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Impact of dietary supplementation with olive and thyme phenols on alpha-tocopherol concentration in the muscle and liver of adult Wistar rats†

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A preliminary study to evaluate the effect of dietary supplementation with olive phenols (oleuropein, hydroxytyrosol and secoiridoids), thyme phenols and a combination of these (5 mg per kg rat weight per day) on the α-tocopherol concentrations in the muscle and liver of healthy adult Wistar rats over 21 days was conducted. In addition, the excretion of α-tocopherol through the faeces was examined. The results demonstrated that the diet supplemented with some phenolic compounds of olive and thyme increased α-tocopherol ($P < 0.05$) in the liver of female rats, although the α-tocopherol content in the diet of all groups was identical. In addition, a synergic effect between the olive phenols and thyme was observed. Therefore, our study indicates a protective effect of olive and thyme phenols supplemented in the diet on α-tocopherol, resulting in a higher concentration of endogenous α-tocopherol in the rat liver.

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1. Introduction

Vitamin E is an essential micronutrient in the diet of all mammals. It acts as a potent antioxidant in biological systems.[1,2] It is the main chain-breaking antioxidant that prevents the propagation of free radical reactions[3] and their damage to tissues, especially with polyunsaturated fatty acids (PUFA), which are prone to oxidation.[4] Consequently, supplementing animal feed with synthetic vitamin E is a conventional strategy for counteracting oxidative stress to decrease the susceptibility to oxidation,[1] which could affect livestock adversely, especially under intensive breeding systems.[5] Nevertheless, the trend is towards a decrease in the use of synthetic feed additives given consumer concerns over health safety and toxicity[6,7] and due to the increasing consumer interest in organic farming and more sustainable livestock production,[8] but also for emotional reasons.[9] Consequently, the search for natural antioxidants, especially from plants, has intensified in recent years.[7,10]

It has been demonstrated *in vitro* that redox interactions of different kinds of natural antioxidants, such as polyphenols, are capable of regenerating oxidized α-tocopherol.[11–15] In addition, the vitamin E status affected by polyphenols has also been evaluated *in vivo*, obtaining in some cases an increase of α-tocopherol in tissues. In particular, red wine polyphenols;[16,17] flavonoids;[18–21] green tea polyphenols[22–24] anthocyanins[25] and lignans[26] have been the more studied phytochemicals.

A more recent study, although not involving a long-term dietary supplementation, showed that the direct incorporation of different concentrations of grape procyanidin powder to the minced white muscle of fresh Atlantic horse mackerel (*Trauchurus trauchurus*) repaired oxidized α-tocopherol in the medium to long term.[27] Another study found that long-term dietary supplementation with a plant extract from *Lippia* spp. (5 mg verbascoside per kg feed) in pigs for 166 days increased α-tocopherol levels in the *Longissimus dorsi* muscle.[10] The plant extract added to the feed did not contain α-tocopherol, but it was capable of increasing endogenous α-tocopherol, compared with the control diet, indicating a protective effect of verbascoside. With the same type of plant extract, but when given to hares, a significant increase in α-tocopherol in the *Longissimus lumborum* muscle has been observed.[28] Therefore, these studies have shown the potential of some phenolic compounds to regenerate α-tocopherol *via* one-electron reduction of α-tocopheroxyl radicals, which complements endogenous antioxidant systems such as ascorbate and ubiquinol.[29–31]

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Based on this evidence, the main aim of the present study was to check whether the sustained supplementation of the diet of healthy Wistar rats with the most representative olive phenols (oleuropein as a glucoside molecule, secoiridoids as aglycone derivatives of oleuropein, and hydroxytyrosol (HT)), thyme phenols and a combination of olive/thyme phenols increased the α-tocopherol concentration in the muscle tissue and in the liver in comparison with the control group that was fed with the same amount of α-tocopherol but without olive and thyme phenols. Olive and thyme phenols were chosen in the study because they are basic elements in the Mediterranean Diet.[32] Although rat muscle is not a meat-based food, the muscle has been studied because it is the tissue studied in the mentioned literature.[10,28] Furthermore, in the case of the existence of a protective effect of the diet supplementation with phenols on the endogenous α-tocopherol in the liver, this fact could open the way to another approach to the treatment of some fatty liver diseases (*e.g.* NAFLD/NASH), where oxidative stress is considered as one of the main players in their development and progression[33]. However, this is not within the scope of this study. Nowadays, there is no pharmacological treatment for these liver diseases and treating them involves mainly changes in lifestyle and diet.[34] However, patients with NASH or risk factors for NASH who fail to achieve a 5–10% weight reduction over 6–12 months of successful lifestyle changes could be apt candidates for an experimental therapy,[35] the most studied of which is the oral administration of vitamin E.

From the results of two large randomized human control trials (the PIVENS and TONIC studies) analysing the effect of vitamin E at high doses (≥400 IU day^{-1}) in NASH patients, and according to the meta-analysis conducted in ref. 33, vitamin E significantly improves liver function and histological changes in patients with NAFLD/NASH. Furthermore, in patients with cirrhosis[36] or chronic alcohol consumption (ASH),[37] the level of α-tocopherol in the liver decreased significantly, and thus any strategy that increases the α-tocopherol in the liver is interesting.

