *Am J Clin Nutr* 86:604-609.
- National Public Health Institute (1998) The 1997 Dietary Survey of Finnish Adults. Publications of National Public Health Institute B 8/1998. Kansanterveyslaitos, Ravitsemusyksikkö. Helsinki, 1998.
- National Public Health Institute (2003) Finravinto 2002 – tutkimus. The National Findiet 2002 study. Männistö, S., Ovaskainen, M.L. & Valsta, L. (toim.). Kansanterveyslaitoksen julkaisuja B3/2003. Kansanterveyslaitos, Ravitsemusyksikkö. Helsinki 2003.
- Nigdikar, S.V., Williams, N.R., Griffin, B.A. & Howard, A.N. (1998) Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation *in vivo*. *Am J Clin Nutr* 68:258-265.
- Ninfali, P., Mea, G., Giorgini, S., Rocchi, M. & Bacchiocca, M. (2005) Antioxidant capacity of vegetables, spices and dressings relevant to nutrition. *Brit J Nutr* 93:257-266.
- Noguchi, N. (2002) Novel insights into the molecular mechanisms of the antiatherosclerotic properties of antioxidants; the alternatives to radical scavenging. *Free Radic Biol Med* 33:1480-1489.
- Noguchi, N., Gotoh, N. & Niki, E. (1993) Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochim Biophys Acta* 1168:348-357.
- Noguchi, N. & Niki, E. (1998) Dynamics of vitamin E action against LDL oxidation. *Free Radic Res* 28:561-572.
- Nyyssönen, K., Porkkala-Sarataho, E., Kaikkonen, J. & Salonen, J.T. (1997) Ascorbate and urate are strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men. *Atherosclerosis* 130:223-233.
- Okamura, N., Haraguchi, H., Hashimoto, K. & Yagi, A. (1994) Flavonoids in *Rosmarinus officinalis* leaves. *Phytochemistry* 37:1463-1466.
- O'Leary, K.A., de Pascual-Tereasa, S., Needs, P.W., Bao, Y.P., O'Brien, N.M. & Williamson, G. (2004) Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2). *Mutat Res* 551:245-254.

- Olthof, M.R., Hollman, P.C.H. & Katan, M.B. (2001) Chlorogenic acid and caffeic acid are absorbed in humans. *J Nutr* 131:66-71.
- Panagiotakos, D.B., Pitsavos, C., Chrysohoou, C., Kokkinos, P., Toutouzias, P. & Stefanadis, C. (2003) The J-shaped effect of coffee consumption on the risk of developing acute coronary syndromes: the CARDIO2000 case-control study. *J Nutr* 133:3228-3232.
- Parejo, I., Viladomat, F., Bastida, J. *et al* (2002) Comparison between the Radical Scavenging Activity and Antioxidant Activity of Six Distilled and Nondistilled Mediterranean Herbs and Aromatic Plants. *J Agric Food Chem* 50:6882-6890.
- Patrono, C. & FitzGerald, G.A. (1997) Isoprostanes: Potential markers of oxidant stress in atherothrombotic disease. *Arterioscler Thromb Vasc Biol* 17:2309-2315.
- Payet, B., Sing, A.S.C. & Smadja, J. (2005) Assessment of Antioxidant Activity of Cane Brown Sugars by ABTS and DPPH Radical Scavenging Assays: Determination of Their Polyphenolic and Volatile Constituents. *J Agric Food Chem* 53:10074-10079.
- Pellegrini, N., Serafini, M., Salvatore, S., Del Rio, D., Bianchi, M. & Brighenti, F. (2006) Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* methods. *Mol Nutr Food Res* 50:1030-1038.
- Peters, U., Poole, C. & Arab, L. (2001) Does tea affect cardiovascular disease? A meta-analysis. *Am J Epidemiol* 154:495-503.
- Pignatelli, P., Ghiselli, A., Buchetti, B. *et al* (2006) Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. *Atherosclerosis* 188:77-83.
- Pinchuk, I. & Lichtenberg, D. (2002) The mechanism of action of antioxidants against lipoprotein peroxidation, evaluation based on kinetic experiments. *Prog Lipid Res* 41:279-314.
- Pizzale, L., Bortolomeazzi, R., Vichi, S., Überegger, E. & Conte, L.S. (2002) Antioxidant activity of sage (*Salvia officinalis* and *S. fruticosa*) and oregano (*Origanum onites* and *O. onites*) extracts related to their phenolic compound content. *J Sci Food Agric* 82:1645-1651.
- Plumb, G.W., Garcia-Conesa, M.T., Kroon, P.A., Rhodes, M., Ridley, S. & Williamson, G. (1999) Metabolism of chlorogenic acid by human plasma, liver, intestine and gut microflora. *J Sci Food Agric* 79:390-392.
- Prior, R. L., Wu, X. & Schaich, K. (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:4290-4302.
- Radeke, H.H, Cross, A.R, Hancock, J.T *et al* (1991) Functional expression of NADPH oxidase components (alpha- and beta-subunits of cytochrome b558 and 45-kDa flavoprotein) by intrinsic human glomerular mesangial cells. *J Biol Chem* 266:21025-21029.
- Rahman, K. (2007) Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging* 2:219-236.
- Rechner, A.R., Kuhnle, G., Bremner, P., Hubbard, G.P., Moore, K.P. & Rice-Evans, C.A. (2002b) The metabolic fate of dietary polyphenols in humans. *Free Radic Biol Med* 33:220-235.

