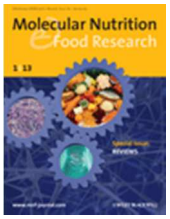

Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats.

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID:	Draft
Wiley - Manuscript type:	Food & Function
Date Submitted by the Author:	n/a
Complete List of Authors:	López de las Hazas, Maria-Carmen; University of Lleida, Food Technology Rubió, Laura; University of Lleida, Food Technology Krotonoulas, Aristotelis; IMIM-Institut, Hospital del Mar d'Investigaciones Mèdiques, de la Torre, Rafael; IMIM-Institut, Hospital del Mar d'Investigaciones Mèdiques, Solà, Rosa; Universitat Rovira i Virgili, Unitat de Recerca en Lípids i Arteriosclerosis Motilva, Maria J.; University of Lleida, Food Technology
Keywords:	Biodistribution, Dose-uptake, Hydroxytyrosol, Phenol metabolites, Tissue distribution

Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats

Maria-Carmen López de las Hazas[1], Laura Rubió[1], Aristotelis Krotonoulas[2], Rafael de la Torre[2,3], Rosa Solà[4], María-José Motilva[1*]

[1] Food Technology Department, Agrotecnio Research Center, Escuela Técnica Superior de Ingeniería Agraria, University of Lleida, Av/Alcalde Rovira Roure 191, 25198 Lleida, Spain

[2] Human Pharmacology and Clinical Neurociences Research Group, IMIM-Institut, Hospital del Mar d'Investigaciones Mèdiques, Doctor Aiguader 88, 08003 Barcelona, Spain. Pompeu Fabra University (CEXS-UPF), Dr. Aiguader 80, 08003 Barcelona, Spain

[3] Cardiovascular Risk and Nutrition Research Group, REGICOR Study Group, IMIM-Research Institute Hospital del Mar, Barcelona, Spain

[4] Unitat de Recerca en Lípids i Arteriosclerosis, CIBERDEM, St. Joan de Reus University Hospital, IISPV, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, C/ Sant Llorenç 21, 43201 Reus, Spain

***Correspondence**: Fax: + 34 973 702596; E-mail: motilva@tecal.udl.es (Dr. Maria-José Motilva)

Abbreviations: VOO: Virgin Olive Oil, HT: Hydroxytyrosol; HT-S: hydroxytyrosol sulfate; HVAlc: Homovanillic alcohol; HVAC. Homovanillic Acid; HVAlc-S: Homovanillic alcohol sulfate; HVAc-S. Homovanillic Acid Sulfate; HT 4-Gluc:

Hydroxytyrosol 4 glucuronide; HT 3-Gluc: Hydroxytyrosol 3 glucuronide; HVAlc-gluc: Homovanillic alcohol glucuronide

Keywords: Biodistribution / Dose-uptake / Hydroxytyrosol / Phenol metabolites / Tissue disposition

Abstract

The aim of the present study was to investigate the relationship between the hydroxytyrosol (HT) dose intake and the HT metabolites tissue uptake in order to assess if the HT levels detected in tissues after the administration of different doses are relevant and sufficiently compatible with those that have been reported as *in vitro* modulators of biological functions. Rats were given a refined olive oil enriched with HT at different doses (1, 10 and 100 mg/kg) and after 5 hours they were sacrificed. Plasma samples and different organs as liver, kidney, heart and brain were obtained, and HT metabolites were analyzed by UPLC-MS/MS. The results showed that HT and its metabolites had a dose-dependent response in both the plasma and tissue deposition for all the organs analyzed. It is noteworthy the appearance of the native bioactive form of HT in circulation and the accumulation in the liver and kidney. The detection of greater amounts of HT sulfate conjugates even at nutritionally-relevant human doses indicates that the bioactivities of these metabolites are also worthy of future research in order to understand the clinical implications of olive oil phenolics, specially to prevent certain hepatic and renal diseases.

