Olive Oil Phenols Are Absorbed in Humans[1]

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ABSTRACT Animal and in vitro studies suggest that olive oil phenols are effective antioxidants. The most abundant phenols in olive oil are the nonpolar oleuropein- and ligstroside-aglycones and the polar hydroxytyrosol and tyrosol. The aim of this study was to gain more insight into the metabolism of those phenols in humans. We measured their absorption in eight healthy ileostomy subjects. We also measured urinary excretion in the ileostomy subjects and in 12 volunteers with a colon. Subjects consumed three different supplements containing 100 mg of olive oil phenols on separate days in random order. Ileostomy subjects consumed a supplement with mainly nonpolar phenols, one with mainly polar phenols and one with the parent compound oleuropein-glycoside. Subjects with a colon consumed a supplement without phenols (placebo) instead of the supplement with oleuropein-glycoside. Ileostomy effluent and urine were collected for 24 h after supplement intake. Tyrosol and hydroxytyrosol concentrations were low (< 4 mol/100 mol of intake) in the ileostomy effluent, and no aglycones were detected. We estimated that the apparent absorption of phenols was at least 55–66% of the ingested dose. Absorption was confirmed by the excretion of tyrosol and hydroxytyrosol in urine. In ileostomy subjects, 12 mol/100 mol and in subjects with a colon, 6 mol/100 mol of the phenols from the nonpolar supplement were recovered in urine as tyrosol or hydroxytyrosol. In both subject groups, 5–6 mol/100 mol of the phenols was recovered from the polar supplement. When ileostomy subjects were given oleuropein-glycoside, 16 mol/100 mol was recovered in 24-h urine, mainly in the form of hydroxytyrosol. Thus, humans absorb a large part of ingested olive oil phenols and absorbed olive oil phenols are extensively modified in the body. J. Nutr. 132: 409–417, 2002.

KEY WORDS: • *phenols* • *olive oil* • *absorption* • *ileostomy* • *humans*

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 (1). Oleic acid, the main fatty acid in olive oil, has favorable effects on blood cholesterol concentration, compared with saturated fat (2), and on LDL oxidizability (3). However, extra virgin olive oil also contains phenolic compounds with antioxidant activity, which are potentially beneficial against LDL oxidation (4–8). The most abundant phenols in extra virgin olive oil are the nonpolar 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 (**Fig. 1**).

A Mediterranean diet rich in olive oil supplies ~10–20 mg of phenols per day (6). 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 (9). A human study also showed that tyrosol and hydroxytyrosol are excreted in urine (10). 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 -aglycones are scarce. Using isolated perfused rat intestine, Edgecombe et al. (11) 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 this 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 leaves and olives (12). A major problem in studying the absorption of phenols in humans is their degradation by microorganisms in the colon, which results in an overestimation of the absorbed amount when fecal excretion is measured. Therefore, we determined the absorption in healthy ileostomy subjects with a complete small intestine. To obtain 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. Therefore, we also determined

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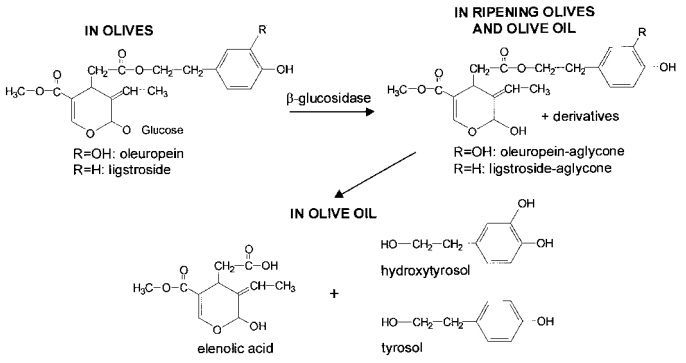

FIGURE 1 Structures of phenols present in olives and olive oil, their degradation into aglycones during ripening, and hydrolysis of aglycones into tyrosol and hydroxytyrosol.

the urinary excretion of tyrosol and hydroxytyrosol in subjects with a colon.