Furthermore, the biological metabolites of different olive and thyme phenols were determined in the present study to assess changes in their concentration in the muscle tissue and liver related to the phenol diet supplementation. In addition, the goal of combining secoiridoids (the main phenols in virgin olive oil) with thyme phenols was to evaluate possible synergic effects, as also studied by our colleagues,[38] who discovered that the presence of thyme jointly with olive phenols in the diet enhances the bioavailability of olive phenolic compounds in rat plasma. To the best of our knowledge, the potential protective effect of these particular phenolic compounds on endogenous α-tocopherol has not been studied *in vivo*.

2. Materials and methods

2.1. Animals and experimental procedure

The animal procedures were conducted in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments and approved by the Animal Ethics Committee at the University of Lleida (CEEA 10-06/14, 31st July 2014). Forty-eight male and female Wistar rats weighing between 300–350 g were obtained from Charles River Laboratories (Barcelona, Spain). They were separated into six groups with 8 rats in each group (4 males, 4 females): Control diet, Oleuropein (OLE), Secoiridoids (SEC), Hydroxytyrosol (HT), Secoiridoids combined with thyme phenols (SEC + T), and Thyme phenols (T) (Table S1, ESI†). During the 21-day experiment, the animals were housed two per cage in a temperature (21 ± 1 °C) and humidity-controlled (55 ± 10%) room with a 12 h light/dark cycle. Food and water were available *ad libitum*. The secoiridoid and thyme phenol extracts were obtained from an olive cake and dried thyme (*Thymus zygis*), respectively. In both cases, the phenolic extracts were obtained with an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA) using the method described in ref. 32. The phenolic extracts were analyzed by liquid chromatography (UPLC-MS/MS) in order to determine the concentration of secoiridoids (expressed as the dialdehydic form of elenolic acid linked with hydroxytyrosol, 3,4-DHPEA-EDA) in the case of the olive cake, and the concentration of rosmarinic acid and the flavonoid thymusin, in the case of thyme. Once these phenolic compounds had been quantified, the extracts were mixed with the feed to prepare the proposed dose.

For the supplemented diets, commercial feed pellets Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories, Santa Perpètua de Mogoda, Spain) (specification sheet in Fig. S1, ESI†) were crushed in an industrial mill and mixed with Milli-Q water containing the equivalent of 5 mg of phenols per kg rat weight in 16 g of crushed pellet (average daily consumption per rat). New pellets were prepared and freeze-dried. Food and animals were weighed every week for 2 days to adjust the weekly dose of the phenolic compound to 5 mg per kg rat weight per day. All treatments involved the same daily dose of vitamin E (120 IU vitamin E per kg of feed), which is included within the formula of the commercial feed used (ESI Fig. S1†). On the twenty-first day, the rats were sacrificed by intracardiac puncture after isoflurane anesthesia (ISOFlo, Veterinaria Esteva, Bologna, Italy). External oblique muscle (abdominal region) and liver samples were extracted. The samples were frozen immediately in liquid nitrogen and then stored at −80 °C until they were analyzed. Furthermore, the faeces from the 21st day were collected.

2.2. Determination of α-tocopherol in muscle, liver and faeces

The sample pre-treatment was based on the method proposed in ref. 39 with modifications contingent on the reduction of the acetone volume used in the extraction washes. In detail, the method comprised the following steps: (i) lyophilization of the samples (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain); (ii) the weighing of 100 mg of lyophilized sample; (iii) homogenization in 5 mL of acetone (Scharlau, Sentmenat, Spain) with a homogenizer (Polytron PT 1200 E, Kinematica, Luzem, Switzerland) for 15 seconds; (iv) centrifugation of the

homogenate at 3600 rpm for 10 minutes at 15 °C, and (v) transfer of the supernatant to a new test tube. The pellet was re-extracted two more times with 5 mL of acetone each time. All the supernatant fractions were reduced to dryness under N_2 (N_2 LCMS Nitrogen Generator, Claind, Lenno, Italy; Reacti-Vap model 18780, Rockford, USA). The residue was resuspended with 1 mL of *n*-hexane 96% (Scharlau, Sentmenat, Spain) and filtered with a 0.45 μm membrane filter (NYL1345200, Scharlau, Sentmenat, Spain) and 20 μL was injected into the HPLC system.

α-Tocopherol was analyzed by liquid chromatography (HPLC) according to the method described in ref. 40. The HPLC equipment consisted of a Waters 600 Controller pump, Waters 717 plus Autosampler and Waters 2475 Multi λ Fluorescence Detector (Waters Corporation, Milford, Massachusetts, USA). A silica column was used (Luna® 5 μm Silica C18(2) 100 Å 250 × 4.60 mm). The mobile phase was hexane–isopropanol (99 : 1). Detection of α-tocopherol was conducted through fluorescence analysis (λ_{ex}: 295 nm/λ_{em}: 330 nm) and the quantification was based on the external calibration curve. The recovery of α-tocopherol in the different matrices studied was between the range 95–100%.