In recent years, hydroxytyrosol (HT) and its derivatives have led to a great interest from the virgin olive oil (VOO) producers and manufacturers of nutraceutical supplements. The increasing interest in HT is mainly due to the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) inform that established a cause-and-effect relationship between the consumption of olive oil polyphenols and protection of LDL particles from oxidative damage [1]. Based on this positive opinion, the health claim "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*" was included in the list of health claims [2], being the only authorized health claim in the European Union regarding polyphenols and health.

In relation to the mechanisms by which olive phenolic compounds can exercise their cardioprotective activity it is becoming more evident that polyphenols exert their cellular protection by interacting with intracellular signaling pathways involved in pathological processes [3]. After absorption, plasma proteins can be targets of the phenolic metabolites, and there are also possible interactions with proteins in specific target organs in the human body [4]. Therefore, along with the effort to elucidate molecular targets of HT, knowledge about its organ distribution and tissue uptake may increase comprehension of its beneficial effects on health. For this, it is necessary to assess if the HT levels detected in tissues are relevant and sufficiently compatible with those that have been reported as *in vitro* modulators of biological functions. In relation to the metabolism and tissue uptake of HT, previous studies have been performed in rats, either using intravenously injected [^{14}C] hydroxytyrosol [5] or by oral administration of an olive phenol extract [6]. Although the pharmacokinetic response and tissue distribution of HT was thoroughly investigated in these aforementioned studies, no information is available in the literature regarding the dose-dependent uptake of HT and its main metabolites into target tissues.

In this context, the present study aimed to investigate the relationship between the HT dose intake and tissue uptake in rats, and thus, providing complementary information in relation to the target/dose relationship. For this purpose, liver, kidney, brain and heart were collected after the administration of three different doses: 1 mg HT/kg rat weight (HT-1) compatible with human dietary habits [7], and two higher doses (10 mg/kg rat weight: HT-10 and 100 mg/kg rat weight: HT-100) that could mimic medium and high exposures of HT through dietary supplements. Detailed materials and methods are contained in the Supporting Information.

Results of the plasma analysis showed that free HT and its metabolites were detected and were measurable after the three administered doses (**Table 1**), except for the glucuronide conjugates, which were only detected at the highest doses (HT-10 and HT-100). In the control plasma (vehicle), none of these compounds were detected, which validates them as products of HT metabolism. Sulfation was the most relevant conjugation pathway at the three administered doses compared with the glucuronide conjugates. It is important to highlight that the free form of HT was detected in the plasma after the administration of all three doses and that the recovery of free HT appeared to rise in a dose-dependent fashion (from 0.05 µmol/L HT-1 to 12.9 µmol/L HT-100) as with the metabolites (p *trend*<0.001).

The dose effect on the tissue uptake of HT was first studied on two metabolic tissues (liver, kidney) obtained five hours after the intake of the vehicle (refined olive oil) and the different doses of HT (**Table 2**). In general, the nature of the HT metabolites was similar to the plasma and a significant dose-dependent uptake was observed for all metabolites studied (p *trend*<0.001) except for HVAlc in the kidney (**Table 2**). Additionally, significant differences were observed for the metabolites quantified at all HT doses compared to the vehicle group (p<0.001).

Interestingly, the free active form of HT was detected in both liver and kidney. If we compare our results with those that have been reported as *in vitro* modulators of biological functions, the concentrations of HT tested *in vitro* cell lines showed significant effects in the range of 5-10 μM in the case of kidney [8] and in the range of 1−5 μM for hepatic cell lines [9], which were much higher than those detected in our study (2-83 nM) (**Table 2**). Despite that, recent studies have reported that HT possesses *in vivo* hepatoprotective effects [10-12]. So it can be hypothesized that HT metabolites may be active per se, or provide a pool for local or systemic regeneration of HT *in vivo*. HT metabolites, which might also show biological activities, probably explain the modulation of other pathways/mechanisms from than those previously reported related to direct antioxidant/scavenging mechanisms [8,13]. Indeed, the most recent hypothesis regarding the mechanism of action of polyphenols is that they can exert protective effects modulating signal transduction, cell signaling, gene expression and cellular communication in several pathways [14].

