# EFFECTS OF PLANT STEROLS AND OLIVE OIL PHENOLS ON SERUM LIPOPROTEINS IN HUMANS

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**Maud Nelly Vissers**

**Proefschrift**

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 23 oktober 2001
des namiddags om vier uur in de Aula

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Effects of plant sterols and olive oil phenols on serum lipoproteins in humans
Thesis Wageningen University – With references – With summary in Dutch

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# Stellingen

1. Wanneer men in het Mediterrane gebied geraffineerde in plaats van extra vierge olijfolie had gegeten, had 'de zeven landen studie' dezelfde resultaten opgeleverd.
   *dit proefschrift*

2. Wetenschappelijke tijdschriften zouden artikelen van interventie-studies zonder controlegroep bij voorbaat af moeten wijzen.
   *dit proefschrift*

3. De 'mythe' rond de beschermende werking van antioxidanten kan niet worden opgehelderd voordat aan ziekte gerelateerde markers zijn gevonden.

4. De volgende generatie pilgebruiksters moet er een generatie pillen op achteruit gaan.
   *BMJ 2001;323:131-134*

5. Vanuit gezondheidskundig oogpunt is de acceptatie van overgewicht onder jongeren minder wenselijk.
   *n.a.v. NRC Handelsblad 9 juni 2001*

6. Juist door de ontwikkelingen binnen de genetische wetenschap moet ieder mens het morele recht op onwetendheid over zijn of haar genetisch profiel kunnen behouden.

7. Ter preventie van hart- en vaatziekten moeten televisieuitzendingen van belangrijke voetbalwedstrijden worden voorafgegaan door de waarschuwing: "niet geschikt voor mannen met een zwak hart".
   *BMJ 2000;321:1552-1554*

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Stellingen behorend bij het proefschrift:
**'Effects of plant sterols and olive oil phenols on serum lipoproteins in humans'**

Maud Vissers
Wageningen, 23 oktober 2001

*Aan mijn ouders*

# Abstract


**Effects of plant sterols and olive oil phenols on serum lipoproteins in humans**

*PhD thesis by Maud N. Vissers, Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands. October 23, 2001.*

The studies described in this thesis investigated whether minor components from vegetable oils can improve health by decreasing cholesterol concentrations or oxidative modification of low-density-lipoprotein (LDL) particles.

The plant sterols β-sitosterol and sitostanol are known to decrease cholesterol concentrations, but it is not clear whether other chemically related structures have similar effects. We examined the cholesterol-lowering effects of concentrates of β-sitosterol and 4,4'-dimethylsterols from rice bran oil and triterpene alcohols from sheanut oil. Plant sterols from rice bran oil lowered serum LDL cholesterol by 9%. This was probably due to β-sitosterol rather than the 4,4'-dimethylsterols. Triterpene alcohols did not affect serum cholesterol concentration.

Oxidative modification of LDL is hypothesised to play a role in the development of atherosclerosis. Extra virgin olive oil contains phenols with antioxidant activity that could prevent oxidative modification of LDL. Three weeks of consumption of phenol-rich olive oil or a single dose of olive oil phenols did not decrease LDL oxidisability, neither in fasting plasma nor postprandial plasma samples. We showed that olive oil phenols reduce LDL oxidisability *in vitro*, but only in amounts that are much higher than can be reached by olive oil consumption *in vivo*.

The first requirement for an *in vivo* action of a dietary antioxidant in humans is that it enters the blood circulation. We therefore studied the absorption and urinary excretion of olive oil phenols in humans. We found that apparent absorption of the ingested olive oil phenols was more than 55-66 mol%. Absorption was confirmed by the urinary excretion of at least 5 mol% tyrosol and hydroxytyrosol. A further requirement for a dietary antioxidant to prevent oxidative modification of LDL is that it becomes present in the circulation or in LDL in a form with antioxidant activity. In the body olive oil phenols are extensively metabolised. The antioxidant activity of these phenol metabolites is unknown. To determine the antioxidant activity of olive oil phenols *in vivo* future studies should focus on the antioxidant activity of the metabolites actually present in plasma rather than on the *in vitro* antioxidant activity of the phenols as present in the olive oil.

In conclusion, although the olive oil phenols are well absorbed, the amount of phenols in olive oil and their consequent attainable plasma concentration in humans is probably too low to reduce LDL oxidisability. Furthermore, our studies provide no evidence that 4,4'-dimethylsterols from rice bran oil or triterpene alcohols from sheanut oil are able to decrease cholesterol concentrations. Thus, there are no indications that the minor components from vegetable oils described in this thesis have important effects on serum lipoproteins.

# Contents

# Introduction

## BACKGROUND

Worldwide, cardiovascular diseases are the most common cause of death and a substantial source of chronic disability and health costs (Assmann *et al*, 1999). In the Netherlands coronary heart disease accounted for 38% of all cardiovascular diseases in 1997 (Konings-Dalstra & Reitsma, 1999). Coronary heart disease is characterised by insufficient blood supply to the heart muscle as a result of narrowed coronary arteries. The underlying cause is atherosclerosis; lipids accumulate in the artery and the artery vessel wall becomes enlarged and inflexible (atherogenic plaque). Risk factors for coronary heart disease include elevated LDL cholesterol concentrations, high blood pressure, obesity, diabetes, and lifestyle factors such as smoking and physical inactivity (Assmann *et al*, 1999). The oxidisability of LDL may also play a role in the development of atherosclerosis (Diaz *et al*, 1997; Steinberg *et al*, 1989). The studies described in this thesis focus on the cholesterol lowering effect of plant sterols and on the protective effects of olive oil phenols against LDL oxidation.

This introduction provides background information on the chemistry of plant sterols and olive oil phenols and the current knowledge of their health effects. Finally, this introduction will describe the rationale and outline of this thesis.

## HEALTH EFFECTS AND CHEMISTRY OF PLANT STEROLS

High LDL or total cholesterol concentrations are established risk factors for coronary heart disease. In the Netherlands 20-35% of adult population has a plasma total cholesterol concentration above 6.5 mmol/L (de Graaf & Stalenhoef, 2000). In people with a total cholesterol concentration above 6 mmol/L a decrease of 10% in total cholesterol has been predicted to reduce the risk of coronary heart disease by 10-50% (Kannel *et al*, 1986).

Plant sterols or phytosterols are used as cholesterol-lowering agents in foods (Jones & Raeini-Sarjaz, 2001). The interest for plant sterols as cholesterol lowering agents already exists since the early 1950's. For a short period they were used in capsules to treat high blood cholesterol concentrations before the introduction of pharmacological agents with higher efficacy and patients acceptance. There has been renewed interest in plant sterols since the late 1980's with the development of food processing technologies whereby they can be incorporated into margarine and other food products (Lichtenstein & Deckelbaum, 2001). These products rich in plant sterols can be consumed as part of a cholesterol lowering diet.

Sterols are essential constituents of cell membranes in animals and plants. Plant sterols are synthesised from squalene in plants and are structurally similar to cholesterol. Two types of plant sterols are the 4,4'-dimethylsterols like cycloartenol, intermediate products of plant sterol synthesis (Goodwin 1980), and the 4-desmethylsterols like β-sitosterol which are end products of plant sterol synthesis. Plant sterols can be converted into stanols, such as sitostanol, by hydrogenation, but they hardly occur in nature (**Figure 1.1**).

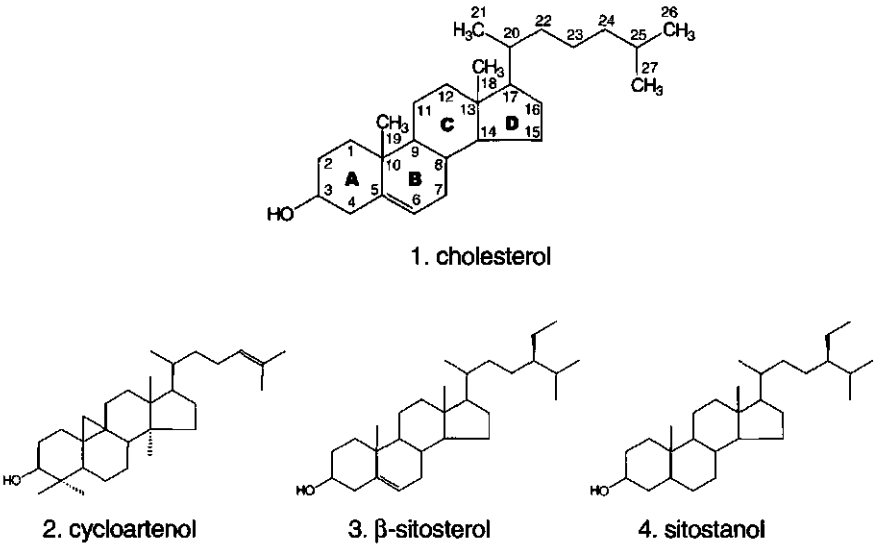


**Figure 1.1** Structures of various types of sterols. Cholesterol (1) is the sterol of mammalian cells. Plant sterols are synthesised in plants. The intermediate products are the 4,4'-dimethylsterols like cycloartenol (2), while 4-desmethylsterols, such as β-sitosterol (3), are the end products of plant sterol synthesis. Plant sterols can be converted into stanols, such as sitostanol (4).

Plant sterols are naturally present in the diet, and β-sitosterol, campesterol, and stigmasterol are the most common ones. The main dietary sources are vegetable oils and margarine. Intake level of plant sterols is with 200-300 mg/day comparable to the usual intake of about 300 mg/day of cholesterol. Intake of the stanols is much lower, about 30 mg/day (De Vries *et al*, 1997; Normen *et al*, 2001).

Plant sterols themselves are like cholesterol potentially atherogenic, but they are poorly absorbed which reduces their atherogenic potential. Cholesterol is absorbed in the body for 40-50%, whereas β-sitosterol is absorbed for about 5%, campesterol for 15%, and sitostanol for less than 1% ( Heinemann *et al*, 1993; Jones *et al*, 1997).

A daily intake of 2 g of plant sterols has been shown to decrease total cholesterol concentrations by 9-14% (Law 2000). Plant sterols may decrease blood cholesterol concentrations by inhibiting cholesterol absorption via micelles in the intestine. Plant sterols competitively replace dietary and biliary cholesterol from the micelles. Instead of being absorbed, cholesterol is precipitated and excreted via the faeces. When cholesterol absorption is decreased, the hepatic cholesterol pool reduces, resulting in enhanced cholesterol synthesis by the liver. At the same time LDL receptors are up-regulated, which leads to lower LDL cholesterol concentrations in blood (Miettinen & Gylling, 1997).

The cholesterol lowering effect of β-sitosterol and sitostanol has been well established (Miettinen *et al*, 1995; Weststrate & Meijer, 1998; Law 2000). However, it is not known if and to what extent other types of plants sterols reduce plasma cholesterol concentrations.

## HEALTH EFFECTS AND CHEMISTRY OF OLIVE OIL PHENOLS

The Mediterranean diet, which is high in olive oil, fruits, vegetables, grains, and legumes and relatively low in meat, has been associated with a reduced incidence of coronary heart disease in epidemiological studies (Keys *et al*, 1986). Olive oil is the main source of fat in the Mediterranean area where it is consumed in relatively high amounts; the average daily intake is about 50 g/day in Italy, Greece, and Spain (Boskou 2000; Helsing 1995). Replacing dietary saturated fatty acids with monounsaturated oleic acid from olive oil decreases plasma LDL concentrations, which presumably contributes to the low incidence of coronary heart diseases (Katan *et al*, 1995).

However, not only a high concentration of LDL cholesterol, but also the oxidisability of LDL might increase the risk of coronary heart disease. The LDL-oxidation-hypothesis states that when LDL particles accumulate in the artery wall, they can undergo progressive oxidation. Oxidised LDL may recruit circulating monocytes which undergo modification into macrophages once in the intima of the arterial wall. Oxidised LDL can be internalised by macrophages by means of the scavenger receptors on the surfaces of these cells. The internalisation of oxidised LDL leads to the accumulation of cholesterol esters in macrophages and these become so-called foam cells. These foam cells are the start of atherogenic plaques (**Figure 1.2**) (Diaz *et al*, 1997; Steinberg *et al*, 1989). Dietary antioxidants, such as vitamin E, may inhibit atherogenesis by reducing the oxidation of LDL and subsequently the accumulation of LDL in macrophages. (Steinberg 1991; Esterbauer *et al*, 1991; Princen *et al*, 1995). Phenols that are present in extra virgin olive oil are such antioxidants *in vitro* (Visioli & Galli, 1994; Visioli *et al*, 1995). As a consequence, olive oil may reduce the risk of atherosclerosis through the antioxidant activities of its phenols, besides the cholesterol lowering effect of monounsaturated oleic acids. However, the ability of olive oil phenols to decrease LDL oxidation *in vivo* still requires confirmation.

Extra virgin olive oil has a high concentration of phenols due to the way olive oil is extracted: Olives are crushed to burst the fruit cells and to crush the kernel, resulting in an olive paste. The paste is then mixed with water, a process which is called malaxation, to promote the coalescing of small oil droplets into larger ones. This facilitates the separation of the oil and water phases. The final stage in the process, centrifugation of the oil, separates the water and the olive residue (Kiritsakis & Markakis, 1987). Because olive oil is obtained without using heating or solvents, the process is referred as 'cold pressing'. This cold press method enables extra virgin olive oil to retain the phenols. These phenols are responsible for the specific taste of olive oil (Visioli & Galli, 1998). On the other hand, refined olive oil does not contain phenols. During refining the oil is extracted by alkaline agents to reduce the excessive acidity of the oil and to get rid of the free fatty acids and metals. At the same time many phenols are lost. The following deodorisation step, when the oil is heated at 190-210°C for 1-3 hours, results in additional losses (Belitz & Grosch, 1987).

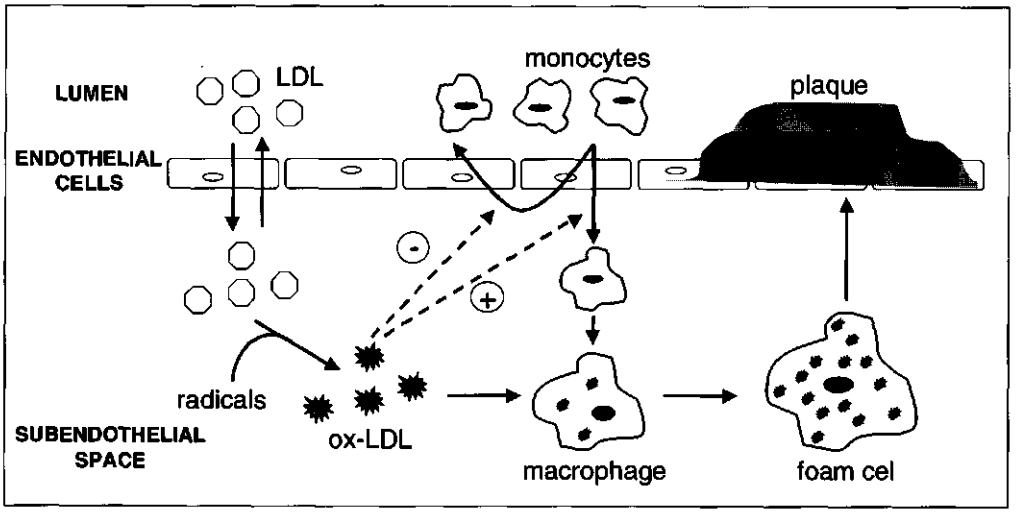


**Figure 1.2** LDL particles become trapped in an artery, where they can be oxidised. Oxidised LDL recruits circulating monocytes which become macrophages. Oxidised LDL is internalised by these macrophages. This leads to the accumulation of cholesterol esters in macrophages, which become so-called foam cells. These foam cells are the start of atherogenic plaques (Steinberg *et al*, 1989; Diaz *et al*, 1997).

The most abundant phenols in extra virgin olive oil are the largely non-polar oleuropein- and ligstroside-aglycones and their derivatives. The aglycones are formed in olives by the enzymatic removal of glucose from the polar parent compound oleuropein-glycoside. The various derivatives of those aglycones differ mainly in their ring structure, which can either be open or closed in two different forms (Personal communication from Dr. S. van Boom). The end products of the hydrolysis of oleuropein- and ligstroside-aglycones are the polar compounds hydroxytyrosol and tyrosol, respectively (**Figure 1.3**).

The antioxidant effect of the olive oil phenols has been observed in a number of *in vitro* and animal studies. However, it is not clear whether these phenols can also protect LDL from oxidation in humans. To date, studies in humans have been inconclusive (Nicolaiew *et al*, 1998; Ramirez-Tortosa *et al*, 1999; Bonanome *et al*, 2000; Visioli *et al*, 2000).

To act as potential antioxidants in humans, the olive oil phenols have to be absorbed from the intestines, to pass the intestinal wall, and to enter the blood circulation. In other words, the phenols have to be bioavailable. Bioavailability can be defined as 'the fraction of the ingested nutrient that is utilised for normal physiological functions or storage' (Jackson 1997). Once entered to the blood circulation phenols are metabolised or transformed into other compounds (Williamson *et al*, 2000; Hollman & Katan, 1998; Manna *et al*, 2000). For antioxidant activity in the body and the potential to prevent oxidative modification of LDL, it is essential that these metabolites of ingested phenols retain their antioxidant activity in plasma. Thus, insight into the kinetics of phenol metabolism in humans is required to understand the potential health effects that olive oil phenols may have in humans.

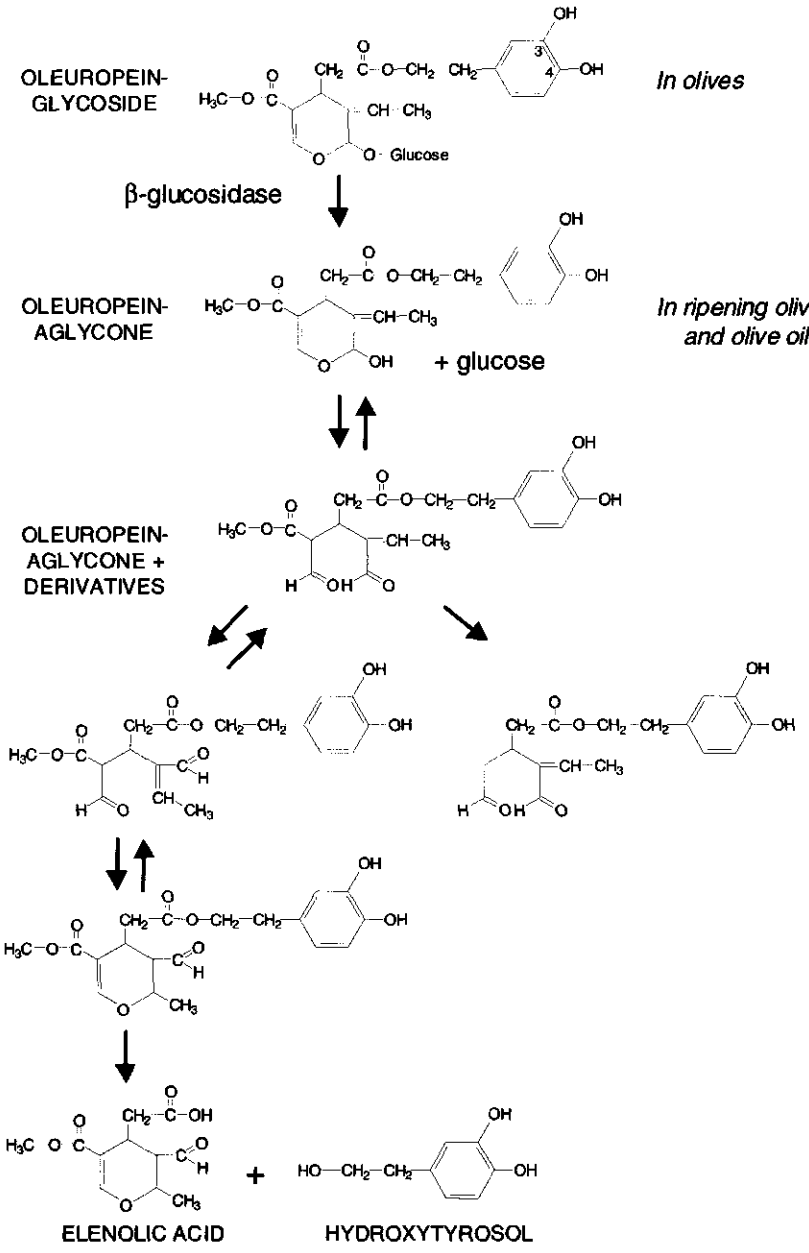


**Figure 1.3** Structures of oleuropein-glycoside and –aglycones, and their degradation into hydroxytyrosol (Personal communication from Dr. S. van Boom). Hydrolysis of ligstroside-glycoside into tyrosol is similar; only the hydroxyl group at carbon 3 of the benzene ring is absent. Oleuropein- and ligstroside-glycosides are mainly present in unripe olives, while their aglycones and tyrosol and hyxroxytyrosol are present in olive oil.

## RATIONALE AND OUTLINE OF THIS THESIS

The studies described in this thesis were designed to investigate *whether minor components from vegetable oils can improve health by decreasing cholesterol concentrations or by preventing oxidative modification of low-density-lipoprotein (LDL) particles.* This objective was subdivided into three main questions:

1. *What is the effect of various plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum cholesterol concentrations in humans?*
   Plant sterols are minor constituents in vegetable oils and are present in the unsaponifiable fraction. The most common plant sterols in the human diet are 4-desmethylsterols β-sitosterol, campesterol, and stigmasterol, and these are found in edible vegetable oils, such as corn, soybean and rapeseed oil. Beta-sitosterol can be converted by hydrogenation into a saturated counterpart sitostanol, which rarely occurs in nature. The cholesterol lowering effect of β-sitosterol and sitostanol has been well established (Miettinen *et al*, 1995; Weststrate & Meijer, 1998). Sterols with other structures, such as the 4,4'-dimethylsterols, may vary in their potential to reduce plasma cholesterol concentrations. *Chapter 2* describes a study on the effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum cholesterol concentrations in humans.

2. *Do phenols from extra virgin olive oil decrease LDL oxidisability in humans?*
   A high intake of olive oil has been proposed as an explanation for the low incidence of coronary heart disease in Mediterranean countries, but it is unclear whether olive oil offers specific benefits beyond a low saturated fat content. Olive oil phenols may dissolve into plasma LDL particles and protect them from becoming atherogenic by oxidative modification. In *chapter 3* we investigated the effect on the susceptibility of LDL to oxidation and other markers of oxidation when healthy humans consumed phenol-rich extra virgin olive oil for three weeks. In *chapter 4* we studied whether a single dose of olive phenols could decrease the postprandial susceptibility of LDL to oxidation. *Chapter 5* describes an *in vitro* study designed to mimic the exposure of olive oil phenols in plasma to LDL in order to estimate the minimum plasma concentration of olive oil phenols needed to reduce oxidative modification of LDL.

3. *To what extent are olive oil phenols absorbed and how are they metabolised?*
   The first requirement for a potential antioxidant *in vivo* is that it enters the blood circulation and that it retains its antioxidant capacity. Phenols are extensively metabolised in the human body (Williamson *et al*, 2000; Hollman & Katan, 1998; Manna *et al*, 2000). Therefore, the kinetics and metabolism of olive oil phenols is critical for our understanding of their potential as antioxidants in the human body. In *chapter 6* we estimate the apparent absorption of the various phenols from extra virgin olive oil in healthy ileostomy subjects with a complete small intestine. We also determined urinary excretion of the phenols. As

the ileostomy model does not take into account the influence of colonic bacteria in the metabolism of those phenols, we also determined the urinary excretion of tyrosol and hydroxytyrosol in subjects with a colon.

*Chapter 7* reviews the potential health effects of phenols from extra virgin olive oil, based on human and animal intervention trials. *Chapter 8* presents the concluding remarks.

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# Effect of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in humans

Maud N Vissers, Peter L Zock, Gert W Meijer, Martijn B Katan

*American Journal of Clinical Nutrition* 2000;72:1510-1515

## ABSTRACT

**Background:** Intake of unsaponifiable compounds from edible oils, such as plant sterols, can lower serum cholesterol concentrations in humans. However, little is known about effects of other chemically related unsaponifiables in edible oils, such as triterpene alcohols.

**Objective:** We studied the effects of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on cholesterol concentrations in healthy, normolipemic volunteers.

**Design:** Twenty-eight men and 32 women consumed 29 g/d of 3 margarines for 3 wk each on a crossover double-blind basis. A margarine based on sunflower oil was used as the control. Concentrates of plant sterols from rice bran oil or triterpene alcohols from sheanut oil were added to make 2 experimental margarines with the same fatty acid composition as the control margarine.

**Results:** Intake of 2.1 g plant sterols/d from rice bran oil decreased total cholesterol by 0.19 mmol/L (95% CI, -0.31, -0.07 mmol/L) and LDL cholesterol by 0.20 mmol/L (95% CI, -0.30, -0.10 mmol/L). HDL cholesterol and triacylglycerol concentrations did not change significantly. Intake of 2.6 g triterpene alcohols/d from sheanut oil did not significantly affect lipoprotein concentrations in all subjects combined.

**Conclusion:** We found that 2.1 g of plant sterols/d from rice bran oil lower serum total cholesterol by 5% and LDL cholesterol by 9% in normolipemic humans, whereas triterpene alcohols from sheanut oil did not significantly affect lipoprotein concentrations in all subjects combined. The effect of rice bran oil sterols is probably due to β-sitosterol and other 4-desmethylsterols and not to 4,4'-dimethylsterols.

## INTRODUCTION

Plant sterols or phytosterols are minor constituents of vegetable oils present in the unsaponifiable fraction. Large doses of plant sterols inhibit cholesterol absorption in humans and cause a modest decrease in serum cholesterol concentration (Lees *et al*, 1977; Pollak 1985; Jones *et al*, 1997). The most common plant sterols in the human diet are the 4-desmethylsterols β-sitosterol, campesterol, and stigmasterol, which are found in edible vegetable oils, such as corn, soybean and rapeseed (canola) oil. Like cholesterol, these plant sterols are unsaturated with a double bond at carbon 5 (**Figure 2.1**). Plant sterols can be converted into stanols by hydrogenation: β-sitosterol is transformed into its saturated counterpart sitostanol. Stanols rarely occur in nature. The cholesterol lowering effect of β-sitosterol and sitostanol has been well established (Miettinen *et al*, 1995; Weststrate & Meijer, 1998). Sterols with other structures may vary in their potential to reduce plasma cholesterol concentrations.

Plant sterols are synthesised from squalene; one of the first intermediate products is cycloartenol, a 4,4'-dimethylsterol (**Figure 2.1**; Goodwin 1980). Rice bran oil contains these 4,4'-dimethylsterols such as cycloartenol and 24-methylene cycloartanol as ferulic acid esters (oryzanol) (Rukmini & Raghuram, 1991). In addition rice bran oil contains a mixture of ferulic acid esters of 4-desmethylsterols, such as β-sitosterol and campesterol, which are the end products of plant sterol synthesis from squalene. The results of animal and some human studies suggest that rice bran oil may reduce plasma cholesterol concentrations (Raghuram & Rukmini, 1995; Sugano & Tsuji, 1997; de Deckere & Korver, 1996).

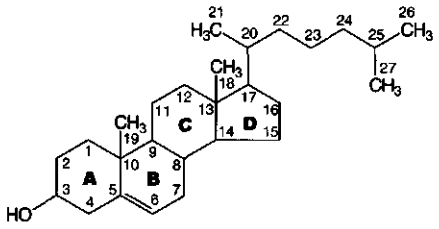
Another class of unsaponifiables is the triterpene alcohols. Strictly speaking, triterpene alcohols are not plant sterols, but there are similarities in their structures (**Figure 2.1**). Sheanut oil contains approximately 8% unsaponifiable material, which is a mixture of fatty acid and cinnamic acid esters of such triterpene alcohols as α-amyrine, butyrospermol, lupeol, and β-amyrine (Peers 1977). Little is known about the effect of sheanut oil on cholesterol concentrations (Weststrate & Meijer, 1998).

We examined the effects of plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum lipoprotein concentrations in healthy humans in order to define the structural elements responsible for such effects.

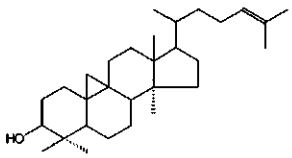
## SUBJECTS AND METHODS

### Subjects

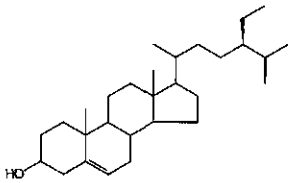
Subjects were recruited via publicity in local newspapers and posters in university buildings and dormitories. We carefully explained the study protocol to the subjects before they gave their written informed consent. The study protocol was approved by the Medical Ethical Committee of Wageningen University.



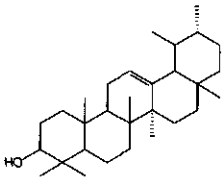
1. cholesterol



2. cycloartenol,
a typical 4,4'-dimethylsterol



3. β-sitosterol,
a typical 4-desmethylsterol



4. α-amyrin,
a typical triterpene alcohol

**Figure 2.1** Structures of plant sterols and triterpene alcohols in rice bran oil and sheanut oil compared to the structure of cholesterol (1). Rice bran oil contains the 4,4'-dimethylsterols cycloartenol (2) and 24-methylene cycloartanol, and the 4-desmethylsterols β-sitosterol (3) and campesterol. Sheanut oil contains the triterpene alcohols α-amyrine (4), butyrospermol, lupeol and β-amyrine. In triterpene alcohols the cyclopentane ring specific for sterols is replaced by 2 cyclohexane rings.

Subjects were eligible if they were >18 y of age, did not use drugs known to affect concentrations of serum lipoproteins, and were not pregnant, lactating, or following a prescribed diet. Volunteers filled out a medical questionnaire that was reviewed by an independent physician. Persons with a history of gastrointestinal, liver or kidney disease were excluded, as were those with glucosuria, proteinuria, anemia, or a serum concentration of total cholesterol >7.5 mmol/L or of fasting triacylglycerol >2.3 mmol/L. Thirty-two women and 28 men aged 18-59 y were enrolled in the study.

## Design and treatment

The 9-wk study was conducted on a multiple cross-over double-blind basis. Subjects consumed 29 g/d of 3 margarines for 3 wk each. The subjects were stratified according to age and sex and then randomly allotted to 1 of the 6 possible treatment sequences. A commercially available diet margarine (Flora; Van den Bergh Foods, Purfleet, United Kingdom) was used as the control margarine and served as the base for preparing the 2 experimental margarines (Unilever Research Vlaardingen, the Netherlands). Concentrates of plant sterols from rice bran oil or triterpene alcohols from sheanut oil were added so that each margarine had the same fatty acid composition (**Table 2.1**). The margarines were provided in tubs that contained on average 28.9 g. The subjects consumed the contents of 1 tub over 1 d. They were not allowed

to use the margarine for baking or frying; they usually consumed it as a spread on bread for breakfast, lunch and in-between meals or added it to prepared hot meals. The mean intake of total plant sterols (in free sterol equivalents) was 0.06 g/d from the control margarine, 2.1 g/d from the rice bran oil margarine, and 2.6 g/d from the sheanut oil margarine (as triterpene alcohols).

**Table 2.1** Compositions of the experimental margarines.

| Component | Control | Rice bran[a] | Sheanut[a] |
|---|---|---|---|
| | | *% by wt* | |
| Fat phase (fat + sterols + other esters) | 70 | 82 | 86 |
| Total fatty acids | 70 | 71 | 75 |
| Saturated | 16 | 14 | 19 |
| Monounsaturated | 15 | 19 | 20 |
| Polyunsaturated | 40 | 38 | 35 |
| Total known plant sterols[b] | 0.2 | 7.3 | 0.4 |
| 4,4'-dimethylsterols | <0.1 | 3.4 | <0.1 |
| Monomethylsterols | <0.1 | 0.4 | <0.1 |
| 4-desmethylsterols | 0.2 | 3.5 | 0.4 |
| Triterpene alcohols[b] | – | – | 8.9 |
| Other unknown sterol-related compounds | – | 1.2 | – |
| Phenolic acids | | | |
| Ferulic acid | – | 2.9 | – |
| Cinnamic acid | – | – | 2.1 |
| Water phase | 30 | 18 | 14 |
| Lithium chloride[c] | 997 | 859 | 999 |

[a] Mean of 3 batches.

[b] Amounts as free sterol or triterpene alcohol equivalents.

[c] µmol/100 g.

The subjects were asked to maintain their usual diet and lifestyle. All subjects kept daily records of illness and deviations from the protocol and they returned empty margarine tubs. Ninety-six percent of the tubs were returned empty; diaries kept by the subjects and anonymous questionnaires administered after the trial showed that 99.5% of the scheduled amount of the control margarine, 97.3% of the rice bran oil margarine, and 99.1% of the sheanut oil margarine was consumed. To check adherence independently we added 285 µmol lithium chloride to each 30 g margarine and determined plasma lithium concentrations (by inductively coupled plasma mass spectrometry, model Elan 6000 spectrometer; Perkin-Elmer Corp, Norwalk, CT). Mean plasma lithium concentration increased to 4.6 ± 1.3 µmol/L which was about 5 times the baseline concentration of 0.9 ± 0.3 µmol/L in similar subjects who did not consume added lithium chloride (de Roos *et al*, 2001). This confirmed adherence to the protocol.