MATERIALS 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 (13–15). 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 >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 four 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 (16). The 12 healthy subjects (8 females and 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 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. The design, duration and setting of the studies in subjects with and without a colon were essentially similar. Each subject consumed a single dose of three 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 d 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 (d 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 three 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 nonpolar aglycones (16), 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 three different supplements containing 100 mg of olive oil phenols. Ileostomy subjects consumed a supplement with mainly nonpolar phenols, one with mainly polar phenols and one with the parent compound oleuropein-glycoside. Subjects with a colon consumed a supplement with mainly nonpolar 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 1**).

Nonpolar supplement. The supplement with nonpolar 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 nonpolar phenols. Ileostomy subjects drank 17.2 g of this supplement (Table 1). Subjects with a colon consumed 37 g of mayonnaise prepared with 14.5 g of the supplement (Table 1). Subjects with a colon consumed the supplement incorporated into a mayonnaise so as to equalize the fat intake in composition between breakfasts. This was essential to study the effect of the olive oil phenols on LDL oxidizability properly.

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 (Table 1). Subjects with a colon drank 8.7 g of this supplement (Table 1) plus consumed 37 g of mayonnaise prepared with 14.5 g of olive oil without phenols to equalize fat intake between breakfasts.

Oleuropein-glycoside. The supplement containing oleuropein-glycoside was commercially available in capsules (Solgar Laboratories, Leonia, NJ). Ileostomy subjects swallowed four capsules, which was 1.9 g (Table 1). Oleuropein-glycoside was the only component from olives that could be supplied in a food grade pure form. We only supplied ileostomy subjects with this supplement to get more information about the absorption and metabolism of such compounds.

Placebo supplement. Subjects with a colon consumed a placebo supplement that consisted of 37 g of mayonnaise prepared with 14.5 g of olive oil without phenols. They were supplied with this supplement to compare the effects of the olive oil phenols on the LDL oxidizability with a control.

Analyses of phenols. We measured the phenol concentration of the supplements with an HPLC method based on the method of Montedoro et al. (17). 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 1; **Fig. 2**). The 12 major peaks in the HPLC chromatogram were quantified using the area/μg at λ = 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 (personal communications, S. van Boom, Unilever Research Vlaardingen, The Netherlands).

Collection of ileostomy effluent and urine. On d 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 minimize degradation of the contents by residual bacterial flora. During the night subjects changed the ileostomy bags one to three times.

Subjects collected all urine during 1 d in plastic bottles of 0.5 L

TABLE 1

The phenol composition of the four supplements consumed by ileostomy subjects and/or subjects with a colon

Supplement	Total phenol concentration in supplement	Supplement intake	Intake of individual phenols					
			Tyrosol (#2)[1]	Hydroxy tyrosol (#1)[1]	Ligstroside-aglycone (#6, #11, #12)[1,2]	Oleuropeine-aglycones (#3, #4, #5, #7, #8, #9, #10)[1,2]	Oleuropein-glycoside	Total intake
	mmol/kg	*g*	*μmol*					
Nonpolar supplement								
Ileostomy subjects	22	17.2	116	3	208[3]	44	0	371
Subjects with a colon	26	14.5	123	9	199	51	0	382
Polar supplement								
Ileostomy subjects	49	10.3	150	198	29	121	0	498
Subjects with a colon	60	8.7	166	235	23	103	0	526
Oleuropein-glycoside[4]								
Ileostomy subjects	100	1.9	0	0	0	0	190	190
Placebo supplement[5]								
Subjects with a colon	0	14.5	0	0	0	0	0	0

[1] Supplements were analyzed by HPLC. The numbering of the various phenols in this table refer to the numbering of the peaks in Figure 2.
[2] Ten derivatives of the aglycones were separated and the peaks identified (Personal communications: S. van Boom, Unilever Research, Vlaardingen, The Netherlands). The various derivatives differ mainly in their ring structure, which can be either open or closed in two different forms.
[3] The ligstroside-aglycones in the nonpolar supplement for ileostomy subjects consisted of 180.3 μmol of ligstroside aglycone derivative #6 and 28.1 μmol the ligstroside-aglycone derivatives #11 and #12 (Fig. 2*A*). Only the ligstroside-aglycone derivative #6 was analyzed in ileostomy effluent (Table 3); the amounts of #11 and #12 were too low to be quantified.
[4] Only consumed by ileostomy subjects.
[5] Only consumed by subjects with a colon.