Besides the metabolic tissues, two target tissues (brain and heart) were studied showing a lower deposition of HT metabolites (**Table 3**). Regarding the brain, some of the HT metabolites were also detected in the vehicle group suggesting that the low concentrations detected could be produced endogenously from dopamine metabolism [15]. It is remarkable, however, that at the highest dose (HT-100), HT-S, HV-Ac, HVAc-S and HVAlc-S presented significant increases compared to the other administered doses, indicating that only at higher doses some metabolites from the HT intake could cross the blood-brain barrier. In fact, dose-dependent accumulation of HT-S and HVAlc-S was observed in the brain ($p\ trend<0.001$) (**Table 3**). A previous study reported that the administration of 100 mg/kg of HT in mice enhanced cytoprotection and the resistance of dissociated brain cells to oxidative stress [16]. Thus, it could be

hypothesized that the accumulation of these metabolites in the brain after a pharmacological dose of HT could exert a neuro-protective activity in the central nerve system. In the present study, the free form of HT was not detected in the brain, in contrast to a previous study, in which 100 mg/kg of HT was administered through the femoral vein [17] and considerable amounts of free HT were detected in the brain. Compared with the oral route of our study, intravenous administration could explain the higher exposure of HT to the brain tissue and its detection in its free form.

In contrast to other tissues, HT metabolites were not detected in the heart in HT-1 and HT-10 groups (**Table 3**), being HT-S the main metabolite quantified after the HT-100. Unlike in the liver and kidney, free HT was not detected in the heart. However, free HT and its metabolites were detected in plasma even at the nutritionally-relevant dose (**Table 1**), which could explain the cardioprotective effects of VOO phenols previously described at different circulation targets. Reported *in vivo* studies indicate that HT can reduce endothelial activation [18], inhibit platelet aggregation [19] and reduce the plasma-reduced homocysteine concentration [20], effects that have been associated with cardiovascular protection and could be related to the presence of circulating HT and its metabolites.

Interestingly, in samples obtained from HT-100 group, a significant gender effect was observed in the plasma (**Table 1**), liver and kidney (**Table 2**). Plasmatic concentrations of free HT and its main metabolites HT-S and HVAc were significantly higher ($p<0.05$) in females (**Table 1**). Similarly, the concentrations of free HT and metabolites (HT-S, HVAc, HVAc-S and HVAlc-S) were significantly higher ($p<0.05$) in the liver from females, this being the tissue where the gender effect was more striking (**Table 2**). No gender effect was observed in the uptake of HT metabolites in the brain and heart (data not shown). Our results are in line with previous studies in which

genistein and daidzein, two major isoflavones in soy, also presented an enhanced oral bioavailability in females than in males [21]. The main reason that could explain these differences seems to be the different expression profiles of metabolizing enzymes and efflux transporters in the main metabolic disposition organs, such as the intestine, liver and kidney [22]. In other studies, pulsatile versus continuous release of growth hormone in male and female rats, respectively, has been suggested as a major reason for the sex-dependent differences in the expression profiles of the hepatic phase I metabolizing enzymes [23].

Summarizing, our study showed that HT and its metabolites could be accumulated in a dose-dependent manner basically in the liver, kidney and brain and were detected in these tissues even at nutritionally-relevant human doses, a dose that was not previously studied in tissue disposition. The detection of free HT in liver and kidney is noteworthy. To date, this appears to be the only biologically active form, and thus, it provides relevant information for optimizing the potential applications of HT to prevent certain hepatic and renal diseases. However, the detection of greater amounts of HT metabolites in tissues, specifically the sulfate conjugates, indicates that the bioactivities of these metabolites are also worthy of future research in order to understand the clinical implications of olive oil phenolics. The obtained results regarding the target/dose relationship of HT and its metabolites, together with the literature data on the biological effects, allow increasing our understanding of the health beneficial effects of HT.