In each 3-wk period, intakes of energy, fatty acids and cholesterol were estimated by a 24-h recall. Intake was similar for each treatment, although cholesterol intake was slightly higher in the period in which the control margarine was consumed (**Table 2.2**). Body weights were measured on day 18 of each 3-wk period. Mean body weight did not differ significantly between treatments: 70.4 ± 9.4 kg with the control margarine, 70.2 ± 9.2 kg with the rice bran oil margarine, and 70.1 ± 9.1 kg with the sheanut oil margarine.

**Table 2.2** Dietary intake of energy, fatty acids, and cholesterol of the subjects while on the 3 study margarines.[a]

| | Margarine | | |
|---|---|---|---|
| | Control | Rice bran oil | Sheanut oil |
| Energy | | | |
| MJ/d | 11.8 ± 3.9[b] | 11.8 ± 3.6 | 11.4 ± 3.7 |
| kcal/d | 2820 ± 941.6 | 2819 ± 854.4 | 2719.8 ± 870.5 |
| Fat (% of energy) | 34.9 | 35.2 | 34.4 |
| Saturated fatty acids | 12.1 | 12.2 | 12.1 |
| Monounsaturated fatty acids | 11.2 | 11.4 | 10.6 |
| Polyunsaturated fatty acids | 9.2 | 9.4 | 9.5 |
| Cholesterol (mg/d) | 225.9 ± 190.2 | 188.6 ± 125.5 | 175.0 ± 109.1 |

[a] Each subject consumed each margarine for 3 wk in random order. Values are based on one 24-h recall per person in each 3-wk period. $n = 60$.
[b] Mean ± SD.

## Blood sampling

Two venous blood samples were taken from subjects after an overnight fast at the end of each 3-wk period, one on day 18 and another on day 21. Serum was obtained by centrifugation at 1187 x *g* for 10 min at 4°C and stored at -80°C. Samples were analysed enzymatically for total and HDL cholesterol and triacylglycerol concentrations (Siedel *et al*, 1983; Warnick *et al*, 1982; Fossati & Prencipe, 1982). The mean bias for control samples provided by the Centers for Disease Control and Prevention in Atlanta was -1% for total and HDL cholesterol and 10% for triacylglycerol. The within-run CV ranged from 0.5% to 1.1%. LDL cholesterol concentrations were calculated (Friedewald *et al*, 1972). For each subject the serum lipoprotein values of day 18 and 21 of each period were averaged before statistical analysis.

## Margarine analyses

The fatty acid compositions of the 3 margarines were analysed by methanolysis of the fatty acids in a sample of margarine extracted with a methanolic NaOH solution using boron

trifluoride as catalyst. The methyl esters were extracted with hexane and analysed on a gas chromatograph (Hewlett Packard, Wilmington, DE) with a CP Wax 58 column (Chrompack, Middelburg, The Netherlands). Analysis of plant sterols and triterpene alcohols was done by saponifying the margarine with KOH and extracting the unsaponifiable part into diisopropylether. After extraction and derivation with 1% TMCS (trimethylsilyl chloride) in BSTFA [*N,O-bis*(trimethylsilyl)trifluoroacetamide] and *N,N*-dimethylformamide, the fractions were analysed on a gas chromatograph (Hewlett Packard) by means of splitless injection with hydrogen as carrier gas. In the case of the 4,4'-dimethylsterols, the response factor compared with cholesterol was 0.87 on the basis of 5 concentrations of cycloartenol. For this reason, we also analysed the plant sterols by using on-column injection with hydrogen as carrier gas and with helium as carrier gas. The response factors were 0.91 for hydrogen and 0.98 for helium as carrier gas. We therefore used the data from the on-column injection with helium as carrier gas.

### Statistical analyses

The data were analysed by two-factor, repeated-measures analysis of variance with interaction by using the general linear models (GLM) subprogram of SAS (SAS Institute Inc. 1989). Because the interaction between sex and margarine was significant for total and LDL cholesterol the analysis was also performed for men and women separately. Tukey's procedure was used for pair-wise comparisons of the margarines and for calculation of 95% CI's of the differences in plasma lipoprotein concentrations between 2 margarines.

## RESULTS

All 60 subjects completed the study. None of the subjects used medications that could have affected the results. Three subjects became ill during the period in which they consumed the rice bran oil margarine. Excluding the data of these subjects from the analyses did not alter the results. We therefore present results of analyses that included all subjects. Because there was a significant interaction between sex and margarine for total cholesterol and LDL cholesterol, we also present the results of men and women separately.

### Serum lipids and lipoproteins

In all subjects combined rice bran oil margarine decreased total cholesterol by 0.19 mmol/L (95% CI: -0.31, -0.07 mmol/L) and LDL cholesterol by 0.20 mmol/L (95% CI: -0.30, -0.10 mmol/L) compared with the control margarine (**Table 2.3**). Of the 60 subjects, 44 showed lower concentrations and 15 showed higher concentrations of LDL cholesterol with rice bran oil margarine than with control margarine (**Figure 2.2**) Rice bran oil margarine did not affect HDL cholesterol or triacylglycerol concentrations (**Table 2.3**).

**Table 2.3** Serum lipid and lipoprotein concentrations at the end of the 3 margarine periods and the differences between experimental and control margarine.[a]

| | Lipid and lipoprotein values | | | Differences from control margarine (95% CI) | |
|---|---|---|---|---|---|
| | Control | Rice bran oil | Sheanut oil | Rice bran oil | Sheanut oil |
| Total cholesterol[b] | | | | | |
| Men | 4.15 ± 0.89[c] | 4.01 ± 0.93 | 4.01 ± 0.80 | -0.14 (-0.31, 0.03) | -0.14 (-0.31, 0.03) |
| Women | 4.32 ± 0.62 | 4.08 ± 0.67[d,e] | 4.35 ± 0.75 | -0.24 (-0.41, -0.07) | 0.03 (-0.14, 0.19) |
| All | 4.24 ± 0.76 | 4.05 ± 0.80[d,e] | 4.19 ± 0.79 | -0.19 (-0.31, -0.07) | -0.05 (-0.17, 0.07) |
| HDL cholesterol | | | | | |
| Men | 1.37 ± 0.30 | 1.35 ± 0.31 | 1.32 ± 0.30 | -0.02 (-0.08, 0.04) | -0.05 (-0.11, 0.01) |
| Women | 1.64 ± 0.38 | 1.64 ± 0.41 | 1.65 ± 0.40 | 0.00 (-0.06, 0.07) | 0.01 (-0.05, 0.08) |
| All | 1.51 ± 0.37 | 1.50 ± 0.39 | 1.50 ± 0.39 | -0.01 (-0.05, 0.03) | -0.02 (-0.06, 0.03) |
| LDL cholesterol[b] | | | | | |
| Men | 2.42 ± 0.70 | 2.27 ± 0.71[d] | 2.29 ± 0.60[d] | -0.15 (-0.28, -0.03) | -0.13 (-0.26, -0.01) |
| Women | 2.30 ± 0.47 | 2.06 ± 0.48[d,e] | 2.30 ± 0.59 | -0.24 (-0.39, -0.10) | -0.003 (-0.15, 0.14) |
| All | 2.36 ± 0.59 | 2.16 ± 0.60[d,e] | 2.29 ± 0.59 | -0.20 (-0.30, -0.10) | -0.06 (-0.16, 0.03) |
| Triacylglycerol | | | | | |
| Men | 0.77 ± 0.39 | 0.86 ± 0.54 | 0.88 ± 0.53 | 0.09 (-0.06, 0.24) | 0.11 (-0.04, 0.26) |
| Women | 0.84 ± 0.47 | 0.83 ± 0.42 | 0.87 ± 0.44 | -0.01 (-0.08, 0.07) | 0.03 (-0.04, 0.10) |
| All | 0.81 ± 0.43 | 0.85 ± 0.48 | 0.87 ± 0.48 | 0.04 (-0.04, 0.12) | 0.07 (-0.01, 0.15) |

[a] All participants (28 men and 32 women) were included in the analysis. They consumed each margarine in random order for 3 wk each. To convert total, HDL, and LDL cholesterol values to mg/dL, multiply by 38.67. To convert triacylglycerol values to mg/dL, multiply by 88.54.

[b] Significant interaction between sex and margarine, $P = 0.03$.

[c] Mean ± SD

[d] Significantly different from the control margarine, $P < 0.05$ (adjusted with Tukey's procedure for multiple comparisons).

[e] Significantly different from sheanut oil margarine, $P < 0.05$ (adjusted with Tukey's procedure for multiple comparisons).

Sheanut oil margarine did not affect total, LDL, or HDL cholesterol or triacylglycerol concentrations compared with the control margarine in all subjects combined (**Table 2.3**). Thirty-three subjects showed lower concentrations and 25 showed higher concentrations of LDL cholesterol with sheanut oil margarine than with the control margarine (**Figure 2.2**). In men, sheanut oil margarine decreased LDL cholesterol by 0.13 mmol/L (95% CI: -0.26, -0.01 mmol/L) and non-significantly decreased total cholesterol by 0.14 mmol/L (95% CI: -0.31, 0.03 mmol/L) (**Table 2.3**).

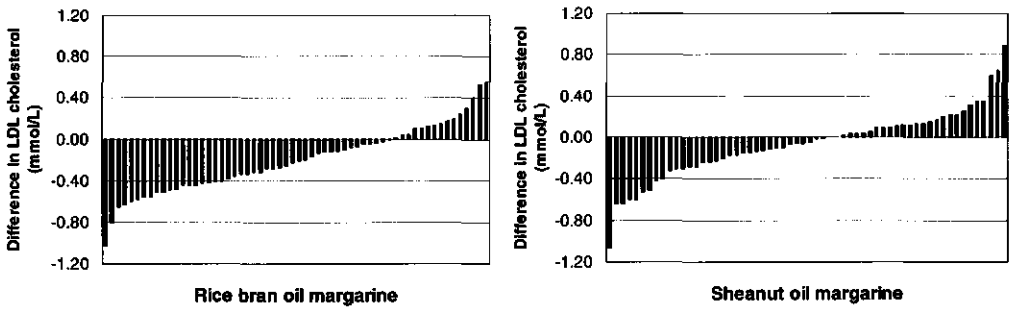



**Figure 2.2** Individual differences in serum LDL cholesterol concentration between the end of 3 wk consumption of experimental margarine (rice bran oil margarine or sheanut oil margarine) and the end of 3 wk consumption of control margarine.

## DISCUSSION

The results of this study show that 2.1 g of plant sterols/d from rice bran oil lowers serum total cholesterol by 5% and LDL cholesterol by 9% in normolipemic subjects whereas triterpene alcohols from sheanut oil have no or much smaller effects.

Earlier studies on rice bran oil could not disentangle the effects of the fatty acids from those of plant sterols (Suzuki & Oshima, 1970a; Suzuki & Oshima, 1970b; Raghuram *et al*, 1989; Lichtenstein *et al*, 1994). In these studies intake of rice bran oil sterols was 4-9 times lower than in our study, and these studies addressed the consumption of complete oils, not their plant sterols per se. One other study investigated the effects of concentrated rice bran oil sterols in humans and found - in contrast with our study - no significant effect on cholesterol concentrations in normo- and mildly hypercholesterolemic subjects (Weststrate & Meijer, 1998).

Our study cannot directly answer the question whether 4,4'-dimethylsterols alone affect cholesterol concentrations; half of the plant sterols supplied by the rice bran oil margarine were 4,4'-dimethylsterols (1.0 g/d) and half were 4-desmethylsterols (1.0 g/d, mainly β-sitosterol and campesterol). The rice bran oil margarine in the study of Weststrate and Meijer, who found no effect on cholesterol concentrations, supplied mainly 4,4'-dimethylsterols such as cycloartenol and 24-methylene cycloartanol (1.1 g/d) and much less 4-desmethylsterols including

β-sitosterol (0.5 g/d) (Weststrate & Meijer, 1998). This suggests that 4,4'-dimethylsterols may have no effect on cholesterol concentrations. Any effect of rice bran oil sterols is therefore probably due to 4-desmethylsterols. This notion is also supported by the studies of Hendriks *et al* (1998) and Sierksma *et al* (1999) in which 0.8 g of 4-desmethylsterols/d from soybean oil lowered cholesterol concentrations by 4.9% and 3.8% respectively. In our study 1.0 g of 4-desmethylsterols/d decreased cholesterol concentrations by 5%, the same extent as observed in these 2 studies. Also in rats a combination of the 4,4'-dimethylsterol cycloartenol and the 4-desmethylsterol β-sitosterol did not lower cholesterol concentrations more than did β-sitosterol alone (Ikeda *et al*, 1985). Thus, any effect of rice bran oil sterols on cholesterol is probably due to the 4-desmethylsterols, with 4,4'-dimethylsterols having no or a much smaller effect. However, this issue can be settled only in a study that directly tests the effects of 4,4'-dimethylsterols.

Plant sterols probably decrease plasma cholesterol concentrations by inhibiting cholesterol absorption in the small intestine (Lees *et al*, 1977; Mattson *et al*, 1982; Miettinen & Vanhanen, 1994; Gylling & Miettinen, 1994). The 4-desmethylsterol β-sitosterol and cholesterol are more similar in structure than are the 4,4'-dimethylsterols and cholesterol. The 4,4'-dimethylsterols have 2 extra methyl groups at carbon 4, a methyl group at carbon 14, and an extra cyclopropyl ring at carbons 9 and 10 compared with 4-desmethylsterols (**Figure 2.1**). Therefore, β-sitosterol may be more effective than are 4,4'-dimethylsterols in competing with cholesterol for incorporation in mixed micelles, which is the supposed mechanism for the cholesterol absorption-inhibiting action of plant sterols (Jones *et al*, 1997). In addition, Heinemann *et al* (1993) suggested that increasing the side chain substitution of cholesterol decreases its absorbability in humans. For example, β-sitosterol has a side chain substitution of an ethyl group compared to cholesterol (**Figure 2.1**) and is absorbed less than is cholesterol. Heinemann *et al* (1993) also indicated an inverse relation between the absorbability of plant sterols and their efficiency in inhibiting cholesterol absorption. Compared with cholesterol the 4,4'-dimethylsterol cycloartenol does not have a side chain substitution but has an additional double bond at carbon 24, and 24-methylene cycloartanol has a methylene group at carbon 24. Hence, these 4,4'-dimethylsterols might be more absorbable and therefore less effective in inhibiting cholesterol absorption than the 4-desmethylsterol β-sitosterol. No data are available in the literature with respect to the absorption of the 4,4'-dimethylsterols in humans. However, in rats the absorption rate of cycloartenol was 4-fold higher than that of β-sitosterol (Ikeda *et al*, 1985). Thus, the differential effects of 4-desmethylsterols and 4,4'-dimethylsterols on serum cholesterol concentrations may be explained by several structural differences.

In our study, intakes of 4-desmethysterols and 4,4'-dimethylsterols were 5-10-fold higher than in a normal diet, and potential adverse effects of such relatively high intakes need to be considered carefully. In patients with homozygous sitosterolemia a high percentage of the 4-desmethylsterol β-sitosterol is absorbed from the intestine; this is believed to account for the plant sterol accumulation in plasma and early atherosclerosis in such patients (Salen *et al*, 1992). Homozygous sitosterolemia is an extremely rare condition, but heterozygotes occur

more frequently and such heterozygotes might theoretically hyperabsorb plant sterols. One study found that plasma plant sterol concentrations in heterozygous subjects were 2-3 times higher than in control subjects and 10-20 times lower than in homozygous subjects (Hidaka *et al*, 1990). Two other studies (Beaty *et al*, 1986; Salen *et al*, 1992) found normal plasma concentrations in heterozygous subjects. These data suggest that there is no reason to expect important adverse effects of foods rich in plant sterols in heterozygous subjects. Weststrate and Meijer (1998) showed that an intake of 1.5 g β-sitosterol/d and 0.8 g campesterol/d compared to a control (<0.1 g/d) increased plasma β-sitosterol concentration from 3.3 to 4.6 mg/L and plasma campesterol concentration from 7.0 to 12.1 mg/L. These concentrations are within the range of normal values (Ling & Jones, 1995). Thus, adverse effects of high intakes of 4-desmethylsterols such as β-sitosterol and campesterol seem unlikely, but long-term observational data are still desirable to confirm the safety of high intakes, especially for subjects who carry the sitosterolemia gene or genes.

We did not analyse plasma plant sterol concentrations in our study, and we are not aware of other data with respect to the absorption of the 4,4'-dimethylsterols such as cycloartenol in humans. If cycloartenol is as highly absorbed in humans as in rats (Ikeda *et al*, 1985) it could be a reason to avoid fortification of food with rice bran sterols because these are rich in 4,4'-dimethylsterols.

Sheanut oil margarine did not significantly affect lipoprotein and triacylglycerol concentrations in all subjects combined. This is in line with the results of Weststrate and Meijer (1998). The unsaponifiables of sheanut oil consist of triterpene alcohols and minor amounts of 4-desmethylsterols and 4,4'-dimethylsterols (Peers 1977). Like 4,4'-dimethylsterols, triterpene alcohols have 2 extra methyl groups at carbon 4 compared to 4-desmethylsterols. Also, in triterpene alcohols the cyclopentane ring specific for sterols is absent; instead triterpene alcohols have 2 cyclohexane rings (ring D; **Figure 2.1**). A sterol structure with a cyclopentane ring might be a minimum requirement for inhibiting cholesterol absorption in the intestine. This may explain why triterpene alcohols do not lower serum cholesterol concentrations.

In our study, there was a significant interaction between sex and margarine for total and LDL cholesterol. Sheanut oil margarine slightly lowered total and LDL cholesterol concentrations in men but not in women. However, the sex-specific analyses were not planned a priori and the difference between men and women was not very large. Therefore, this finding may have been due to chance. Only one postmenopausal woman, aged 59 y, participated in our study and omitting her data did not change the conclusions (data not shown). Therefore our data refer to pre-menopausal women.

Only normocholesterolemic subjects participated in our study, and we cannot answer the question of what the effect would have been in hypercholesterolemic subjects. If we calculated treatment effect for tertiles on the basis of the study entry value for total cholesterol concentration, the response in the lowest tertile (range 3.15-4.16 mmol/L) was -0.16 ± 0.3 mmol/L, in the mid tertile (range 4.17-4.71 mmol/L) -0.32 ± 0.3 mmol/L, and in the highest tertile (range 4.74-7.32 mmol/L) -0.10 ± 0.5 mmol/L. There were no significant differences between

tertiles. We therefore estimate that the effect of plant sterols from rice bran oil is similar in normo- and hypercholesterolemic subjects. However, this issue can be settled only in a study that directly tests the effect of plant sterols from rice bran oil in hypercholesterolemic subjects.

In summary, plant sterols from rice bran oil lowered serum total and LDL cholesterol concentrations in normolipemic humans, whereas triterpene alcohols from sheanut oil did not affect the average cholesterol concentration in all subjects combined. The effect of rice bran oil sterols on serum cholesterol concentrations is probably due to the 4-desmethylsterol β-sitosterol and not to 4,4'-dimethylsterols such as cycloartenol and 24-methylene cycloartanol. Because rice bran oil contains mainly 4,4'-dimethylsterols and less 4-desmethylsterols, it might not be an efficient dietary source of cholesterol lowering plant sterols.

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# Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers

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## ABSTRACT

**Objective**: We studied whether consumption of phenol-rich extra virgin olive oil affects the susceptibility of low density lipoprotein (LDL) to oxidation and other markers of oxidation in humans.

**Design**: Randomised cross-over intervention trial, stratified according to sex, age, and energy intake.

**Setting**: Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands.

**Subjects**: Forty-six healthy men and women completed the study.

**Intervention**: Subjects consumed two diets supplying 69 g/d of extra virgin olive oil either rich or poor in phenols for 3 wk each. The mean difference in phenol intake between the treatments was 18 mg/d. Vitamin E intake was low during the whole study. Fasting blood samples were taken twice at the end of each period.

**Results**: Resistance of LDL and high density lipoprotein (HDL) to oxidation was not affected by treatment. The mean lag time of copper-induced formation of conjugated dienes was 1.6 min shorter in LDL and 0.4 min longer in HDL after the high phenol diet. Other markers of antioxidant capacity in plasma were also not affected: mean lipid hydroperoxides were 0.07 µmol/L higher, mean malondialdehydes were 0.001 µmol/L higher, mean protein carbonyls were 0.001 nmol/mg protein lower, and the mean ferric reducing ability of plasma (FRAP) was 0.006 mmol/L higher after the high phenol diet. All 95% confidence intervals enclosed zero. Serum cholesterol concentrations were not affected by the treatment.

**Conclusion**: Consumption of 18 mg/d of phenols from extra virgin olive oil for 3 wk did not affect LDL or HDL oxidation or other markers of antioxidant capacity in fasting plasma samples.

## INTRODUCTION

The Mediterranean diet, with olive oil as the major fat source, has been shown in epidemiological studies to be associated with a reduced incidence of coronary heart disease (Keys *et al*, 1986). The replacement of dietary saturated fatty acids with monounsaturated oleic acid from olive oil decreases plasma LDL concentrations, which presumably contributes to the low incidence of coronary heart diseases (Katan *et al*, 1995). It has also been suggested that a high-monounsaturated fat diet lowers the risk of coronary heart disease by producing oleic acid-enriched LDL particles, which are more resistant to oxidative modification (Bonanome *et al*, 1992; Reaven *et al*, 1991; Aviram & Eias, 1993). Oleic acid, however, may not be the only component of olive oil protecting LDL from oxidation; some types of extra virgin olive oil contain phenols with antioxidative properties. These phenols are formed during ripening of olives by hydrolysis of the parent compound oleuropein (**Figure 3.1**), and they are, in contrast to phenols from, for example, tea and wine, partly lipid-soluble.

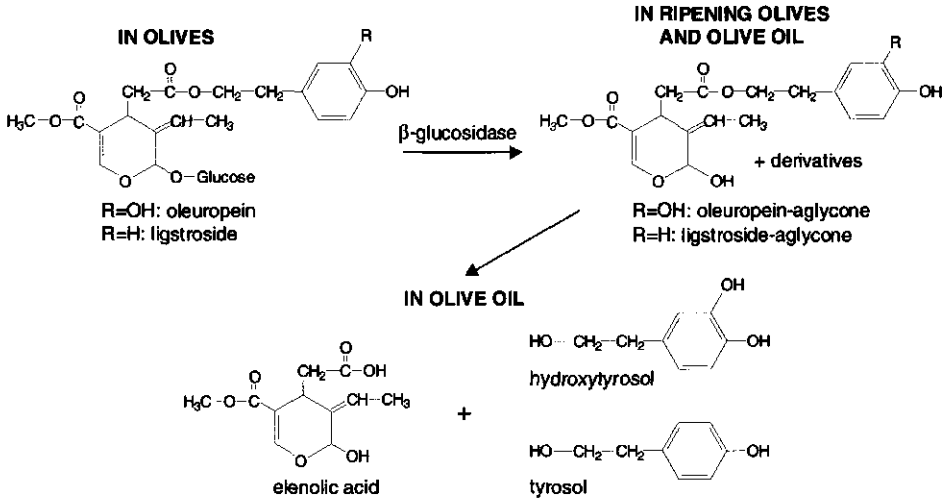


**Figure 3.1** Structures of various phenols and their degradation into tyrosol and hydroxytyrosol in olives and extra virgin olive oil.

The oxidative modification hypothesis of atherosclerosis suggests that LDL particles are oxidatively modified and then taken up by macrophages inside the arterial wall. Such cholesterol-laden macrophages form the start of atherosclerotic plaques. Dietary antioxidants may therefore inhibit atherogenesis by inhibiting oxidation of LDL and accumulation of LDL in macrophages (Witztum & Steinberg, 1991). When olive oil is ingested, the lipid-soluble phenols are possibly taken up by LDL particles in plasma. Thus, these phenols may protect LDL particles from becoming atherogenic by oxidative modification. The aim of this study was to determine whether consumption of phenols from olive oil affects the susceptibility of LDL to oxidation and other markers of oxidation in normolipemic humans.

## SUBJECTS AND METHODS

### Subjects

Subjects were recruited via publicity in local newspapers and posters in university buildings and student apartments. We carefully explained the study protocol before subjects gave their written informed consent. The study protocol was approved by the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University.

Subjects were eligible if they were older than 17 years, did not use any drugs known to affect concentrations of serum lipids, were not pregnant, not lactating, and not on a prescribed diet. Volunteers filled out a medical questionnaire that was reviewed by an independent physician. Persons with a history of gastrointestinal, liver or kidney disease were excluded, as were those with glucosuria, proteinuria, anaemia, a serum concentration of total cholesterol >7.0 mmol/L, or fasting triglycerides >2.3 mmol/L, and serum values of liver enzymes >30 U/L for alanine aminotransferase (ALT), or >30 U/L for aspartate aminotransferase (AST). Thirty-two women and 17 men, 18-58 y, were enrolled in the study. Three subjects withdrew during the study; one because of illness unrelated to the treatment, and two because of personal reasons. Forty-six subjects completed the study. None of the subjects used medications that could have affected the results.

### Design and treatment

The study consisted of two 3-wk treatment periods, during which each subject consumed 2 diets, one with phenol-rich extra virgin olive oil and one with phenol-poor extra virgin olive oil, in random order (cross-over). Before the study subjects were stratified according to sex, age, and energy intake and then randomly allocated to 1 of 2 sequence groups. During the 2 wk before the study (run-in) and 2 wk in-between the treatment periods (wash-out) subjects consumed diets without olives, olive oil and olive oil products.

The high phenol olive oil was prepared from Tsunati olives with a high content of phenols. The low phenol olive oil was prepared from Koroneiki olives; most of the phenols were removed by washing with hot water. Vitamin E was added to the high phenol olive oil to obtain the same vitamin E content in both oils. The 2 olive oils had a similar fatty acid composition (data not shown). The phenol concentration in the experimental oils was determined as described by Montedoro *et al* (1993). The high phenol olive oil contained 308 mg/kg of phenols (mean intake 21 mg/d), of which 2% was tyrosol, 1% was hydroxytyrosol, 72% were oleuropein-aglycones, and 25% were ligstroside-aglycones. The low phenol olive oil contained 43 mg/kg of phenols (mean intake 3 mg/d), of which 16% was tyrosol, 2% was hydroxytyrosol, 13% were oleuropein-aglycones, and 69% were ligstroside-aglycones. The mean difference in phenol intake between the high and low phenol diet was 18 mg/d.

Before the trial, energy intake of individual subjects was estimated by a food frequency questionnaire (Feunekes *et al*, 1993). Each subject received the amount of olive oil that fulfilled energy needs. Energy intakes were subdivided into 4 levels, ranging from 7.5 to 17.5 MJ/d. The

amount of olive oil per day varied from 55 g to 102 g, with a mean intake of 69 g. Forty percent of the daily oil was incorporated into a mayonnaise (78 w/w% olive oil), 30% was incorporated into sauces for hot meals, and 30% in cookies and raisin rolls baked by a local bakery. We recorded body weights twice weekly and adjusted the intake of olive oil when necessary so as to maintain a stable weight. Over the duration of the trial average body weight increased by 0.3 ± 0.9 kg (range -1.2 to 2.8 kg). Body weight did not differ between treatments; it was 68.0 ± 9.6 kg on the high phenol diet, and 67.8 ± 9.5 kg on the low phenol diet.

Each study diet was assigned a colour code that was used for labelling all foods supplied during the trial. In this way, subjects were blinded to the type and the sequence of the olive oils. On weekdays at noon, hot meals were served and eaten at the department in the presence of the researchers. These meals supplied at least 50% of the experimental olive oil. Foods that contained the remaining part of the olive oil were consumed at home. Foods containing the experimental olive oil were weighed or counted for each subject. During the whole study subjects followed instructions for a background diet low in vitamin E. A margarine with a low vitamin E content was supplied (Van den Bergh Foods, United Kingdom). Apart from this, subjects were asked to maintain their usual diet. During the study subjects were not allowed to take vitamin supplements or aspirin, which is a radical scavenger (Kuhn *et al*, 1995; Hermann *et al*, 1999). Therefore they were provided with acetaminophen (paracetamol). Subjects were asked to maintain their usual pattern of physical activity and not to change their smoking habits, consumption of coffee, or use of oral contraceptives. All subjects kept daily records of illness and deviations from the protocol. Diaries and anonymous questionnaires administered after the trial showed that consumption of experimental olive oil was 90% of the scheduled amount for the low phenol diet and 87% for the high phenol diet. To check adherence, we added 6.8 µmol/g of lithium chloride to the mayonnaise. The daily intake of lithium was 190-360 µmol/d, depending on the level of energy intake and consequently the amount of olive oil consumed per day. Mean plasma lithium concentrations after olive oil consumption was 4.7 ± 1.3 µmol/L, which was about 5 times the concentration of 0.9 ± 0.3 µmol/L in similar subjects that did not consume added lithium chloride (De Roos *et al*, 2001). This confirmed adherence of the subjects to the protocol.

## Blood sampling

Venous blood samples were taken twice after a 12-h fast at the end of each 3-wk period (days 17 and 21). Plasma or serum was immediately obtained by low speed centrifugation and stored at -80°C. For the determination of the concentration of serum lipids, the liver enzymes, uric acid, and the ability of plasma to reduce iron (III) (FRAP), the 2 samples of each subject at the end of each period were analysed separately and the results were averaged before statistical analysis. For the determination of the susceptibility of LDL and HDL to oxidation, the 2 samples obtained at the end of each period were pooled before analytical analyses. For all other analyses, only the last samples obtained at the end of each period were used.

### Lipoprotein isolation

Plasma lipoproteins were isolated by density gradients ultracentrifugation in a SW 41Ti rotor (Beckman Instruments, Palo Alto, USA) for 24 h at 10°C (Redgrave *et al*, 1975). LDL was isolated in a density range of 1.019-1.063 g/mL, and HDL in a density range of 1.063-1.210 g/mL. Density gradients solutions contained 0.1 mM $Na_2EDTA$ to inhibit metal ion catalysed LDL and HDL oxidation during the isolation procedure.

### Markers of oxidisability

The susceptibility of LDL and HDL to copper-mediated oxidation was determined by monitoring the formation of conjugated dienes, essentially as described by Princen *et al* (1992). Malondialdehyde in plasma was determined as described by Wong *et al* (1987), except that the HPLC eluent was monitored using fluorescence detection. The excitation wavelength was 537 nm and the emission wavelength was 554 nm. Lipid hydroperoxides in plasma were determined by the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA USA). Protein carbonyls in plasma were determined by an ELISA method as described by Buss *et al* (1997). The ability of plasma to reduce iron (III) (FRAP) was determined by the method of Benzie and Strain that measures the reduction of ferric to ferrous iron in the presence of antioxidants (Benzie & Strain, 1996).

### Vitamin and uric acid concentrations in serum

The concentrations of lycopene, β-carotene, retinol, and α-tocopherol in serum samples were determined by high performance liquid chromatography (HPLC, Waters Instruments, Milfort, USA). Serum was deproteinised with ethanol-internal standard solution (1:1 v/v) and extracted with hexane. A part of the hexane layer was evaporated after centrifugation and reconstituted in the mobile phase. Samples to determine retinol and α-tocopherol were injected into a Resolve C18-5 µm column (30 cm x 3.9 mm; Waters Instruments, Milfort, USA) with methanol/ dichloromethane/acetonitrile (10:20:70, v/v) as the mobile phase, with detection at 325 nm and 292 nm, respectively. Samples to determine lycopene and β-carotene were injected into a Sperisorb 5 µm ODS-2 column (25 cm x 4.6 mm; Waters Instruments, Milfort, USA) with methanol/dichloromethane/acetonitrile (30:20:50, v/v) as the mobile phase, with detection at 472 nm and 450 nm, respectively. Calibration was performed by a single standard solution on the same way as a sample. Serum uric acid was determined by UA plus kit (Boehringer, Mannheim, Germany).

### Plasma lipids and liver enzymes

Plasma lipids were analysed enzymatically (Siedel *et al*, 1983; Warnick *et al*, 1982; Fossati & Prencipe, 1982). Mean bias for control samples provided by the Centers of Disease Control in Atlanta was -1% for total and HDL cholesterol and 10% for triglycerides. The coefficient of

variation within runs ranged from 0.5 to 1.1%. LDL cholesterol concentrations were calculated (Friedewald *et al*, 1972). Alanine and aspartate aminotransferase were measured at 37°C using Abbott Spectrum reagents (Bergmeyer *et al*, 1978). The mean bias for 'Monitrol' control sera (Baxter Dade, Switzerland) ranged from 0 to 2%. The coefficient of variation within runs ranged from 2 to 8%.

### Statistical analyses

The data were analysed by ANOVA using the General Linear Models (GLM) of the Statistical Analyses System (SAS Institute Inc. 1989). The Tukey method was used for calculation of 95% confidence limits of the differences between the 2 diets.