2.3. Sample pre-treatment for phenolic metabolite analysis

The muscle and liver samples were sequentially pretreated with a combination of liquid–solid extraction (LSE) combined with microelution solid-phase extraction (μSPE).[41] Briefly, for the analysis of LSE, 60 mg of freeze-dried samples was mixed with 50 μL of ascorbic acid (1%), 100 μL of phosphoric acid (4%), and 50 μL of catechol 20 ppm (as the internal standard). The samples were treated four times with 400 μL of water/methanol/phosphoric acid 4% (94 : 4.5 : 1.5, v/v/v). In each extraction, the sample was sonicated for 30 s, maintaining it in ice to avoid heating, and then centrifuged. The supernatants were collected (approx. 1600 μL), and an aliquot of 350 μL of the supernatant was diluted with 350 μL of phosphoric acid 4% (resulting volume: 700 μL, tissue mixture). This 700 μL was used in the μSPE procedure described below. OASIS hydrophilic–lipophilic balance (HLB) μElution plates 30 μm (Waters, Milford, MA, USA) were used. The cartridges were conditioned sequentially by using 250 μL of methanol and acidified Milli-Q water (Milli-Q water at pH 2 with diluted hydrochloric acid). After loading the tissue mixture (700 μL), the plates were washed with 200 μL of Milli-Q water and 200 μL of 5% methanol. The retained phenolic compounds were then eluted with 2 × 50 μL of methanol and injected into an UPLC-MS/MS system.

2.4. Chromatographic analysis of phenolic compounds

The phenolic compounds were analysed by using an UPLC system, coupled to a PDA detector, Acquity Ultraperformance™ liquid chromatography and a triple quadrupole detector (TQD™) mass spectrometer from Waters (Milford, MA, USA), as reported in our previous study.[41] The column was an Acquity UPLC™ HSST3 from Waters (100 mm, 2.1 mm i.d., 1.8 μm particle size). The mobile phase was 0.2% (v/v) acetic acid as solvent A and methanol as solvent B, with a flow rate of 0.4 mL min^{-1}. The gradient was performed as follows: 0–6 min, 3–15% B; 6–14 min, 15–70% B; 14–17 min, 70–100% B; 17–18 min, 100–3% B; 18–20 min, 3% B isocratic. The injection volume was 2.5 μL. The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode, and data were collected by selected reaction monitoring (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L h^{-1} and desolvation gas flow rate, 800 L h^{-1}; desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (>99% purity) were used as the nebulizing and collision gases, respectively. The SRM transitions, individual cone voltages and collision energies were optimized for each analyte by injection of each standard compound into a mixture of methanol/water (1 : 1, v/v) at a concentration of 10 mg L^{-1}. Two SRM transitions were studied to find the most abundant product ions, selecting the most sensitive transition for quantification and the second one for identification purposes. When standard phenolic compounds were not available, the SRM parameters were selected by analyzing the real sample in full-scan mode in MS and in daughter-scan mode in tandem MS. When standard compounds were not available, the phenolic compound was tentatively quantified with a phenol standard with a similar chemical structure. The chromatographic analysis for the metabolites derived from thyme was performed according to our previous study.[32]

2.5. Statistical analysis

IBM SPSS Statistics software v.20 was used for the statistical analysis with a level of significance set at 95%. The normality of the data was evaluated according to the Shapiro–Wilk test. In the case of normal data a one-way ANOVA test was performed followed by a Tukey or Games-Howell *post-hoc* test in the case of ANOVA significance. In the case of non-normal data the Kruskal–Wallis test was performed and the Dunn-Bonferroni *post-hoc* test was performed in the case of Kruskal–Wallis test significance.

3. Results and discussion

Table 1 summarizes the animal performance including the body weight, feed intake and liver weight reported according to diet treatment and gender. For each gender, no statistical significant differences were detected between the control group and any of the treatments. In addition, no animal suffered from any disease or even died.

3.1. α-Tocopherol content in the liver

The absorption of vitamin E from the intestinal lumen is dependent upon processes necessary for digestion of dietary fats and uptake into enterocytes. Vitamin E absorption requires the presence of bile acids for micelle formation. In addition, vitamin E needs pancreatic enzymes for its lipid hydrolysis to aid in the absorption of vitamin E in entero-

Table 1 Animal performance (mean ± standard deviation) according to diet treatment and rat gender

	Control		HT		SEC		OLE		SEC + T		T	
Parameter	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Body weight (g)	426 ± 27	395 ± 25	437 ± 19	384 ± 22	438 ± 10	388 ± 22	411 ± 31.9	395 ± 6.3	422 ± 26	387 ± 26	441 ± 27	394 ± 35
Feed intake (g daily)	18.7 ± 1.2	14.3 ± 1.2	18.1 ± 1.3	14.0 ± 0.9	17.9 ± 0.8	14.1 ± 1.2	17.8 ± 0.5	14.4 ± 0.8	18.5 ± 0.5	14.7 ± 0.5	18.1 ± 0.8	13.8 ± 0.2
Liver weight (g)	8.4 ± 1.5	7.1 ± 1.1	7.6 ± 0.8	7.3 ± 0.7	8.9 ± 1.7	7.1 ± 1.0	7.1 ± 0.8	6.7 ± 0.8	9.4 ± 2.4	7.0 ± 0.4	11.1 ± 1.0	7.6 ± 0.3

♂: male; ♀: female; HT (hydroxytyrosol), SEC (secoiridoids), OLE (oleuropein), SEC + T (secoiridoids + thyme), T (thyme).

cytes.[42] In the intestinal mucosa, chylomicrons are formed as vehicles for transport, consisting of triglycerides, free and esterified cholesterol, carotenoids, phospholipids, proteins and apolipoproteins (apoB48) and fat soluble vitamins (including vitamin E).[43] Chylomicrons are catabolized in the circulation by the endothelial-bound lipoprotein lipase enzyme. Chylomicron catabolism leads to some transfer of vitamin E to tissues (*i.e.* muscle tissue, discussed below). After partial delipidation (due to lipoprotein lipase) and acquisition of apoE, chylomicron remnants are taken up by the liver parenchymal cells. These remnants taken up by the liver likely contain a major portion of absorbed tocopherols.[42]