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 (18,19). 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 lyophilized, 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 ~40°C, pooled by subjects, homogenized, and stored at −80°C until analysis. Ileostomy and urine samples collected before breakfast (presupplement 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 to assess possible losses of phenols during gastrointestinal transit. Gastric juice and duodenal fluid were obtained from two 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 of human gastric juice plus 10 mL of water at 37°C for 0.5 and 2 h (20,21). We also incubated 3 mg of tyrosol, hydroxytyrosol and oleuropein-glycoside in 3 mL of human duodenal fluid plus 9 mL of water at 37°C for 1 and 4 h, corresponding to the average and maximal transit time in the small intestine, respectively (22). 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 d 4, they connected an ileostomy bag to their ileostoma containing 30 mg of tyrosol mixed into 3 g of 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 of hydroxytyrosol mixed into 3 g of strawberry jam and with a bag containing 30 mg of oleuropein-glycoside mixed into 3 g of strawberry jam. Strawberry jam was also used as a vehicle for phenolic compounds in another study with ileostomy subjects because of its viscosity (13). Strawberry jam itself does not contain olive oil phenols. The contents of each ileostomy bag were stored and analyzed 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 lyophilized effluent with 50 mL dimethylformamide 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 1580 × *g* for 10 min. We silylated 0.25 mL of the supernatant with 0.25 mL of *N,O-bis*(trimethylsilyl)-trifluoroacetamide with 10 g/L 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 × 0.25 mm CP-SIL5-CB low bleed MS column (Chrompak Internation BV, Middelburg, The Netherlands). We used helium as carrier gas at a flow rate of 20 cm/s. We used the same oven conditions, temperatures of injection port, transfer line and detector as described by Bai et al. (23), 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

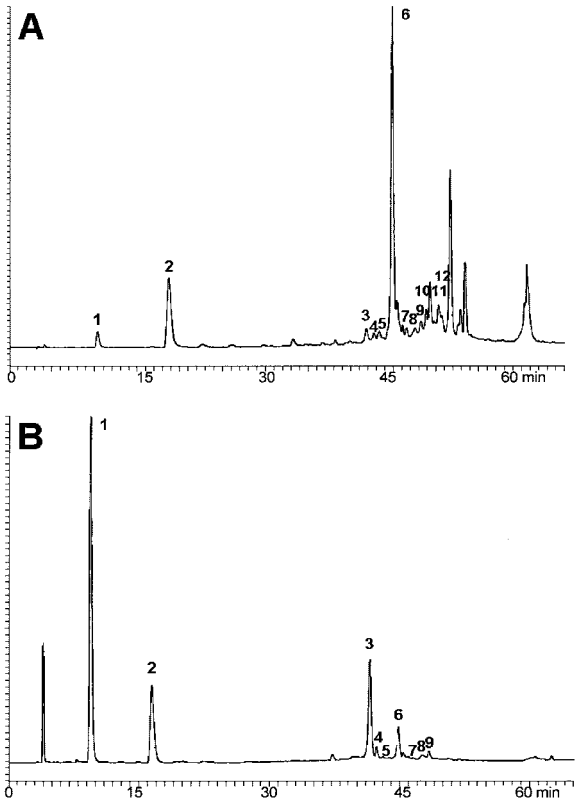

FIGURE 2 Chromatogram of various identified phenols in the nonpolar (*A*) and the polar (*B*) supplement. The numbering of the peaks refer to the numbering of the various phenols in Table 1: 1 = hydroxytyrosol; 2 = tyrosol; 3, 4, 5, 7, 8, 9 and 10 = oleuropein-aglycones; 6, 11 and 12 = ligstroside-aglycones.

m/z for hydroxytyrosol. We obtained calibration curves by six injections of different concentrations of two 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 gram of lyophilized ileostomy effluent. Addition of 38.5 μg tyrosol and 37.0 μg hydroxytyrosol per gram of ileostomy effluent yielded a recovery of 114.4 ± 12.8% for tyrosol and 115.6 ± 9.2% for hydroxytyrosol.