- Rechner, A.R., Kuhnle, G., Hu, H.L. *et al* (2002b) The metabolism of dietary polyphenols and the relevance to circulating levels of conjugated metabolites. *Free Radic Res* 36:1229-1241.
- Rechner, A.R., Spencer, J.P., Kuhnle, G., Hahn, U. & Rice-Evans, C. (2001) Novel biomarkers of the metabolism of caffeic acid derivatives *in vivo*. *Free Radic Biol Med* 30:1213-1222.
- Rechner, A.R., Smith, M.A., Kuhnle, G. *et al* (2004) Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radic Biol Med* 36:212-225.
- Reddy, V.C., Sagar, V.G.V, Sreeramulu, D., Venu, L. & Raghunath, M. (2005) Addition of milk does not alter the antioxidant activity of black tea. *Ann Nutr Metab* 49:189-195.
- Rice-Evans, C.A. Formation of free radicals and mechanism of action on normal biochemical processes and pathological states. In: *Free Radical Damage and its Control*. Rice-Evans, C.A & Burdon, R.H. (eds.). Elsevier Science B.V., Amsterdam, The Netherlands, 1994.
- Rice-Evans, C. Plant polyphenols: free radical scavengers or chain-breaking antioxidants? In: *Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives*. Rice-Evans, C., Halliwell, B. (eds.). Portland Press, London, UK, 1995.
- Rice-Evans, C., Miller, N.J. & Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 20:933-956.
- Richelle, M., Turini, M.E., Guidoux, R., Tavazzi, I., Metairon, S. & Fay, L.B. (1999) Urinary isoprostane excretion is not confounded by the lipid content of the diet. *FEBS Lett* 459:259-262.
- Rimm, E., Katan, M., Ascherio, A., Stampfer, M.J. & Willet, W.C. (1996) Relation between intake of flavonoids and risk of coronary heart disease in male health professionals. *Ann Intern Med* 125:384-389.
- Roberts, L.J. & Morrow, J.D. (1997) The generation and actions of isoprostanes. *Biochim Biophys Acta* 1345:121-135.
- Rodríguez-Meizoso, I., Marin, F.R., Herrero, M. *et al* (2006). Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization. *J Pharm Biomed Anal* 41:1560-1565.
- Sadik, C.D., Sies, H. & Schewe, T. (2003) Inhibition of 15-lipoxygenases by flavonoids: structure-activity relations and mode of action. *Biochem Pharmacol* 65:773-781.
- Satué-Gracia, M.T., Heinonen, M. & Frankel, E.N. (1997) Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *J Agric Food Chem* 45:3362-3367.
- Scalbert, A., Manach, C., MOrand, C., Remesy, C. & Jimenez, L. (2005) Dietary polyphenols and the prevention of diseases. *Crit Rew Food Sci Nutr* 45:287-306.
- Scalbert, A. & Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. *J Nutr* 130:2073S-2085S.
- Schlesier, K., Harwat, M., Böhm, V. & Bitsch, R. (2002) Assessment of antioxidant activity by using different *in vitro* methods. *Free Radic Res* 36:177-187.