## RESULTS

### Markers of oxidisability

Resistance of LDL and HDL to copper-mediated oxidation and other markers of antioxidant capacity or oxidative processes were not affected by treatment (**Table 3.1**). The lag time of copper-induced formation of conjugated dienes was 1.6 min shorter in LDL and 0.4 min longer in HDL after the high phenol diet. Maximum rate of diene formation in LDL and HDL was similar after both treatments (**Table 3.1**).

Other oxidation markers were also not affected. Plasma lipid hydroperoxides and malondialdehydes, both markers of lipid peroxidation, were respectively 0.07 and 0.001 µmol/L higher after the high phenol diet. Protein carbonyls, a marker of protein oxidation in plasma, were 0.001 nmol/mg protein lower after the high phenol diet. The ferric reducing ability of plasma (FRAP), a marker for the antioxidant capacity of plasma, was 0.006 mmol/L higher after the high phenol diet. All 95% confidence intervals enclosed zero (**Table 3.1**).

### Vitamin and uric acid concentrations in serum

Serum uric acid, lycopene, retinol, β-carotene, or α-tocopherol concentrations were not affected by the treatment (**Table 3.2**). The concentration of α-tocopherol in our subjects was 19.4 ± 5.0 µmol/L, which is relatively low compared to normal plasma vitamin E concentrations from 11 to 37 µmol/L (Cohn 1997). This can be explained by the low vitamin E content in the diet. This suggested adherence of the subjects to the protocol.

### Serum lipids and liver enzymes

Mean total cholesterol concentration was 0.06 mmol/L lower (not significant) on the high phenol diet than on the low phenol diet. Concentrations of LDL, HDL, triglycerides, and the liver enzymes alanine and aspartate aminotransferase also did not differ between the high and low phenol diet (**Table 3.3**).

**Table 3.1** Oxidisability of LDL and HDL, and other markers of oxidation at the end of the high and the low phenol diet ($n = 46$).

| | Low phenol diet | High phenol diet | Differences between high and low phenol diet (95% CI) |
|---|---|---|---|
| LDL oxidisability[a] | | | |
| Lag time (min) | 110.2 ± 25.0 | 108.6 ± 20.4 | -1.6 (-8.2, 5.0) |
| Maximum rate (nmol dienes/min/mg LDL protein) | 12.0 ± 2.6 | 11.8 ± 2.3 | -0.1 (-0.6, 0.4) |
| HDL oxidisability | | | |
| Lag time (min) | 69.3 ± 47.5 | 69.7 ± 50.5 | 0.4 (-12.8, 13.5) |
| Maximum rate (nmol dienes/min/mg HDL protein) | 4.6 ± 2.2 | 4.4 ± 2.2 | -0.1 (-0.3, 0.03) |
| Malondialdehyde (μmol/L) | 0.68 ± 0.15 | 0.69 ± 0.13 | 0.001 (-0.03, 0.03) |
| Lipid hydroperoxides (μmol/L) | 0.36 ± 0.52 | 0.44 ± 0.54 | 0.07 (-0.07, 0.21) |
| Protein carbonyls (nmol/mg protein) | 0.23 ± 0.12 | 0.23 ± 0.12 | -0.001 (-0.02, 0.02) |
| Ferric Reducing Ability of Plasma (mmol/L) | 1.05 ± 0.18 | 1.06 ± 0.18 | 0.006 (-0.01, 0.02) |

Values are means ± SD. Participants (15 men and 31 women) consumed both diets in random order for 3 wk each.
[a] $n = 44$

**Table 3.2** Serum concentration of uric acid, lycopene, β-carotene, retinol, and α-tocopherol at the end of the high and the low phenol diet ($n = 46$).

| | Low phenol diet | High phenol diet | Differences between high and low phenol diet (95% CI) |
|---|---|---|---|
| Uric acid (μmol/L) | 260.6 ± 56.1 | 264.4 ± 58.0 | 3.8 (-2.2, 9.8) |
| Lycopene (μmol/L)[a] | 0.59 ± 0.20 | 0.61 ± 0.19 | 0.01 (-0.05, 0.08) |
| β-carotene (μmol/L)[a] | 0.54 ± 0.23 | 0.52 ± 0.24 | -0.02 (-0.08, 0.04) |
| Retinol (μmol/L) | 2.51 ± 0.55 | 2.47 ± 0.70 | -0.04 (-0.2, 0.1) |
| α-tocopherol (μmol/L) | 19.77 ± 4.58 | 18.97 ± 5.37 | -0.8 (-2.1, 0.5) |

Values are means ± SD. Participants (15 men and 31 women) consumed both diets in random order for 3 wk each.
[a] $n = 40$

**Table 3.3** Serum lipid and lipoprotein cholesterol concentrations at the end of the high and the low phenol diet ($n = 46$).

| | Low phenol diet | High phenol diet | Differences between high and low phenol diet (95% CI) |
|---|---|---|---|
| *Lipoproteins (mmol/L)* | | | |
| Total cholesterol[a] | 4.25 ± 0.83 | 4.19 ± 0.76 | -0.06 (-0.15, 0.04) |
| HDL cholesterol[a] | 1.54 ± 0.36 | 1.52 ± 0.37 | -0.01 (-0.06, 0.04) |
| LDL cholesterol[a] | 2.29 ± 0.65 | 2.26 ± 0.59 | -0.04 (-0.12, 0.05) |
| Triglycerides[b] | 0.92 ± 0.35 | 0.90 ± 0.32 | -0.02 (-0.08, 0.04) |
| *Liver enzymes (U/L)* | | | |
| Alanine aminotransferase | 20.3 ± 7.0 | 20.7 ± 7.7 | 0.3 (-1.0, 1.6) |
| Aspartate aminotransferase | 21.7 ± 4.7 | 21.8 ± 5.1 | 0.1 (-1.0, 1.2) |

Values are means ± SD. Participants (15 men and 31 women) consumed both diets in random order for 3 wk each.
[a] To convert to mg/dL, multiply by 38.67.
[b] To convert to mg/dL, multiply by 88.54.

## DISCUSSION

Our data show that consumption of 18 mg/d of phenols from olive oil for 3 wk does not affect *in vitro* susceptibility of LDL to oxidation or other markers of oxidation in fasting blood of healthy volunteers.

Our results are in line with two other human studies that addressed the effect of minor components in olive oil on the susceptibility of LDL to oxidation in fasting blood (Nicolaiew *et al*, 1998; Bonanome *et al*, 2000). In these studies, extra virgin olive oil versus high oleic acid sunflower oil or refined olive oil also did not affect the *in vitro* susceptibility of LDL to oxidation in fasting plasma samples. Other human studies on the effect of olive oil on LDL oxidation addressed the effects of oleic acid rather than olive oil phenols *per se* (Reaven *et al*, 1991; Bonanome *et al*, 1992; Tsimikas *et al*, 1999). *In vitro*, the olive oil phenols hydroxytyrosol and oleuropein strongly inhibited LDL oxidation (Visioli *et al*, 1995; Visioli & Galli, 1994). Also, tyrosol, oleuropein, and extracts of minor components from extra virgin olive oil decreased the oxidation of LDL as assessed by oxysterol formation (Berra *et al*, 1995; Caruso *et al*, 1999). We realise that LDL oxidation *ex vivo* is different from LDL oxidation *in vitro*. Phenols or antioxidants might get lost during isolation of LDL from plasma by centrifugation, a process that is absent when doing experiments *in vitro*. However, in rabbits and rats, non-tocopherol antioxidants from olive oil showed a favourable effect on the susceptibility of LDL to oxidation ( Scaccini *et al*, 1992; Wiseman *et al*, 1996; Coni *et al*, 2000). This suggests that during isolation phenols might

stay in or attached to lipoprotein particles where they might affect the resistance of LDL to oxidation. Thus, results of animal and *in vitro* studies suggest a protective effect of phenols from olive oil on LDL oxidation, but such an effect was not observed in fasting blood samples of humans in the present and other studies (Nicolaiew *et al*, 1998; Bonanome *et al*, 2000).

One explanation might be that our study did not address postprandial effects. If phenol clearance is fast, phenol concentrations might be elevated in the first hours after a meal and during that time protect LDL from oxidation, but not after 12 h of fasting as in our study. Visioli *et al* actually found that the phenols tyrosol and hydroxytyrosol were mostly excreted within 24 h after intake of 50 mL extra virgin olive oil, which indicates that clearance of phenols from plasma is fast (Visioli *et al*, 2000). Furthermore, Bonanome *et al* found a significant postprandial effect 2 h after intake of 100 g of extra virgin olive oil on plasma antioxidant capacity, but they did not include a control group (Bonanome *et al*, 2000). Nicolaïew *et al*, on the other hand, did not find a significant effect on the lag time or maximum rate of LDL oxidation of extra virgin olive oil 6 h after intake (Nicolaiew *et al*, 1998). The proper study of postprandial effects requires more insight into the kinetics of phenol transport and metabolism in man and more studies are needed on this aspect.

We also did not find an effect of phenols from extra virgin olive oil on the susceptibility of HDL to oxidation. Like modified LDL, oxidatively modified HDL is suggested to increase intracellular cholesterol accumulation (Nagano *et al*, 1991; Gesquiere *et al*, 1997; Bonnefont-Rousselot *et al*, 1995). The physiological role of HDL oxidation is still unclear, but in our study 18 mg/d of phenols for 3 wk did not influence the susceptibility of HDL to oxidation in fasting plasma samples.

Furthermore, we did not find an effect of olive oil phenols on other markers of lipid and protein oxidation (lipid hydroperoxides, malondialdehyde, protein carbonyls, and FRAP). The average plasma values of the oxidation markers in our study were in line with those found in other studies. Lag time of LDL oxidation was about 110 min, which is relatively high (Esterbauer & Jürgens, 1993). This can be explained by methodological differences and the high dose of oleic acid that increases the resistance of LDL to oxidative modification (Bonanome *et al*, 1992; Reaven *et al*, 1991; Tsimikas *et al*, 1999). Mean plasma lipid hydroperoxide and malondialdehyde concentrations and FRAP were similar to values found by others (Wong *et al*, 1987; Benzie & Strain, 1996). Protein carbonyls were slightly higher in our subjects than in healthy subjects in the study by Buss *et al*, but much lower than in 23 critically ill subjects in the same study (Buss *et al*, 1997). Thus, plasma values of the markers of oxidation were within previously reported ranges.

The phenol oleuropein-aglycone and some of its derivatives (**Figure 3.1**) from extra virgin olive oil are lipid-soluble, while oleuropein, tyrosol and hydroxytyrosol are water-soluble (unpublished data, Unilever Research Vlaardingen). We therefore hypothesised that the lipid-soluble aglycones, which are the main phenols in olive oil, may accumulate in LDL and HDL particles and provide better protection of LDL against oxidation than other, more water-soluble, dietary compounds like phenols from tea and wine, for example. The amount of phenols

needed to protect LDL and HDL from oxidation is unknown. Also, data about the amounts of other antioxidants needed to protect LDL from oxidation are scarce. Vitamin E is the most important lipid-soluble antioxidant in the body (Princen *et al*, 1995; Jialal *et al*, 1995). In one dose-response study Princen *et al* suggested that intake of 25 mg/d of vitamin E for 2 wk was sufficient to reduce the susceptibility of LDL to oxidation (Princen *et al*, 1995). In another dose-response study, Jialal *et al* investigated the effects of vitamin E intake in doses of 60, 200, 400, 800, 1200 mg/d. They found that at least 400 mg/d of vitamin E was needed to reduce the susceptibility of LDL to oxidation (Jialal *et al*, 1995). Thus, the minimum dose of vitamin E needed to reduce the susceptibility of LDL to oxidation is as yet unclear. However, if 25 mg/d of vitamin E is the minimum effective amount and if olive oil phenols and vitamin E would be equally effective, then the amount of 18 mg/d in our study might have been too low to detect an effect on LDL oxidisability. Moreover, vitamin E might partition better into LDL particles and thus be more effective in reducing the susceptibility of LDL to oxidation than phenols from olive oil. This implies that even more dietary phenols would be needed to detect an effect. Thus, the amount of 18 mg/d of olive oil phenols in our study, a very high intake in terms of practical realistic diets, might have been too low to affect LDL oxidisability.

More is known about the *in vitro* than about the *in vivo* antioxidant activity of olive oil phenols. *In vitro* dose-response studies by Visioli and co-workers demonstrated that pre-incubation of LDL with 10 µmol/L oleuropein or 10 µmol/L hydroxytyrosol inhibited the decrease of vitamin E in LDL during copper-mediated oxidation and delayed the formation of conjugated diene formation, lipid peroxides, and thiobarbituric acid-reacting substances (Visioli *et al*, 1995; Visioli & Galli, 1994). These studies showed that the protection of vitamin E from oxidation was correlated with the concentration of oleuropein or hydroxytyrosol, and that a concentration of 1 µmol/L provided much less protection than a concentration of 10 µmol/L (Visioli *et al*, 1995; Visioli & Galli, 1994). Visioli *et al* suggested that olive oil phenols retard the oxidation of the natural vitamin E pool in LDL and in this way indirectly protect LDL from oxidation. We do not know the plasma concentration of the various phenols that were reached in our study, but it is unlikely that it reached 1 µmol/L after a whole-body dose of 18 mg/d (about 50 µmol) for 3 wk. Thus, on the basis of *in vitro* data the concentration of phenols in LDL was probably too low to produce detectable effects on LDL oxidisability.

It remains possible that amounts of olive oil phenols higher than the 18 mg/d fed in our study can affect LDL oxidisability. However, we supplied the maximum dose that is achievable with practical every-day diets. Olives were specially selected to obtain a large difference in phenol concentration between the experimental oils, and the subjects consumed large amounts of olive oil (69 g/d). The average daily intake of phenols from olive oil is not exactly known, but estimations from the Mediterranean diet indicate that 10-20 mg/d of total phenols may be supplied by olive oil (Visioli *et al*, 1995). According to our data, this is probably not enough to affect markers of antioxidant status in fasting plasma samples.

It is conceivable that the background diet of our subjects contained too high an amount of antioxidants to allow additional antioxidant effects of the phenols of extra virgin olive oil. Our

subjects consumed a background diet low in vitamin E, which was confirmed by the low plasma vitamin E concentrations. Furthermore, subjects were not allowed to take vitamin supplements. However, it is possible that tissue stores of vitamin C and vitamin E, for example, after the run-in period were still considerable, and it may require depletion periods to show an effect. Thus, olive oil phenols might theoretically still affect oxidisability of fasting LDL in subjects who are depleted in dietary antioxidants.

The oxidative modification hypothesis of atherosclerosis is an attractive one, but it is still not proven that dietary antioxidants prevent coronary heart disease (Zock & Katan, 1998). Only a few studies have reported the relation between markers of oxidation and coronary heart disease, and the results of these studies are inconsistent (van de Vijver *et al*, 1998; de Rijke *et al*, 1995; Cominacini *et al*, 1993; Croft *et al*, 1992). Thus, none of the oxidation markers have yet been validated as risk factors for atherosclerosis, although it may be that markers more representative of *in vivo* changes, like oxidised LDL antibodies, may be more sensitive to changes in LDL antioxidant content. Much work remains to be done here. In addition, randomised clinical trials with hard endpoints are needed to prove that dietary antioxidants, such as the phenols from olive oils, may offer protection against coronary heart disease.

The serum concentrations of total and LDL cholesterol did not differ between the 2 diets. It has been suggested that minor components, such as phenols, may be responsible for a cholesterol-lowering effect (Matsumoto *et al*, 1998). We therefore studied the effects of olive oil phenols on serum cholesterol concentrations. Our results are in line with those of Nicolaïew *et al*, who found that a diet with extra virgin olive oil and a diet with oleic-acid-rich sunflower oil produced the same cholesterol concentrations (Nicolaïew *et al*, 1998). Thus, the cholesterol-lowering effect of olive oil is probably not due to its phenolic compounds.

We also measured the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Phenols from extra virgin olive oil are responsible for the bitter taste of extra virgin olive oil. This bitter taste might be a warning from nature for possible adverse or toxic effects. We therefore measured ALT and AST as indicators of such effects. In our study the serum concentrations of ALT and AST did not differ between the 2 diets. This suggests that phenols from olive oil have no adverse affects on liver integrity.

In conclusion, we did not find an effect of a high intake of phenol-rich olive oil on the susceptibility of LDL and HDL to oxidation and other markers of oxidation in fasting plasma. The natural concentration of phenols in olive oil might be too low or their clearance from plasma too fast to produce an effect on markers of oxidation in the post-absorptive phase.

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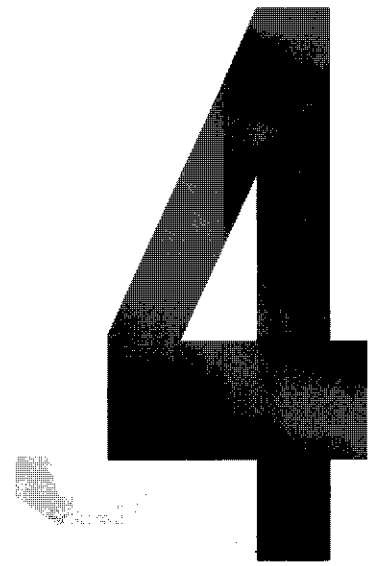

# Effect of consumption of phenols from olives and extra virgin olive oil on LDL oxidisability in healthy humans

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Annet JC Roodenburg, Karel PAM van Putte, Martijn B Katan

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## ABSTRACT

A high intake of olive oil has been proposed as an explanation for the low incidence of coronary heart disease in Mediterranean countries, but it is unclear whether olive oil offers specific benefits beyond a low content of saturated fat. Some types of extra virgin olive oil are rich in non-polar phenols, which might be taken up by plasma LDL particles and protect these from becoming atherogenic by oxidative modification. In a pilot study we found that consumption of 47 g fortified olive oil containing 31 mg phenols significantly increased the lag time of LDL oxidation from 112 ± 5 min before to 130 ± 7 min 2 h after the meal. However, this study was not controlled, and in the current study we therefore investigated whether olive oil phenols increase the lag time of LDL oxidation in postprandial samples when compared with a control group.

Twelve healthy men and women consumed 4 different olive oil supplements with a meal on 4 separate occasions: one similar to the supplement in the pilot study (positive control); one containing mainly non-polar olive oil phenols; one containing mainly polar olive oil phenols; and one without phenols (placebo). Lag time significantly increased 2 h after the meals with the positive control (8 ± 2 min), the polar phenols (8 ± 2 min), and the placebo (8 ± 2 min), but not after the non-polar phenols (-0.4 ± 3 min). Increases were not statistically different between supplements.

These results indicate that the lag time of LDL oxidation is increased after consumption of a meal. This increase is probably due to non-specific meal or time effects and not to phenols from olives or olive oil. Furthermore, these findings stress the need for adequate controlled studies to avoid misinterpretations of the data.

## INTRODUCTION

The Mediterranean diet, with olive oil as the major fat source, has been shown in epidemiological studies to be associated with a reduced incidence of coronary heart disease (Keys *et al*, 1986). The replacement of dietary saturated fatty acids with monounsaturated oleic acid from olive oil decreases plasma LDL concentrations, which presumably contributes to the low incidence of coronary heart diseases (Katan *et al*, 1995). It has also been suggested that a high-monounsaturated fat diet lowers the risk of coronary heart disease by producing oleic acid-enriched LDL particles, which are more resistant to oxidative modification (Reaven *et al*, 1991; Bonanome *et al*, 1992; Mata *et al*, 1997). Oleic acid, however, may not be the only component of olive oil protecting LDL from oxidation. Some types of extra virgin olive oil contain phenols with antioxidative properties. These phenols are aglycones formed in olives by removal of glucose from the parent compound oleuropein or ligstroside, and they are less polar. End products of hydrolysis of ligstroside- and oleuropein-aglycones are tyrosol and hydroxytyrosol which are polar (**Figure 4.1**).

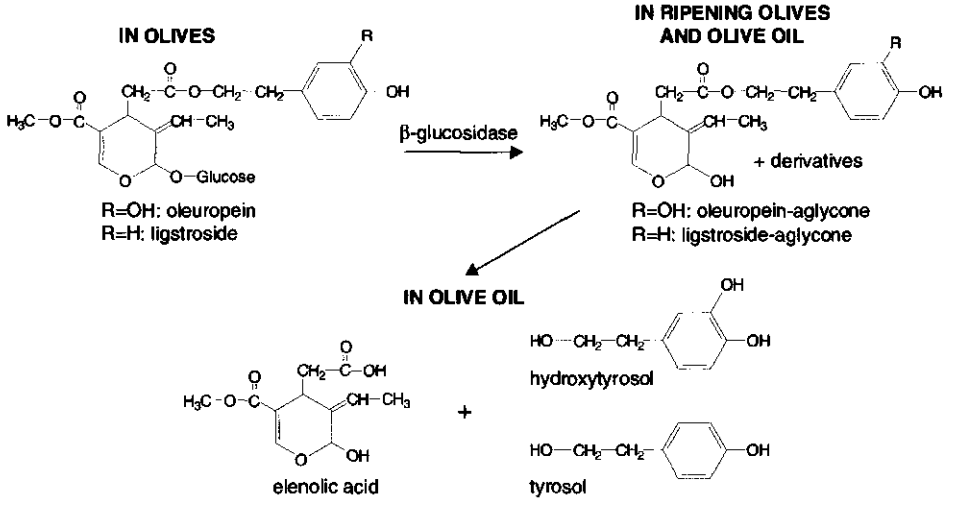


**Figure 4.1** Structures of phenols present in olives and olive oil, their degradation into aglycones during ripening, and hydrolysis of aglycones into tyrosol and hydroxytyrosol.

Oxidation of LDL may be an important risk factor for atherosclerosis, the underlying cause of coronary heart disease, and olive oil phenols may be particularly effective in preventing oxidation of LDL. Due to their relatively lipophilic nature the olive oil phenols might directly dissolve into LDL particles where they can protect LDL from oxidation. Results of animal (Wiseman *et al*, 1996; Scaccini *et al*, 1992; Coni *et al*, 2000) and *in vitro* studies (Visioli *et al*, 1995; Visioli & Galli, 1994; Salami *et al*, 1995; Caruso *et al*, 1999) do suggest a protective effect of phenols from olive oil on LDL oxidation. Some human studies suggest that such an effect may also apply to the practical *in vivo* situation. Visioli and co-workers found that administration

of phenol-rich oils resulted in a dose-dependent decrease in urinary excretion of $F_2$-isoprostanes, which indicates less overall oxidation of arachidonic acid (Visioli *et al*, 2000). Results of other human studies, on the other hand, are inconsistent (Nicolaiew *et al*, 1998; Ramirez-Tortosa *et al*, 1999; Bonanome *et al*, 2000; Vissers *et al*, 2001). In a previous human study (Vissers *et al*, 2001) we did not find an effect on LDL oxidisability in fasting blood after subjects had consumed extra virgin olive oil that was naturally high in phenols (21 mg/d) for 3 wk. From that study we concluded that a dose of 21 mg/d of phenols was not high enough to affect markers of oxidation, or that we missed an effect because we measured blood samples taken at least 12 h after the last meal. Because the metabolism of olive oil phenols might be fast, we investigated effects on LDL oxidation in plasma taken shortly after intake of olive oil phenols in a pilot study. In this pilot study 10 healthy men and women consumed in one meal 47 g of olive oil fortified with a phenol-rich extract from olives containing 31 mg phenols. The mean lag time of LDL oxidation increased from 112 ± 5 min before to 130 ± 7 min 2 h after the meal (95% CI of change, 5.3 to 30.8 min) (**Figure 4.2**). However, this study did not include a concurrent control group, and the observed decrease in LDL oxidisability may have been due to non-specific meal or time effects. Therefore we performed a controlled intervention study on whether olive oil phenols affect LDL oxidisability directly after intake.

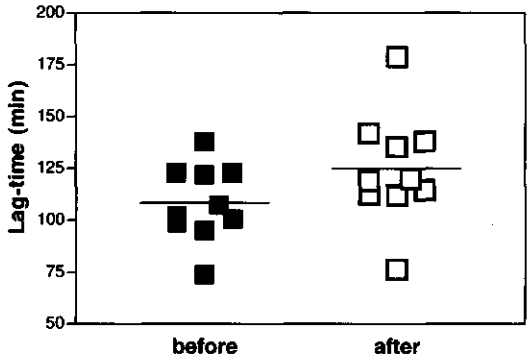


**Figure 4.2** Individual lag times of LDL oxidation in samples taken before and 2 h after intake of the phenol-rich supplement in the pilot study ($n$ = 10). In this pilot study subjects started with a 7-d run-in period in which they were not allowed to consume olives, olive oil, or olive oil products. On day 8 they consumed 47 g fortified olive oil containing 31 mg phenols with breakfast at our department. The subjects consumed their breakfast within half an hour and did not eat or drink anything else except water until 3 h after supplement intake.

## METHODS

### Subjects

The study protocol was approved by the Medical Ethical Committee of Wageningen University. The protocol was fully explained to the participants before they gave their written informed consent.

Twelve healthy subjects (8 females, 4 males) were recruited, with a mean age of 22 years (range 20-28 years), and with a mean body mass index of 21.8 kg/$m^2$ (range 18.3-27.1 kg/$m^2$).

Subjects had no history of gastrointestinal, liver or kidney disease, did not use any drugs known to affect concentrations of serum lipids, and were not pregnant, lactating, or on a prescribed diet. Subjects were all healthy as judged by normal blood count, the absence of glucose and protein in urine, and a medical questionnaire, which was reviewed by an independent physician. Subjects had a serum concentration of total cholesterol <7.0 mmol/L, and fasting triglycerides <2.3 mmol/L.

### Design and Treatment

Each subject consumed a single dose of 4 different supplements, in random order (cross-over). The study consisted of a 4-wk treatment period, during which subjects were not allowed to consume olives, olive oil, olive oil products, and products extremely rich in vitamin E, like peanut butter and nuts. At the end of each week, on day 8, 15, 22, and 29, we provided the subjects with a supplement between 8:00 and 9:30 h at our department. The supplement was consumed together with a standard breakfast, containing bread, tomato, cucumber, tuna, ham, cheese, milk or buttermilk, and tea or coffee with or without sugar and evaporated milk. Products were low in vitamin E to prevent any effect of vitamin E on LDL oxidation. The breakfast were not protein-free, but protein content was kept as low as possible to minimise the possible binding of phenols to proteins in the intestines. To this end, we instructed the subjects not to consume more than 1 slice of ham or cheese and not to drink more than 150 mL milk or buttermilk. The breakfasts contained 3.0 ± 0.8 MJ as energy, and 26 ± 7 g protein. The breakfast with the positive control contained 4.2 ± 0.8 MJ as energy because of the higher dose of olive oil in this supplement. The subjects consumed breakfast within half an hour. Each subject consumed exactly the same amount of breakfast on each of the 4 occasions. The only exception was that subjects were allowed to consume some more cucumber or salad with one of the supplements (positive control), in order to facilitate the intake of the higher amount of olive oil in this supplement than in the other 3 supplements. Tea, coffee or other foods and drinks were not allowed until the last blood sample had been taken. Blood samples were taken before, and ½ h and 2 h after the experimental breakfast. All subjects kept daily records of illness and deviations from the protocol.

## Supplements

**The positive control supplement** was similar to the supplement consumed in the pilot study in which we found a postprandial effect on the lag time. The supplement consisted of 65 g mayonnaise prepared with 47 g olive oil, water, egg yolk powder, vinegar, pizza herbs, mustard, salt and pepper. This extra virgin olive oil was fortified with a phenol-rich olive fraction. The total phenol concentration in the fortified oil was 674 mg/kg. Thus, the supplement contained 31.1 mg of phenols (**Table 4.1**).

**The supplement with non-polar phenols** consisted of 37 g mayonnaise prepared with 14.5 g phenol-enriched olive oil, sour cream and small amounts of water, egg yolk powder, vinegar, mustard, honey, pizza herbs, salt and pepper. The phenols were extracted from extra virgin olive oil by food-grade ethanol, which was evaporated in a later stage. The total phenol concentration of the olive oil used in this supplement was 6876 mg/kg, thus the supplement provided 100 mg of olive oil phenols (**Table 4.1**).

**The supplement with polar phenols** was not a mayonnaise because the olive oil extract was watery and not suitable to prepare a mayonnaise. The supplement was extracted from extra virgin olive oil by reversed osmosis. Subjects drank 8.7 g of this supplement containing a total phenol concentration of 11470 mg/kg. Thus, the supplement provided 100 mg of phenols (**Table 4.1**). Subjects also consumed 37 g mayonnaise prepared with 14.5 g olive oil without phenols to equalise fat intake between breakfasts. The mayonnaise was similar to the placebo supplement.

**The placebo supplement** also consisted of 37 g mayonnaise prepared with 14.5 g olive oil without phenols and vitamin E, sour cream and small amounts of water, egg yolk powder, vinegar, mustard, honey, pizza herbs, salt and pepper. The olive oil without phenols and vitamin E was prepared by stripping it several times.

The phenol concentration of the fortified oil was measured with an HPLC method based on the method of Montedoro *et al* (1993). With this HPLC method hydroxytyrosol and tyrosol and 10 derivatives of the oleuropein- and ligstroside-aglycones were separated, and the peaks of the aglycones were identified using HPLC-MS-MS (**Table 4.1**, **Figure 4.3**). The 12 major peaks in the HPLC chromatogram were quantified using the area/µg at $\lambda$=280 nm of the individual compounds hydroxytyrosol, tyrosol and oleuropein or of mixtures of aglycones. The aglycone reference mixture was obtained by enzymatic hydrolysis of oleuropein by β-glucosidase. The various derivatives differ mainly in their ring structure, which can either be open or closed in two different forms (van Boom *et al*, in preparation). Because some peaks could not be identified, the concentrations of phenols in the experimental oils might be an underestimation of the total amount of phenolic substances from olive oil. Thus intake of phenols might have been even higher.

**Table 4.1** The amount of the various phenols and vitamin E present in the supplements, analysed by HPLC. The placebo did not contain any phenols or vitamin E.

| Peak number and type of phenol [a] | Phenol intake | | |
|---|---|---|---|
| | Positive control (mg/47 g oil) | Non-polar (mg/14.5 g oil) | Polar (mg/8.7 g extract) |
| Hydroxytyrosol (1) | 3.7 | 1.4 | 36.2 |
| Tyrosol (2) | 1.2 | 16.9 | 22.9 |
| Oleuropein-aglycone (peaks 3, 4, 5, 7, 8, 9, 10) [a, b] | 19.0 | 19.0 | 34.1 |
| Ligstroside-aglycone (peaks 6, 11, 12) [a, b] | 7.2 | 62.8 | 6.9 |
| Total phenols | 31.1 | 100.1 | 100.1 |
| Alpha-tocopherol | 10.0 | 0.1 | 0.0 |

[a] 10 derivatives of the aglycones were separated and the peaks identified (van Boom *et al*, in preparation). The various derivatives differ mainly in their ring structure, which can be either open or closed in two different forms.

[b] The numbering of the various phenols in this table refer to the numbering of the peaks in **Figure 4.3.**

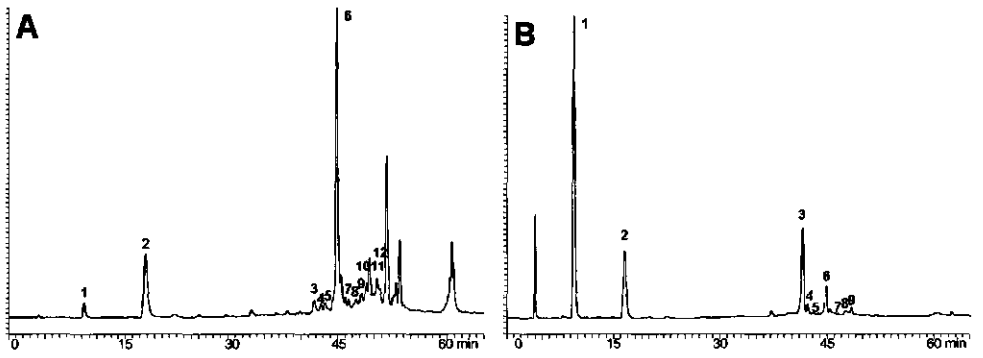


***Figure 4.3*** *Chromatogram of various identified phenols in the non-polar (A) and the polar (B) supplement.* The numbering of the peaks refer to the numbering of the various phenols in **Table 4.1:** 1 = hydroxytyrosol; 2 = tyrosol; 3, 4, 5, 7, 8, 9, 10 = oleuropein-aglycones; 6, 11, 12 = ligstroside-aglycones.

## Blood sampling

Venous blood samples for determination of LDL oxidisability were taken using vacuum tubes containing EDTA. Blood was sampled before and ½ and 2 h after breakfast. Plasma was prepared within 30 min by centrifugation at 4°C for 10 min at 2,500 g, and 10 µL of a 600 g/L sucrose solution was added per mL plasma (Kleinveld *et al*, 1992). Samples were stored at -80°C until analysis less than 3 months later.