Analytical methods: oleuropein-glycoside and ligstroside-aglycone derivative in ileostomy effluent. We dissolved 0.5 g of lyophilized 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 × *g* at 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, Japan) column (4.6 × 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) semiquantitatively, using the peak height per milligram of 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 nonpolar supplement (Fig. 2*A*, #6). 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 ~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 were 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 nonpolar and polar supplements. Two additional larger peaks in the nonpolar supplement could not be identified (Fig. 2*A*) and, therefore, were not taken into account. We measured every ileostomy sample with and without the addition of the nonpolar supplement or the oleuropein-glycoside supplement to compare the time of the peaks found in the effluent with those present in the supplement. Addition of 110 mg of nonpolar supplement per gram of lyophilized ileostomy effluent yielded a recovery of 58 ± 9%. Addition of 15 mg oleuropein-glycoside supplement per gram of lyophilized ileostomy effluent yielded a recovery of 86 ± 21%. We present the estimated apparent absorption with and without correction for these analytical losses.

Analytical methods: tyrosol and hydroxytyrosol in urine. We added 3 mg of β-glucuronidase dissolved in 200 μL phosphate buffer pH 5 (Sigma, St. Louis, MO) to 1 mL of urine, and incubated the mixture at 37°C for 24 h. Subsequently, we added 1 mg/L of α-naphthol as internal standard and extracted the urine twice with ethyl acetate. The organic phase was evaporated completely under nitrogen. We dissolved the residue in a mixture of dimethylformamide and *N,O-bis*(trimethylsilyl)-trifluoroacetamide with 10 g/L 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 limits, i.e., the concentration producing a peak height three times the standard deviation of the baseline noise, were 0.04 μmol/L for tyrosol and 0.05 μmol/L for hydroxytyrosol. Addition of 0.15 mg and 1.6 mg tyrosol per liter of urine yielded a recovery of 100 ± 6% and 99 ± 4%, respectively. Addition of 0.25 mg and 2.5 mg hydroxytyrosol per liter of urine yielded a recovery of 103 ± 17% and 105 ± 8%, respectively.

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

RESULTS

Composition of the supplements. Chromatograms of the phenol composition of the nonpolar and polar supplements are presented in Figure 2, *A* and *B*. The main phenol in the nonpolar 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

TABLE 2

The 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[1]		Duodenal fluid[1]		Ileostomy effluent[2]
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)

1 Mean of triplicate analyses.

2 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).

present in the oleuropein-glycoside supplement (data not shown).

Stability of phenols. Olive oil phenols seemed stable in gastric juice and in duodenal fluid (**Table 2**). 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 2).

Excretion of phenols in ileostomy effluent. Excretion of tyrosol and hydroxytyrosol in ileostomy effluent was low. It was highest on the nonpolar supplement rich in aglycones, but it was always <4 mol/100 mol of total phenol intake (**Table 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 (**Fig. 3**). The ileostomy effluent of subjects who had consumed the nonpolar supplement contained a minor amount of a compound with the retention time of the major component of this supplement, the ligstroside-aglycone derivative #6 (arrow; Fig. 3). However, the size of both these peaks was <25% of the size of the peaks seen when one daily dose of oleuropein-glycoside or nonpolar supplement was mixed into blank ileostomy effluent and analyzed. Such in vitro mixing studies showed that the detection limit for oleuropein-glycoside and for the ligstroside-aglycone derivative #6 was ~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 nonpolar supplement (Table 3).

We did not detect other aglycones and derivatives in ileostomy effluent after subjects had consumed the nonpolar 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 nonquantifiable aglycones in the ileostomy effluent was at most equal to the amount ingested (Table 3).