- Schwab, U., Törrönen, A., Toppinen, L. *et al* (2002) Betaine supplementation with a low calorie diet decreases plasma homocysteine concentrations but does not affect body weight, body composition, resting energy expenditure or serum lipid concentrations. *Am J Clin Nutr* 76:961–967.
- Schwedhelm, E., Maas, R., Troost, R. & Böger, R.H. (2003) Clinical pharmacokinetics of antioxidants and their impact on oxidative stress. *Clin Pharmacokinet* 42:437-459.
- Seifried, H.E., Anderson, D.E, Fisher, E.I. & Milner, J.A. (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. *J Nutr Biochem* 18:567-579.
- Serafini, M., Laranjinha, J.A., Almeida, L.M. & Maiani, G. (2000) Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. *J Nutr Biochem* 11:585-590.
- Serra-Majem, L., Roman, B. & Estruch, R. (2006) Scientific evidence of interventions using the Mediterranean diet: a systematic review. *Nutr Rev* 64:27S-47S.
- Sesso, H.D., Gaziano, J.M., Liu, S. & Buring, J.E. (2003) Flavonoid intake and the risk of cardiovascular disease in women. *Am J Clin Nutr* 77:1400-1408.
- Shahidi, F. Natural Antioxidants: An Overview. In: *Natural Antioxidants*. Shahidi, F. (ed.). AOCS Press, Illinois, US, 1997.
- Shan, B., Cai, Y.Z., Sun, M. & Corke, H. (2005) Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agric Food Chem* 53:7749-7759.
- Shaw, L.M., Stromme, J.H., London, J.L. & Theodorsen, L. (1983) International Federation of Clinical Chemistry. Scientific Committee, Analytical Section. Expert Panel on Enzymes. IFCC methods for measurement of enzymes. Part 4. IFCC methods for gamma-glutamyltransferase [(gamma-glutamyl)-peptide: amino acid gamma- glutamyltransferase, EC 2.3.2.2]. IFCC Document, Stage 2, Draft 2, 1983-01 with a view to an IFCC Recommendation. *J Clin Chem Clin Biochem* 10:633–646.
- Silva, F.A.M., Borges, F., Guimarães, C., Lima, J.L.F.C., Matos, C. & Reis, S. (2000) Phenolic acids and derivatives: studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *J Agric Food Chem* 48:2122-2126.
- Singleton, V.L., Orthofer, R. & Lamuela-Raventós, R.M. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152-178.
- Singleton, V.L. & Rossi, J.A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *J Am Enol Vitic* 16:144-158.
- Siquet, C., Paiva-Martins, F., Lima, J.L.F.C., Reis, S. & Borges, F. (2006) Antioxidant profile of dihydroxy- and trihydroxyphenolic acids – A structure-activity relationship study. *Free Radic Res* 40:433-442.
- Soobrattee, M.A., Neergheen, V.S., Luximon-Ramma, A., Aruoma, O.I. & Bahorun, T. (2005) Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutat Res* 579:200-213.
- Statistics Finland (2006) Causes of death. Electronic reference, retrieved 6.11.2007 from: http://pxweb2.stat.fi/database/StatFin/Ter/ksyyt/ksyyt_fi.asp

- Steinbeck, M.J., Appel, W.H. Jr, Verhoeven, A.J. & Karnovsky, M.J. (1994) NADPH-oxidase expression and in situ production of superoxide by osteoclasts actively resorbing bone. *J Cell Biol* 126:765-772.
- Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. & Witztum, J.L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320:915-924.
- Stocker, R. & Keaney, J.F.J. (2004) Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84:1381-1478.
- Stoclet, J.-C., Chataigneau, T., Ndiaye, M. *et al* (2004) Vascular protection by dietary polyphenols. *Eur J Pharmacol* 500:299-313.
- Sung, H., Nah, J., Chun, S., Park, H., Yang, S.E. & Min, W.K. (2000) *In vivo* antioxidant effect of green tea. *Eur J Clin Nutr* 54:527-529.
- Tavani, A., Spertini, L., Bosetti, C. *et al* (2006) Intake of specific flavonoids and risk of acute myocardial infarction in Italy. *Publ Health Nutr* 9:369-374.
- Teissedre, P.L., Frankel, E.N., Waterhouse, A.L., Peleg, H. & German, J.B. (1996) Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. *J Sci Food Agric* 70:55-61.
- The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 330:1029-1035.
- Tijburg, L.B.M., Wiseman, S.A., Meijer, G.W. & Weststrate, J.A. (1997) Effects of green tea, black tea and dietary lipophilic antioxidants on LDL oxidizability and atherosclerosis in hypercholesterolaemic rabbits. *Atherosclerosis* 135:37-47.
- Trichopoulou, A., Vasilopoulou, E. & Lagiou, A. (1999) Mediterranean diet and coronary heart disease: Are antioxidants critical? *Nutr Rev* 57:253-256.
- Tsang, C., Higgins, S., Duthie, G.G. *et al* (2005) The influence of moderate red wine consumption on antioxidant status and indices on oxidative stress associated with CHD in healthy volunteers. *Brit J Nutr* 93:233-240.
- U.S. Food and Drug Administration (2006) Food additive status list. Electronic reference, retrieved 6.1.2008 from:
<http://www.cfsan.fda.gov/~dms/opa-appa.html>
- Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. & Mazur, M. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160:1-40.
- Van den Berg, R., Haenen, G.R.M.M., Van den Berg, H. & Bast, A. (1999) Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem* 66:511-517.
- Van het Hof, K.H., Wiseman, S.A., Yang, C.S. & Tijburg, L.B. (1999) Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proc Soc Exp Biol Med* 220:203-209.
- Vartiainen, E., Jousilahti, P., Alfthan, G., Sundvall, J., Pietinen, P. & Puska, P. (2000) Cardiovascular risk factor changes in Finland, 1972–1997. *Int J Epidemiol* 29:49-56.
- Viña, J., Borrás, C. & Miquel, J. (2007) Theories of aging. *IUBMB Life* 59:249-254.
- Vita, J.A. (2005) Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *Am J Clin Nutr* 81:292-297.
- Voutilainen, S., Nurmi, T., Mursu, J. & Rissanen, T.H. (2006) Carotenoids and cardiovascular health. *Am J Clin Nutr* 83:1265-1271.