### LDL isolation and LDL oxidation

Plasma lipoproteins were isolated by density gradient ultracentrifugation in an SW 41Ti rotor (Beckman Instruments, Palo Alto, USA) for 24 h at 10°C (Redgrave *et al*, 1975). LDL was isolated in a density range of 1.019-1.063 g/mL. Density gradients solutions contained 0.1 mmol/L $Na_2EDTA$ to inhibit metal ion catalysed LDL oxidation during the isolation procedure.

The susceptibility of LDL to copper-mediated oxidation was determined in duplicate by monitoring the formation of conjugated dienes, as described by Princen *et al* (1992). Determinations before, and ½ h and 2 h after supplement intake were done per 2 supplements of one subject in one single run. The duplicates of these determinations were done in another run.

### Statistical analyses

The duplicate values obtained from each subject were averaged before data analysis.

LDL-oxidisability before, and ½ and 2 h after intake of the supplement were compared using Student's paired t-tests. To compare differences in LDL oxidisability between the various supplements we applied ANOVA using the General Linear Models (GLM) of the Statistical Analyses System (SAS Institute Inc. 1989). Before statistical analysis, the lag time and maximum rate of each individual subject before supplement intake were subtracted from the lag time and maximum rate at ½ h or 2 h after intake. Then, these differences were compared among the various supplements. The positive control, the supplement also used in the pilot study, was statistically compared with the placebo supplement without phenols. The supplements containing polar or non-polar phenols were statistically compared with each other and with the placebo supplement only. The Tukey method was used to adjust for multiple comparisons and to calculate the 95% confidence limits of the differences between the supplements. Data are presented as mean ± SE.

## RESULTS

One subject did not consume the breakfast containing the non-polar phenol-supplement because of personal reasons not related to the study.

The mean lag time increased significantly both at ½ h and 2 h after the meals with the positive control (10 ± 4 min at ½ h and 8 ± 2 min at 2 h), the polar phenols (4 ± 1 min at ½ h and 8 ± 2 min at 2 h), and the placebo (7 ± 3 min at ½ h and 8 ± 2 min at 2 h), but not after the non-polar phenols (4 ± 2 min at ½ h and -0.4 ± 3 min at 2 h) (**Table 4.2**). The maximum rate of diene formation was not affected by any of the 4 supplements (**Table 4.3**).

The increase in lag time and maximum rate did not differ significantly between the positive control supplement and the placebo supplement, neither at ½ h nor at 2 h after intake. Also, the increases did not significantly differ between the supplement with polar or non-polar phenols and the placebo supplement (**Table 4.4**).

**Table 4.2** The lag time of LDL oxidation (min) before intake and ½ h and 2 h after intake of the 4 experimental supplements and differences in lag time ½ h and 2 h after compared to before intake of the supplements ($n = 12$).

| Supplement | Lag time of LDL oxidation | | | Differences in lag time compared to t=0 | |
|---|---|---|---|---|---|
| | Before supplement intake (t = 0) | ½ h after supplement intake (t = ½) | 2 h after supplement intake (t = 2) | t = ½ (95% CI) | t = 2 (95% CI) |
| Placebo | 76 ± 2 | 82 ± 4[a] | 84 ± 3 | 7 (0.4, 14.2) | 8.0 (3.5, 11.9) |
| Polar phenols | 74 ± 2 | 78 ± 2 | 82 ± 3 | 4 (1.7, 6.8) | 8.0 (2.9, 13.3) |
| Non-polar phenols | 79 ± 3[a] | 83 ± 3[a] | 79 ± 4[a] | 4 (-0.3, 8.3) | -0.4 (-6.9, 6.1) |
| Positive control[b] | 74 ± 3 | 83 ± 4 | 82 ± 4 | 10 (1.8, 17.5) | 8.0 (3.2, 12.4) |

Values are means ± SE. All subjects (4 men, 8 women) were included in the analyses. Each subject consumed each supplement in random order.

[a] $n = 11$: there was not enough plasma of one subject to do the analyses (placebo supplement, t = ½); and one subject was not able to consume the non-polar phenol-supplement.

[b] Positive control was the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

**Table 4.3** The maximum rate of LDL oxidation (nmol conjugated dienes/min/mg LDL protein) before intake and ½ h and 2 h after intake of the 4 experimental supplements and differences in maximum rate ½ h and 2 h after compared to before intake of the supplements ($n = 12$).

| Supplement | Maximum rate of LDL oxidation | | | Differences in maximum rate compared to t=0 | |
|---|---|---|---|---|---|
| | Before supplement intake (t = 0) | ½ h after supplement intake (t = ½) | 2 h after supplement intake (t = 2) | t = ½ (95% CI) | t = 2 (95% CI) |
| Placebo | 23.4 ± 0.7 | 22.4 ± 0.9[a] | 21.9 ± 0.8 | -0.9 (-2.0, 0.16) | -1.6 (-2.9, -0.2) |
| Polar phenols | 22.5 ± 0.7 | 21.5 ± 0.8 | 21.4 ± 0.4 | -1.0 (-2.5, 0.42) | -1.1 (-2.3, 0.16) |
| Non-polar phenols | 21.4 ± 0.7[a] | 21.0 ± 0.6[a] | 21.8 ± 0.9[a] | -0.4 (-1.4, 0.6) | 0.4 (-0.5, 1.4) |
| Positive control[b] | 22.9 ± 0.6 | 22.4 ± 0.7 | 22.6 ± 0.7 | -0.5 (-1.8, 0.9) | -0.3 (-1.4, 0.9) |

Values are means ± SE. All subjects (4 men, 8 women) were included in the analyses. Each subject consumed each supplement in random order.

[a] $n = 11$: there was not enough plasma of one subject to do the analyses (placebo supplement, t = ½); and one subject was not able to consume the non-polar phenol-supplement.

[b] Positive control was the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

**Table 4.4** The difference in lag time and maximum rate of LDL oxidation between the supplements with non-polar phenols, polar phenols, and the placebo supplement and between the positive control and the placebo supplement: ½ h and 2 h after compared to before intake of the supplements ($n = 12$).

| Supplement | Difference in lag time compared to t=0 (min) | | Difference in maximum rate compared to t=0 (nmol conjugated dienes/ min/mg LDL protein) | |
|---|---|---|---|---|
| | t = ½ (95% CI) | t = 2 (95% CI) | t = ½ (95% CI) | t = 2 (95% CI) |
| Polar phenols vs. placebo | -3.0 (-10.8, 4.8) | 0.4 (-7.5, 8.4) | -0.1 (-2.1, 2.0) | 0.5 (-1.6, 2.5) |
| Non-polar phenols vs. placebo | -3.3 (-11.3, 4.7) | -8.0 (-16.2, 0.1) | 0.5 (-1.5, 2.6) | 2.0 (-0.1, 4.1) |
| Polar vs. non-polar phenols | 0.3 (-7.5, 8.1) | 8.4 (0.3, 16.6) | -0.6 (-2.7, 1.4) | -1.5 (-3.6, 0.6) |
| Positive control[a] vs. placebo | 2.3 (-8.8, 13.5) | 0.2 (-3.8, 4.1) | 0.5 (-1.3, 2.2) | 1.3 (0.0, 2.5) |

Values are means ± SE. All subjects (4 men, 8 women) were included in the analyses. Each subject consumed each supplement in random order.

[a] Positive control was the same supplement as used in the pilot study in which we found an increased lag time of 18 min.

## DISCUSSION

Our study shows that consumption of a high dose of phenols from olives and extra virgin olive oil does not affect LDL oxidisability in postprandial blood samples. We found that the increase in lag time after intake of a meal with phenol-rich olive oil was not different from the increase after intake of a meal with placebo olive oil. Thus, the decrease in LDL oxidisability that we observed in the pilot study was not due to phenolic substances but probably to non-specific effects of the meal or to changes in LDL oxidisability over the day.

### Adequate control groups

Our findings stress the need for adequate control groups to avoid misinterpretations of the data. In the pilot study we found that the phenol-rich breakfast decreased LDL oxidisability, which would have suggested a positive effect of the phenols on LDL oxidisability. However, if compared with a control group, the effect turned out to be a non-specific meal or time effect rather than an effect of the phenols. The importance of a control group should thus not be underestimated, and the results of studies should be interpreted very carefully when no control group is included. Thus, for a conclusive effect of dietary components on antioxidative markers, including a control group is essential.

## Olive oil phenols and LDL oxidation

Our findings are similar to those from the studies of Nicolaïew *et al* (1998) and Bonanome *et al* (2000) in which extra virgin olive oil also did not affect the lag time of the *ex vivo* susceptibility of LDL to oxidation as compared to the control group, both in fasting and in postprandial plasma samples. In contrast to what we found, those studies did not show an increase in lag time after eating a meal. A reason for that might be that blood samples were taken 6 h after the oral fat load in the study of Nicolaiew *et al* instead of the 2 h in our study (Nicolaiew *et al*, 1998). However, Bonanome *et al* measured LDL oxidisability 30, 60, 120, 240 and 360 min after intake of 100 mL olive oil, and did not find an effect on lag time on all these time points (Bonanome *et al*, 2000).

Bonanome *et al* did find an increase of total plasma antioxidant capacity, another marker than lag time, in postprandial plasma samples 2 h after intake of a single dose of 100 mL extra virgin olive oil (Bonanome *et al*, 2000). However, they did not include a control group and this effect might therefore, like in our study, also be a non-specific meal or time effect.

Other human studies addressed long-term effects of phenols from olive oil in fasting blood and not immediate effects in postprandial blood (Nicolaiew *et al*, 1998; Ramirez-Tortosa *et al*, 1999; Bonanome *et al*, 2000; Vissers *et al*, 2001). Ramirez-Tortosa *et al* (1999) showed a decreased LDL oxidisability after 3 months of consumption of extra virgin olive oil compared to refined olive oil. However, LDL oxidation was assessed as the amount of thiobarbituric acid-reactive substances (TBARS) formed after incubation for 24 h with various concentrations of copper ions instead of assessing LDL oxidation as the lag time as in the current study. The value of TBARS as a measure of *in vivo* oxidation is questionable (Meagher & FitzGerald, 2000), but Ramirez-Tortosa *et al* also found that extra virgin olive oil reduced uptake by macrophages of oxidised LDL in fasting blood compared to refined olive oil. Other human studies did not find an effect on LDL oxidisability in fasting blood (Nicolaiew *et al*, 1998; Bonanome *et al*, 2000; Vissers *et al*, 2001). Taken together, results from human studies are thus inconsistent.

## Increased lag time in postprandial samples

Few data are available on the diurnal variation of the lag time. Studies of LDL oxidation have mainly focussed on the fasting state. In the postprandial state triglyceride concentrations in the LDL fraction increase (Cohn *et al*, 1988; Lechleitner *et al*, 1994), which is thought to increase the susceptibility of LDL to oxidation and thus, in contrast to what we found, decrease the lag time (Regnstrom *et al*, 1992). But the type of fatty acids in the supplement might also affect LDL oxidisability. Olive oil is rich in oleic acid, which reduces LDL oxidisability (Reaven *et al*, 1991). VLDL or chilomicrons might exchange lipid molecules with other lipoproteins (Zilversmit 1995), like LDL, and thereby decrease the LDL oxidisability in postprandial plasma. Unfortunately we did not measure the fatty acids composition of the LDL fractions. However, other studies did not show an effect of boluses of up to 100 g olive oil on the lag time of LDL oxidation in postprandial samples ( Nicolaiew *et al*, 1998; Nielsen *et al*, 2000; Bonanome *et al*, 2000). Thus,

an acute effect of an oleic acid bolus on LDL composition and subsequent LDL oxidisability is not likely. Furthermore, fat intake was similar among all supplement groups and could thus not have affected the results of the phenols on LDL oxidisability.

We did not measure vitamin E concentrations in plasma. However, we think it is not likely that vitamin E could have confounded the results. The amount of vitamin E that subjects consumed from the supplement was at maximum 10 mg in the positive control supplement. One dose-response study suggested that a daily ingestion for 2 wk of at least 25 mg vitamin E was needed to reduce LDL susceptibility (Princen *et al*, 1995). Another study even suggests that much higher daily doses are needed (Jialal *et al*, 1995). Furthermore, Dimitrov *et al* demonstrated in a kinetic study that a single dose of as much as 440, 880, and 1320 mg vitamin E hardly elevated plasma vitamin E concentrations 2 h after intake, and that a peak is reached as late as 12-24 h after ingestion (Dimitrov *et al*, 1991). Thus, we think it is unlikely that the single dose of at most 10 mg of vitamin E materially influenced plasma vitamin E concentrations and LDL oxidisability. Furthermore, the amount of vitamin E in the background diet was 2.3 ± 1.0 mg, which is probably too low to affect LDL oxidisability 2 h after intake.

The pizza herbs mainly consisted of oregano, which is known to have antioxidant properties *in vitro* (Tsimidou *et al*, 1995). However, subjects consumed only 0.37 g of the herbs, and each supplement contained exactly the same amount and type of the herbs, which thus could not have affected the differences in effects on LDL oxidisability between the supplements.

Thus, our results indicate that phenols from extra virgin olive oil do not affect LDL oxidisability, but a non-specific time or meal effect might be responsible for the decreased LDL oxidisability after the meal. Future research should more precisely determine the 24-h time course of LDL oxidisability, including effects of meals.

### Dose of phenols

The plasma concentration of phenols needed to protect LDL from oxidation *in vivo* is unknown. *In vitro* dose-response studies by Visioli and co-workers demonstrated that pre-incubation of LDL with 10 µmol/L oleuropein or 10 µmol/L hydroxytyrosol protected vitamin E from oxidation, but that a concentration of 1 µmol/L provided much less protection (Visioli *et al*, 1995; Visioli & Galli, 1994). We do not know the plasma concentration of the various phenols that were reached in our studies, but a single dose of 100 mg (about 275 µmol) does probably not result in a plasma concentration higher than 1 µmol/L (Hollman *et al*, 1997). Thus, it is likely that the plasma concentrations in our studies were too low to produce an effect on the *ex vivo* oxidation of LDL. Furthermore, estimations of the average daily intake of phenols from olive oil in the Mediterranean diet indicate that 10-20 mg/d of total phenols may be supplied by olive oil (Visioli *et al*, 1995), which is at least 5 times less than the amount given by us in a single dose. Therefore the phenol content of olive oil may be too low to produce a measurable effect on LDL oxidisability *in vivo*.

### Pharmacokinetics of phenols from olive oil

In the pilot study we measured the LDL oxidisability 2 h after intake of the meals (Bonanome *et al*, 2000). We found an intriguing increase in lag time and we therefore repeated measurement at this time point in the current study. In this study we also measured the effect at ½ h after intake because Hollman and co-workers found a plasma peak concentration of quercetin (Hollman *et al*, 1997), a compound that is comparable with the olive oil aglycones, ½ h after intake of a quercetin-rich breakfast. However, we can not be sure that olive oil phenols were present in plasma or LDL ½ h or 2 h after intake. Thus, more insight into the time course of plasma phenol concentrations after intake is required to conclude whether olive oil phenols can add a positive health effect in humans. Future research should focus more on the kinetics and metabolism of phenols and other potential antioxidants in humans.

### Conclusion

We did not find an effect of a large single dose of olive oil phenols on plasma LDL oxidisability. More insight into the kinetics of phenol transport and metabolism in humans is required to study the potential health effects of olive oil phenols in humans.

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# The effect of olive oil phenols and extracts on LDL oxidation in an *in vitro* system

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## ABSTRACT

It has been suggested that phenols present in olive oil contribute to the health effects of the Mediterranean life-style. Olive oil antioxidants, and in particular the *ortho*-dihydroxy phenol hydroxytyrosol, increase the resistance of low density lipoproteins (LDL) against oxidation *in vitro*. Human dietary intervention studies have failed to demonstrate a consistent effect of olive oil consumption on LDL resistance to oxidation when determined *ex vivo*.

To close this gap, a method has been used that mimics the exposure of LDL to olive oil phenols in its natural environment, i.e. in plasma. Plasma was incubated with individual olive oil phenols and with olive oil extracts rich in *ortho*-dihydroxy phenols, oleuropein-aglycone and hydroxytyrosol, and/or *mono*-hydroxy phenols, ligstroside-aglycone and tyrosol. LDL was subsequently isolated and challenged for its resistance against copper-mediated oxidation.

The results show that olive oil phenols increase LDL resistance. As predicted from their structural features, the *ortho*-dihydroxy phenols (hydroxytyrosol and oleuropein-aglycone) are more efficient than their *mono*-hydroxy counterparts (tyrosol and ligstroside-aglycone) in protecting against lipid peroxidation. However, the concentration of antioxidants required to inhibit LDL oxidation when added to whole plasma was substantially higher compared to previously published data where antioxidants had been added directly to isolated LDL. Furthermore, the hydrophilic oleuropein-glycoside only marginally increased LDL resistance, whereas its lipophilic aglycone counterpart is a more potent antioxidant in this model system.

In conclusion, this *in vitro* study has shown that extra virgin olive oil phenols can provide protection to LDL in plasma. The explanation that *in vitro* studies show protective effects in contrast to the lack effect in the majority of human studies may be due to the fact that the dose of the phenols and thus their plasma concentration in humans was too low to reduce *ex vivo* LDL oxidisability. Further studies on the bioavailability, metabolism and other mechanisms of action of olive oil phenols are needed for a better understanding of the potential health benefits that extra virgin olive oil may provide.

## INTRODUCTION

In Mediterranean countries, where olive oil is the major source of fat, rates of coronary heart disease are relatively low compared with more Northern European societies (Keys *et al*, 1986). In addition to the favourable effects of the monounsaturated fatty acids on blood cholesterol concentrations (Katan *et al*, 1995) and on LDL oxidisability (Reaven *et al*, 1991), animal and *in vitro* studies suggest that the high concentration of phenolic antioxidants in extra virgin olive oil also may contribute to the healthy nature of this diet ( Scaccini *et al*, 1992; Visioli & Galli, 1994; Visioli *et al*, 1995; Salami *et al*, 1995; Wiseman *et al*, 1996; Visioli *et al*, 1998; Caruso *et al*, 1999; Coni *et al*, 2000). The most abundant phenols in extra virgin olive oil are the less polar oleuropein- and ligstroside-aglycones. They are formed in olives by enzymatic removal of glucose from the parent compound oleuropein-glycoside. Hydroxytyrosol and tyrosol are end products produced from the hydrolysis of oleuropein- and ligstroside-aglycones, respectively, and they are polar compounds (**Figure 5.1**).

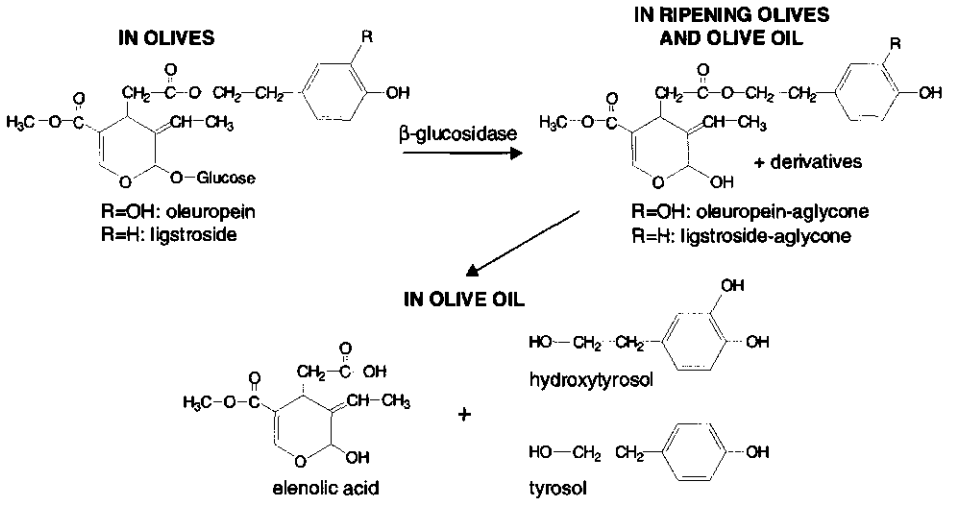


**Figure 5.1** The main phenols present in olives and olive oil. The main phenols in olive oil are the aglycones, which are derived from oleuropein-glycoside by enzymatic degradation. Tyrosol and hydroxytyrosol are derived from ligstroside- and oleuropein-aglycones by hydrolysis.

Oxidation of LDL is believed to increase the risk of atherogenesis, the underlying cause of coronary heart disease. Olive oil phenols may be particularly effective in preventing oxidation of LDL. Due to their relatively non-polar nature, they may directly associate with LDL particles. This association may lead to an increased resistance of LDL against oxidation and this could be one of the potential mechanisms by which extra virgin olive oil may benefit health. This hypothesis is supported by *in vitro* experiments, where it has been demonstrated that oleuropein-glycoside and hydroxytyrosol increase the resistance of LDL against copper-induced oxidation (Visioli & Galli, 1994; Visioli *et al*, 1995). However, in the majority of human

studies (Nicolaiew *et al*, 1998; Bonanome *et al*, 2000; Vissers *et al*, 2001a; Vissers *et al*, 2001b) no effect of phenols present in extra virgin olive oil on *ex vivo* LDL oxidisability could be found in fasting or postprandial plasma samples. An explanation for this apparent discrepancy may be that the concentrations in human plasma were too low to influence *ex vivo* LDL susceptibility. Alternatively, human plasma olive oil phenols may also associate with other plasma proteins than LDL. If those phenols are directly added to the isolated LDL fraction, LDL is the major protein source to which they can bind. Finally, phenols may dissociate from LDL during isolation from plasma.

The aim of the present *in vitro* study was to evaluate the effect of olive oil phenols on the resistance of LDL to oxidation. In order to mimic the *in vivo* situation as closely as possible, plasma was incubated with olive oil phenols. LDL was subsequently isolated and the resistance of LDL to oxidation was evaluated by monitoring copper-induced lipid peroxidation. In addition to pure olive oil compounds, we also examined the effects of structurally similar compounds, other dietary antioxidants like vitamin E and tea catechins, and 3 olive oil extracts with different phenolic compositions in order to determine whether not only phenol concentration but also the antioxidant properties influence the resistance of LDL to oxidation.

## MATERIALS AND METHODS

### Tested antioxidants

The following antioxidants or olive oil extracts were investigated for their ability to protect LDL against copper-induced oxidation and their antioxidant properties: tyrosol (Acros, New Jersey, USA); oleuropein-glycoside (Roth, Karlsruhe, Germany); DL alpha-tocopherol (Merck, Darmstadt, Germany); green tea extract (Lipton, New Jersey, US). Gallic acid, 3,4-di-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and catechin were obtained from Sigma, Saint Louis, USA. Hydroxytyrosol, 2 non-polar extracts and a polar extract derived from extra-virgin olive oil were prepared at Unilever Research Vlaardingen, the Netherlands.

Two batches of hydroxytyrosol were prepared according to the method described by Baraldi *et al* (1983). The purity of the 2 batches was determined to be at least 93%.

Oleuropein- and ligstroside-aglycones (**Figure 5.1**) and their derivatives were not available in the pure form. Therefore, we prepared several olive oil extracts, in which the various phenols were present in varying amounts (**Table 5.1**).

The phenol composition was determined by HPLC according to Montedoro *et al* (1993). With this HPLC method it was possible to identify hydroxytyrosol, tyrosol and 10 derivatives of the oleuropein- and ligtroside-aglycones. The aglycones were identified with HPLC-MS-MS. The 12 major peaks in the HPLC chromatogram were quantified using the area/µg at $\lambda$=280 nm of the individual compounds hydroxytyrosol, tyrosol and oleuropein or of mixtures of aglycones. The aglycone reference mixture was obtained by enzymatic hydrolysis of oleuropein by β-glucuronidase. The various derivatives differ mainly in their ring structure, which can either be

open or closed in two different forms (van Boom *et al*, in preparation). The total phenol concentration in each extract was standardised to a concentration of 1000 µmol/L.

**Table 5.1** The relative amount (%) of the various phenols in the tested olive oil extracts.

| *Description of the extract*[a] | *Tyrosol* | *Hydroxytyrosol* | *Ligstroside-aglycone* | *Oleuropein-aglycone* |
|---|---|---|---|---|
| Non-polar extract 1 | 21 | 26 | 14 | 39 |
| Non-polar extract 2 | 31 | 2 | 53 | 14 |
| Polar extract | 31 | 45 | 4 | 20 |

[a] The phenol concentrations was determined by HPLC according to Montedoro *et al* (1993) and van Boom *et al* (in preparation). The total phenol concentration in each extract was standardised to a concentration of 1000 µmol/L. The polar extract was extracted from extra virgin olive oil by reversed osmosis. The non-polar extracts (1 and 2) were extracted from extra virgin olive oil with ethanol, which was later removed by evaporation.

## LDL isolation and the measurement of the resistance of LDL to copper-induced oxidation

Plasma was prepared by centrifugation at room temperature for 10 min at 2,500 g, and 10 µl of a 600 g/L sucrose solution was added per mL plasma (Kleinveld *et al*, 1992). Plasma was stored at -80°C until use. Plasma was mixed with the compounds and extracts tested for 30 min at room temperature. A control plasma was treated in the same way. The olive oil extracts were added directly, whereas alpha-tocopherol was dissolved in ethanol, and all other antioxidants were dissolved in water prior to addition to plasma. The concentrations of all compounds and extracts tested were 1000 µmol/L. For a selection of these compounds (the 2 non-polar extracts, the polar extract, hydroxytyrosol, tyrosol, oleuropein-glycoside, and DL alpha-tocopherol) the minimal concentration required to increase LDL resistance to oxidation was determined by incubating plasma with a concentration range: 0, 50, 100, 250, 500, and 1000 µmol/L. For the green tea extract the concentrations were 0, 80, 160, 320, 640, and 960 µmol/L.

LDL was subsequently isolated by density gradient ultracentrifugation using an SW 41Ti rotor (Beckman Instruments, Palo Alto, USA), 36,000 *g* for 24 h at 10°C (Redgrave *et al*, 1975). Density gradient solutions contained 0.1 mmol/L $Na_2EDTA$ to prevent metal ion catalysed LDL oxidation during the isolation procedure. The LDL-fraction was isolated in a density range of 1.019-1.063 g/mL.

The protein concentration of the LDL fraction was determined as approximately 500 µg/mL (Lowry *et al*, 1951). This fraction was initially diluted to a concentration of 200 µg/mL with gradient solution (density was 1.019 g/mL) and was further diluted with PBS to a concentration of 50 µg per mL in a thermostat controlled cuvette at a temperature of 30°C. The oxidation of LDL was initiated by the addition of 10 µL of 5 mmol/L $CuCl_2$ in a total volume of 1 mL. This

method was essentially the same as described by Princen *et al* (1992). The formation of conjugated dienes was monitored spectrophotometrically at a wavelength of 234 nm at 2 min intervals for a period of 400 min or until oxidation was complete, using a UV-Vis spectrophotometer equipped with a thermostat controlled multicuvette holder (Cary 50 Spectrophotometer, Varian Inc., Mulgrave, Victoria, Australia). The oxidation profile of each LDL sample was plotted against time. The lag time and the maximum rate of oxidation were determined as described by Esterbauer *et al* (1992). Each condition was tested in duplicate.

### Determination of alpha-tocopherol levels in LDL

A small aliquot of the LDL fraction that had been incubated with DL-alpha-tocopherol, was stored at -80ºC until analysis of its concentration in alpha-tocopherol. Butylated Hydroxy Toluene (BHT) was added to prevent oxidation. Alpha-tocopherol in LDL was determined by HPLC on a 5-µm column (Lichrospher RP-18; Merck, Darmstadt, Germany). Alpha-tocopheryl acetate (Merck) was used as an internal standard. The mobile phase consisted of methanol: isopropanol:water (50:50:8), by vol) and the flow rate was 0.6 mL/min. Alpha-tocopherol was detected by UV-Vis spectrometry at 292 nm, and alpha-tocopheryl acetate at 284 nm.

### Statistical analyses

The duplicate values were averaged before data analysis. We applied the Least Square Means of the General Linear Models (GLM) of the Statistical Analyses System (SAS Institute Inc. 1989) to analyse whether the various tested phenols significantly affected the absolute values of the lag time and maximum rate of LDL oxidation compared to control-treated plasma (one-tailed, $P<0.05$). Control-treated plasma was included in each experiment as either a monoplo or duplo and the observed lag time and maximum rate were adjusted for run as an independent variable. The Dunnett multiple comparison test was used to adjust for multiple comparisons.

For the concentration-dependent oxidisability of LDL we compared each concentration with the control-treated plasma by using ANOVA, with dose and run as independent variables.

## RESULTS

### Effect of olive oil phenols, extracts and other dietary antioxidants on LDL oxidisability

The effects of pre-incubation of plasma with antioxidants at a concentration of 1000 µmol/L on the lag time of copper-induced LDL oxidation are presented in **Figure 5.2**.

Hydroxytyrosol, 3,4-dihydroxyphenylacetic acid and catechin all increased the lag time of the LDL oxidation process compared to LDL derived from control treated plasma, indicating that an *ortho*-dihydroxy structure on the phenol ring is important in determining antioxidant function. The *mono*-hydroxy phenols (tyrosol and 4-hydroxyphenyl acetic acid) were, on the other hand, ineffective in affording protection. This was also the case for alpha-tocopherol, whereas gallic

acid slightly increased the lag time. Green tea extract was most efficient in delaying the lag time compared to all other ingredients examined in this study. All 3 olive oil extracts, normalised to a concentration of 1000 µmol of phenols per litre, were shown to increase LDL resistance to *in vitro* oxidation.

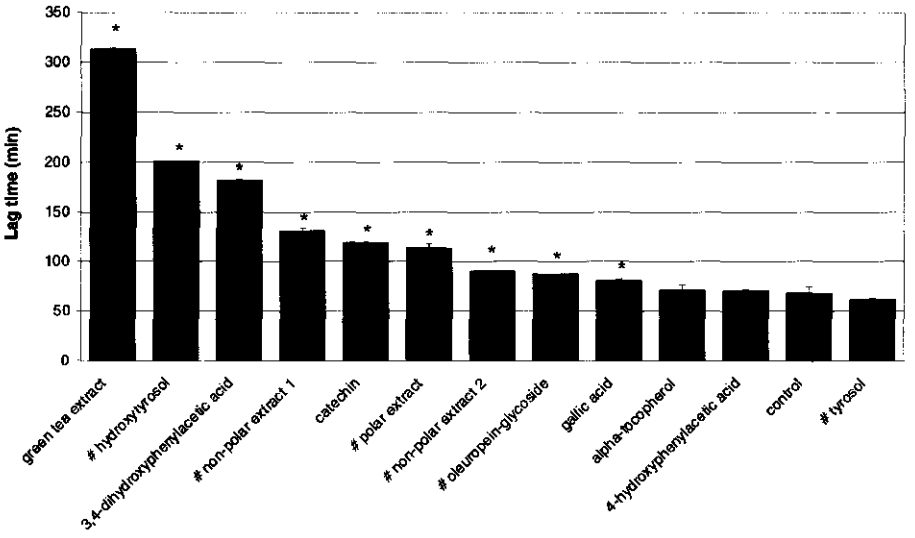


**Figure 5.2** *Effect of all compounds and extracts on the lag time of copper-mediated LDL oxidation.* Plasma was incubated with the compounds at a concentration of 1000 µmol/L. LDL was isolated by means of density gradient ultracentrifugation and submitted to oxidation by the addition of cupric ions. The formation of conjugated dienes was monitored spectrophotometrically and the lag time of the oxidation process was determined as described in detail in Materials and Methods. The results presented are the mean lag time ± SD of 2 independent determinations. Compounds indicated with # are present in olives or olive oil. * = Significant increase in lag time compared to the control samples.

The effect of all compounds or extracts tested on the maximum rate of oxidation during the propagation phase at a concentration of 1000 µmol/L were also determined. Green tea extract and 3,4 dihydroxyphenyl acetic acid slightly, albeit significantly, reduced the oxidation rate compared to control plasma (18.6 nmol conjugated dienes/min/mg LDL protein for the green tea extract ($P$=0.004) and 19.3 nmol conjugated dienes/min/mg LDL protein for 3,4-dihydroxy phenylacetic acid ($P$=0.02) versus 22.5 nmol conjugated dienes/min/mg LDL protein for the control plasma). All other components did not significantly alter the oxidation rate (data not shown).

For some of the compounds investigated, the minimum concentration required to increase LDL resistance to oxidation was determined by incubating plasma with concentrations ranging from 0 – 1000 µmol/L. The results are shown in **Figure 5.3**.