Under these assumptions, at least 55–66% of the phenols from the nonpolar supplement had been absorbed; 55% if we corrected for the analytical loss of the phenols plus their degradation within the ileostomy bag, and 66% if we did not. Apparent absorption was somewhat higher for the polar supplement and for oleuropein-glycoside (Table 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, because tyrosol and hydroxytyrosol were major components of the polar supplement and likely metabolites of the aglycones administered (Fig. 1). Indeed, both subject groups excreted 5–6 mol/100 mol of the phenols from the polar supplement into urine in the form of tyrosol or hydroxytyrosol. Ileostomy subjects excreted 12 mol/100 mol of the phenols from the nonpolar supplement into urine in the form of tyrosol or hydroxytyrosol. For subjects with a colon, this figure was 6 mol/100 mol (**Table 4**; **Fig. 4**). Of oleuropein-glycoside administered to ileostomy subjects, 16

TABLE 3

Intake of tyrosol, hydroxytyrosol, aglycones and oleuropein from the three supplements consumed by 8 ileostomy subjects and subsequent excretion in ileostomy effluent over 24 h

		Excretion[1]							Total apparent absorption as proportion of intake[2]
Supplement	Intake	Tyrosol	Hydroxy-tyrosol	Ligstroside-aglycon	Oleuropein-aglycone	Oleuropein-glycoside	Total excretion	Total apparent absorption[2]	
	μmol								mol/100 mol
Nonpolar supplement	371	5.6	4.4	<73[3]	<44[4]	n.a.[5]	<127	>205	>55–66
Polar supplement	498	1.4	1.8	<29[4]	<121[4]	n.a.[5]	<153	>343	>68–69
Oleuropein-glycoside	190	1.0	2.2	n.a.	n.a.	<48[6]	<51	>125	>66–73

[1] Values include the final but not the prebreakfast sample. Mean excretion in prebreakfast samples was <0.3 μmol.

[2] 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 value 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 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 methods).

[3] 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 1, footnote 3) = 45 μmol. We assumed that the excretion of the nonquantifiable aglycones of #11 and #12 in the ileostomy effluent was equal to the amount ingested, which was 28 μmol (Table 1, footnote 3). Thus, the amount of ligstroside-aglycones in ileostomy effluent after intake of the nonpolar supplement is minimally 45 + 28 = 73 μmol.

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

[5] Oleuropein-glycoside was not present in the supplement and, therefore, not analyzed in these ileostomy samples.

[6] 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.

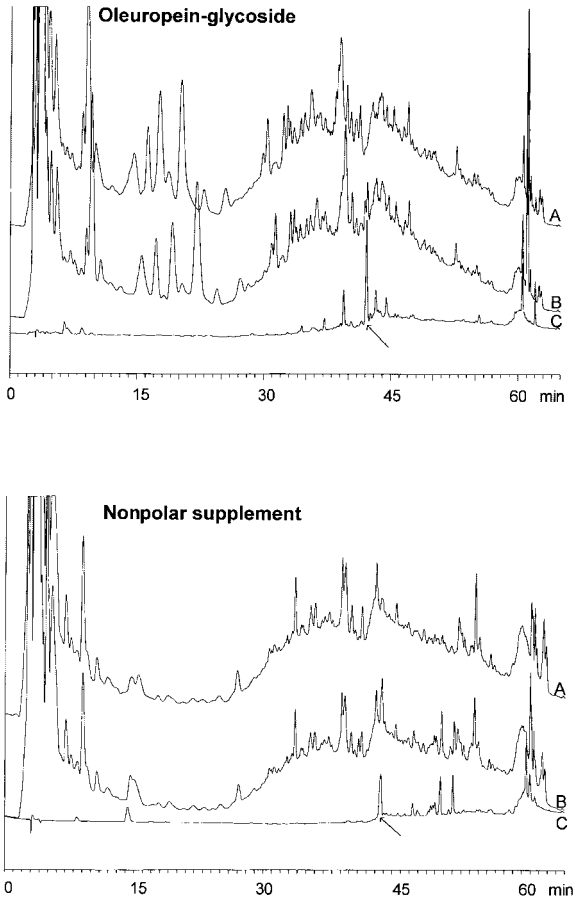

FIGURE 3 HPLC chromatogram of ileostomy effluent after subjects ingested the oleuropein-glycoside (upper chromatogram) and the nonpolar supplement (lower chromatogram). Ileostomy effluent was analyzed with (line B) and without the addition of the oleuropein-glycoside or nonpolar supplement (line A). We added 50% of the amount of ingested supplement to the total amount of lyophilized ileostomy effluent excreted during 24 h. Line C is the chromatogram of the supplement. The arrow indicates oleuropein-glycoside or ligstroside-aglycone derivative #6.