- Wang, M., Kikuzaki, H., Zhu, N., Sang, S., Nakatani, N. & Ho, C.-T. (2000) Isolation and Structural Elucidation of Two New Glycosides from Sage (*Salvia officinalis* L.) *J Agric Food Chem* 48:235-238.
- Watson, L. & Dallwitz, M.J. (2007) 1992 onwards. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Electronic reference, retrieved 6.11.2007 from: <http://delta-intkey.com/angio/www/labiateae.htm>
- Williams, R.J., Spencer, J.P.E. & Rice-Evans, C. (2004) Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med* 36:838-849.
- Williamson, G. & Manach, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* 81:243-255.
- Winter, J., Moore, L.H., Dowell Jr, V.R. & Bokkenheuser, V.D. (1989) C-ring cleavage of flavonoids by human intestinal bacteria. *Appl Environ Microbiol* 55:1203-1208.
- Witztum, J.L. (1994) The oxidation hypothesis of atherosclerosis. *Lancet* 344:793-795.
- Wright, J.S., Johnson, E.R. & DiLabio, G.A. (2001) Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J Am Chem Soc* 123:1173-1183.
- Yamakoshi, J., Kataoka, S., Koga, T. & Ariga, T. (1999) Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* 142:139-149.
- Yang, R., Le, G., Li, A., Zheng, J. & Shi, Y. (2006) Effect of antioxidant capacity on blood lipid metabolism and lipoprotein lipase activity of rats fed a high-fat diet. *Nutrition* 22:1185-1191.
- Yeomans, V.C., Linseisen, J. & Wolfram, G. (2005) Interactive effects of polyphenols, tocopherol and ascorbic acid on the Cu²⁺-mediated oxidative modification of human low density lipoproteins. *Eur J Nutr* 44:422-428.
- Yesil-Celiktas, O., Girgin, G., Orhan, H., Wichers, H.J., Bedir, E. & Vardar-Sukan, F. (2007) Screening of free radical scavenging capacity and antioxidant activities of *Rosmarinus officinalis* extracts with focus on location and harvesting times. *Eur Food Res Technol* 224:443-451.
- Yildirim, A., Mavi, A., Oktay, M., Kara, A., Algur, O.F. & Bilaloglu, V. (2000) Comparison of antioxidant and antimicrobial activities of tilia (*Tilia Argentea Desf Ex DC*), sage (*Salvia Triloba L.*), and black tea (*Camellia Sinensis*) extracts. *J Agric Food Chem* 48:5030-5034.
- Yochum, L., Kushi, L.H., Meyer, K. & Folsom, AR. (1999) Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. *Am J Epidemiol* 149:943-949.
- Young, J.F., Dragstedt, L.O., Haraldsdottir, J. *et al* (2002) Green tea extract only affects markers of oxidative status postprandially: lasting antioxidant effect of flavonoid-free diet. *Brit J Nutr* 87:343-355.
- Zgórká, G. & Glowniak, K. (2001) Variation in free phenolic acids in medicinal plants belonging to the Lamiaceae family. *J Pharm Biomed Anal* 26:79-87.
- Zheng, W. & Wang, S.Y. (2001) Antioxidant activity and phenolic compounds in selected herbs. *J Agric Food Chem* 49:5165-5170.

ORIGINAL PUBLICATIONS I-V

Reproduced with the kind permission of the original copyright holders:

- I.** European Food Research and Technology.
© Springer Science and Business Media, 2002.
- II.** Food Chemistry.
© Elsevier Ltd., 2003.
- III.** Journal of Agricultural and Food Chemistry.
© American Chemical Society, 2006.
- IV.** (Submitted in Journal of Agricultural and Food Chemistry.)
- V.** Journal of Agricultural and Food Chemistry.
© American Chemical Society, 2006.