No organ (and the liver is not an exception) functions as storage of α-tocopherol. In the liver, newly absorbed dietary lipids are incorporated into nascent very low density lipoproteins (VLDL) enriched preferentially in RRR-α-tocopherol due to the hepatic cytosolic α-tocopherol transfer protein (α-TTP). RRR-α-tocopherol is the naturally occurring stereoisomer with the highest biological activity, being the main form of vitamin E in humans due to its physicochemical and biological characteristics (*i.e.* α-tocopherol provides optimal structural interactions with the membrane phospholipids). A number of studies have supported that the liver is responsible for the control and release of RRR-α-tocopherol into human plasma, and therefore to the peripheral tissues.[43,44]

As we have now briefly described the tocopherol absorption and transport to the liver, let's comment on the liver α-tocopherol content detected in our study. No significant differences between treatments in the mean concentration of α-tocopherol in the liver (combined sexes) (μg g^{-1} liver) were seen (Fig. 1). Nevertheless, by analyzing the results according to rat gender (Fig. 1) an important sexual dimorphism was detected. We observed that the diet supplementation with the particular olive phenols (OLE and HT) and the combination of these with thyme phenols (SEC + T), produced an increase of the α-tocopherol concentration in the female liver compared with the control ($P < 0.05$). This finding is well-established in the literature.[45–48] Comparing our results with the literature available, we have only found studies on male rodents which, depending on the polyphenol studied, showed a significant increase in α-tocopherol liver concentration[18,20,25,26] or not[21,22,49] in comparison with the control group.

Finally, in this section, although it is not within the scope of our study, we considered it adequate to mention briefly fatty liver diseases (*e.g.* NAFLD/NASH). The mechanisms leading to hepatic steatosis and subsequently NASH have remained

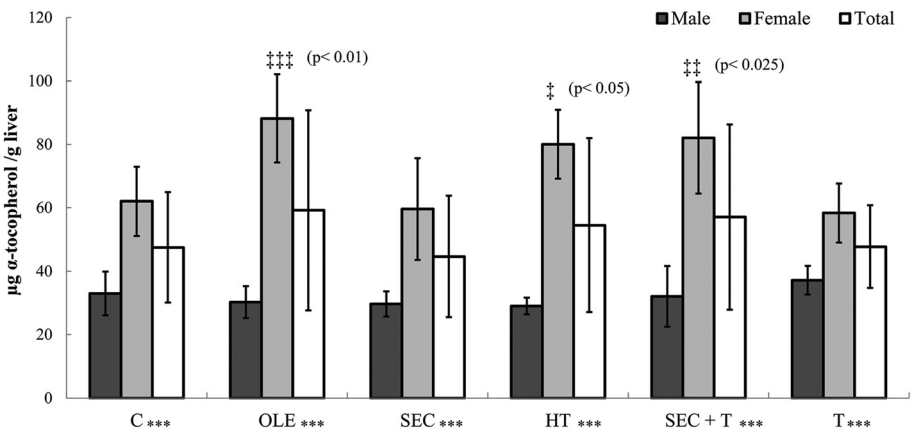

Fig. 1 Total α-tocopherol in the liver for each treatment, separated by gender for each treatment (mean ± SD). C (control), OLE (oleuropein), SEC (secoiridoids), HT (hydroxytyrosol), SEC + T (secoiridoids + thyme), T (thyme). Statistically significant differences by gender for each treatment: ***($P < 0.01$). Statistically significant differences by treatment compared to the control: ‡($P < 0.05$), ‡‡($P < 0.025$), ‡‡‡($P < 0.01$).

poorly understood, but are often characterized by a "two-hit mechanism".[50] The "first-hit", triggered by obesity and insulin resistance, results in excess hepatic lipid accumulation causing hepatic steatosis and injury. Steatotic livers are vulnerable to suffer "second-hits", mediated by inflammation and/or oxidative stress, resulting in NASH. Elevated oral doses of vitamin E have been used to treat these liver pathologies in humans but its results and safety are controversial.[51] Nevertheless, a study[52] in an obese mouse model with induced NASH episode through LPS (lipopolysaccharides) action demonstrated that the hepatic accumulation of α-tocopherol mitigates the liver damage produced by NASH. In addition, in patients with cirrhosis[36] or chronic alcohol consumption (ASH),[37] the level of α-tocopherol in the liver decreased significantly, and thus any strategy to increase the α-tocopherol in the liver is interesting.

3.2. α-Tocopherol content in muscle tissue

The muscle tissue (jointly with the brain) has a low vitamin E turnover in contrast to the fast turnover in plasma or in liver tissue. There are no specific transport proteins for vitamin E in plasma, and consequently lipoproteins are mainly responsible for the vitamin E transport. Therefore the major mechanisms for the delivery of vitamin E to tissues are also the major mechanisms for the delivery of lipids to the tissues.[43] Additionally, vitamin E delivery to tissues can also take place *via* the LDL receptor. Nevertheless, muscle tissue, which receives most of their analys during the delipidation cascade, probably obtains tocopherols mainly as a result of lipoprotein lipase activity[43] commented above.