This study was supported by the Spanish Ministry of Education and Science (AGL2012-40144-C03-03, AGL2012-40144-C03-02 and AGL2012-40144-C03-01

projects), by the University of Lleida through the M.C. López de las Hazas and L. Rubió grant. CIBERobn is an iniciative of Instituto de Salud Carlos III (ISCIII).

The authors are grateful for the generous gift of hydroxytyrosol from Seprox Biotech, Madrid, Spain. The authors declare no conflict of interest.

References

[1] EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), Scientific opinion on the substantiation of health claims related to polyphenols in olive. Pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* 2011, 9 , 2033 Available online: www.efsa. europa.eu/efsajournal.

[2] COMMISSION REGULATION (EU) No 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. *Official Journal of the European Union L136*.

[3] Konstantinidou, V., Covas, M.I., Muñoz-Aguayo, D., Khymenets, O., et al., In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *FASEB J.* 2010, *24*, 2546 – 2557.

[4] Rohn, S., Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins. *Food Res. Int.* 2014, *65*, 13-19.

[5] D'Angelo, E., Manna, C., Migliardi, V., Mazzoni, O., et al., Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug. Metabol. Dis*. 2001, *29*, 1492-1498.

[6] Serra, A., Rubió, L., Borràs, X., Macià, A., et al., Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Mol Nutr. Food Res.* 2012*, 56*, 486–496.

[7] Kotronoulas, A., Pizarro, N., Serra, A., Robledo, P., et al., Dose-dependent metabolic disposition of hydroxytyrosol and formation of mercapturates in rats. *Pharmacol. Res.* 2013, *77*, 47–56.

[8] Deiana, M., Incani, A., Rosa, A., Atzeri, A., et al., Hydroxytyrosol glucuronides protect renal tubular epithelial cells against H(2)O(2) induced oxidative damage. *Chem. Biol. Interact*. 2011, *193*, 232–239.

[9] Giordano, E., Davalos, A., Nicod, N., Visioli, F., Hydroxytyrosol attenuates tunicamycin-induced endoplasmic reticulum stress in human hepatocarcinoma cells. *Mol. Nutr. Food Res*. 2014, *58*, 954–962.

[10] Zhao, B., Ma, Y., Xu, Z., Wang, J., et al., Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via inactivating AKT and nuclear factor-kappa B pathways. *Cancer Lett*. 2014, *347*, 79–87.

[11] Pan, S., Liu, L., Pan, H., Ma, Y., et al., Protective effects of hydroxytyrosol on liver ischemia/reperfusion injury in mice. *Mol. Nutr. Food Res*. 2013, *57*, 1218–1227.

[12] Kim, M.S., Koppula, S., Sung, S.J., Lee, S.R., et al., Olea europaea Linn (Oleaceae) Fruit Pulp Exhibits Hypocholesterolemic and Hepatoprotective Effects via Regulation of Peroxisome Proliferation-Activated Receptor Alpha in High-Fat Diet-Fed Rats. *Trop. J. Pharm. Res*. 2014, *12*, 13-31.

[13] Khymenets, O., Fito, M., Tourino, S., Munoz-Aguayo, D., et al. Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial

health effects after olive oil ingestion. *Drug Metab. Dispos*. 2010, *38*, 1417-1421.

[14] Kang, N.J., Shin, S.H., Lee, H.J., Lee, K.W., Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis. *Pharmacol. Ther.* 2011, *130*, 310–324.

[15] Gallardo, E., Madrona, A., Palma-Valdés, R., Trujillo, M., et al., The effect of hydroxytyrosol and its nitroderivatives on catechol-O-methyl transferase activity in rat striatal tissue. *RSC Adv.* 2014, *4*, 61086-61091.