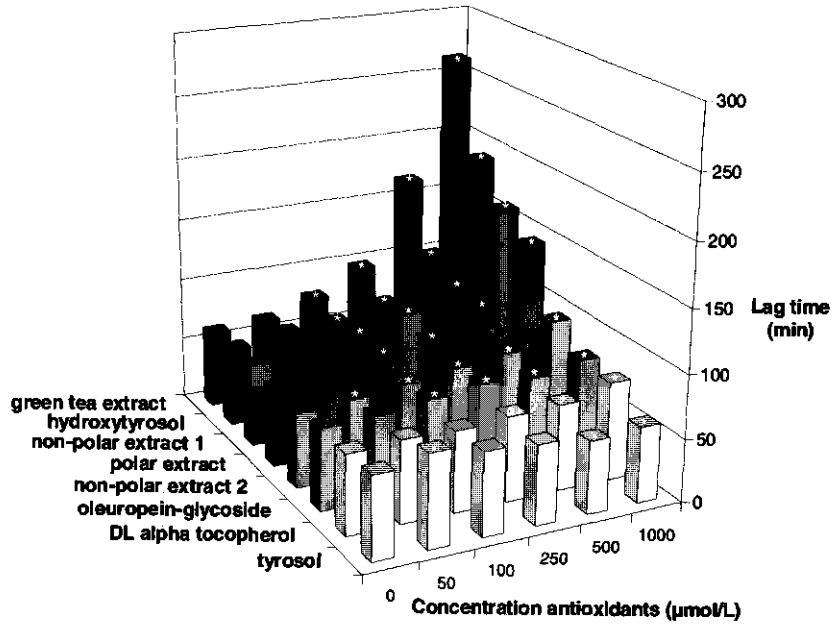


**Figure 5.3** Effect of antioxidant concentration on the lag time of copper-induced LDL oxidation. Plasma was incubated with the antioxidants in concentrations varying between 0 and 1000 µmol/L. LDL was isolated and its resistance to oxidation was determined as described in Materials and Methods. The results presented are the mean of 2 independent determinations of the lag time. * = Significant increase in lag time compared to control samples.

For most olive oil components a minimum concentration of 100 µmol/L was required to significantly increase the lag time, and at this concentration the increases in lag time were comparable for these components. However, when higher concentrations were used, the increase in lag time varied considerably between the ingredients. Green tea extract, hydroxytyrosol, non-polar extract 1 and the polar extract all progressively increased the lag time with increasing concentrations. In contrast increasing doses of non-polar extract 2 and oleuropein-glycoside only moderately influenced the lag time.

The maximum rates of LDL oxidation during the propagation phase were not studied for the various concentrations of the compounds and extracts tested as for the lag time, because pre-incubation of plasma with those compounds at a concentration of 1000 µmol/L had no major effect on the maximum rate of LDL oxidation.

## Effect of incubation of plasma with alpha-tocopherol on its levels in LDL

Due to its lipophilicity, vitamin E is incorporated in lipoproteins such as LDL. The effect of incubating plasma with alpha-tocopherol on its partitioning in LDL was evaluated. The results in

**Figure 5.4** show that alpha-tocopherol was incorporated dose-dependently in LDL. Despite the nearly five-fold increase in vitamin E content of the LDL particles following an incubation of plasma with 1000 µmol/L alpha-tocopherol, only a marginal increased protection of LDL against oxidation was observed (Figures 2 and 3).

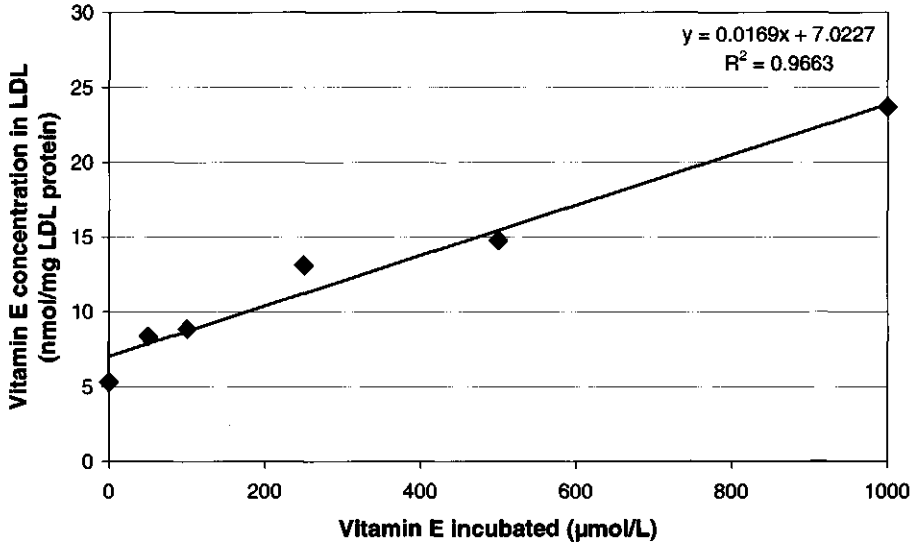


**Figure 5.4** Vitamin E content in LDL isolated from plasma that was incubated with vitamin E concentrations ranging from 0 to 1000 µmol/L. LDL was isolated by means of density centrifugation and the vitamin E content in LDL was determined by HPLC as described in Materials and Methods. Vitamin E concentrations are expressed in nmoles per mg LDL protein.

## DISCUSSION

The results of the present study show that LDL isolated from plasma that has been incubated with the *ortho*-dihydroxy phenol hydroxytyrosol and with extracts rich in hydroxytyrosol, have an increased resistance of LDL to copper-induced oxidation. However, the *mono*-hydroxy counterparts, tyrosol and tyrosol-rich olive oil extracts appeared to have little effect on the susceptibility of LDL to oxidation.

We compared the effects of the various phenols to determine whether structural features influence antioxidant activity. The importance of the *ortho*-dihydroxy feature in antioxidant function is confirmed in this study, since hydroxytyrosol, 3,4-dihydroxy-phenylacetic acid and catechin all efficiently increased the lag time of the LDL oxidation process. From the literature it is known that *mono*-hydroxy polyphenols have weak antioxidant properties (Rice-Evans *et al*, 1996), which was also the case for tyrosol and 4-hydroxyphenylacetic acid in the present study. In addition to pure individual phenols, olive oil extracts and green tea extract were also tested.

The polar and non-polar olive oil extracts as well as green tea extract all contained phenols, including *ortho*-dihydroxy phenols. Indeed all extracts increased LDL resistance. A positive relationship between the concentrations of hydroxytyrosol and oleuropein-aglycone in the extract and the lag time could be observed: non-polar extract 1 and the polar extract both with a relatively high content of oleuropein-aglycone and hydroxytyrosol were more effective in protecting LDL from oxidation than non-polar extract 2 with a relatively low oleuropein-aglycone and hydroxytyrosol content (**Table 5.1**, **Figure 5.2**).

Only green tea extract and 3,4 dihydroxyphenyl acetic acid slightly reduced the oxidation rate of the propagation phase of the LDL oxidation process, whereas all other antioxidants or antioxidant-rich extracts did not influence this oxidation rate. This indicates that, similar to the loss of antioxidants endogenously present in LDL, the majority of supplementary antioxidants is destroyed during the lag phase and that, after complete consumption of the antioxidants present in LDL, other constituents such as lipids become prone to copper-mediated oxidation (Esterbauer *et al*, 1992). It should be noted that we would expect the maximum rate of oxidation to be affected when the lipid composition in the LDL particle had changed, which is not the case in this *in vitro* system.

Of all antioxidants evaluated, alpha-tocopherol was the most lipophilic. Alpha-tocopherol levels in isolated LDL increased approximately five-fold after incubation of plasma with 1000 µmol/L of this antioxidant, but only marginally increased LDL resistance. In contrast, human studies have reported vitamin E (DL alpha-tocopherol) supplementation protects LDL from oxidation (Princen *et al*, 1995; Jialal *et al*, 1995). The concentrations of vitamin E in isolated LDL in the current *in vitro* study were similar to the concentrations of vitamin E in isolated LDL in a human study by Princen *et al* (1995) (5.3-23.7 nmol/mg LDL protein and 13.2-29.6 nmol/mg LDL protein, respectively). However, the amounts added to plasma in the current *in vitro* study were much higher (0-1000 µmol/L) compared to the plasma concentrations of the subjects in the human study of Princen *et al* (23.6-61.2 µmol/L) who consumed vitamin E supplements for minimally 2 wk. This indicates that under these *in vitro* conditions, the plasma alpha-tocopherol concentration needs to be much higher to end up with similar concentrations in LDL after isolation compared to the *in vivo* situation. A likely explanation for this difference may be that after ingestion vitamin E is incorporated in LDL through the alpha-tocopherol transfer protein (Stocker & Azzi, 2000), which is not present in an *in vitro* situation. In addition, the location of vitamin E within the LDL particle after incubation may differ from vitamin E that is incorporated via the metabolic route. This might influence its effectiveness in protecting lipids in LDL particles against oxidation. Comparison with other *in vitro* studies also reveals several discrepancies. Some studies showed no or limited protecting effects of vitamin E (Vinson *et al*, 1995), whereas another *in vitro* study (Esterbauer *et al*, 1989) showed a protective effect of vitamin E. Differences in experimental conditions, such as incubation time or higher vitamin E concentrations in LDL, make it difficult to compare these *in vitro* studies.

The amounts of antioxidants required to inhibit LDL oxidation when added to whole plasma were substantially higher compared to experiments where antioxidants are directly added to

isolated LDL (Visioli & Galli, 1994; Visioli *et al*, 1995). An approximately 100 fold higher concentration of antioxidants needs to be added to plasma in order to achieve a similar inhibitory effect compared to the condition where antioxidants are directly added to isolated LDL. When phenols are directly incubated with isolated LDL they can directly trap radicals generated in the system. Furthermore, they have no other alternative than to associate with LDL, and this leads to a substantially higher accumulation of phenols in LDL compared to the plasma incubation method. In plasma, olive oil phenols might bind to a number of other proteins present. In fact, the majority of the polyphenol quercetin indeed binds to plasma proteins of which serum albumin is the major binding site (Boulton *et al*, 1998). Phenols are less lipophilic than tocopherol and carotenoids, therefore integration within the core of the LDL particle is unlikely. Also, a loss of phenols during the isolation procedure might lead to reduced concentration in the final experimental conditions (Carbonneau *et al*, 1997). Fito *et al* (2000) compared the effect on LDL oxidisability of plasma that was spiked with antioxidants before analyses with that of isolated LDL that was incubated with phenols directly. They showed that plasma needed to be spiked with substantially higher amounts of olive oil phenols in order to achieve an increase in LDL resistance to oxidation. Our observations are in line with those observed by Fito and co-workers. They found that 160 mg caffeic acid equivalents per L of plasma, which equals to about 900 µmol of olive oil phenols per L of plasma, extended the lag time by about 110%, which is comparable to the increases in lag time observed in the current study.

The ability of olive oil phenols to associate with LDL may be an important factor determining LDL resistance, which is suggested by the different effects of oleuropein-glycoside and its aglycone. Like its aglycone, oleuropein-glycoside is equipped with a hydroxytyrosol moiety but is considerably more hydrophilic due to its glycosylation and is, therefore, expected to show less association with LDL. Indeed oleuropein-glycoside only slightly increased the LDL resistance whereas the oleuropein-aglycone rich extract resulted in a considerable protection of LDL against oxidation.

Feeding trials in rabbits and rats demonstrated that phenolic antioxidants from olive oil showed a favourable effect on the susceptibility of LDL to oxidation (Scaccini *et al*, 1992; Wiseman *et al*, 1996; Coni *et al*, 2000;). During these feeding trials, the animals were fed with considerable amounts of olive oil for a prolonged period. In the study reported by Wiseman *et al* (1996), rabbits were fed for a 6-wk period with an experimental diet rich in extra virgin olive oil. This probably leads to much higher plasma levels of olive oil phenols leading to the accumulation of sufficient amounts of phenols in LDL that provide protection in the *ex vivo* determination of LDL resistance.

In contrast most human intervention studies (Nicolaiew *et al*, 1998; Bonanome *et al*, 2000; Vissers *et al*, 2001a; Vissers *et al*, 2001b), have failed to demonstrate a protective effect of olive oil consumption on *ex vivo* LDL oxidisability in either fasting and postprandial plasma samples. Only the study of Ramirez-Tortosa *et al* (1999) showed a decrease in LDL oxidisability after 3 months of consumption of extra virgin olive oil compared to refined olive oil. However, the

method for measurement of LDL oxidation described in this study was completely different and cannot be compared with our own study and the other published studies. One possible explanation for the lack of effect in the majority of the human studies is that the dose of the phenols was too low to reduce LDL oxidisability. We do not know the plasma concentrations of the various phenols that were reached in these human studies but, based on results of a study with quercetin, a dose of maximally 100 mg (about 275 µmol) did probably not result in a plasma concentration higher than 1 µmol/L (Hollman *et al*, 1997). Daily intake of olive oil phenols in the Mediterranean is estimated to be 10-20 mg (Visioli *et al*, 1995), which implies that the level of phenols in olive oil is too low to produce meaningful effects on LDL oxidisability within the normal range of consumption. Thus, it is likely that the plasma concentrations in humans were too low to produce an effect on the *ex vivo* oxidation of LDL.

Data concerning intestinal absorption of phenols from olive oil is scarce. Furthermore, it is not known whether sufficient phenols accumulate in LDL particles to provide protection from oxidation. Until now, only Bonanome *et al* (2000) recovered hydroxytyrosol and tyrosol in LDL, albeit, with a high variability among subjects. Phenols are probably present in the conjugated or metabolised form in plasma or LDL. Recently, it has been shown that tyrosol and hydroxytyrosol were mainly excreted in urine in the conjugated form (Visioli *et al*, 2000a) and also that the metabolite *O*-methylated hydroxytyrosol was excreted in high amounts after phenol-rich olive oil had been consumed (Visioli *et al*, 2000b). Conjugation, such as glucuronidation and sulfation, renders phenols more hydrophilic and this might reduce their antioxidant capacity (Morand *et al*, 1998; Manach *et al*, 1998). The antioxidant capacity of the metabolite *O*-methylated hydroxytyrosol is not known, but for quercetin it has been demonstrated that the magnitude of prolonging the lag phase for 3'-*O*-methylquercetin is about half of that of its aglycone (Manach *et al*, 1998). Future research should therefore focus more on the kinetics and metabolism of phenols in humans.

In conclusion, the present *in vitro* study shows that olive oil phenols and olive oil extracts rich in phenols increase the resistance of LDL against peroxidation. The *ortho*-dihydroxyphenol and oleuropein-aglycone are more efficient than their *mono*-hydroxy counterparts in protecting LDL. The ability of phenols to partition in LDL is also expected to be a relevant factor for the antioxidant efficacy. Glycosylated phenols, such as oleuropein-glycoside, are very hydrophilic and only marginally increase LDL resistance, whereas a mixture rich in the aglycone counterparts display more antioxidant activity in this model system. The explanation that *in vitro* studies show protective effects whereas the majority of human studies do not, may be due to the fact that the dose of the phenols and thus their plasma concentration in humans was too low to reduce LDL oxidisability. This implies that the level of phenols in olive oil is too low to produce meaningful effects on *ex vivo* LDL oxidisability within the normal range of olive oil consumption. However, more *in vivo* studies are needed to confirm these protective effects. In addition, it is plausible that olive oil antioxidants may play a role in other mechanisms of actions related to coronary heart disease. Thus, we need to increase our knowledge on the

bioavailability, metabolism and other mechanisms of action of olive oil phenols for a better understanding of the potential health benefits that extra virgin olive oil may provide.

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# Apparent absorption and urinary excretion of olive oil phenols in humans

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*Submitted for publication*

## ABSTRACT

Animal and *in vitro* studies suggest that olive oil phenols are effective antioxidants. The most abundant phenols in extra virgin olive oil are the non-polar oleuropein- and ligstroside-aglycones which can be hydrolysed into the polar hydroxytyrosol and tyrosol.

Aim of this study was to get more insight into the absorption and metabolism of olive oil phenols in humans. We therefore measured their absorption and urinary excretion in 8 healthy ileostomy subjects. To assess the influence of colonic bacteria in the metabolism of those phenols we also determined urinary excretion in 12 subjects with a colon. Subjects consumed single doses of 3 different supplements containing 100 mg of olive oil phenols on separate days in random order. Ileostomy subjects consumed a supplement with mainly non-polar phenols, one with mainly polar phenols, and one with the parent compound oleuropein-glycoside. Subjects with a colon consumed a supplement with mainly non-polar phenols, one with mainly polar phenols, and one without phenols (placebo). Ileostomy effluent and urine were collected for 24 h after supplement intake.

Tyrosol and hydroxytyrosol concentrations were low (<4 mol% of intake) in the ileostomy effluent, and no aglycones could be detected. We estimated that the apparent absorption of phenols was at least 55-66 mol% of the ingested dose.

Absorption was confirmed by the excretion of tyrosol and hydroxytyrosol with urine. In ileostomy subjects 12 mol% and in subjects with a colon 6 mol% of the phenols from the non-polar supplement was recovered in urine as tyrosol or hydroxytyrosol. In both subject groups 5 to 6 mol% of the phenols was recovered from the polar supplement. When ileostomy subjects were given oleuropein-glycoside 16 mol% was recovered in 24-h urine, mainly in the form of hydroxytyrosol.

We conclude that humans absorb a large part of ingested olive oil phenols. Furthermore, oleuropein-glycoside and oleuropein- and ligstroside-aglycones are probably hydrolysed into hydroxytyrosol and tyrosol and further metabolised after absorption.

## INTRODUCTION

Olive oil is the principle fat source of the traditional Mediterranean diet, a diet that has been associated with a low incidence of coronary heart disease (Keys *et al*, 1986). Oleic acid, the main fatty acid in olive oil, has favourable effects on blood cholesterol concentration, when compared to saturated fat (Katan *et al*, 1995), and on LDL oxidisability (Reaven *et al*, 1991). However, extra virgin olive oil also contains phenolic compounds with antioxidant activity, which are potentially beneficial against LDL oxidation (Wiseman *et al*, 1996; Scaccini *et al*, 1992; Visioli *et al*, 1995; Salami *et al*, 1995; Caruso *et al*, 1999). The most abundant phenols in extra virgin olive oil are the non-polar oleuropein- and ligstroside-aglycones and their derivatives. They are formed in olives by enzymatic removal of glucose from the polar parent compound oleuropein-glycoside. End products of hydrolysis of oleuropein- and ligstroside-aglycones in olive oil are the polar compounds hydroxytyrosol and tyrosol, respectively (**Figure 6.1**).

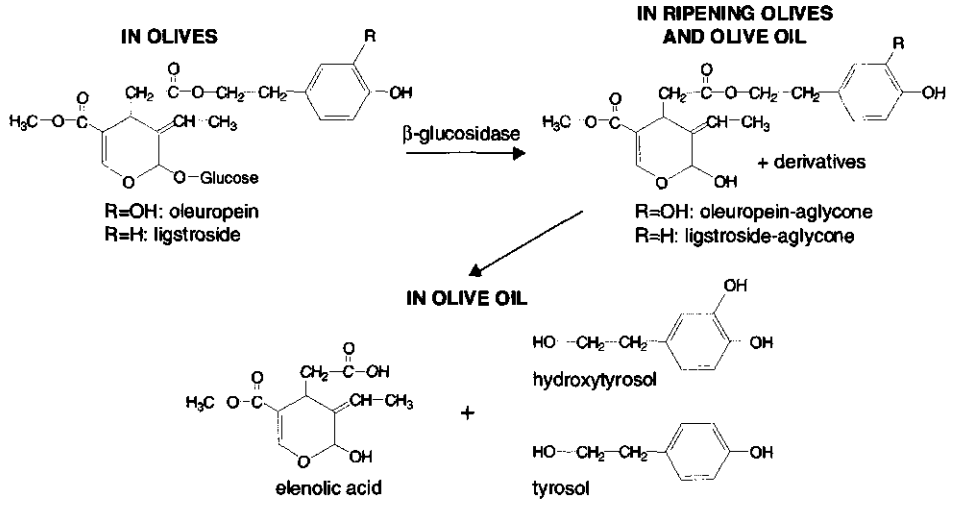


**Figure 6.1** Structures of phenols present in olives and olive oil, their degradation into aglycones during ripening, and hydrolysis of aglycones into tyrosol and hydroxytyrosol.

A Mediterranean diet rich in olive oil supplies about 10-20 mg of phenols per day (Visioli *et al*, 1995). However, it is unknown to what extent olive oil phenols are absorbed. An *in vitro* study in Caco-2 cells showed that hydroxytyrosol is transported via passive diffusion in a dose dependant manner (Manna *et al*, 2000). A human study also showed that tyrosol and hydroxytyrosol are excreted in urine (Visioli *et al*, 2000a). These results suggest that these simple olive oil phenols are absorbed in the intestine after ingestion, but it is unclear to what extent tyrosol and hydroxytyrosol are absorbed in humans. Furthermore, data on the absorption of oleuropein- and ligstroside-glycosides and –aglycone are scarce. Using an isolated perfused rat intestine, Edgecombe *et al* (2000) found that oleuropeine-glycoside was poorly absorbed.

However, no information is available on the extent of absorption of oleuropein- and ligstroside-glycosides and -aglycones in humans.

In the present study we estimated the apparent absorption of the various phenols from extra virgin olive oil and that of oleuropein-glycoside, the parent compound of the phenols present in olive leafs and olives (Soler-Rivas *et al*, 2000). A major problem in studying the absorption of phenols in humans is their degradation by micro-organisms in the colon, which results in an overestimation of the absorbed amount when faecal excretion is measured. We therefore determined the absorption in healthy ileostomy subjects with a complete small intestine. To get more insight into the metabolism we also determined urinary excretion of the phenols. However, the ileostomy model does not take into account the influence of colonic bacteria in the metabolism of those phenols. We therefore also determined the urinary excretion of tyrosol and hydroxytyrosol in volunteers with a colon.

## SUBJECTS AND METHODS

### Subjects

The study protocol was approved by the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University. We fully explained the protocol to the participants before they gave their written informed consent.

**Ileostomy subjects.** We recruited ileostomy subjects from a group of volunteers who successfully participated in previous studies at our division (Olthof *et al*, 2001; De Roos *et al*, 1998; Hollman *et al*, 1995). Ileostomy subjects have had their colon completely removed and the terminal ileum brought out onto the anterior abdominal wall as a fistula. Exclusion criteria were resection of more than 50 cm of the terminal ileum; signs of diseases related to the gastrointestinal tract; an ileostomy that did not function properly; use of drugs that influenced gastrointestinal transit; present illness; and pregnancy or lactation. Four men and 4 women, with a mean age of 57 y (range 37-75 y) and a mean body mass index of 25.2 kg/m$^2$ (range 22.1-28.8 kg/m$^2$), were eligible and willing to participate. All subjects had had a total colectomy for ulcerative colitis or polyposis coli 8-31 y ago.

**Subjects with a colon.** Subjects with a colon were volunteers who also participated in a larger study on the effect of phenols on LDL oxidation in which urine was collected (Vissers *et al*, 2001). The 12 healthy subjects (8 females, 4 males) had a mean age of 22 y (range 20-28 y), a mean body mass index of 21.8 kg/m$^2$ (range 18.3-27.1 kg/m$^2$), serum cholesterol concentrations <7.0 mmol/L, and fasting triglycerides <2.3 mmol/L. Subjects had no history of gastrointestinal, liver or kidney disease, did not use any drugs known to affect concentrations of serum lipids, and were not pregnant, lactating, or on a prescribed diet.

Both subject groups with an ileostoma and with a colon were healthy as judged by normal blood count, the absence of glucose and protein in urine, and a medical questionnaire, which was reviewed by an independent physician.

## Study designs

Design, duration, and setting of the studies in subjects with and without a colon were essentially similar. Each subject consumed a single dose of 3 different supplements, on separate days in random order (cross-over). Both studies consisted of a 3-wk treatment period, during which subjects were not allowed to consume olives or olive oil. At the end of each week, on days 8, 15, and 22, subjects consumed one of the provided supplements together with a standard breakfast. All subjects kept daily records of illness and deviations from the protocol.

**Ileostomy subjects.** We delivered the supplement and the breakfast at the subjects' home on the day before each supplement intake (days 7, 14, and 21). Breakfast consisted of bread, cheese, ham, strawberry jam, honey, milk, buttermilk, tea or coffee, plus the supplement. Each subject consumed exactly the same amount of breakfast on each of the 3 occasions. Tea, coffee or other foods and drinks were not allowed until lunch.

**Subjects with a colon.** The study design for subjects with a colon was the same as that for ileostomy subjects, except that the supplements were incorporated into a mayonnaise and that breakfast also contained cucumber, tomato, and tuna. Furthermore, subjects with a colon consumed the supplements at our department instead of at home. Tea, coffee or other foods and drinks were not allowed until 2 h after supplement intake.

Subjects with a colon consumed a fourth supplement containing 31 mg of mainly non-polar aglycones (Vissers *et al*, 2001), but because of its low dose of phenols compared with the other supplements we do not present the urine excretion data.

## Supplements

Subjects consumed single doses of 3 different supplements containing 100 mg of olive oil phenols. Ileostomy subjects consumed a supplement with mainly non-polar phenols, one with mainly polar phenols, and one with the parent compound oleuropein-glycoside. Subjects with a colon consumed a supplement with mainly non-polar phenols, one with mainly polar phenols, and one without phenols (placebo). There were minor batch differences in phenol concentration between supplements for ileostomy subjects and those for subjects with a colon (**Table 6.1**).

**Non-polar supplement.** The supplement with non-polar phenols was extracted from extra virgin olive oil with food-grade ethanol, which was evaporated at a later stage. This produced a dark green oil-like fluid rich in non-polar phenols. Ileostomy subjects drank 17.2 g of this supplement. Subjects with a colon consumed 37 g mayonnaise prepared with 14.5 g of the supplement (**Table 6.1**).

**Polar supplement.** The supplement with polar phenols was extracted from extra virgin olive oil by reversed osmosis. This produced a clear watery fluid rich in polar phenols. Ileostomy subjects drank 10.3 g of this supplement. Subjects with a colon drank 8.7 g of this supplement (**Table 6.1**) plus 37 g mayonnaise prepared with 14.5 g olive oil without phenols to equalise fat intake between breakfasts.

**Table 6.1** The phenol composition of the 4 supplements consumed by ileostomy subjects ($n = 8$) and/or subjects with a colon ($n = 12$). Supplements were analysed by HPLC.

| Supplement | Total phenol concentration in supplement (mmol/kg) | Intake supplement (g) | Intake of individual phenols (µmol) | | | | | Total intake (µmol) |
|---|---|---|---|---|---|---|---|---|
| | | | tyrosol (#2)[a] | hydroxy-tyrosol (#1)[a] | ligstroside-aglycones (#6, #11, #12)[a,b] | oleuropein-aglycones (#3, #4, #5, #7, #8, #9, #10)[a,b] | oleuropein-glycoside | |
| Non-polar supplement | | | | | | | | |
| for ileostomy subjects | 21.5 | 17.2 | 116.4 | 2.8 | 208.4[c] | 43.8 | 0 | 371.4 |
| for subjects with a colon | 26.3 | 14.5 | 122.5 | 9.1 | 199.1 | 51.3 | 0 | 382.0 |
| Polar supplement | | | | | | | | |
| for ileostomy subjects | 48.5 | 10.3 | 150.2 | 197.6 | 29.2 | 120.9 | 0 | 497.9 |
| for subjects with a colon | 60.4 | 8.7 | 165.9 | 235.1 | 22.7 | 102.7 | 0 | 526.4 |
| Oleuropein-glycoside[d] | 100 | 1.9 | 0 | 0 | 0 | 0 | 190 | 190 |
| Placebo supplement[e] | 0 | 14.5 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] The numbering of the various phenols in this table refer to the numbering of the peaks in **Figure 6.2.**

[b] Ten derivatives of the aglycones were separated and the peaks identified (van Boom *et al*, in preparation). The various derivatives differ mainly in their ring structure, which can be either open of closed in two different ways.

[c] The ligstroside-aglycones in the non-polar supplement for ileostomy subjects consisted of 180.3 µmol of ligstroside-aglycone derivative #6 and 28.1 µmol of the ligstroside-aglycone derivatives #11 and #12 (**Figure 6.2A**). Only the ligstroside-aglycone derivative #6 was analysed in ileostomy effluent (**Table 6.3**), the amounts of #11 and #12 were too low to be quantified.

[d] Only consumed by ileostomy subjects

[e] Only consumed by subjects with a colon

**Oleuropein-glycoside.** The supplement containing oleuropein-glycoside was commercially available in capsules (Solgar Laboratories, Leonia N.J., USA). Ileostomy subjects swallowed 4 capsules, which was 1.9 g (**Table 6.1**).

**Placebo supplement.** Subjects with a colon consumed a placebo supplement that consisted of 37 g mayonnaise prepared with 14.5 g olive oil without phenols.

**Analyses of phenols.** We measured the phenol concentration of the supplements with an HPLC method based on the method of Montedoro *et al* (1993). With this HPLC method hydroxytyrosol and tyrosol and 10 derivatives of the oleuropein- and ligstroside-aglycones were separated, of which the peaks were identified with HPLC-MS-MS (**Table 6.1**, **Figure 6.2**). The 12 major peaks in the HPLC chromatogram were quantified using the area/µg at $\lambda$ = 280 nm of the individual compounds (hydroxytyrosol, tyrosol and oleuropein) or mixtures (aglycones). The aglycone reference mixture was obtained by enzymatic hydrolysis of oleuropein by β-glucosidase. The various derivatives differ mainly in their ring structure, which can either be open or closed in two different forms (van Boom *et al*, in preparation).

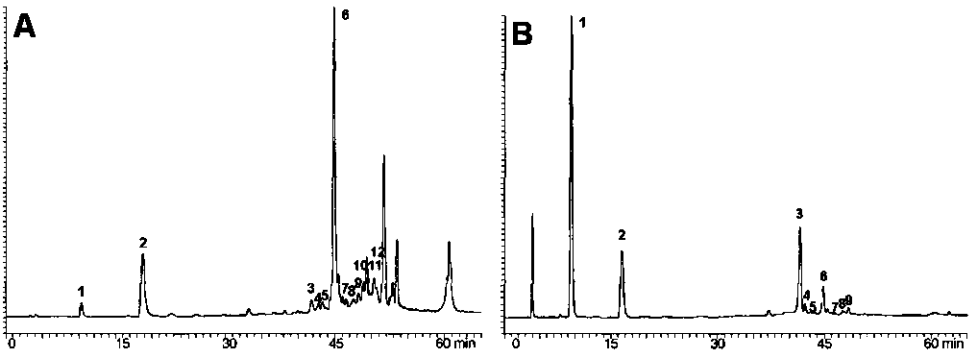


**Figure 6.2** Chromatogram of various identified phenols in the non-polar (A) and the polar (B) supplement. The numbering of the peaks refer to the numbering of the various phenols in **Table 6.1**: 1 = hydroxytyrosol; 2 = tyrosol; 3, 4, 5, 7, 8, 9, 10 = oleuropein-aglycones; 6, 11, 12 = ligstroside-aglycones.

## Collection of ileostomy effluent and urine

On days 8, 15, and 22 subjects collected a sample of ileostomy effluent and/or urine just before intake of the supplement and breakfast. After intake of the supplement they collected all ileostomy effluent and/or urine for 24 h. Ileostomy subjects changed the ileostomy bag every 2 h during the daytime. They immediately stored collected ileostomy effluent in a polystyrene box containing dry ice (-80°C) to minimise degradation of the contents by residual bacterial flora. During the night subjects changed the ileostomy bags 1 to 3 times.

Subjects collected all urine during 24 h in plastic bottles of 0.5 L containing 0.13 g thymol (#8167, Merck, Amsterdam, The Netherlands) as a preservative and stored the bottles with urine on dry ice immediately after voiding. We checked the completeness of urine collection by assessment of recovery of 250 µmol lithium chloride in urine (Sanchez-Castillo *et al*, 1987a;

Sanchez-Castillo *et al*, 1987b). Therefore, subjects drank a solution of 250 µmol lithium chloride in 10 mL of tap water daily, starting 7 d before the first urine collection. Urinary recovery of lithium was 98 ± 15%, which indicated good compliance in collecting urine.

## Sample preparation

The filled ileostomy bags were frozen with liquid nitrogen, the plastic bags were removed, and the frozen contents were lyophilised, ground to pass through a 0.5 mm sieve, and stored at -20°C until analysis. All urine samples were thawed in a water bath of approximately 40°C, pooled by subjects, homogenised, and stored at -80°C until analysis. Ileostomy and urine samples collected before breakfast (pre-supplement sample) and the final collection 24 h after intake of the supplement were prepared separately.

## Stability of phenols in gastrointestinal fluids

We evaluated the stability of the phenols in gastric juice and duodenal fluid *in vitro* in order to assess possible losses of phenols during gastrointestinal transit. Gastric juice and duodenal fluid were obtained from 2 fasted healthy volunteers with a colon by means of a probe and stored at -20°C. We incubated 1 mg of tyrosol (Fluka, Buchs, Switzerland), hydroxytyrosol (prepared by Unilever Research Vlaardingen), and oleuropein-glycoside (Roth, Karlsruhe, Germany) in 1 mL human gastric juice plus 10 mL water at 37°C for 0.5 and 2 h (Roxburgh *et al*, 1992; Jebbink *et al*, 1992). We also incubated 3 mg of tyrosol, hydroxytyrosol, and oleuropein-glycoside in 3 mL human duodenal fluid plus 9 mL water at 37°C for 1 and 4 h, corresponding to the average and maximal transit time in the small intestine, respectively (Malagelada *et al*, 1984). All measurements were done in triplicate.