In the present study, the mean concentration of α-tocopherol in the muscle (combined sexes) (μg g^{-1} muscle) showed no significant differences between treatments (Fig. 2), although dietary supplementation with SEC + T and T resulted in a higher concentration of α-tocopherol in rat muscle. When the concentration of α-tocopherol in the muscle was analysed according to the gender (Fig. 2), no significant differences were detected except in the HT and SEC groups for males and females respectively, where the concentrations of α-tocopherol were lower than in the control group. These results are not in agreement with two previous studies that detected an increase in the concentration of α-tocopherol in the muscle after long-term dietary supplementation with a plant extract titrated in verbascoside in hares (240 days)[28] and pigs (166 days)[10]. Our first hypothesis is that 21 days of diet supplementation with phenols may not be long enough to produce a significant increase in the α-tocopherol concentration in the muscle. For example, one study[53] in growing pigs did not establish differences of α-tocopherol muscle concentration in response to green tea polyphenols added to the diet over a 35 day period. Nevertheless, in our study to explain the lack of differences in α-tocopherol muscle concentration, a plausible reason could be the composition of the rat muscle fibre. Alpha-tocopherol is deposited in greater amounts in skeletal muscles rich in fibre types with high oxidative capacity (fibres type I, red/slow muscle) than in muscle fibre types with low oxidative capacity (fibres type II, white/fast muscle).[54] Rat muscle is characterized by a low proportion of type I fibres, approximately only 6%.[55] Additionally, the characteristics of the rat muscle (low intramuscular fat) in relation to hare or pig muscle could explain the results observed in our study that showed no statistical differences in the α-tocopherol deposition in rat muscle in relation to dietary phenol supplementation.

3.3. α-Tocopherol content in faeces

The main route for eliminating α-tocopherol from the body is through faeces, mainly as a result of incomplete intestinal absorption. Secretion from mucosal cells, desquamation and biliary excretion also contribute to faecal α-tocopherol.[56,57] In order to assess whether the higher concentration of α-tocopherol in the liver in some treatments (Fig. 1) could be related to greater efficiency in the absorption of dietary α-tocopherol, the rat faeces were studied. The results of the analysis of the α-tocopherol content in the faeces for each treatment as group means (combined sexes) or separated by gender are shown in Fig. 3. Analyzing

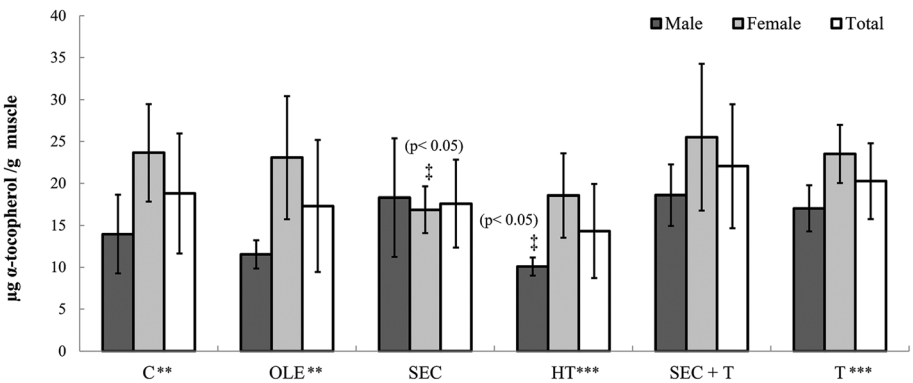

Fig. 2 Total α-tocopherol in muscle tissue for each treatment, separated by gender for each treatment (mean ± SD). C (control), OLE (oleuropein), SEC (secoiridoids), HT (hydroxytyrosol), SEC + T (secoiridoids + thyme), T (thyme). Statistically significant differences by gender for each treatment: **($P < 0.025$), ***($P < 0.01$). Statistically significant differences by treatment compared to the control: ‡($P < 0.05$).

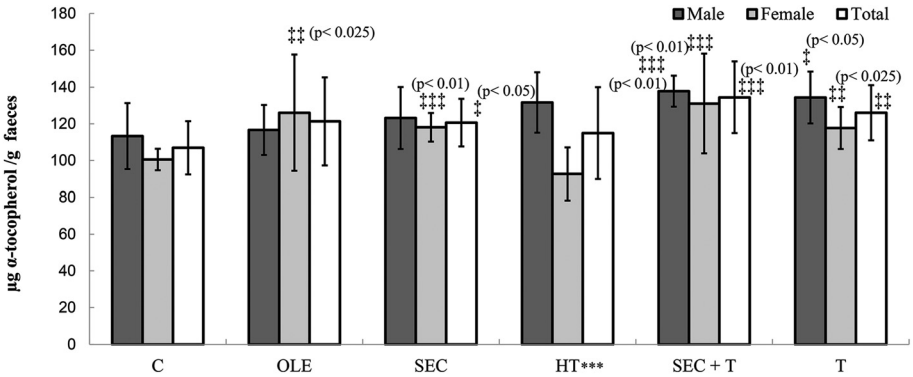

Fig. 3 Total α-tocopherol content in faeces for each treatment, separated by gender for each treatment (mean ± SD). C (control), OLE (oleuropein), SEC (secoiridoids), HT (hydroxytyrosol), SEC + T (secoiridoids + thyme), T (thyme). Statistically significant differences by gender for each treatment: ***($P < 0.01$). Statistically significant differences by treatment compared to the control: ‡($P < 0.05$), ‡‡($P < 0.025$), ‡‡‡($P < 0.01$).