[16] Schaffer, S., Podstawa, M., Visioli, F., Bogani, P., et al., Hydroxytyrosol-Rich Olive Mill Wastewater Extract Protects Brain Cells in Vitro and ex Vivo. *J. Agric. Food Chem*. 2007, *55*, 5043-5049.

[17] Wu, Y.T., Lin, L.C, Tsai, T.H., Measurement of free hydroxytyrosol in microdialysates from blood and brain of anesthetized rats by liquid chromatography with fluorescence detection. *J. Chromatogr. A.* 2009, *1216*, 3501–3507.

[18] Carluccio M.A., Siculella, L., Ancora, M.A., Massaro, M. et al., Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: Antiatherogenic properties of Mediterranean diet phytochemicals. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2003, *23*, 622-629.

[19] Visioli F., Caruso D.,Virgin Olive Oil Study (VOLOS): Vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. *Eur.J. Clin. Nutrition*, 2005, *44*, 121-127.

[20] Priora R., Summa D., Administration of minor polar compound-enriched extra virgin olive oil decreases platelet aggregation and the plasma concentration of reduced homocysteine in rats. *J. Nutr*. 2008, *138*, 36-41.

[21] Kulkarni, K.H., Yang, Z., Niu, T., Hu, M., Effects of estrogen and estrus cycle on pharmacokinetics, absorption, and disposition of genistein in female Sprague-Dawley rats. *J Agric Food Chem*. 2012, *60*, 7949–7956.

[22] Buckley, D.B., Klaassen, C.D., Mechanism of gender-divergent UDP-glucuronosyltransferase mRNA expression in mouse liver and kidney. *Drug Metab. Dispos*., 2009, *37*, 834–840.

[23] Waxman D.J., Holloway M.G., Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol. Pharmacol.* 2009, *76*, 215–228.

Table 1. Concentration (µmol/L plasma) of HT and its metabolites in rat plasma after 5 hours of the administration of different doses of HT and the vehicle (refined oil). Effect of gender in plasma concentration at HT-100 dose.

Compound (µmol/L)	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	0.05 ±0.03[a]	0.99 ± 0.66[a]	12.9 ± 5.05[b]	8.38 ± 4.10*	17.4 ± 8.40*
HT-S	n.d.	0.12 ± 0.05[a]	1.28 ± 0.85[a]	16.5 ± 6.40[b]	9.82 ± 3.82*	22.7 ± 8.40*
HT 4'-Gluc	n.d.	n.d.	0.18 ± 0.12[a]	1.87 ± 1.03[b]	1.77 ± 1.13	1.93 ± 0.86
HT 3'-Gluc	n.d.	n.d.	0.11 ± 0.10[a]	1.18 ± 0.69[b]	0.91 ± 0.49	1.50 ± 0.68
HVAc	n.d.	0.05 ± 0.09[a]	0.53 ± 0.37[a]	7.65 ± 2.86[b]	2.87 ± 0.63*	11.2 ± 5.35*
HVAc-S	n.d.	0.28 ± 0.15[a]	2.17 ± 1.08[a]	14.9 ± 5.42[b]	13.3 ± 5.01	16.6 ± 5.83
HVAlc	n.d.	0.12 ± 0.09[a]	0.14 ± 0.10[a]	0.18 ± 0.16[a]	0.09 ± 0.09	0.25 ± 0.17
HVAlc-S	n.d.	0.09 ± 0.03[a]	0.38 ± 0.16[b]	2.21 ± 1.09[c]	2.32 ± 0.65	2.16 ± 1.26
HVAlc-Gluc	n.d.	n.d.	0.12 ± 0.06[a]	0.57 ± 0.32[b]	0.74 ± 0.43	0.48 ± 0.18

HT: hydroxytyrosol; HT-S: hydroxytyrosol-sulfate; HT 4-Gluc: hydroxytyrosol 4' glucuronide; HT 3-Gluc: hydroxytyrosol 3' glucuronide; HVAc: homovainillic acid; HVAc-S: homovainillic acid-sulfate; HVAlc: homovainillic alcohol; HVAlc-S: homovainillic alcohol-sulfate; HVAlc-Gluc: homovainillic alcohol-glucuronide.