We studied the stability of tyrosol, hydroxytyrosol, and oleuropein-glycoside in ileostomy effluent in a separate experiment. Two ileostomy subjects did not consume olives, olive oil, and olive oil products for 4 d. On day 4 they connected an ileostomy bag to their ileostoma containing 30 mg tyrosol mixed into 3 g strawberry jam. They collected ileostomy effluent into this bag for 2 h during which they regularly kneaded the content to mix the strawberry jam with the ileostomy fluid. They then repeated the process with a bag containing 30 mg hydroxytyrosol mixed into 3 g strawberry jam and with a bag containing 30 mg oleuropein-glycoside mixed into 3 g strawberry jam. The contents of each ileostomy bag were then stored and analysed as described below.

We could not study the *in vitro* stability of the various aglycones, because these were not available in the pure form.

## Analytical methods

### Tyrosol and hydroxytyrosol in ileostomy effluent.

We extracted 0.25-1.0 g of lyophilised effluent with 50 mL dimethylformamide (DMF), dispersed the suspension with a Polytron mixer (model PT10/35, Kinematica AG, Lucerne, Switzerland) at

medium speed for 1 min, and equilibrated it for 30 min. The suspension was shaken after 15 and 30 min, and then centrifuged at 3000 rpm (1580 x *g*) for 10 min. We silylated 0.25 mL of the supernatant with 0.25 mL of *N,O-bis*(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane, heated the extract at 70°C for 45 min, and splitlessly injected 1 µl of the effluent extract on a GC-MS apparatus (GC model 5890, MSD 5971, Hewlett-Packard GmbH, Waldbronn, Germany) that was equipped with a 25 m x 0.25 mm CP-SIL5-CB low bleed MS column (Chrompak Internation B.V., Middelburg, The Netherlands). We used helium as carrier gas at a flow rate of 20 cm/sec. We used the same oven conditions, temperatures of injection port, transfer line and detector as described by Bai *et al* (1998), except that the final oven temperature of the program was extended to 27 min. We applied selected ion monitoring for quantification of phenols and recorded target ions at 179.1 *m/z* for tyrosol and at 267.1 *m/z* for hydroxytyrosol. Peaks were identified based on retention times and qualifier ions that were recorded at 282.2 *m/z* for tyrosol and at 370.1 *m/z* for hydroxytyrosol. We obtained calibration curves by 6 injections of different concentrations of 2 standards before and after the samples, and we performed calculations by the external standard method. We carried out all determinations in duplicate. The detection limits were 10.1 nmol for tyrosol and 7.1 nmol for hydroxytyrosol per g lyophilised ileostomy effluent. Addition of 38.5 µg tyrosol and 37.0 µg hydroxytyrosol per g ileostomy effluent yielded a recovery of 114.4 ± 12.8% for tyrosol and 115.6 ± 9.2% for hydroxytyrosol.

**Oleuropein-glycoside and ligstroside-aglycone derivative in ileostomy effluent.**

We dissolved 0.5 g of lyophilised ileostomy effluent in 9.5 mL water/methanol (40:60, v/v) and extracted it at 75°C under nitrogen for 1 h. We sonicated the suspension for 5 min, shook it with 2 mL hexane, and centrifuged it for 10 min (4500 x *g*, 7°C). Hexane was removed and the water-phase was filtered through a 0.45 µm filter. We injected 25 µl of the water-phase onto an HPLC with an Inertsil ODS-3 (GL Sciences Inc, Tokyo) column (4.6 x 250 mm, 5 µm particle size) using a gradient of 2% acetic acid in water and methanol as mobile phase, at a flow rate of 1 mL/min. We measured the phenolic compounds at 280 nm with a Waters 996 diode-array detector (Waters, Milford, MA) semi-quantitatively, using the peak height/mg supplement for quantification.

We quantified oleuropein-glycoside in the ileostomy effluent after subjects had ingested the oleuropein-glycoside supplement. Furthermore, we quantified only one ligstroside-aglycone derivative in the ileostomy effluent after subjects had ingested the non-polar supplement (**Figure 6.2A**, #6). In order to determine the detection limit we added various doses of the supplements as consumed by subjects to blank ileostomy effluent without olive oil phenols. We estimated from these measurements that the detection limit in ileostomy effluent of the oleuropein-glycoside and the ligstroside-aglycone derivative corresponded with about 25% of their amount ingested. This corresponded with 48 µmol/24 h for the oleuropein-glycoside and 45 µmol/24 h for the ligstroside-aglycone derivative. The detection limits of other aglycones and derivatives in the ileostomy effluent was higher than 100% of their amount ingested. We

therefore could not quantify those other aglycones and derivatives in ileostomy effluent after subjects had consumed the non-polar and polar supplements. Two additional larger peaks in the non-polar supplement could not be identified (**Figure 6.2A**) and were therefore not taken into account. We measured every ileostomy sample with and without the addition of the non-polar supplement, the polar supplement, or the oleuropein-glycoside supplement in order to compare the time of the peaks found in the effluent with those present in the supplement. Addition of 110 mg non-polar supplement per gram lyophilised ileostomy effluent yielded a recovery of 58 ± 9%. Addition of 15 mg oleuropein-glycoside supplement per g lyophilised ileostomy effluent yielded a recovery of 86 ± 21%. We present the estimated apparent absorption with and without correction for these analytical losses.

**Tyrosol and hydroxytyrosol in urine**

We added 3 mg of β-glucuronidase dissolved in 200 µl phosphate buffer pH 5 (Sigma, St. Louis, MO, USA) to 1 mL of urine, and incubated the mixture at 37°C for 24 h. Subsequently we added 1 µg/mL of α-naphthol as internal standard and extracted the urine twice with ethyl acetate. The organic phase was evaporated to dryness under nitrogen. We dissolved the residue in a mixture of dimethylformamide and *N,O-bis*(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane and heated it for 45 min at 70°C. We performed GC-MS analysis and quantification of the phenols in the same way as for the ileostomy samples. The target and qualifier ions for α-naphthol were recorded at 216.0 *m/z* and 200.95 *m/z*, respectively. We carried out all determinations in duplicate. The detection limit, i.e. the concentration producing a peak height 3 times the standard deviation of the baseline noise, was 0.04 µmol/L for tyrosol and 0.05 µmol/L for hydroxytyrosol. Addition of 0.15 µg and 1.6 µg tyrosol per mL urine yielded a recovery of 100 ± 6% and 99 ± 4%, respectively. Addition of 0.25 µg and 2.5 µg hydroxytyrosol per mL urine yielded a recovery of 103 ± 17% and 105 ± 8%, respectively.

Oleuropein-glycoside and the ligstroside-aglycone were not analysed in urine.

## RESULTS

### Composition of the supplements

Chromatograms of the phenol composition of the non-polar and polar supplements are presented in **Figure 6.2A and 6.2B**. The main phenol in the non-polar supplement was the ligstroside-aglycone derivative (#6) whereas the main phenols in the polar supplement were hydroxytyrosol (#1), tyrosol (#2), and an oleuropein-aglycone derivative (#3). Oleuropein-glycoside was the only phenol present in the oleuropein-glycoside supplement (data not shown).

## Stability of phenols

Olive oil phenols appeared stable in gastric juice and in duodenal fluid. Tyrosol and oleuropein-glycoside were also reasonably stable in ileostomy effluent: Incubation of these phenols with ileostomy effluent for 2 h yielded a mean recovery of 76% for tyrosol 81% for oleuropein-glycoside. Mean recovery for hydroxytyrosol added to ileostomy effluent was only 51%: 29% for one subject and 72% for the other (**Table 6.2**).

**Table 6.2** Percentage of tyrosol, hydroxytyrosol, and oleuropein-glycoside recovered after incubation with human gastric juice or duodenal fluid *in vitro*, or with ileostomy effluent *ex vivo*.

| | Gastric juice[a] | | Duodenal fluid[a] | | Ileostomy effluent[b] |
|---|---|---|---|---|---|
| Supplement | 0.5 h | 2 h | 1 h | 4 h | 2 h |
| | | | (%) | | |
| Tyrosol | 113 | 128 | 99 | 95 | 76 (76-77) |
| Hydroxytyrosol | 96 | 112 | 89 | 79 | 51 (29-72) |
| Oleuropein-glycoside | 99 | 98 | 101 | 98 | 81 (79-82) |

[a] Mean of triplicate analyses

[b] Mean (range) of recoveries in ileostomy bags on the bodies of two subjects. Analytical recovery after addition to lyophilized effluents was 114% for tyrosol, 116% for hydroxytyrosol, and 86% for oleuropein-glycoside (see Analytical methods).

## Excretion of phenols in ileostomy effluent

Excretion of tyrosol and hydroxytyrosol in ileostomy effluent was low. It was highest on the non-polar supplement rich in aglycones, but it was always less then 4 mol% of total phenol intake (**Table 6.3**).

The ileostomy effluent of subjects who had ingested the oleuropein-glycoside supplement contained a small amount of material with the retention time of oleuropein-glycoside (**Figure 6.3**). The ileostomy effluent of subjects who had consumed the non-polar supplement contained a minor amount of a compound with the retention time of the major component of this supplement, the ligstroside-aglycone derivative #6 (**Figure 6.4**). However, the size of both these peaks was less than 25% of the size of the peaks seen when one daily dose of oleuropein-glycoside or non-polar supplement was mixed into blank ileostomy effluent and analysed. Such *in vitro* mixing studies showed that the detection limit for oleuropein-glycoside and for the ligstroside-aglycone derivative #6 was about 25% of the administered daily dose per 24-h collection of ileostomy fluid (data not shown). This means that the amount excreted in the ileostomy effluent was less than could be reliably identified and quantified. We therefore assumed that 25% of the ingested dose was the maximum amount present in the ileostomy effluent. Thus, maximally 48 µmol oleuropein-glycoside was present in the ileostomy effluent after subjects had ingested the oleuropein supplement, and maximally 45 µmol ligstroside-

aglycone derivative #6 was present after subjects had ingested the non-polar supplement (**Table 6.3**).

We could not detect other aglycones and derivatives in ileostomy effluent after subjects had consumed the non-polar and polar supplements, but these peaks were also not detectable reliably if a full dose of supplement was mixed into blank ileostomy fluid. We therefore assumed that the excretion of these non-quantifiable aglycones in the ileostomy effluent was at most equal to the amount ingested (**Table 6.3**).

Under these assumptions at least 55 to 66 mol% of the phenols from the non-polar supplement had been absorbed; 55 mol% if we corrected for the analytical loss of the phenols plus their degradation within the ileostomy bag, and 66 mol% if we did not. Apparent absorption was somewhat higher for the polar supplement and for oleuropein-glycoside (**Table 6.3**).

## Excretion of phenols in urine

The low excretion of olive oil phenols into ileostomy effluent suggested that a large proportion was absorbed. We therefore expected to find tyrosol and hydroxytyrosol in urine, as tyrosol and hydroxytyrosol were major components of the polar supplement, and likely metabolites of the aglycones administered (**Figure 6.1**). Indeed, both subject groups excreted 5-6 mol% of the phenols from the polar supplement into urine in the form of tyrosol or hydroxytyrosol. Ileostomy subjects excreted 12 mol% of the phenols from the non-polar supplement into urine in the form of tyrosol or hydroxytyrosol. For subjects with a colon this figure was 6 mol% (**Table 6.4, Figure 6.5**). Sixteen mol% of oleuropein-glycoside administered to ileostomy subjects was recovered in urine, largely in the form of hydroxytyrosol (**Table 6.4, Figure 6.5**). Thus, 5 to 16 mol% of total phenols ingested was found back in urine in the form of tyrosol or hydroxytyrosol. Oleuropein- and ligstroside aglycones were not measured. Therefore, we do not know how much of these phenols was excreted unchanged with the urine.

The placebo supplement did not contain olive oil phenols. Nevertheless, the urine of one subject contained 12.2 µmol/24 h of tyrosol and 3.6 µmol/24 h of hydroxytyrosol. As a consequence, the mean total excretion in urine after the placebo supplement was 2.6 µmol/24 h. Excluding this subject from analysis resulted in mean excretion of 0.43 ± 0.92 µmol/24 h for tyrosol and 0.97 ± 0.97 µmol/24 h for hydroxytyrosol. This subject reported that he had not eaten olives, olive oil or olive oil products in the days before ingestion of the placebo supplement. One explanation might be that tyrosol and hydroxytyrosol are not unique to olives and olive oil.

**Table 6.3** Intake of tyrosol, hydroxytyrosol, aglycones and oleuropein-glycoside from the 3 supplements consumed by ileostomy subjects ($n = 8$) and subsequent mean excretion in ileostomy effluent over 24 h.

| Supplement | Intake (μmol) | Excretion[a] (μmol) | | | | | | Total apparent absorption[b] (μmol) | Total apparent absorption as proportion of intake[b] (mol%) |
|---|---|---|---|---|---|---|---|---|---|
| | | tyrosol | hydroxy-tyrosol | ligstroside-aglycones | oleuropein-aglycones | oleuropein-glycoside | Total excretion | | |
| Non-polar supplement | 371 | 5.6 | 4.4 | <73[c] | <44[d] | n.a.[e] | <127 | >205 | >55 - 66 |
| Polar supplement | 498 | 1.4 | 1.8 | <29[d] | <121[d] | n.a.[e] | <153 | >343 | >68 - 69 |
| Oleuropein-glycoside | 190 | 1.0 | 2.2 | n.a. | n.a. | <48[f] | <125 | >125 | >66 - 73 |

[a] Mean ± SD, values include the final but not the prebreakfast sample. Mean excretion in prebreakfast samples was <0.3 μmol.

[b] We estimated the total apparent absorption with and without the correction for analytical loss plus degradation within the ileostomy bag. Both estimates are presented: the lowest percentage is corrected and the highest value is not corrected for these losses. Total losses were 24% for tyrosol, 49% for hydroxytyrosol, and 19% for oleuropein-glycoside (**Table 6.2**). We do not know the degradation of the quantified ligstroside-aglycone #6 within the ileostomy bag. We therefore only corrected for analytical loss which was 42% (see Analytical method).

[c] We only quantified the ligstroside-aglycone derivative #6. Because the amount of this ligstroside-aglycone was below detection limit, we estimated that maximally 25% of the dose ingested was present in ileostomy effluent, which is 25% of 180 μmol (**Table 6.1** footnote 3) = 45 μmol. We assumed that the excretion of the non-quantifiable aglycones of #11 and #12 in the ileostomy effluent was equal to the amount ingested, which was 28 μmol (**Table 6.1**, footnote 3). Thus, the amount of ligstroside-aglycones in ileostomy effluent after intake of the non-polar supplement is minimally 45 + 28 = 73 μmol.

[d] We could not quantify these aglycones in ileostomy effluent after subjects ingested the non-polar and polar supplement because of their low dose in the supplement. We postulated the maximum excretion of these non-analysed aglycones in the ileostomy effluent as 100% of the amount ingested.

[e] Oleuropein-glycoside was not present in the supplement and therefore not analysed in these ileostomy samples.

[f] Because the amount oleuropein-glycoside was below detection limit, we estimated that maximally 25% of the dose ingested was present in ileostomy effluent, which is 25% of 190 μmol = 48 μmol oleuropein-glycoside.

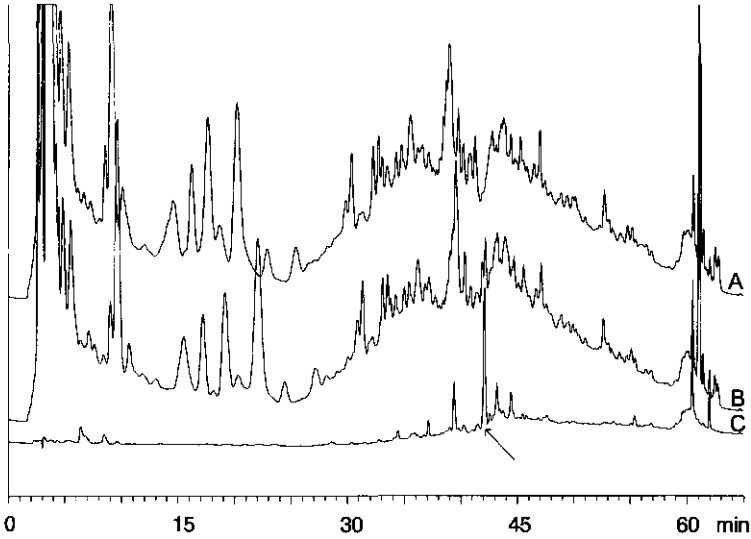


**Figure 6.3** HPLC chromatogram of ileostomy effluent after subjects ingested the oleuropein-glycoside supplement. Ileostomy effluent was analysed with (B) and without the addition of the oleuropein-glycoside supplement (A). We added 50% of the amount of ingested supplement to the total amount of lyophilised ileostomy effluent excreted during 24 h. Line C is the chromatogram of the supplement. The arrow indicates oleuropein-glycoside.

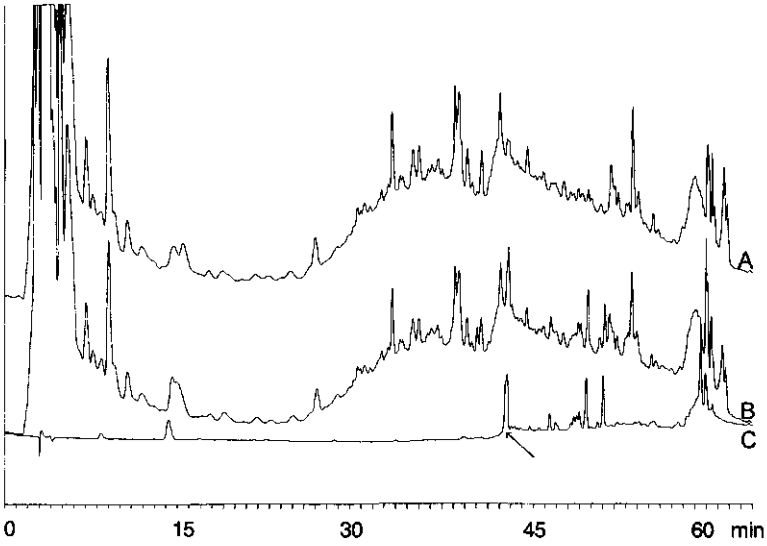


**Figure 6.4** HPLC chromatogram of ileostomy effluent after subjects ingested the non-polar supplement. Ileostomy effluent was analysed with (B) and without the addition of the non-polar supplement (A). We added 50% of the amount of ingested supplement to the total amount of lyophilised ileostomy effluent excreted during 24 h. Line C is the chromatogram of the supplement. The arrow indicates the ligstroside-aglycone derivative #6.

**Table 6.4** Intake of total phenols from the 4 supplements consumed by ileostomy subjects ($n = 8$) and/or subjects with a colon ($n = 12$) and subsequent mean excretion of tyrosol and hydroxytyrosol in urine over 24 h.

| Supplement | Intake (μmol) | Urinary excretion[a] (μmol) | | | Total excretion as proportion of intake (%) |
|---|---|---|---|---|---|
| | | tyrosol | hydroxytyrosol | sum | |
| Non-polar supplement | | | | | |
| ileostomy subjects | 371.4 | 33.5 ± 13.9 | 10.8 ± 4.5 | 44.3 | 12 |
| subjects with a colon | 382.0 | 15.5 ± 4.6 | 6.0 ± 1.7 | 21.5 | 6 |
| Polar supplement | | | | | |
| ileostomy subjects | 497.9 | 4.1 ± 3.1 | 24.7 ± 10.9 | 28.8 | 6 |
| subjects with a colon | 526.4 | 5.6 ± 8.8 | 21.6 ± 4.8 | 27.2 | 5 |
| Oleuropein-glycoside[b] | 190.0 | 1.3 ± 1.5 | 28.4 ± 8.7 | 29.7 | 16 |
| Placebo supplement[c] | 0.0 | 1.4 ± 3.5 | 1.2 ± 1.2 | 2.6 | - |

[a] Mean ± SD, values include the final but not the prebreakfast sample. Mean excretion in prebreakfast samples was <0.4 μmol

[b] Only consumed by ileostomy subjects

[c] Only consumed by subjects with a colon

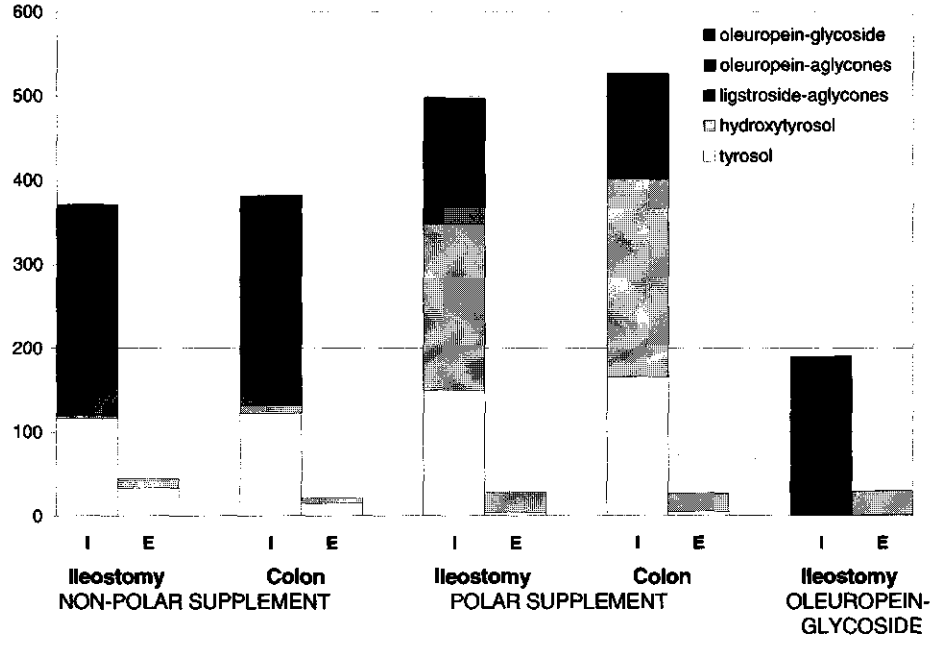


**Figure 6.5** Intake of total phenols from the 3 various supplements consumed by ileostomy subjects ($n = 8$) and/or subjects with a colon ($n = 12$) and subsequent mean excretion of tyrosol, hydroxytyrosol in urine over 24 h. I: intake; E: excretion.

## DISCUSSION

We estimate that at least 55-66 mol% of ingested olive oil phenols is absorbed in our volunteers and that 5-16 mol% is re-excreted as tyrosol and hydroxytyrosol in urine. Thus, only a fraction of ingested olive oil phenols is recovered in urine. However, we did not analyse urinary excretion of the other components of the supplements, namely oleuropein-glycoside, oleuropein- or ligstroside-aglycones. Furthermore, phenols are probably metabolised into other compounds after absorption (Visioli *et al*, 2000b; Williamson *et al*, 2000; Hollman & Katan, 1998). Therefore the figure of 5-16 mol% recovered in urine as tyrosol or hydroxytyrosol supports our finding that humans absorb a major part of the dietary olive oil phenols that they eat.

### Metabolism of phenols

To study the absorption and metabolism of tyrosol and hydroxytyrosol we supplied subjects with phenol-rich supplements prepared from olive oil. A disadvantage of this mixture of phenols is that individual phenols might be converted into hydrolysis products or other metabolites, which is hard to estimate. Therefore, we would have preferred to use pure tyrosol or hydroxytyrosol, but there are not such substances available that are food grade. Pure oleuropein- and ligstroside-aglycones are not commercially available at all. We therefore could not study the absorption of the individual phenols.

We estimated that >55 mol% of ingested olive oil phenols were absorbed in ileostomy subjects, which implies that most, if not all, phenols are absorbed in the small intestine. Subjects with a colon might theoretically also absorb phenols from the colon (Williamson *et al*, 2000; Hollman & Katan, 1998). If this is the case then the urinary excretion of tyrosol and hydroxytyrosol should be increased. However, we found similar or lower levels of tyrosol and hydroxytyrosol in urine of subjects with a colon than subjects without a colon, which confirms that olive phenols are absorbed mainly in the small intestine, rather than in the colon.

An important step in metabolism of olive oil phenols in the body might be that oleuropein-glycoside and oleuropein- and ligstroside-aglycones are split into hydroxytyrosol or tyrosol and elenolic acid (**Figure 6.6**). This was indicated by our finding that 15 mol% of the pure oleuropein-glycoside supplement was excreted in urine as tyrosol and hydroxytyrosol. Oleuropein-glycoside and oleuropein- and ligstroside-aglycone might be split either in the gastrointestinal tract before they are absorbed, or in the intestinal cell, in blood, or in the liver after they are absorbed. Although we did not measure the stability of oleuropein- and ligstroside-aglycones or their derivatives in gastric or duodenal fluid, the stability of oleuropein-glycoside in these fluids (**Table 6.2**) suggests that oleuropein-glycoside, oleuropein- and ligstroside-aglycone are mainly split after they have been absorbed. However, it is possible that the *in vivo* situation is different.

Another important step in metabolism is conjugation to glucuronic acid because tyrosol and hydroxytyrosol are mainly excreted as glucuronides. Glucuronidation possibly takes place in the

enterocytes and in liver cells (**Figure 6.6**) (Scheline 1978; Donovan *et al*, 2001; Spencer *et al*, 1999). Our analysis method did not distinguish between free and conjugated phenols, but other studies showed that about 90% of tyrosol and hydroxytyrosol is excreted in the conjugated form (Visioli *et al*, 2000a; Miro-Casas *et al*, 2001a; Miro-Casas *et al*, 2001b). One more possible metabolic step of dihydroxy-phenols is *O*-methylation, which mainly takes place in the liver (**Figure 6.6**) (Scheline 1978). Recently it was shown that *O*-methylated hydroxytrosol is an important metabolite in urine after intake of olive oil phenols (Manna *et al*, 2000; Visioli *et al*, 2000b; Miro-Casas *et al*, 2001a). Thus, absorbed olive oil phenols are probably extensively modified before the kidneys excrete them.

Our assay could detect tyrosol and hydroxytyrosol only in the free form or conjugated to glucuronic acid, but not as *O*-methylated hydroxytyrosol or other metabolites. It is also possible that part of the ingested phenols remained undetected in urine as aglycones or oleuropein-glycoside. Thus the urine of our subjects probably contained other olive oil phenols and their metabolites which we could not detect.

### Comparisons with previous studies

A study of Visioli *et al* (2000a) showed that when humans ingested a single dose of 50 mL of phenol-rich olive oil they excreted 20-60% of the tyrosol and hydroxytyrosol in that dose into their urine. This is much higher than what we found. However, Visioli *et al* calculated the recovery of tyrosol and hydroxytyrosol in urine as the percentage of tyrosol and hydroxytyrosol intake, but did not take into account the possible hydrolysis of oleuropein- and ligstroside-aglycones in the body. The recovery of 20-60% found in the study of Visioli *et al* (2000) may therefore be an overestimate. Miro-Casas *et al* also found high recoveries: 72% of hydroxytyrosol and oleuropein-like substances were recovered as hydroxytyrosol and 34% of tyrosol and ligstroside-like substances was excreted as tyrosol in urine of 8 subjects (Miro-Casas *et al*, 2001a). A reason for their high recovery might be that they hydrolysed conjugated hydroxytyrosol in tyrosol with HCL instead of with β-glucuronidase as in our study. They could therefore not provide specific information about the type of conjugate (Miro-Casas *et al*, 2001a). It is possible that other types of conjugates were present in urine, or that the ingested aglycones were excreted as well.

We are not aware of human studies on the absorption of oleuropein-glycoside or oleuropein- and ligstroside-aglycones. In a study with an isolated perfused rat intestine, Edgecombe *et al* (2000) found that oleuropeine-glycoside was poorly absorbed from an aqueous solution. However, the validity of this model for the human *in vivo* situation is unclear, and orally ingested oleuropein-glycoside in an oily matrix might be absorbed better (Edgecombe *et al*, 2000).

### Validity of the ileostomy model

In our study subjects collected ileostomy effluent for 24 h. This period should have been long enough to collect all non-absorbed phenols in ileostomy effluent, because transit time of

ingested food through the stomach and small intestine is approximately 8-16 h (Fallingborg *et al*, 1990; Goldberg *et al*, 1996). This is also supported by the amounts of phenols found in ileostomy effluent and urine after 24 h; these amounts were similar to those before supplement intake. Thus, it was probably long enough to collect ileostomy effluent for 24 h.

It is unlikely that high amounts of tyrosol or oleuropein-glycosides were degraded in the ileostomy bag or during analysis in the laboratory. *In vitro* tests with added tyrosol and oleuropein-glycoside showed that these substances are fairly stable in ileostomy effluent; recovery over 2 h was 76-81%. Stability of hydroxytyrosol in ileostomy effluent was more variable, 72% for one person and 29% for the other (**Table 6.2**). Conceivably, the vehicle for hydroxytyrosol was not completely mixed with ileostomy effluent in the bag. Nevertheless, it is possible that hydroxytyrosol is unstable in the ileostomy bag or during sample preparation. Therefore we corrected the estimated absorption for these possible losses in the ileostomy bag or during analysis (**Table 6.3**).

We also assessed possible losses of phenols during gastrointestinal transit by *in vitro* incubation of these substances in gastric juice and duodenal fluid. De Roos *et al* showed that 24-32% of coffee diterpenes, which are other plant components, were lost during incubation with gastric juice *in vitro*. This decrease could not be fully explained by low pH, which indicates that this *in vitro* test might be useful to study the stability of compounds in the gastrointestinal tract (De Roos *et al*, 1998). Our *in vitro* tests showed that oleuropein-glycoside, tyrosol, and hydroxytyrosol are stable compounds and that they are hardly degraded in the gastrointestinal fluids.

## Mechanism of absorption

The mechanism of absorption of olive oil phenols is unclear. Different polarity of oleuropein-glycoside, oleuropein- and ligstroside-aglycones, and tyrosol and hydroxytyrosol probably results in different mechanisms of absorption. Tyrosol and hydroxytyrosol are polar compounds and their transport might occur via passive diffusion (Manna *et al*, 2000). However, it seems less likely that the also polar but much larger oleuropein-glycoside readily diffuses through the lipid bilayer of the epithelial cell membrane. This glycoside is more likely absorbed via a glucose transporter (Edgecombe *et al*, 2000), which is supported by the studies of Hollman *et al* (1995, 1999), who found significant absorption of quercetin glycoside, another phenolic compound, in humans. Another possible mechanisms of absorption of oleuropein-glycoside is via the paracellular passive diffusion (Edgecombe *et al*, 2000). Oleuropeine- and ligstroside-aglycones are less polar compounds, and at present no data are available on their mechanism of absorption. Furthermore, even though our *in vitro* studies with gastric juice and duodenal fluid do not suggest it, it is possible that oleuropein-glycosides, oleuropein- and ligstroside-aglycones are hydrolysed in the gastrointestinal tract. Then tyrosol and hydroxytyrosol are absorbed instead of the whole molecule oleuropein-glycoside or the aglycones. Thus, the mechanism of absorption is not clear for oleuropein- and ligstroside-like substances.

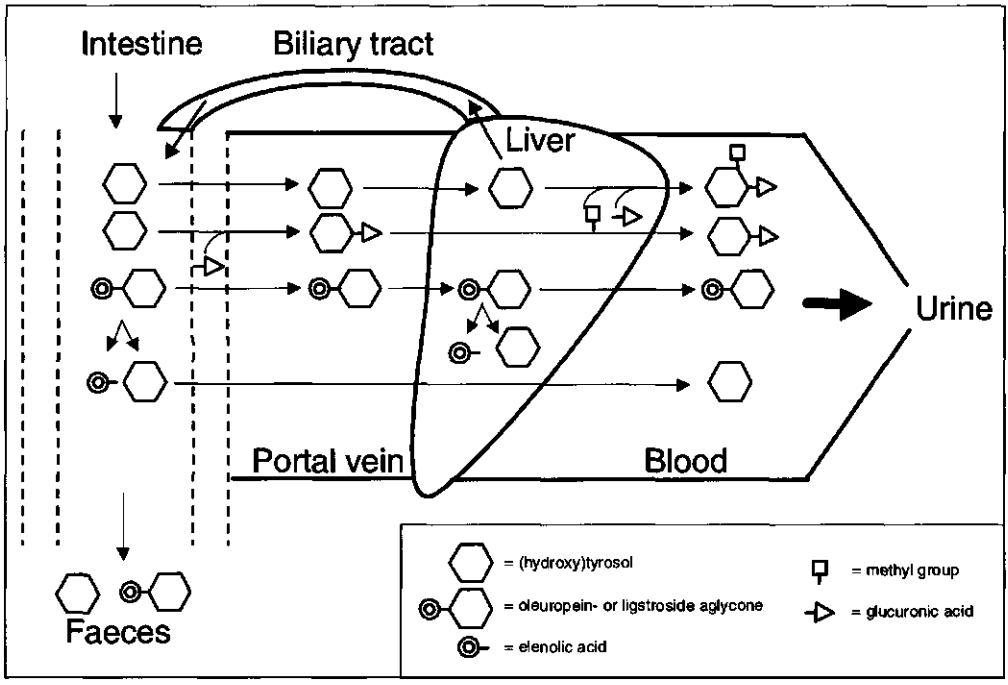


**Figure 6.6** Schematic presentation of the possible metabolism of olive oil phenols. Olive oil phenols are probably absorbed >66 mol%. They can be glucuronidated in the enterocyte as well as in the liver. *O*-methylation of hydroxytyrosol is another important metabolic step, which also takes place in the liver by *catechol-O-methyltransferase. Probably the aglycones are hydrolysed in the body, but is it not known whether* this takes place in the intestine before absorption or in the circulation after absorption. It is also not known whether the aglycones are excreted in urine.