the global data, some treatments (SEC, SEC + T and T) produced a significant increase in the α-tocopherol concentration in the faeces compared with the control group. Nevertheless, the HT group showed no significant increase in the α-tocopherol content in the faeces compared with the control, even though this group showed a significant increase in the α-tocopherol concentration in the liver (Fig. 1). It is noteworthy that the SEC + T group showed a high concentration of α-tocopherol in the liver (Fig. 1) and the highest in faeces (Fig. 3) compared with the control group. A tendency was observed regarding sexual dimorphism (Fig. 3). In general, the α-tocopherol content in the faeces of the male rats was higher than in the faeces of the females, but only the HT group showed significant differences ($P < 0.01$). So, the higher excretion of α-tocopherol in male faeces could partially explain the lower concentration of α-tocopherol observed in the male livers (Fig. 1).

3.4. Plausible synergy between secoiridoids and thyme in α-tocopherol

From the SEC + T group statistical significance in the vitamin E concentration in female rat livers and its almost statistical significance in muscle tissue, it seems possible that an enhanced bioavailability of α-tocopherol could occur in the presence of thyme. These results are in the same line as the synergic findings reported in ref. 38 which detected an increase of olive phenolic compounds' bioavailability in rat plasma due to the presence of thyme in the diet containing olive phenolic compounds.

3.5. Phenolic compounds

There are a number of processes occurring during vitamin E absorption, metabolism and trafficking that could be targeted by phenolics. One mechanism could be the interaction with mixed micelle formation in the small intestine, where the more abundant lipid-soluble phenolics may compete with the incorporation into micelles and the uptake of vitamin E into enterocytes. Nevertheless, some studies suggested that olive phenolic compounds are absorbed from the intestine not through a pathway dependent on the chylomicron formation.[58] While the more simple phenolic compounds, such as phenolic acids, can cross the cell membrane *via* passive diffusion, more complex phenolic compounds, such as flavonoid glycosides, cross the enterocyte cell membrane *via* two active mechanisms: lactase-phlorizin hydrolase (LPH) or active sodium-dependent glucose transporter (SGLT1).[59] Another mechanism could be the competition between phenolic compounds and vitamin E in the liver during the phase II metabolism. Nevertheless, phenolic compounds unlike vitamin E undergo phase II metabolism not only in the liver but also in the enterocyte cytosol.[59] Finally, another relevant mechanism could be the influence of phenolic compounds on the deposition of vitamin E in peripheral tissues and tissues rich in LDL receptors (discussed below).

3.5.1. Liver phenols. Numerous *in vivo* studies have shown the capacity of some phenolic compounds to protect α-tocopherol from oxidation in LDL.[60–62] So, the increase of the LDL phenolic content could account for the increase of the resistance of LDL to oxidation, and the decrease of the *in vivo* oxidized LDL. The tocopherol in LDL can be acquired by tissues with LDL receptors, the liver being one of the tissues with the highest LDL receptors.[42]

In the present study, the average concentration (nmol g^{-1} liver) of the main phenolic metabolites whose concentrations in the liver increased after the dietary phenol supplementation compared with the control group is shown in Table 2, and the proposed metabolic pathways of the olive and thyme phenols are shown in Fig. 4. In contrast to that observed in the muscle (see below), fewer metabolites derived from olive phenols and more metabolites derived from thyme phenols were detected in the liver. The main metabolites quantified from olive phenols in the liver were hydroxytyrosol-3-*O*-sulphate (phase II metabolite) and homovanillic derivatives (mainly homovanillic acid glucuronide), as obtained in our previous study.[41] Nevertheless, the concentration of hydroxytyrosol-3-*O*-sulphate detected in rat livers in the present study was lower than that

Table 2 Main phenolic metabolites (mean±SD) whose concentrations increased, expressed in nmol g^{-1} fresh tissue, before (control) and after the intake of HT (hydroxytyrosol), SEC (secoiridoids), OLE (oleuropein), SEC + T (secoiridoids + thyme), and T (thyme)