Values are mean ± SD (n=8). Values male and female are mean ± SD (n=4).

[abc] mean significant differences between doses in the same row ($p < 0.001$)

* mean significant differences between genders in the same row of HT-100 ($p < 0.05$)

n.d. not detected

Table 2. Concentration (nmol/g fresh tissue) of HT and its metabolites in rat metabolic tissues (liver and kidney) after 5 hours of the administration of different doses of HT and the vehicle (refined oil). Effect of gender in the tissue disposition at HT-100 dose.

Compound (nmol/g fresh tissue)	LIVER					
	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	0.1 ± 0.01[a]	1.11 ± 0.59[a]	17.5 ± 12.9[b]	7.66 ± 1.68*	27.4 ± 11.5*
HT-S	n.d.	0.02 ± 0.01[a]	1.26 ± 0.70[a]	27.4 ± 26.5[b]	8.76 ± 1.84*	46.1 ± 26.6*
HT 4-Gluc	n.d.	n.d.	n.d.	0.21 ± 0.09	0.16 ± 0.12	0.26 ± 0.03
HT 3-Gluc	n.d.	n.d.	n.d.	0.22 ± 0.14	0.14 ± 0.07	0.31 ± 0.14
HVAc	n.d.	n.d.	0.03 ± 0.05[a]	2.67 ± 3.20[b]	0.50 ± 0.17*	4.85 ± 3.46*
HVAc-S	n.d.	0.04 ± 0.02	0.56 ± 0.33[a]	6.71 ± 3.28[b]	4.92 ± 3.65*	8.49 ± 1.63*
HVAlc	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HVAlc-S	n.d.	n.d.	0.32 ± 0.13[a]	5.23 ± 5.28[b]	1.80 ± 0.27*	8.66 ± 5.74*
HVAlc-Gluc	n.d.	n.d.	n.d.	0.04 ± 0.03	0.02 ± 0.01	0.07 ± 0.02
Compound (nmol/g fresh tissue)	**KIDNEY**					
	Vehicle	HT-1	HT-10	HT-100		
				Mean value	Male	Female
HT	n.d.	n.d.	2.73 ± 2.33[a]	33.9 ± 10.3[b]	26.1 ± 3.28*	41.8 ± 10.3*
HT-S	0.14 ± 0.16[a]	0.48 ± 0.1[a]	10.1 ± 5.74[b]	82.8 ± 38.9[c]	78.9 ± 5.70	86.8 ± 29.7
HT 4-Gluc	n.d.	0.02 ± 0.05[a]	0.25 ± 0.12[b]	0.89 ± 0.39[c]	0.88 ± 0.09	1.09 ± 0.10
HT 3-Gluc	n.d.	0.02 ± 0.05[a]	0.25 ± 0.12[b]	0.99 ± 0.45[c]	0.94 ± 0.07	1.04 ± 0.31
HVAc	0.26 ± 0.17[a]	0.33 ± 0.13[a]	1.26 ± 0.76[b]	7.90 ± 2.25[c]	6.73 ± 2.89	9.07 ± 1.65
HVAc-S	1.22 ± 0.51[a]	0.98 ± 0.31[a]	2.32 ± 0.74[b]	6.90 ± 1.33[c]	8.49 ± 2.98	5.30 ± 0.05
HVAlc	0.23 ± 0.27[a]	0.21 ± 0.22[a]	0.46 ± 0.17[b]	0.30 ± 0.30[a]	0.20 ± 0.23	0.40 ± 0.01
HVAlc-S	0.26 ± 0.02[a]	0.45 ± 0.09[a]	3.23 ± 1.38[b]	20.1 ± 4.98[c]	19.3 ± 4.63	21.9 ± 12.7
HVAlc-Gluc	n.d.	0.14 ± 0.03[a]	0.22 ± 0.03[b]	0.39 ± 0.06[c]	0.37 ± 0.10	0.41 ± 0.07