## Conclusion

We found that humans absorb a large part of the ingested olive oil phenols, mainly in the small intestine. The human body seems able to hydrolyze oleuropein- and ligstroside-aglycones into hydroxytyrosol and tyrosol and to metabolise these phenols extensively, probably after absorption from the small intestine.

## ACKNOWLEDGEMENTS

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# Bioavailability and antioxidant effects of olive oil phenols in humans: a review

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*Submitted for publication*

## ABSTRACT

**Objective:** To review the bioavailability and antioxidant effects of phenols from extra virgin olive oil.

**Search Strategy:** We searched the MEDLINE database for the years 1966-2001. To review the bioavailability of olive oil phenols, we selected *in vitro*, animal, and human studies that studied the absorption, metabolism and urinary excretion of olive oil phenols. We also estimated the intake of the various phenols in the Mediterranean area.

To review the antioxidant effects of olive oil phenols, we included human and animal studies on the effect of olive oil phenols on markers of oxidative processes in the body. We excluded studies without a proper control treatment and studies in which the antioxidant effects of phenols could not be disentangled from those of the fatty acid composition of olive oil.

**Results:** Bioavailability studies in humans show that the absorption of olive oil phenols is probably larger than 55-66 mol%, and that at least 5% is excreted in urine as tyrosol and hydroxytyrosol.

Animal studies suggest that phenol-rich olive oil lowers oxidisability of *ex vivo* LDL particles or lowers markers in urine of oxidative processes in the body. In 5 out of 7 human studies, however, these effects of phenols were not found.

There are no data on the phenol concentrations in plasma that are attainable by intake of olive oil. We estimate that 50 g of olive oil per day provides about 2 mg or ~13 µmol of hydroxytyrosol-equivalents per day, and that the plasma concentration of olive oil phenols with antioxidant potential resulting from such an intake can be at most 0.06 µmol/L. This is much lower than the minimum concentrations of these phenols (50-100 µmol/L) required to show antioxidant activity *in vitro*.

**Conclusion:** Although phenols from olive oil seem to be well absorbed, the content of olive oil phenols with antioxidant potential in the Mediterranean diet is probably too low to produce a measurable effect on LDL oxidisability or other oxidation markers in humans. The available evidence does not suggest that consumption of phenols in the amounts provided by dietary olive oil will protect LDL against oxidative modification to any important extent.

## INTRODUCTION

Oxidation of Low Density Lipoproteins (LDL) is hypothesised to play an important role in the development of atherosclerosis, an underlying factor of cardiovascular diseases. LDL oxidation might be prevented or reduced by intake of antioxidants such as vitamin E. Other compounds with potential antioxidant effect are dietary phenols. Phenols are compounds with an aromatic ring structure with one or more hydroxyl groups. Phenols with two or more hydroxyl groups show antioxidant capacity *in vitro*, whereas phenols with one hydroxyl group have little or none (*chapter 5*; Rice-Evans *et al*, 1996). Extra virgin olive oil contains phenols with either one or two hydroxyl groups (**Figure 1.3**, page 16). Today olive oil is marketed as being healthier than other vegetable oils because of the presence of these phenols, but a pertinent question is whether this suggestion or claim is correct?

This article reviews the evidences from human and animal studies on the potential of olive oil phenols to protect LDL against oxidation. Three important questions in this context are: 1) Can olive oil phenols affect oxidative processes in the human body?; 2) How much of the phenolic compounds from olive oil are absorbed in the human body?; and 3) How are they subsequently metabolised? We first describe the possible effects of olive oil on coronary heart disease, the chemistry of the olive oil phenols, and their estimated intake.

### Olive oil and the risk of coronary heart disease

Keys and coworkers showed that in the period between 1960 and 1975 men in Southern European countries such as Italy, Greece, and Yugoslavia had a much lower incidence of coronary heart disease than men in Northern Europe. These differences between countries could be largely explained by differences in the ratio of monounsaturated to saturated fatty acids in the diet (Keys *et al*, 1986). This suggested that in particular the type of dietary fat in the Mediterranean area, mainly olive oil, protects against coronary heart disease, because olive oil has a high ratio of monounsaturated to saturated fatty acids.

Controlled dietary trials in humans have showed that replacement of dietary saturated fatty acids with monounsaturated oleic acid (C18:1n-9) from olive oil decreases plasma LDL concentrations, which presumably contributes to the low incidence of coronary heart diseases (Katan *et al*, 1995). It has also been suggested that a high-monounsaturated fat diet lowers the risk of coronary heart disease by producing LDL particles that are enriched in oleic acid at the expense of linoleic acid (C18:2n-6). Such a change in fatty acid composition renders LDL particles more resistant to oxidative modification (Reaven *et al*, 1991; Bonanome *et al*, 1992; Berry *et al*, 1992; Mata *et al*, 1997).

Oleic acid, however, may not be the only component of olive oil protecting LDL from oxidation; in particular the phenols in extra virgin olive oil could be effective antioxidants. The oxidative modification hypothesis of atherosclerosis states that LDL particles are oxidatively modified and then taken up by macrophages inside the arterial wall. Dietary antioxidants might inhibit atherogenesis by inhibiting oxidation of LDL and accumulation of LDL in macrophages

(Witztum & Steinberg, 1991). When olive oil is ingested, the phenols might dissolve into or attach to LDL particles in plasma, where they may prevent LDL from oxidation.

## Chemistry and content of phenols in olive oil

The types of phenols in extra virgin olive oil are different from those of the olive fruit. The olives mainly contain the polar glycosides oleuropeine and ligstroside. Oleuropein is the ester of elenolic acid with 3,4'-dihydroxyphenylethanol (hydroxytyrosol), and ligstroside is the ester of elenolic acid with 4-hydroxyphenylethanol (tyrosol). Oleuropein and ligstroside are the parent compounds of the less polar oleuropein- and ligstroside-aglycones. Oleuropein- and ligstroside-aglycones are formed by removal of the glucose moiety from the oleuropein- and ligstroside-glycoside by β-glucosidase during ripening. Those aglycones and their various derivatives are the most abundant phenols in olive oil. The derivatives differ mainly in their ring structure, which can either be open or closed in two different forms (unpublished data, personal communication from Dr. S. van Boom). The polar compounds hydroxytyrosol and tyrosol are the end products of hydrolysis of oleuropeine- and ligstroside-aglycone or their derivatives in olives and olive oil (**Figure 1.3** page 16).

The concentration of phenols in olive oil varies from 50 to 800 mg/kg (Visioli & Galli, 1995), with a mean value for commercial olive oils of approximately 180 mg/kg (unpublished data, personal communications from Dr. S. van Boom). The phenol concentration in olive oil depends on variety, climate, area of growth, latitude, and ripeness of the olive. The phenols, and in particular the *ortho*-diphenols, have been demonstrated to contribute considerably to the oxidation stability of the oil (Gutfinger 1981; Papadopoulos & Boskou, 1991; Visioli & Galli, 1998; Rice-Evans *et al*, 1996). *Ortho*-diphenols are the phenols with two adjacent hydroxyl groups to the ring structure: hydroxytyrosol and oleuropein and its derivatives (**Figure 1.3**, page 16).

## Intake of phenols from olive oil

Intake of olive oil in the Mediterranean countries is estimated to be 30-50 g/d, based on the *per capita* disappearance of 10-20 kg of olive oil per year in Greece, Italy, and Spain (Helsing 1995; Boskou 2000; Food and Agricultural Organization 2000). A daily consumption of 50 g olive oil with a concentration of 180 mg/kg of phenols results in an estimated intake of about 9 mg of olive oil phenols per day. This is similar to the intake of flavonols and catechins from apples (about 10 mg/d), but lower than that of catechins from tea (50 mg/d) (Arts *et al*, 2001) or phenolic acids from coffee (200 mg/d) (Radtke *et al*, 1998). However, it is more relevant to express the amount of dietary antioxidants in moles rather than milligrams, because the antioxidant activity depends on the number of reactive OH groups. In six Greek olive oils, which were specifically analysed to measure the phenol content by HPLC, tyrosol and hydroxytyrosol was comprised on average 10 weight% (range 5-16%) and aglycones 90 weight% (range 84-96%) (unpublished data, personal communication from Dr. S. van Boom). Based on these figures and assuming a phenol intake of 9 mg/d in Mediterranean countries, we can estimate

that about 1 mg (6 µmol) is derived from hydroxytyrosol and tyrosol and about 8 mg (23 µmol) from the aglycones. Then, total phenol intake in the Mediterranean area is about 29 µmol. However, this does not yet represent the amount of effective olive oil phenols. Part of the phenols in olive oil are ligstroside-aglycones and tyrosol, which are *mono*-phenols (figure 1) with little or no antioxidant capacity (Rice-Evans *et al*, 1996; *chapter 5*). Of the 6 analysed Greek olive oils, the mean percentage of diphenols was 44 mol% (range 39-51 mol%). Thus, the intake of phenols with antioxidant capacity is about 0.44 x 29 = 13 µmol, which is equivalent to 2 mg of hydroxytyrosol per day. Thus, if the 6 Greek oils are considered as representative for the Mediterranean area, then the intake in that area can be estimated to be 2 mg hydroxytyrosol-equivalents per day.

## METHODS

To identify studies on the health effects and metabolism of olive oil phenols we searched the MEDLINE database (National Library of Medicine, Bethesda, MD) for the years 1966-2001 using the following keywords: phenol*, polyphenol*, olive oil, tyrosol, hydroxytyrosol, oleuropein, antioxidant, oxidation, absorption, bioavailability, and metabolism. We also searched the ISI Web of Science Citation Databases for articles that cited two well-known publications on this topic (Visioli *et al*, 1995; Wiseman *et al*, 1996).

To address the bioavailability of olive oil phenols we reviewed animal and human studies on the absorption, metabolism and urinary excretion of olive oil phenols.

We selected human and animal intervention studies that examined the effect of consumption of olive oil phenols on oxidation markers in plasma. We excluded studies in which we could not disentangle the antioxidant effects of phenols from those of the fatty acid composition of olive oil, studies without a control diet, and studies in which the amount of ingested phenols was not reported or could not be estimated. Human studies were stratified according to measurement of oxidation markers in fasting versus postprandial blood.

We specifically extracted data on the lag time of LDL oxidisability and combined these in a random-effects model assuming heterogeneity (DerSimonian & Laird, 1986). To this end we extracted or estimated for each study the differences in lag time between the high and low phenol treatment, and the standard error of these differences. A model assuming equal sampling variances for each study, i.e., each study having equal weight, and a fixed-effects model assuming homogeneity, yielded similar results.

**Table 7.1A** Human studies on the antioxidant effect of olive oil phenols in fasting blood samples

| Treatment | Subjects (*n*) | Dose of phenols | Design | Oxidation marker | Result (high vs low phenol treatment) | Direction of phenol effect[a] | Ref. |
|---|---|---|---|---|---|---|---|
| Extra virgin olive oil versus oleic acid rich sunflower oil | 10 | 16 vs 0 mg/d[b] | 3 wk crossover | - lag phase of LDL oxidation (min)<br>- max. rate of LDL oxidation (μmol/min/g LDL protein)<br>- total conjugated diene formation (μmol/g LDL protein) | 59 vs 64<br>11 vs 11<br>483 vs 485 | 0/-<br>0<br>0 | (1) |
| Extra virgin versus refined olive oil | 24 | 33 vs 3 mg/d<br>Vit E:<br>12 vs 8 mg/d | 3 mo crossover | - rate of TBARS formation ((nmol TBARS/mg LDL protein)/(μmol $Cu^{2+}$/L))<br>- macrophage-uptake of oxidised LDL (% of LDL uptake by U937 macrophages) | 12 vs 15<br>35 vs 46 | +<br>+ | (2) |
| Extra virgin versus refined olive oil | 14 | tyrosol + hydroxytyrosol: 0.4 mg.d | 1 mo crossover | - lag phase of LDL oxidation (min)<br>- peroxidation rate (nmol $O_2$ uptake/min) | 40 vs 47<br>13 vs 15 | 0/-<br>0/+ | (3) |
| Phenol-rich versus phenols-poor extra virgin olive oil | 46 | 21 vs 3 mg/d | 3 wk crossover | - lag phase of oxidation (min)<br>  - in LDL<br>  - in HDL<br>- max. rate of oxidation (μmol/min/g LDL protein)<br>  - in LDL<br>  - in HDL<br>- malondialdelhyde (μmol/L)<br>- lipid hydroperoxides (μmol/L)<br>- protein carbonyls (nmol/mg protein)<br>- Ferric Reducing Ability of Plasma (mmol/L) | <br>109 vs 110<br>70 vs 69<br><br>12 vs 12<br>4.4 vs 4.6<br>0.7 vs 0.7<br>0.4 vs 0.4<br>0.2 vs 0.2<br>1.1 vs 1.1 | <br>0<br>0<br><br>0<br>0<br>0<br>0<br>0<br>0 | (4) |

**Table 7.1A (continued)** Human studies on the antioxidant effect of olive oil phenols in fasting blood samples

| Treatment | Subjects (*n*) | Dose of phenols | Design | Oxidation marker | Result (high vs low phenol treatment) | Direction of phenol effect[a] | Ref. |
|---|---|---|---|---|---|---|---|
| Phenol-rich versus phenols-poor extra virgin olive oil | 25 | 22 vs 3 mg/d | 3 wk crossover | - lag phase of plasma oxidation (min) | 113 vs 111 | 0 | (5) |
| | | | | - max. rate of plasma oxidation (µmol/min/g LDL protein) | 0.2 vs 0.2 | 0 | |
| | | | | - malondialdelhyde (µmol/L) | 0.6 vs 0.6 | 0 | |
| | | | | - lipid hydroperoxides (µmol/L) | 0.5 vs 0.7 | 0/+ | |
| | | | | - protein carbonyls (nmol/mg protein) | 0.2 vs 0.2 | 0 | |
| | | | | - Ferric Reducing Ability of Plasma (mmol/L) | 1.1 vs 1.1 | 0 | |

[a] 0 = no effect, + = protective effect, - = negative effect, 0/+ = no significant effect in the protective direction, 0/- = no significant effect in the negative direction.
[b] Intake of olive oil and phenols was estimated from the amount of MUFA in the diet and in the oil. We assumed that all MUFA in the diet was derived from olive oil. This estimate is therefore the maximum olive oil intake per day.
(1): (Nicolaiew *et al*, 1998); (2): (Ramirez-Tortosa *et al*, 1999); (3): (Bonanome *et al*, 2000); (4):(Vissers *et al*, 2001a); (5): Moschandreas *et al* (submitted).

**Table 7.1B** Human studies on the antioxidant effect of olive oil phenols in postprandial blood samples

| Treatment | Subjects (*n*) | Dose of phenols | Design | Oxidation marker | Result | Direction of phenol effect[a] | Ref. |
|---|---|---|---|---|---|---|---|
| Extra virgin olive oil versus oleic acid rich sunflower oil | 10 | 16 mg/d + 12 mg[b] on last day | 3 wk crossover, blood sampling 6 h after intake on last day | Δ compared to t=0 | high vs low[c] | | (1) |
| | | | | - lag phase of LDL oxidation (min) | 4 vs 3 | 0/+ | |
| | | | | - max. rate of LDL oxidation (μmol/min/g LDL protein) | -1 vs -1 | 0 | |
| | | | | - total conjugated diene formation (μmol/g LDL protein) | -30 vs -24 | 0/+ | |
| Olive oil with different amounts of phenolic extract | 6 | - 24 mg<br>- 49 mg<br>- 73 mg<br>- 98 mg | 4 single doses, 24 h urinary collection | urinary excretion of 8-isoprostaglandines $F_{2\alpha}$ | (pg/mg creatinine) | | (2) |
| | | | | - 24 mg | 273 | | |
| | | | | - 49 mg | 228 | | |
| | | | | - 73 mg | 180 | | |
| | | | | - 98 mg | 184 | + | |
| Olive oil with extracts high in polar or non-polar phenols versus olive oil low in phenols | 12 | 100 vs 0 mg | 3 single doses, blood sampling at 0, ½ and 2 h | Δ compared to t=0 | high vs low[d] | | (3) |
| | | | | - lag phase of LDL oxidation (min) | | | |
| | | | | - t=½ h | 4 vs 7 | 0/- | |
| | | | | - t=2 h | 4 vs 8 | 0/- | |
| | | | | - max. rate (μmol/min/g LDL protein) | | | |
| | | | | - t=½ h | -1 vs -1 | 0 | |
| | | | | - t=2 | 0 vs -2 | 0 | |

[a] 0 = no effect, + = protective effect, - = negative effect, 0/+ = no significant effect in the protective direction, 0/- = no significant effect in the negative direction.

[b] Intake of olive oil and phenols was estimated from the amount of MUFA in the diet and in the oil. We assumed that all MUFA in the diet was derived from olive oil. This estimate is therefore the maximum olive oil intake per day.

[c] In the postprandial study of Nicolaïew *et al* (1998) we subtracted the lag times of t=0 from the lag times at t=6, and then calculated the difference in lag time between consumption of the high and low phenol oil.

[d] In this study the effects of 3 various supplements were compared with each other: one containing mainly non-polar olive oil phenols; one containing mainly polar olive oil phenols; and one without phenols (placebo). The effects between the supplements that contained mainly non-polar or polar olive oil phenols did not significantly differ. We therefore here present the mean lag-time and maximum rate of LDL oxidation after consumption of the supplements high in non-polar and polar phenols compared to t=0.

(1): (Nicolaiew *et al*, 1998); (2): (Visioli *et al*, 2000a); (3): (Vissers *et al*, 2001b).

**Table 7.2** Animal studies on the antioxidant effect of olive oil phenols

| Treatment | Species (*n*) | Dose of phenols | Duration | Oxidation marker | Result (high vs low phenol oil) | Direction of phenol effect[a] | Ref. |
|---|---|---|---|---|---|---|---|
| Extra virgin olive oil versus refined olive oil and Trisun high oleic sunflower oil | rabbits (24) | 8.7 vs 0.1 vs 0 mg[b] | 6 wk consumption | - lag phase of LDL oxidation (min)<br>- max. rate (µmol/min/g LDL protein)<br>- plasma malondialdehyde (nmol/L) | 283 vs 218[c]<br>6.1 vs 6.4<br>610 vs 560[d] | +<br>0/+<br>- | (1) |
| Olive mill waste water extract dissolved in ethanol versus ethanol | rats (12) | hydroxytyrosol: 83 vs 0 µg | 6 d consumption + 4 d 20 min/d exposure to smoke 24-h urine collection on day 0, 2, 4 | Δ urinary excretion of 8-isoprostaglandines $F_{2\alpha}$ compared to t=0 (pg/mg creatinine)<br>- day 2<br>- day 4 | <br>-4 vs 82<br>84 vs 145 | <br>+<br>0/+ | (2) |
| Olive mill waste water extract dissolved in water/ethanol solution versus water/ethanol solution | rats (3) | hydroxytyrosol: 83 vs 0 µg | one single dose, blood sampling at 0, 15, 30, 90, 240 min | plasma antioxidant capacity: $Cu^{2+} \rightarrow Cu^{+}$ with uric acid as reference (mEq uric acid)<br>- t=0 min<br>- t=15 min<br>- t=30 min<br>- t=90 min<br>- t=240 min | <br>205[e]<br>233 vs 210<br>198 vs 208<br>190 vs 210<br>250 vs 208 | <br><br>0/+<br>0/-<br>0/-<br>+ | (3) |

[a] 0 = no effect, + = protective effect, - = negative effect, 0/+ = no significant effect in the protective direction, 0/- = no significant effect in the negative direction.

[b] Estimated phenol intake (personal communication from R. Leenen and A. Roodenburg, Unilever Research Vlaardingen, the Netherlands).

[c] Data on the refined olive oil and Trisun high oleic sunflower oil were combined because of their low phenol content.

[d] Plasma malondialdehyde was significantly reduced in the refined olive oil group.

[e] The values are estimated from the figures presented in the article of Visioli *et al* (2001).

(1): (Wiseman *et al*, 1996); (2): (Visioli *et al*, 2000b); (3): (Visioli *et al*, 2001).

## RESULTS

We found ten publications that addressed absorption, metabolism or urinary excretion of olive oil phenols: four animal (Bai *et al*, 1998; Coni *et al*, 2000; Tuck *et al*, 2001; Visioli *et al*, 2001), and six human studies (*chapter 6;* Visioli *et al*, 2000a; Visioli *et al*, 2000c; Bonanome *et al*, 2000; Miro-Casas *et al*, 2001a; Miro-Casas *et al*, 2001b). One study by our group (*chapter 6*) showed that apparent *in vivo* absorption of the ingested olive oil phenols was more than 55-66 mol% in humans. Also, a study in rats showed that bioavailability of radiolabeled tyrosol and hydroxytyrosol was 71-99% compared to intravenously administered tyrosol and hydroxytyrosol (Tuck *et al*, 2001). Seven studies showed that of the total amount of ingested phenols at least 5% was excreted in urine as tyrosol and hydroxytyrosol (*chapter 6*; Visioli *et al*, 2000a; Visioli *et al*, 2000c; Visioli *et al*, 2001; Tuck *et al*, 2001; Miro-Casas *et al*, 2001a; Miro-Casas *et al*, 2001b), mainly conjugated to glucuronic acid (Visioli *et al*, 2000c; Miro-Casas *et al*, 2001a; Miro-Casas *et al*, 2001b) and in the *O*-methylated form (Visioli *et al*, 2000a).

Ten published papers addressed the antioxidant effects of consumption of phenol-rich olive oil compared to consumption of phenol-poor olive oil. Seven human studies (**Table 7.1**) and three animal studies (**Table 7.2**) investigated the effects of olive oil phenols on oxidation markers in blood and urine. Five studies compared the effects between phenol-rich and phenol-poor olive oil in fasting blood samples (**Table 7.1A**) and three in postprandial blood samples (**Table 7.1B**).

The three animal studies showed a lower LDL oxidisability or a lower concentration of oxidation products in urine after consumption of phenol-rich olive oil than after consumption of phenol-poor olive oil (**Table 7.2**). In 5 out of 7 human studies, however, these effects of phenols were not found (**Table 7.1A** and **7.1B**). The only oxidation marker that could quantitatively be combined across studies was the lag time of LDL oxidation (**Figure 7.1**). Although animal studies suggest a protective effect, human studies suggest that olive oil phenols reduced the lag time somewhat and thus increased rather than decreased LDL oxidisability. The combined estimate of the difference in lag time of LDL oxidisability between high and low phenol treatment was -3.7 ± 2.2 min (mean ± SE) (**Figure 7.1**).

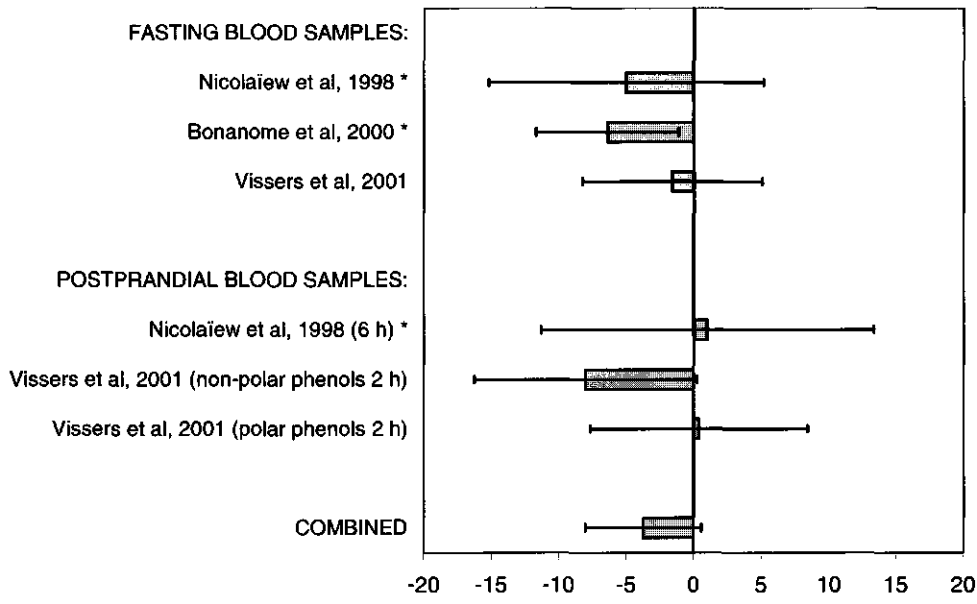


**Figure 7.1** The effect of olive oil phenols on the lag time of LDL oxidisability compared to a control group of monounsaturated vegetable oil in humans. Bars are mean changes, and the lines are 95% CI's. We estimated the standard error of the difference in studies with an asterisk by adding the variances of the groups consuming high or low phenol oil. In the postprandial study of Nicolaïew *et al* (1998) we subtracted the lag times of t=0 from the lag times at t=6, and then calculated the difference in lag time between consumption of the olive oil and sunflower oil. CI's were calculated by 1.96 * SE.

## DISCUSSION

### Bioavailability

The first requirement for a dietary compound to be a potential *in vivo* antioxidant in humans is that it enters the blood circulation. Animal and human studies show that olive oil phenols are well absorbed. Absorption is confirmed by the recovery of tyrosol and hydroxytyrosol with urine after intake of olive oil phenols. A further requirement for protection against oxidative LDL modification is that the ingested compound becomes available in plasma or LDL in a form with antioxidant capacity, because the original antioxidant activity of an absorbed compound may alter upon metabolism. Thus, insight into the kinetics and metabolism of olive oil phenols is needed to assess their potential for increasing the antioxidant capacity of LDL or plasma in the human body.

The amount and form in which the olive oil phenols are present in plasma or are excreted in urine may give insight into their metabolism in the human body. At present, the plasma phenol concentration that can be reached after consumption olive oil is not known. One study measured the amounts of hydroxytyrosol and tyrosol in LDL of humans, but the variability was

too high to provide reliable estimates of concentrations in LDL (Bonanome *et al*, 2000). Bai *et al* (1998) found only low concentrations of plasma hydroxytyrosol concentrations in rats after oral administration of a high single dose of hydroxytyrosol. However, Bai *et al* analysed hydroxytyrosol without prior deconjugation, which may cause underestimation of total hydroxytyrosol in plasma. Likewise, Coni *et al* (2000) also analysed olive oil phenols without deconjugation in plasma of rabbits that consumed extra virgin olive oil for six weeks. Thus, reliable data on plasma concentration of olive oil phenols are scarce. An alternative is to look at olive oil phenols excreted in urine; these may provide information on the form in which phenols are present in plasma. The reported figure for the recovery of ingested olive oil phenols as tyrosol and hydroxytyrosol in urine range between 5 and 72%, most of them conjugated to glucuronic acid (*chapter 6*; Visioli *et al*, 2000a; Visioli *et al*, 2000c; Miro-Casas *et al*, 2001a; Miro-Casas *et al*, 2001b). This wide range is probably due to the various approaches to calculate urinary excretion and to different analytical analyses. For instance, Visioli *et al* (2000c) measured the percentage recovery in urine of total ingested tyrosol and hydroxytyrosol without taking into account the production of additional hydroxytyrosol and tyrosol from ingested oleuropein- and ligstroside-aglycones in the body. Their reported recovery of 20-60% may thus be an overestimate. Miro-Casas *et al* found a recovery for hydroxytyrosol of 72% of total ingested hydroxytyrosol-like substances, after acidic hydrolysis instead of enzymatic hydrolysis in the chemical analysis. They could therefore not provide specific information about the type of conjugate, and it is possible that other types of conjugated metabolites were present in urine (Miro-Casas *et al*, 2001a). We found a recovery of 5-16 mol% of total ingested phenols (*chapter 6*), which is lower than reported by others. However, our finding may be an underestimate because we did not measure metabolites of olive oil phenols, such as *O*-methylated hydroxytyrosol (3-hydroxy-4-methoxyphenylethanol) in urine (Manna *et al*, 2000; Visioli *et al*, 2000a). Taken together, data on urinary excretion indicate that at the very least least 5% of ingested olive oil phenols is recovered in urine as (glucuronidated) tyrosol and hydroxytyrosol. The remaining phenols are probably metabolised into other compounds, such as *O*-methylated hydroxytyrosol (Manna *et al*, 2000; Visioli *et al*, 2000a). Thus, olive oil phenols may be present in plasma mainly in the glucuronidated and *O*-methylated form. However, this suggestion needs to be confirmed by analyses of phenols and their metabolites in plasma. Therefore, development of methods to analyse these phenols in plasma is needed.

### Studies on LDL oxidisability and other markers of oxidation

Animal studies suggest that olive oil phenols protect LDL against oxidation as indicated by decreased LDL oxidisability or other markers of oxidation (**Table 7.2**). In contrast, four human studies do not point to protective effects of olive oil phenols on LDL oxidisability. Our meta-analysis (**Figure 7.1**) suggested that the lag time of *ex vivo* LDL oxidation after high phenol treatment was 3.7 min lower (95% CI, -8.0 to 0.6 min) than after low phenol treatment, which does not suggest decreased susceptibility of LDL to oxidation.

Some other studies do suggest protective effect of olive oil phenols as indicated by markers of oxidation other than the lag time of LDL oxidation. Visioli *et al* (2000a) found that administration of phenol-rich oils resulted in a dose-dependent decrease in urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$, an $F_2$-isoprostane, which indicates less overall oxidation of arachidonic acid. Ramirez *et al* (1999) showed a decreased LDL oxidation rate as measured by thiobarbituric acid-reactive substances and a reduced *in vitro* uptake of oxidised LDL by macrophages in fasting blood after 3 mo of consumption of extra virgin olive oil compared to refined olive oil. Furthermore, Bonanome *et al* (2000) found an increase of antioxidant capacity of postprandial plasma samples measured by a crocin-bleaching test 2 h after intake of 100 mL extra virgin olive oil. However, this study did not include a control group and the effect might therefore be due to a non-specific meal or time effect. Thus, results of human studies on the effects of olive oil phenols on various markers of oxidation are inconsistent, with most studies showing no effect.

## Are phenols antioxidants *in vivo*?

How can we explain that animal and *in vitro* studies suggest antioxidant effects of olive oil phenols, while most human studies do not find effects? Possible explanations include the dose and plasma concentration of phenols, type of oxidation marker, and metabolism of the phenols in the body. In addition we should consider the possibility of publication bias. We speculate that negative outcomes of human studies may have a higher chance of being published than negative outcomes of animal studies, either because human studies are more expensive and difficult, which increases the pressure to publish for investigators, or because they are more likely to be accepted for publication. We found only three animal studies that specifically studied the antioxidant effect of consumption of phenol-rich olive oil compared to consumption of phenol-poor olive oil. It is conceivable that more animal studies have been carried out but remained unpublished because of negative results. However, we are aware that this is pure speculation, and it is quite possible that laboratory animals simply react differently to olive oil phenols than humans.

Differences in the doses of phenols fed might also explain discrepant results between animal and human studies. An important question is whether the doses and thus the plasma concentration of antioxidant phenols in humans was high enough to affect the markers of LDL oxidation. Unfortunately, reliable data on plasma concentration of olive oil phenols are lacking. We can put a ceiling on attainable plasma concentrations as follows. Fifty g/d of olive oil provides 2 mg or 13 µmol of hydroxytyrosol-equivalents. If these are absorbed immediately and completely dissolved into a blood compartment of 5 L this would result in a plasma level of 2.6 µmol/L. In reality, absorption is slow and incomplete, and clearance and redistribution will remove phenols from plasma immediately after absorption. We make a more realistic guess at attainable plasma concentrations using data on other phenolic compounds. For instance, intake of 225 µmol of quercetin-glucosides (68 mg quercetin equivalents) from onions, which are absorbed for about 50% (Hollman *et al*, 1995), produced a peak level of 0.75 µmol of quercetin

per L plasma in humans (Hollman *et al*, 1997). This peak rapidly fell off in the hours after consumption. By analogy, intake of 2 mg of hydroxytyrosol-equivalents (13 µmol), which is absorbed for 66% (*chapter 6*), might produce a peak level of 0.06 µmol of hydroxytyrosol-equivalents per L of plasma. In contrast, when olive oil phenols were added directly to plasma *in vitro*, concentrations of 50-100 µmol/L were required to protect LDL from oxidation (*chapter 5*). This is several orders of magnitude higher than our estimate of the maximum concentration attainable after intake of a high dose of olive oil phenols *in vivo*. Other *in vitro* studies demonstrated that pre-incubation of a purified LDL fraction with 10 µmol/L of olive oil phenols prevented oxidation (Grignaffini *et al*, 1994; Visioli *et al*, 1995; Caruso *et al*, 1999). However, phenols react avidly with many proteins, and therefore in whole plasma olive oil phenols will bind to other proteins like albumin, whereas in an purified LDL fraction these phenols can only bind to LDL. Hence, higher concentrations of olive oil phenols would be needed to protect LDL from oxidation in plasma than in a purified LDL fraction. Thus, it is likely that the plasma concentrations of olive oil phenols in the human studies were too low to affect LDL oxidisability. Our estimate of the *in vivo* plasma concentration is of course crude and subject to a number of errors, but it is unlikely to be several orders of magnitude too low, which is the difference between our estimate and the concentrations needed *in vitro*.