	Control	HT	SEC	OLE	SEC + T	T
Muscle Tissue (nmol g^{-1} muscle)						
Hydroxytyrosol 3-*O*-sulphate	0.7 ± 0.6	1.0 ± 0.9‡	2.2 ± 1.2** ‡‡‡	2.0 ± 0.7‡‡‡	0.8 ± 0.6	n.d.
Homovanillic acid sulphate	0.2 ± 0.1	n.d.	1.6 ± 0.8	0.9 ± 0.8‡‡	n.d.	n.d.
Homovanillic acid glucuronide	0.4 ± 0.2	1.3 ± 0.2‡‡	2.4 ± 1.1** ‡‡‡	1.5 ± 0.2‡‡‡	1.4 ± 0.1‡‡‡	n.d.
Oleuropein	n.d.	n.d.	0.3 ± 0.2	0.5 ± 0.4‡‡‡	n.d.	n.d.
Homovanillic alcohol sulphate	n.d.	0.6 ± 0.4*	2.1 ± 1.1** ‡‡‡	1.6 ± 0.5‡‡‡	n.d.	n.d.
Hydroxytyrosol acetate sulphate	n.d.	n.d.	1.3 ± 1.2‡‡‡	0.9 ± 0.8‡‡	0.7 ± 0.5	n.d.
Coumaric acid sulphate	0.3 ± 0.2	n.d.	n.d.	n.d.	0.7 ± 0.2‡‡‡	1.4 ± 0.3‡‡‡
Thymol sulphate	n.d.	n.d.	n.d.	n.d.	39.7 ± 25.3* ‡‡	104 ± 72.6‡‡‡
Hydroxyphenylpropionic acid sulphate	n.d.	n.d.	n.d.	n.d.	6.7 ± 2.8* ‡‡‡	25.9 ± 16.0‡‡‡
Thymol glucuronide	n.d.	n.d.	n.d.	n.d.	1.1 ± 1.1* ‡‡‡	2.54 ± 1.4‡‡‡
Liver Tissue (nmol g^{-1} liver)						
Hydroxytyrosol 3-*O*-sulphate	1.6 ± 0.2	2.6 ± 1.3‡‡‡	3.0 ± 1.3‡‡‡	2.0 ± 0.5‡‡‡	1.2 ± 0.8‡‡	n.d.
Homovanillic alcohol sulphate	0.2± 0.0	0.3 ± 0.2‡‡	0.6 ± 0.3‡‡‡	0.2 ± 0.2	1.2 ± 0.7** ‡‡‡	n.d.
Homovanillic acid glucuronide	2.7 ± 0.8	3.4 ± 1.0‡‡‡	2.8 ± 1.1‡‡‡	3.8 ± 1.0‡‡‡	0.8 ± 0.6	n.d.
Oleuropein	n.d.	n.d.	0.2 ± 0.0* ‡‡‡	0.2 ± 0.1* ‡‡‡	n.d.	n.d.
Coumaric acid sulphate	n.d.	n.d.	n.d.	n.d.	2 ± 0.8‡‡‡	3.0 ± 1.8** ‡‡‡
Hydroxyphenylpropionic acid sulphate	n.d.	n.d.	n.d.	n.d.	9.1 ± 7.2‡‡‡	14.9 ± 10.0** ‡‡‡
Caffeic acid sulphate	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.3** ‡‡‡	1.7 ± 0.6‡‡‡
Ferulic acid sulphate	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.3‡‡‡	0.7 ± 0.6‡‡‡
Coumaric acid glucuronide	n.d.	n.d.	n.d.	n.d.	0.7 ± 0.1‡‡‡	0.8 ± 0.1‡‡‡
Thymol sulphate	n.d.	n.d.	n.d.	n.d.	10.5 ± 9.4‡‡‡	23.0 ± 13.6‡‡‡
Thymol glucuronide	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.3	2.0 ± 2.0‡‡‡
p-Cymene diol glucuronide	n.d.	n.d.	n.d.	n.d.	n.d.	1.7 ± 1.3‡‡‡
Hydroxyphenylacetic acid	0.01 ± 0.00	n.d.	n.d.	n.d.	4.7 ± 2.2‡‡‡	5.6 ± 4.1‡‡‡
Dihydroxyphenylpropionic acid	0.04 ± 0.02	n.d.	n.d.	n.d.	0.4 ± 0.2‡‡	1.2 ± 1.0‡‡‡

Statistically significant difference by gender for each treatment: $^{*}(P < 0.05)$, $^{**}(P < 0.025)$. Statistically significant difference by treatment compared to the control: $^{‡}(P < 0.05)$, $^{‡‡}(P < 0.025)$, $^{‡‡‡}(P < 0.01)$. n.d.: not detected.

reported in our previous study after the acute intake of a single dose of olive phenolic extract (3 g kg^{-1} rat weight) dispersed in water and administered by intragastric gavage. The differences between the two studies could be related to the phenol dose and the manner in which it was administered. In the present study, the phenol was administered by supplementing the diet (21 days) at a low daily dose (5 mg per kg rat weight per day), instead of an acute intake, resulting in a lower accumulation of phenolic metabolites in the liver. In addition, the tissue perfusion with isotonic solution performed to remove the remaining blood irrigating tissues after sacrifice of the rats could also explain this fact.

Thymol sulphate and thymol glucuronide were the main metabolites related to the thyme detected in the liver, which are in agreement with the literature.[38,63–65] Furthermore, *p*-cymene-diol glucuronide was detected in the T group (Table 2) and this is a good biomarker of thyme phenols, providing a good indication of thyme phenolic exposure similar to that observed in human plasma after the intake of a thyme phenol-enriched olive oil.[38] Similar to what was observed to a lesser extent in the muscle (see the next section), sexual dimorphism for some metabolites was noted, favouring the female gender (Table 2).

3.5.2. Muscle tissue phenols. The average concentration (nmols g^{-1} muscle) of the main phenolic metabolites whose concentrations in the muscle increased after the phenol supplementation of the diet related to the control group is shown in Table 2. In contrast to what was observed with the α-tocopherol, after the sustained diet supplementation with different phenols (olive and/or thyme), all experimental groups showed a phenolic deposition in muscle, therefore demonstrating its bioavailability. The main phenolic metabolites related to olive were hydroxytyrosol phase-II metabolites (hydroxytyrosol-3-*O*-sulphate and hydroxytyrosol-acetate sulphate) and homovanillic derivatives (homovanillic acid glucuronide and homovanillic alcohol sulphate) (Fig. 4) which are derived from homovanillic alcohol which, in turn, comes from hydroxytyrosol metabolism.[65] In relation to the main metabolites related to thyme, thymol sulphate showed the largest increase, after sustained dietary supplementation, jointly with hydroxyphenilpropionic acid sulphate and thymol glucuronide (Table 2), similar to what was observed after the sustained intake of olive oil enriched with olive and thyme phenols.[32] Sexual dimorphism has been detected in some phenolic compounds favouring female rats (Table 2). This phenomenon was detected in the SEC and HT group for metabolites derived from olive and in the SEC + T group for the metabolites derived from thyme.