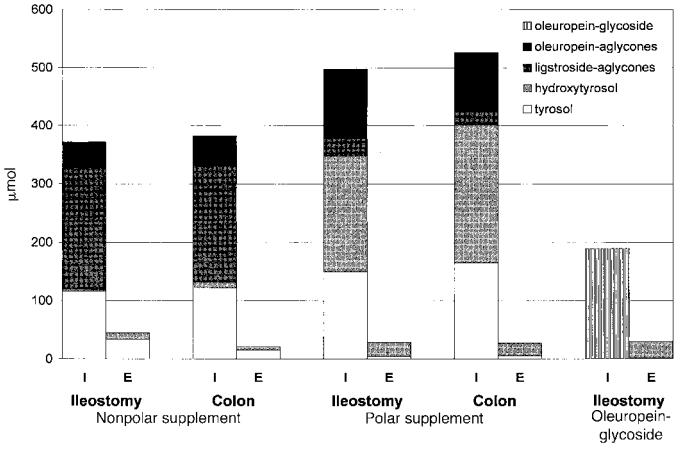

FIGURE 4 Intake of total phenols from the three various supplements consumed by ileostomy subjects (n = 8) and/or subjects with a colon (n = 12) and subsequent excretions of tyrosol, hydroxytyrosol in urine over 24 h. Values are means. I: intake; E: excretion.

mol/100 mol was recovered in urine, largely in the form of hydroxytyrosol (Table 4; Fig. 4). Thus, 5–16 mol/100 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 in 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. Consequently, 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 4

Intake of total phenols from the four supplements consumed by ileostomy subjects and/or subjects with a colon and subsequent excretion of tyrosol and hydroxytyrosol in urine over 24 h

Supplement	Intake	Urinary excretion[1]			Total excretion as proportion of intake
		Tyrosol	Hydroxytyrosol	Sum	
	μmol				%
Nonpolar supplement					
Ileostomy subjects	371	33.5 ± 13.9	10.8 ± 4.5	44.3	12
Subjects with a colon	382	15.5 ± 4.6	6.0 ± 1.7	21.5	6
Polar supplement					
Ileostomy subjects	498	4.1 ± 3.1	24.7 ± 10.9	28.8	6
Subjects with a colon	526	5.6 ± 8.8	21.6 ± 4.8	27.2	5
Oleuropein-glycoside[2]					
Ileostomy subjects	190	1.3 ± 1.5	28.4 ± 8.7	29.7	16
Placebo supplement[3]					
Subjects with a colon	0	1.4 ± 3.5	1.2 ± 1.2	2.6	—

[1] Mean ± SD, n = 8 (ileostomy subjects) or 12 (subjects with a colon). Values include the final but not the prebreakfast sample. Mean excretion in prebreakfast samples was <0.4 μmol.
[2] Only consumed by ileostomy subjects.
[3] Only consumed by subjects with a colon.