HT: hydroxytyrosol; HT-S: hydroxytyrosol-sulfate; HT 4-Gluc: hydroxytyrosol 4' glucuronide; HT 3-Gluc: hydroxytyrosol 3' glucuronide; HVAc: homovainillic acid; HVAc-S: homovainillic acid-sulfate; HVAlc: homovainillic alcohol; HVAlc-S: homovainillic alcohol-sulfate; HVAlc-Gluc: homovainillic alcohol-glucuronide.

Values are mean ± SD (n=8). Values male and female are mean ± SD (n=4).

[abc] mean significant differences between doses in the same row ($p < 0.001$)

* mean significant differences between genders in the same row of HT-100 ($p < 0.05$)

n.d. not detected

Table 3. Concentration (nmol/g fresh tissue) of HT and its metabolites in rat target tissues (heart and brain) after 5 hours of the administration of different doses of HT and the vehicle (refined oil).

Compound (nmol/g fresh tissue)	BRAIN			
	Vehicle	**HT-1**	**HT-10**	**HT-100**
HT	n.d.	n.d.	n.d.	n.d.
HT-S	0.26 ± 0.00 [a]	0.32 ± 0.05 [a]	0.35 ± 0.10 [a]	1.26 ± 0.72 [b]
HT 4-Gluc	n.d.	n.d.	n.d.	0.14 ± 0.03
HT 3-Gluc	n.d.	n.d.	n.d.	0.15 ± 0.02
HVAc	0.34 ± 0.01 [a]	0.39 ± 0.04 [a]	0.32 ± 0.02 [a]	0.40 ± 0.05 [b]
HVAc-S	0.50 ± 0.03 [a]	0.72 ± 0.10 [b]	0.49 ± 0.08 [a]	0.58 ± 0.12 [b]
HVAlc	n.d	n.d.	n.d.	n.d.
HVAlc-S	0.14 ± 0.08 [a]	0.22 ± 0.01 [a]	0.41 ± 0.12 [b]	2.58 ± 1.26 [c]
HVAlc-Gluc	n.d.	n.d.	n.d.	n.d.
Compound (nmol/g fresh tissue)	**HEART**			
	Vehicle	**HT-1**	**HT-10**	**HT-100**
HT	n.d.	n.d.	n.d.	n.d
HT-S	n.d.	n.d.	n.d.	2.73 ± 2.19
HT 4-Gluc	n.d.	n.d.	n.d.	0.02 ± 0.02
HT 3-Gluc	n.d.	n.d.	n.d	n.d
HVAc	n.d.	n.d.	n.d.	0.08 ± 0.10
HVAc-S	n.d.	n.d.	n.d.	0.19 ± 0.22
HVAlc	n.d.	n.d.	n.d.	n.d
HVAlc-S	n.d.	n.d.	n.d.	0.42 ± 0.33
HVAlc-Gluc	n.d.	n.d.	n.d.	n.d

HT: hydroxytyrosol; HT-S: hydroxytyrosol-sulfate; HT 4-Gluc: ydroxytyrosol 4' glucuronide; HT 3-Gluc: hydroxytyrosol 3' glucuronide; HVAc: homovainillic acid HVAc-S: homovainillic acid-sulfate; HVAlc: homovainillic alcohol; HVAlc-S: homovainillic alcohol-sulfate; HVAlc-Gluc: homovainillic alcohol-glucuronide

Values are mean ± SD (n=8).

[abc] mean significant differences between doses in the same row ($p < 0.001$).

n.d. not detected