Finally, taking into account the α-tocopherol concentration in the muscle (Fig. 2) and the phenolic metabolites detected in it (Table 1), the SEC + T and T groups showed higher accumulation of phenol metabolites (mainly due to thymol sulphate)

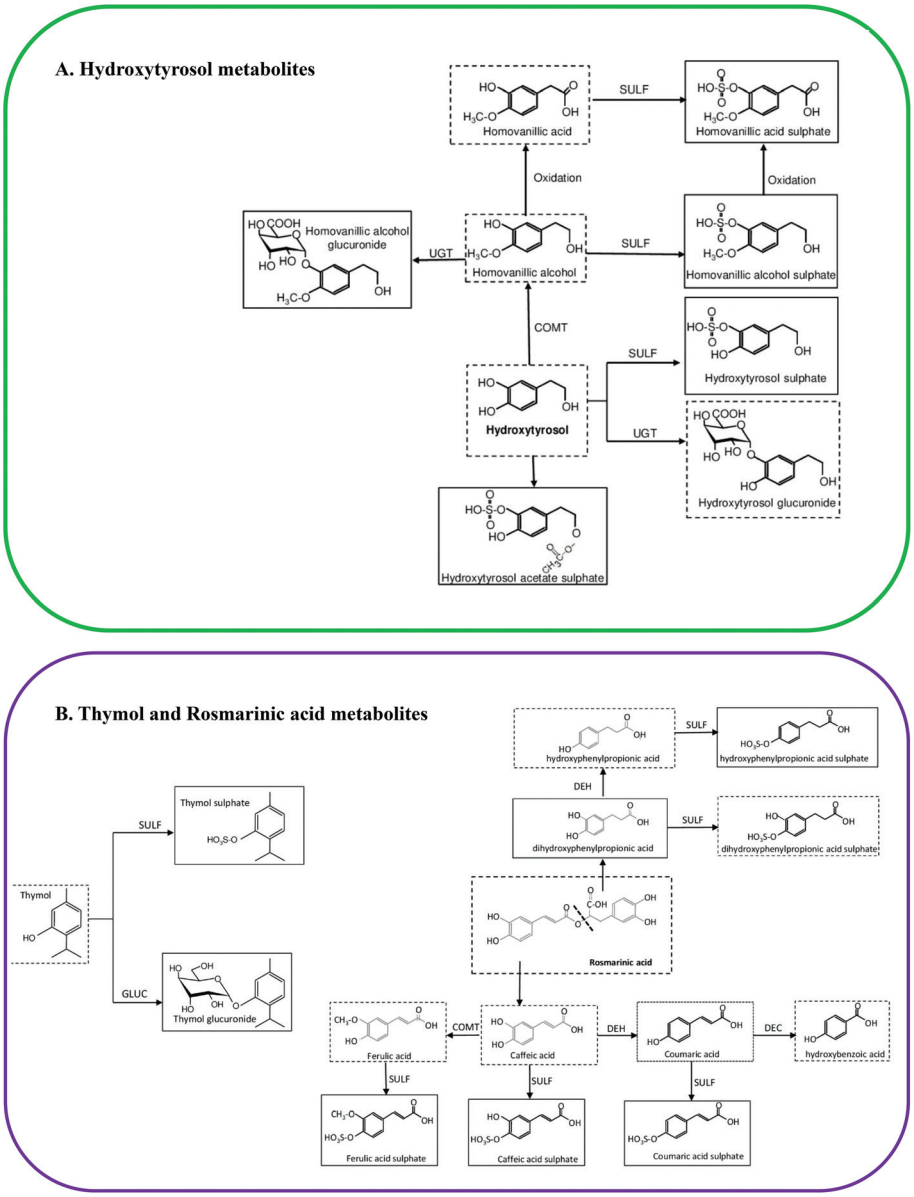

Fig. 4 Proposed metabolic pathways of the main metabolites detected in the study.

and a higher mean α-tocopherol concentration, although it was not large enough to be statistically significant.

4. Conclusions

In summary, giving Wistar rats a sustained dietary supplementation (5 mg per kg rat weight per day) for 21 days demonstrated the ability of some of the olive (OLE and HT) and thyme (T) phenolic compounds to maintain higher levels of α-tocopherol in the female liver, although all diets supplied the same daily dose of α-tocopherol. In addition, a synergic effect between the olive phenols and thyme (SEC + T) was observed. In general, no clear relationship was observed between the increase in the α-tocopherol concentration in the liver and a lower concentration of α-tocopherol in the faeces, indicating a higher efficacy in the α-tocopherol accumulation in the liver that could be related to the dietary supplementation with phenolic compounds. Polyphenols are thus expected to act at the water–lipid interface at the liver tissue level and may be involved in the oxidation regeneration pathway of vitamin E. Nevertheless, in order to find out more features about *in vivo* vitamin E regeneration orchestrated by polyphenols it is necessary to perform future analysis more complex in order to certify/amplify the biological fact presented in this preliminary study.

Conflicts of interest

The authors declare no conflict of interest.

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