The actual concentration of phenols within the core of the LDL particle might be higher if phenols are lipophilic and accumulate inside lipoproteins. The mean water/lipid partition coefficient of the most lipophilic phenols, i.e. the aglycones, is about 0.7 (unpublished data, personal communications Dr. S. Van Boom) which implies that their concentration in the lipid core of LDL will be 1:0.7 = 1.4 times higher than in the surrounding aqueous medium. However, the same partitioning occurs *in vitro*. Even if some phenols show a higher affinity for the inside of the LDL particles, the concentration in the aqueous medium evidently still needs to be about 50 to 100 µmol/L to produce inhibition of conjugated diene formation.

The use of different markers of oxidation might also explain discrepancies in results. Olive oil phenols might act as plasma antioxidant in ways other than dissolved in LDL particles. Visioli *et al* (2000a) measured the urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$ instead of the LDL oxidisability. They found a negative correlation between intake of olive oil phenols and excretion of these markers of oxidative stress, which indicates less overall oxidation of arachidonic acid. The *ex vivo* LDL oxidisability is measured in LDL particles isolated from plasma by centrifugation. For this *ex vivo* analysis it is necessary that all phenols in or attached to LDL are isolated and not lost during centrifugation. Tyrosol and hydroxytyrosol are polar and will not easily dissolve into LDL particles, but *in vivo* they might loosely bind to the surface of the LDL particle (Vinson *et al*, 1995). If so, tyrosol and hydroxytyrosol might get lost during the centrifugation of LDL (Halliwell 2000; Carbonneau *et al*, 1997), and the *ex vivo* method would underestimate *in vivo* effects. Urinary excretion of $F_2$-isoprostanes might be a better marker because those $F_2$-isoprostanes are formed *in vivo*. However, $F_2$-isoprostanes have, like other oxidation markers, not yet been validated as true predictor of coronary heart disease endpoints. It remains possible that phenols from olive oil decrease LDL oxidisability, but that the currently

available markers are not suitable to measure such an effect. Thus, for definitive answers as to the health effects of olive oil phenols through their antioxidant capacity, we need markers of oxidative processes in the body that reliably predict disease risk.

The antioxidant capacity of conjugated or *O*-methylated metabolites of hydroxytyrosol might be less than that of the hydroxytyrosol itself. The glucuronides might have less antioxidant activity than the ingested compounds, as was shown for quercetine-glucuronide compared to quercetin (Manach *et al*, 1998; Day *et al*, 2000). Also, we do not know the antioxidant capacity of *O*-methylated hydroxytyrosol. However, the extra methyl group substitutes an original hydroxyl group, and *O*-methylated hydroxytyrosol is thus not an *ortho*-diphenol. Consequently, it probably has less antioxidant activity than hydroxytyrosol, as was shown for *O*-methylated quercetin compared to quercetin (Manach *et al*, 1998; Yamamoto *et al*, 1999). To determine the true antioxidant activity of olive oil phenols *in vivo*, future studies should focus on the antioxidant activity of the metabolites actually present in plasma rather than on the *in vitro* antioxidant activity of the phenols as present in the olive oil.

## CONCLUSION

Although the olive oil phenols are well absorbed, the amount of olive oil phenols in the diet is probably too low to produce a quantifiable and biological significant effect on LDL oxidisability.

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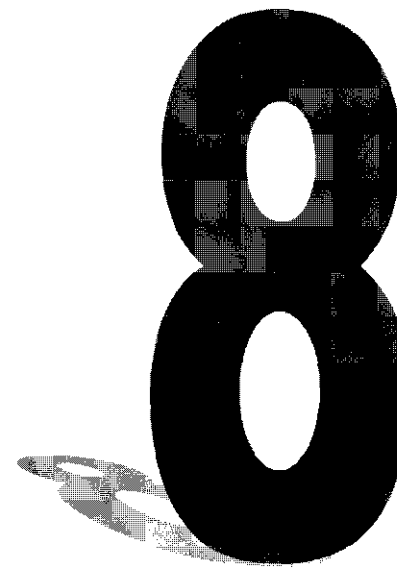

# Concluding remarks

The studies described in this thesis investigated whether minor components from vegetable oils can improve health by decreasing cholesterol concentrations or oxidative modification of low density lipoprotein (LDL) particles. This objective was subdivided into three main questions in the general introduction (*chapter 1*). This chapter summarises the answers to these questions, gives the main conclusion, and gives recommendations for further research.

## MAIN QUESTIONS AND SUMMARY OF CONCLUSIONS

1. *What is the effect of various plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum cholesterol concentrations in humans?*
   Plant sterols from rice bran oil lower serum total and LDL cholesterol concentrations. This effect is probably due to β-sitosterol and not to the 4,4'-dimethylsterols. Triterpene alcohols from sheanut oil do not affect cholesterol concentrations (*chapter 2*). These results indicate that 4,4'-dimethylsterols from rice bran oil and triterpene alcohols from sheanut oil are not able to decrease cholesterol concentrations.

2. *Do phenols from extra virgin olive oil decrease LDL oxidisability in humans?*
   We found that consumption of olive oil phenols does not decrease LDL oxidisability or other markers of antioxidant capacity in humans, neither in fasting plasma samples after 3-wk consumption (*chapter 3*), nor in postprandial samples after a single high dose (*chapter 4*). The *in vitro* study (*chapter 5*) showed that the dose of the phenols and consequently their plasma concentration in humans is probably too low to reduce LDL oxidisability.

3. *To what extent are olive oil phenols absorbed and how are they metabolised?*
   The apparent absorption in the small intestine of humans is more than 55-66 mol% of the ingested olive oil phenols. Absorption was confirmed by urinary excretion of free or glucuronidated tyrosol or hydroxytyrosol of at least 5 mol% of ingested phenols. The human body hydrolyses oleuropein-glycoside into hydroxytyrosol, and the remainder of ingested phenols is probably metabolised into forms that we could not detect in urine (*chapter 6*). The antioxidant activity of the phenol metabolites is unknown.

## CONCLUSION

Although the olive oil phenols are well absorbed, the amount of phenols in olive oil and their consequent attainable plasma concentration in humans is probably too low to reduce LDL oxidisability. Furthermore, our studies provide no evidence that 4,4'-dimethylsterols from rice bran oil or triterpene alcohols from sheanut oil are able to decrease cholesterol concentrations. Consequently, there are no indications that the minor components from vegetable oils described in this thesis have important effects on serum lipoproteins.

## RECOMMENDATIONS FOR FUTURE RESEARCH

### Plant sterols

Abundant research shows that 4-desmethylsterols in vegetable oils, such as β-sitosterol or sitostanol, reduce serum LDL cholesterol concentrations. We found that chemically related structures such as 4,4'-dimethylsterols and triterpene alcohols have less or no effect. It is not clear what exactly determines the efficacy of the plant sterols. To elucidate that, more information is needed about the mechanism of action. It is generally assumed that plant sterols displace cholesterol from the micelles in the intestine (Heinemann *et al*, 1991; Vanhanen & Miettinen, 1992), which transport the cholesterol or plant sterols to the surface of the absorptive enterocytes. However, plant sterols themselves are not absorbed. It is not known whether they enter the enterocyte, temporarily stay there, and are excreted back into the intestinal lumen, or whether the do not enter the enterocyte at all. One approach to elucidate the exact cellular mechanism of action of plant sterols would be absorption studies with *in vitro* cell lines. It may learn us more about their mechanism of action, but also about their safety, the beneficial way of dosing, and the possible influence of other food components.

Although the cholesterol lowering effects of certain plant sterols has been well established, studies with hard clinical end-points are lacking. Long-term prospective studies are needed to confirm that consumption of high doses of sterols can lower the risk of coronary heart disease. Furthermore, such studies will also give information about side effects of such high doses of plant sterols, such as the negative or positive relation with colon cancer (Tseng *et al*, 1996; Awad & Fink, 2000; Normen *et al*, 2001) or other diseases.

### Studies on antioxidants

To study the importance of antioxidant in health, reliable markers of oxidative damage are needed. The currently used markers have not been correlated to clinical end points such as the risk of coronary heart disease. It is thus imperative to find *in vivo* markers for antioxidant capacity that reliably predict the risk of coronary heart diseases. Experiments on whether such markers can be affected by dietary antioxidant components may shed light on the potential of diet to prevent the development of atherosclerosis. The Cu-induced LDL oxidation method has been most widely used to study the potential effects of dietary antioxidant on lipid peroxidation. This LDL oxidation method tests the susceptibility of LDL to copper induced oxidation *in vitro*, which may not be a valid indication of the *in vivo* situation (Heinecke 1998). Also, antioxidants might get lost during the prolonged procedures to isolate LDL from plasma, causing underestimation of the *in vivo* potential (Halliwell 2000). New promising markers of lipid peroxidation *in vivo* are $F_2$-isoprostanes (Roberts & Morrow, 2000) or auto-antibodies against oxidised LDL (Holvoet & Collen, 1998). However, these markers still need to be validated as true predictors of coronary heart disease endpoints.

To establish whether intake of antioxidants can protect against oxidation *in vivo*, we need not only validated markers but we also need to know that the antioxidant retains its antioxidant capacity after absorption into the plasma and after metabolism. It is thus essential to study the metabolism of dietary antioxidants in the human body and the antioxidant capacity of those metabolites to establish a potential health effect, before long-term and expensive intervention trials with antioxidant supplementation are carried out.

## Health effects of olive oil phenols

Our studies suggest that olive oil phenols do not prevent oxidative modification of LDL. It can not be excluded that olive oil antioxidants may play a role in other mechanisms than the protection of oxidative modification of LDL. For instance, *in vitro* studies have shown that hydroxytyrosol might reduce platelet aggregation and exert anti-inflammatory effects (Petroni *et al*, 1994; de la Puerta *et al*, 2000). However, it is not clear whether olive oil phenols do also show these effects in an *in vivo* situation. The concentration of olive oil phenols might also be too low for such actions, and it is not known whether the metabolites of the olive oil phenols in the body also exert similar effects.

## Metabolism of olive oil phenols

We estimated that the dose of olive oil phenols in the Mediterranean diet is too low to reach an effective concentration in plasma. However, we only studied the urinary excretion of the olive oil phenols in the form in which they were ingested. To study antioxidant efficacy it is crucial to know in which form the olive oil phenols become present in plasma. Unfortunately, actual plasma concentrations of olive oil phenols are unknown because there is as yet no reliable analytical method available. Development of such method would facilitate studies on the pharmocokinetics and plasma concentrations of these compounds.

In all human studies subjects were supplied with olive oil or olive oil extracts that contain a mixture of various phenols. A disadvantage of using such a mixture is that conversion of individual phenols into their derivatives cannot be estimated. For instance, hydroxytyrosol in urine could derive directly from ingested hydroxytyrosol, but it might also be derived from hydrolysed oleuropein-aglycones. Pure phenolic substances should be produced and made food-grade to enable studies on the metabolism of individual phenols and their antioxidant potential in the human body.

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# Summary

The studies described in this thesis investigated whether minor components from vegetable oils can improve health by decreasing cholesterol concentrations or oxidative modification of low-density-lipoprotein (LDL) particles.

High LDL or total cholesterol concentrations are established risk factors for coronary heart disease. Plant sterols or phytosterols are used as cholesterol-lowering agents in foods. The plant sterols β-sitosterol and sitostanol are known to decrease cholesterol concentrations, but it is not clear whether other chemically related structures have similar effects.

Oxidation of LDL may also increase the risk of developing coronary heart disease. Dietary antioxidants may inhibit the oxidation of LDL or other oxidative processes in the body. Phenols from extra virgin olive oil are such antioxidants *in vitro*. However, the ability of olive oil phenols to inhibit LDL oxidation *in vivo* still requires confirmation.

This thesis describes one study on the cholesterol lowering effect of plant sterols from rice bran oil and sheanut oil and four studies on the antioxidant activity and metabolism of phenols from extra virgin oil in humans.

## The effect of various plant sterols from rice bran oil and triterpene alcohols from sheanut oil on serum cholesterol concentrations in humans

Plant sterols are minor constituents present in the unsaponifiable fraction of vegetable oils, such as corn, soybean and rapeseed oil. The most common plant sterols in the human diet are the 4-desmethylsterols, such as β-sitosterol. Beta-sitosterol can be converted into a saturated counterpart sitostanol by hydrogenation, although sitostanol rarely occurs in nature. The cholesterol lowering effects of β-sitosterol and sitostanol have been well established. Sterols with other structures may vary in their potential to reduce plasma cholesterol concentrations. In *chapter 2* we studied the effects of a combination of 4-desmethylsterols and 4,4'-dimethylsterols from rice bran oil and of triterpene alcohols from sheanut oil on cholesterol concentrations in healthy, normolipemic volunteers. Sixty healthy volunteers each consumed 30 g/d of 3 margarines for 3 wk. The study was a cross-over double-blind design and had the following treatments: one margarine with plant sterols from rice bran oil; one margarine with triterpene alcohols from sheanut oil; and one margarine without plant sterols. We found that 1.7 g/d of plant sterols from rice bran oil lowered serum total cholesterol by 5% and LDL cholesterol by 9%. This was probably due to β-sitosterol and other 4-desmethylsterols but not to the 4,4'-dimethylsterols. Triterpene alcohols from sheanut oil did not change cholesterol concentrations. These results indicate that 4,4'-dimethylsterols from rice bran oil and triterpene alcohols from sheanut oil are not able to decrease cholesterol concentrations.

## Antioxidant effect of phenols from extra virgin olive oil in healthy humans

A high intake of olive oil has been proposed as an explanation for the reduced incidence of coronary heart disease in Mediterranean countries, but it is unclear whether olive oil offers specific benefits beyond a low saturated fat content. Extra virgin olive oil contains phenols with antioxidant activity and these phenols may protect LDL against oxidation. The most abundant phenols in extra virgin olive oil are the non-polar oleuropein- and ligstroside-aglycones which can be hydrolysed in olive oil into the polar hydroxytyrosol and tyrosol. In *chapter 3* we investigated the effect of consumption of phenol-rich extra virgin olive oil for 3 wk on the susceptibility of LDL to oxidation and other markers of oxidation in fasting blood samples of 46 healthy subjects. The subjects consumed two diets each supplying 69 g/d of extra virgin olive oil that was either rich or poor in phenols. The mean difference in phenol intake between the treatments was 18 mg/d. Vitamin E intake was low during the entire study. We found that consumption of phenol-rich olive oil did not decrease LDL oxidation or other markers of antioxidant capacity.

It is possible that we did not find an effect of olive oil phenols on LDL oxidisability because our study was not able to address postprandial effects. If phenol clearance is fast, phenol concentrations may be elevated in the first few hours after a meal and during that time protect LDL from oxidation, but not after 12 hours of fasting as was the case in our study. Indeed, in a pilot study we found that consumption of 47 g fortified olive oil containing 31 mg phenols significantly increased the lag time of LDL oxidation from 112 ± 5 minutes before to 130 ± 7 minutes 2 h after the meal. However, this pilot study was not placebo-controlled, and in *chapter 4* we therefore investigated the effects of olive oil phenols on the lag time of LDL oxidation in postprandial samples when compared to a placebo group. Twelve subjects consumed 4 different olive oil supplements with a meal on 4 separate occasions: one similar to the supplement in the pilot study containing 31 mg of phenols (positive control); one containing 100 mg of mainly non-polar phenols; one containing 100 mg of mainly polar phenols; and one without phenols (placebo). Lag time significantly increased 2 h after the meals with the positive control and the polar phenols, but also with the placebo. Lag time did not increase after the non-polar phenols, and increases did not differ between supplements. These results indicate that the lag time of LDL-oxidation is increased after consumption of a meal, but this increase is probably due to a non-specific meal or time effect and not to phenols from olives or olive oil.

The phenol content of the experimental olive oils may have been too low to produce an effect on the *ex vivo* oxidation of LDL. We therefore estimated the minimum plasma concentration of olive oil phenols needed to change the LDL oxidisability. *Chapter 5* describes an *in vitro* study that mimics the exposure of LDL to olive oil phenols in plasma. Plasma was incubated with olive oil phenols and olive oil extracts rich in *ortho*-dihydroxy phenols (oleuropein-aglycone and hydroxytyrosol) and/or *mono*-hydroxy phenols (ligstroside-aglycone and tyrosol). LDL was subsequently isolated and its susceptibility to oxidation measured. The results show that olive oil phenols prevent the oxidative modification of LDL. Efficacy in protecting LDL against oxidative modification was consistent with prediction based on their

structural antioxidant features; the *ortho*-dihydroxy phenols, hydroxytyrosol and oleuropein-aglycone, were more efficient than their *mono*-hydroxy counterparts, tyrosol and ligstroside-aglycones. However, the plasma concentration of olive oil phenols required to inhibit LDL oxidation were substantially higher than could be expected to be achieved by dietary consumption.

In *chapter 7* we review the antioxidant effects of olive oil phenols in animal and humans studies. Although animal studies suggest a protective effect, combined human studies suggest that olive oil phenols do not decrease LDL oxidisability. In *chapter 7* we furthermore estimated the daily intake of olive oil phenols in the Mediterranean area. The estimated plasma concentration of olive oil phenols with antioxidant potential resulting from such an intake is much lower than the minimum concentrations of these phenols required to show antioxidant activity *in vitro* as found in *chapter 5*. Thus, the content of phenols in extra virgin olive oil phenols is probably too low to produce a quantifiable effect on LDL oxidisability in humans.

## Absorption and metabolism of olive oil phenols

The first requirement for an *in vivo* action of a potential antioxidant in humans is that it can enter the blood circulation. In *chapter 6* we studied the absorption and urinary excretion of olive oil phenols in 8 ileostomy subjects and 12 subjects with intact colons. The subjects consumed a single dose of 3 different supplements, each containing 100 mg of olive oil phenols on separate days. The treatment order was randomised. Ileostomy effluent and/or urine were collected for 24 h after supplement intake. We found that the apparent absorption was more than 55-66 mol% and the urinary excretion of tyrosol and hydroxytyrosol at least 5 mol% of total ingested phenols.

A further requirement for an antioxidant to protect LDL against oxidation is that it retains its antioxidant activity after absorption into the plasma and after metabolism. In *chapter 7* we reviewed results of studies on the metabolism of olive oil phenols. Olive oil phenols are extensively metabolised in the body by conjugation to glucuronic acid and *O*-methylation. Hydrolysis of oleuropein-glycoside and oleuropein- and ligstroside-aglycones into hydroxytyrosol and tyrosol might be another important step in the metabolism of the olive oil phenols. However, we do not know the antioxidant activity of the conjugated or *O*-methylated metabolites of olive oil phenols. Data from literature suggest that these metabolites may have lower antioxidant properties than their ingested parent compounds.

## Conclusion

Although the olive oil phenols are well absorbed, the amount of phenols in olive oil and their consequent attainable plasma concentration in humans is probably too low to reduce LDL oxidisability. It is unclear whether much higher doses of olive oil phenols would be able to protect LDL from oxidation because these phenols are extensively modified in the human body. The antioxidant activity of phenol metabolites may be less than that of the parent compounds ingested. Thus, the data presented in this thesis do not support the hypothesis that olive oil

phenols reduce LDL oxidation. Furthermore, our studies provide no evidence that 4,4'-dimethylsterols from rice bran oil and triterpene alcohols from sheanut oil are able to decrease cholesterol concentrations. In conclusion, there are no indications that the minor components from vegetable oils described in this thesis have important effects on serum lipoproteins.

# Samenvatting

## ACHTERGROND

Dit proefschrift beschrijft studies waarin we hebben onderzocht of plantensterolen en fenolen uit plantaardige oliën de gezondheid kunnen bevorderen door hun effect op cholesterol in mensen.

Hart- en vaatziekten, zoals een hartinfarct of een beroerte, zijn één van de grootste volksgezondheidsproblemen in welvarende landen. Een hoog cholesterolgehalte bij mensen is één van de risicofactoren voor het ontstaan van hart- en vaatziekten. Bepaalde stoffen in de voeding kunnen het cholesterolgehalte doen dalen waardoor de kans op hart- en vaatziekten afneemt. Plantensterolen oftewel fytosterolen zijn zulke stoffen. Zoals de naam al zegt, komen ze voor in plantaardige voedingsmiddelen, en met name in plantaardige oliën. Van veel voorkomende plantensterolen, zoals β-sitosterol en sitostanol, is bekend dat ze het cholesterolgehalte kunnen verlagen. Het is echter nog onduidelijk of zeldzamere plantensterolen ook het cholesterol verlagen bij mensen. In één van de studies (*hoofdstuk 2*) hebben we daarom gekeken naar het cholesterolverlagende effect van plantensterolen uit rijstkiemolie en uit het vet van een tropische noot (de shea-noot).

Niet alleen een hoog cholesterolgehalte, maar ook de vorm waarin cholesterol in het bloed aanwezig is kan de kans op hart- en vaatziekten verhogen. Cholesterol dat in het bloed circuleert is onder te verdelen in twee soorten, namelijk in het slechte 'low-density-lipoprotein' (LDL) cholesterol en het goede 'high-density-lipoprotein' cholesterol. Wanneer het slechte LDL deeltje in de geoxideerde vorm voorkomt, zou het schade kunnen aanrichten aan de vaatwand, waardoor ophopingen ontstaan die de toevoer van bloed kunnen belemmeren. Bepaalde voedingsstoffen, de zogenaamde antioxidanten, kunnen deze oxidatie van LDL mogelijk tegengaan en daarmee de kans op hart- en vaatziekten verkleinen. Voorbeelden van antioxidanten in de voeding zijn vitamine C en vitamine E. Ook fenolen uit extra vierge olijfolie staan in de belangstelling vanwege hun mogelijke antioxidant activiteit. Het is reeds lang bekend dat olijfolie en andere plantaardige oliën door hun hoge gehalte aan onverzadigde vetzuren het cholesterolgehalte verlagen in vergelijking met harde vetten die veel verzadigd vetzuur bevatten. Hiernaast zou de aanwezigheid van fenolen met een antioxidant-werking extra vierge olijfolie mogelijk nog beter maken dan andere oliën die geen fenolen bevatten. Of olijfolie-fenolen ook daadwerkelijk de oxidatie van LDL deeltjes in het menselijk lichaam kunnen remmen, hebben wij in *hoofdstuk 3 t/m 6* onderzocht.

## PLANTENSTEROLEN

### Het cholesterol verlagende effect van zeldzame plantensterolen uit rijstkiemolie en uit het vet van een tropische noot

Om dit te onderzoeken hebben we aan 60 mensen 3 verschillende margarines te eten gegeven, van elke margarine 30 gram per dag gedurende 3 weken. Eén margarine bevatte de plantensterolen uit rijstkiemolie, één margarine bevatte de plantensterolen uit het vet van de tropische shea-noot, en één margarine zonder plantensterolen die als controle diende.

De plantensterolen uit rijstkiemolie bestonden voor 1 gram uit β-sitosterol en voor 1 gram uit minder bekende plantensterolen waarvan we het effect op het cholesterolgehalte wilden onderzoeken. In deze studie vonden wij dat de plantensterolen uit rijstkiemolie het cholesterolgehalte met 5% deed dalen ten opzichte van de controle margarine. Echter, uit eerder onderzoek bleek dat dit effect even groot was als dat wat je van 1 gram β-sitosterol zou verwachten. Daarom kan het effect van de plantensterolen uit rijstkiemolie waarschijnlijk geheel worden toegeschreven aan β-sitosterol en zijn de minder bekende plantensterolen uit rijstkiemolie dus niet werkzaam. De plantensterolen uit het vet van de tropische shea-noot bleken ook geen effect op het cholesterolgehalte te hebben.

Dit betekent dus dat de zeldzame plantensterolen die wij hebben onderzocht niet of minder effectief het cholesterolgehalte verlagen dan β-sitosterol. Voor toepassing in cholesterolverlagende margarine, waarvan er inmiddels enkele op de markt verkrijgbaar zijn, zijn de minder bekende plantensterolen uit rijstkiemolie en de shea-noot dus niet geschikt.

## FENOLEN UIT EXTRA VIERGE OLIJFOLIE

### Het antioxidant effect van fenolen uit extra vierge olijfolie bij gezonde mensen

Om te onderzoeken of de consumptie van een fenolrijke olijfolie de oxidatie van LDL kon tegengaan hebben we 46 mensen twee verschillende voedingen gegeven: 3 weken een voeding met 69 gram fenolrijke olijfolie per dag en drie weken een voeding met 69 gram fenolarme olijfolie per dag. Aan het einde van de 3 weken namen we bij alle deelnemers bloed af en isoleerden hieruit het LDL cholesterol. Vervolgens testten we de oxideerbaarheid van LDL door het in reageerbuizen bloot te stellen aan pro-oxidanten en te meten hoe lang het in deze test duurt voordat LDL geoxideerd wordt. Zo konden we nagaan of de fenolen uit olijfolie in de voeding ook effectieve antioxidanten in het LDL deeltje zijn. We vonden dat de oxidatie van LDL niet langer duurde bij mensen die de fenolrijke olie hadden gegeten dan bij diegenen die de fenolarme olijfolie hadden gegeten. Met andere woorden, olijfolie-fenolen hadden geen beschermend effect op de oxideerbaarheid van LDL.

In een volgende proef aten 12 mensen 3 keer een supplement van geconcentreerde olijfolie met verschillende soorten fenolen en 1 keer een supplement zonder fenolen (controle-

supplement). Deze 4 verschillende supplementen werden op verschillende dagen samen met het ontbijt gegeten. Bij ieder supplement namen we 3 keer bloed af; 1 keer vóór het ontbijt, 1 keer een half uur na en 1 keer twee uur na het ontbijt. Ook in deze studie vonden we dat de oxidatie van LDL niet langer duurde na het eten van olijfolie-fenolen in vergelijking met het supplement zonder fenolen. En dus liet ook deze studie geen beschermend effect zien van olijfolie-fenolen op de oxideerbaarheid van LDL.

## Absorptie en metabolisme van fenolen uit olijfolie

Omdat we geen effect van de olijfolie-fenolen op de oxidatie van LDL vonden, wilden we weten of de mate en de vorm waarin olijfolie-fenolen in het bloed komen wel voldoende is om daar antioxidant activiteit te hebben. Een voorwaarde voor stoffen om in het bloed terecht te kunnen komen is dat ze eerst door de darmen worden opgenomen. Dit konden we onderzoeken bij mensen met een stoma. Een stoma is een kunstmatige uitgang voor de ontlasting aan het einde van de dunne darm die bij mensen wordt aangelegd als hun dikke darm om medische redenen moet worden verwijderd. Bij mensen met een stoma worden voedingsstoffen die niet door de dunne darm zijn opgenomen direct via de stoma uitgescheiden en opgevangen in een stomazakje. Als we precies weten hoeveel olijfolie-fenolen de mensen hebben gegeten en meten hoeveel er in de stoma-zak is uitgescheiden, kunnen we schatten hoeveel er door de dunne darmen is opgenomen.

Als olijfolie-fenolen eenmaal zijn opgenomen in het bloed kunnen ze middels stofwisselingsprocessen worden omgezet in andere componenten, die mogelijk een andere antioxidant activiteit hebben dan de oorspronkelijke stoffen. Daarom is het dus belangrijk om te weten in welke mate en in welke vorm de fenolen in het bloed voorkomen. Helaas is het nog niet mogelijk om de hoeveelheden aan olijfolie-fenolen en verschillende omzettingsproducten in het bloed te bepalen. Er wordt daarom vaak gekeken naar de uitscheiding van zulke stoffen in de urine, waarin deze stoffen gemakkelijker kunnen worden bepaald. De uitgescheiden olijfolie-fenolen in de urine geven een indicatie over de hoeveelheid en de vorm waarin deze stoffen in het bloed voorkomen.

In *hoofdstuk 6* hebben we bij 8 mensen met een stoma de opname van olijfolie-fenolen in de dunne darm gemeten. Tevens hebben we bij 20 mensen de uitscheiding van fenolachtige stoffen in urine onderzocht. De deelnemers aan de proef kregen op 3 verschillende dagen 's ochtends bij het ontbijt 3 verschillende supplementen met olijfolie-fenolen. Ieder supplement bevatte olijfolie, maar ze verschilden in de soorten olijfolie-fenolen. Gedurende 24 uur na inname van een supplement verzamelden de deelnemers stomavloeistof en/of urine. In de stomavloeistof vonden we minder dan 45% van de ingenomen olijfolie-fenolen terug. Dit betekent dat kennelijk meer dan 55% van de fenolen in de dunne darm wordt opgenomen. In de urine vonden we ongeveer 5% van de ingenomen olijfolie-fenolen terug. Deze resultaten betekenen dat de olijfolie-fenolen waarschijnlijk goed uit de dunne darm worden opgenomen. Omdat we maar 5% van de fenolen in de urine terugvinden, worden ze waarschijnlijk in het lichaam voor een groot deel omgezet in stoffen die we niet in de urine hebben gemeten.

## Wat zeggen deze resultaten over de olijfolie-fenolen nu?

Onze resultaten wijzen erop dat olijfolie-fenolen goed door de darmen worden opgenomen, maar dat ze kennelijk niet de oxidatie van LDL remmen. Hier zijn twee mogelijke verklaringen voor: 1) de hoeveelheid fenolen in olijfolie is te laag om er voldoende van in het bloed te krijgen om oxidatie van LDL te kunnen remmen; of 2) ze worden in het bloed omgezet in andere stoffen met minder of geen antioxidant activiteit.

We konden niet meten hoeveel en in welke vorm fenolen in het bloed voorkomen. We konden wel schatten welke concentratie van fenolen in het bloed minimaal nodig is voor een effect op de oxidatie van LDL. In *hoofdstuk 5* hebben we dat gedaan met behulp van een *in vitro* (reageerbuis) studie. Dat wil zeggen dat we in reageerbuisjes olijfolie-fenolen aan bloed hebben toegevoegd om te meten bij welke concentratie de LDL oxidatie werd beïnvloed. De resultaten laten zien dat olijfolie-fenolen wel degelijk de LDL oxidatie kunnen remmen, maar dat de minimale hoeveelheid die nodig is veel groter is dan wat in bloed met het eten van olijfolie-fenolen kan worden bewerkstelligd. In *hoofdstuk 7* hebben we de dagelijks inname van olijfolie-fenolen in het Middellandse Zeegebied geschat en op basis daarvan een berekening gemaakt hoe hoog de concentratie van olijfolie-fenolen in het bloed zou kunnen zijn. En ook dan blijkt dat de concentratie waarschijnlijk te laag is om een effect op de LDL oxidatie te kunnen bewerkstelligen.

Wij concluderen dan ook dat er op dit moment geen bewijs is dat olijfolie-fenolen de LDL oxidatie in mensen kunnen tegengaan.

# Dankwoord

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# Curriculum vitae

Maud Nelly Vissers was born on January 15, 1972, in Horst, the Netherlands. In 1990 she passed secondary school, atheneum B, at 'Boschveldcollege' in Venray. In the same year she started the study 'Human Nutrition' at Wageningen Agricultural University. As part of that study she conducted research projects on Human Nutrition (at the former Department of Human Nutrition, Wageningen Agricultural University Sep 1994 – May 1995; and at the Institute of Nutrition and Food Hygiene, Beijing, China, Sept – Dec 1995), and on Food Science (Department of Integrated Food Sciences, Wageningen Agricultural University, Feb – Aug 1996; and Mona, division of the dairy company Campina Melkunie, Woerden, the Netherlands, Aug 1996 – Jan 1997). In January 1997 she received the MSc degree and started working as a research assistant on the cholesterol lowering effect of plant sterols at the Division of Human Nutrition, Wageningen University. This project was continued with the PhD project on the metabolism and health effects of olive oil phenols in October 1997. Both the plant sterol and olive oil projects were in collaboration with Unilever Health Institute, Vlaardingen, the Netherlands. For the olive oil project she participated in meetings in Heraklion (1997), Milan (1999), and Athens (1999). She joined the education programme of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). In June 1998 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. She was a member of PhD board of the Division of Human Nutrition and Epidemiology from 1997 through 1999. She was a member of the editoral board of the PhD-newsletter 'Newtrition' and the PhD-excursion committee that organised a study tour to South Africa in 1999. Finally, she was selected to participate in the 7$^{th}$ European Nutrition Leadership Programme, March 2001, Luxembourg.

Since August 2001 she is working as a research associate at the Academic Medical Centre of the University of Amsterdam, the Netherlands.

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