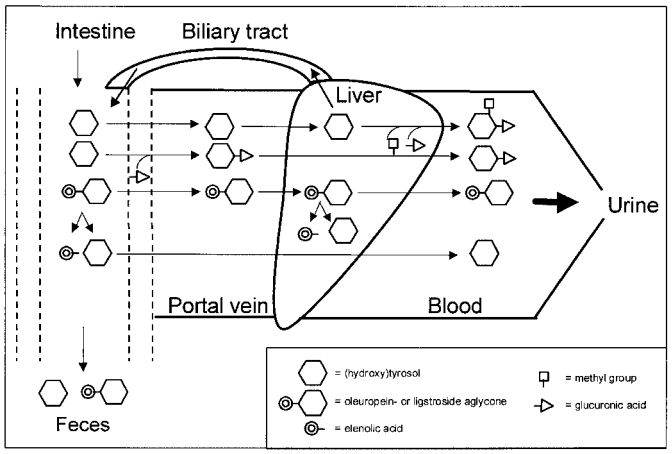

FIGURE 5 Schematic presentation of the possible metabolism of olive oil phenols. Olive oil phenols are probably absorbed > 55 mol/100 mol. They can be glucuronidated in the enterocyte as well as in the liver. *O*-methylation of hydroxytyrosol is another important metabolic step, which also takes place in the liver by catechol-*O*-methyltransferase. Probably the aglycones are hydrolyzed 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.

DISCUSSION

We estimate that at least 55–66 mol/100 mol of ingested olive oil phenols is absorbed in our volunteers and that 5–16 mol/100 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 analyze urinary excretion of the other components of the supplements, namely oleuropein-glycoside, oleuropein- or ligstroside-aglycones. Furthermore, phenols are probably metabolized into other compounds after absorption (24–26). Therefore, the figure of 5–16 mol/100 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/100 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 (25,26). 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 in 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 (**Fig. 5**). This was indicated by our finding that 15 mol/100 mol of the pure oleuropein-glycoside supplement was excreted in urine as tyrosol and hydroxytyrosol. Oleuropein-glycoside and oleuropein- and ligstroside-aglycones might be split either in the gastrointestinal tract before they are absorbed or in the intestinal cell, blood, or 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 2) suggests that oleuropein-glycoside, oleuropein- and ligstroside-aglycones are mainly split after they have been absorbed (Fig. 5). 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 (Fig. 5) (27–29). Our analysis method did not distinguish between free and conjugated phenols, but other studies showed that ~90% of tyrosol and hydroxytyrosol is excreted in the conjugated form (10,30,31). One more possible metabolic step of dihydroxyphenols is *O*-methylation, which mainly takes place in the liver (Fig. 5) (27). Recently it was shown that *O*-methylated hydroxytrosol is an important metabolite in urine after intake of olive oil phenols (9,24,30). The remaining part of the phenols that is not detected in urine is probably extensively metabolized in the body. Thus, absorbed olive oil phenols are 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 that we could not detect.

Comparisons with previous studies. A study of Visioli et al. (10) 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 in their urine. This is much higher than what we found. However, Visioli et al. (10) 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 by Visioli et al. (10), therefore, may be an overestimate. Miro-Casas et al. (30) 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 eight subjects. A reason for their high recovery might be that they hydrolyzed conjugated hydroxytyrosol in tyrosol with HCL instead of with β-glucuronidase as in our study. They, therefore, could not provide specific information about the type of conjugate (30). It is possible that other types of conjugates were present in the urine or that the ingested aglycones were excreted as well. Our results are comparable with the data of Olthof et al. (13). They found that caffeic acid, which is comparable with hydroxytyrosol, was absorbed for 95 ± 4% and that 11% was excreted in urine as such or conjugated to glucuronic acid.

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. (11) found that oleuropeine-glycoside was poorly absorbed from an aqueous solution. However, the validity of this model for humans in vivo is unclear, and orally ingested

oleuropein-glycoside in an oily matrix might be absorbed better (11).

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 nonabsorbed phenols in ileostomy effluent, because transit time of ingested food through the stomach and small intestine is ~8–16 h (32,33). 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 large 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% (Table 2). Stability of hydroxytyrosol in ileostomy effluent was more variable, 72% for one person and 29% for the other (Table 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 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. (14) 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. 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 (9). 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 (11), which is supported by the studies of Hollman et al. (15,34), who found substantial absorption of quercetin glycoside, another phenolic compound, in humans. Other possible mechanisms of absorption of oleuropein-glycoside are via the paracellular route or via transcellular passive diffusion (11). Oleuropeine- and ligstroside-aglycones are less polar compounds, and currently no data are available on their mechanism of absorption. Furthermore, although 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 hydrolyzed 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.

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 metabolize these phenols extensively, probably after absorption from the small intestine.

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