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Hydroxytyrosol in functional hydroxytyrosol-enriched biscuits is highly bioavailable and decreases oxidised low density lipoprotein levels in humans

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ABSTRACT

Hydroxytyrosol (HT) and its derivatives in olive oil protect low-density lipoproteins (LDL) against oxidation. Biscuits could be a convenient alternative to broaden consumers' choice of HT-rich foods, although the biscuit matrix could affect HT bioavailability. We performed a crossover, randomized, double-blind study to evaluate HT bioavailability in HT-enriched biscuits (HT-B) *versus* non-enriched biscuits (C-B), and the effects on oxidative postprandial status. On two separate days, 13 subjects consumed 30 g of C-B or HT-B (5.25 mg HT) after overnight-fasting. Blood and urine were collected at different intervals and analysed by LC–MS-QToF. After HT-B consumption, plasma metabolites peaked between 0.5 and 1 h and were extensively excreted in urine. HT-sulphate and 3,4-dihydroxyphenylacetic acid (DOPAC)-sulphate were the main metabolites, followed by DOPAC and homovanillic acid (HVA). HT-glucuronide, DOPAC-glucuronide, HVA-glucuronide and HVA-sulphate were also detected. Postprandial oxidised-LDL concentrations decreased with HT-B. HT is a promising functional ingredient and, in biscuits, it is highly bioavailable and lowers postprandial oxidised-LDL levels.

1. Introduction

Olive oil is rich in polyphenols, and hence presents biological activity other than that due to its content in monounsaturated fat. The phenolic fraction of virgin or extra virgin olive oil has proved to positively influence cardiovascular health, with evidence strong enough for the European Food Safety Authority (EFSA) to grant the positive health opinion "olive oil polyphenols contribute to the protection of blood lipoproteins (low-density lipoprotein, LDL) from oxidative stress" (EFSA Panel on Dietetic Products & Nutrition & Allergies, 2011). Hydroxytyrosol (HT) is considered as one of the most representative phenols among the phenolic compounds in virgin and extra virgin olive oil. HT is present mainly as a secoiridoid derivative, together with minor amounts of the free form and the acetylated derivative hydroxytyrosyl acetate (Mateos et al., 2001). Other phenols, such as tyrosol and its secoiridoid derivatives, in addition to phenolic acids, flavones, lignans and isochromans, form part of the large phenolic fraction present in virgin or extra virgin olive oils. Secoiridoid derivatives of HT and tyrosol account for 90–95% of the phenolic fraction (Carrasco-Pancorbo et al., 2006), providing bitterness to olive oil (Mateos, Cert, Pérez-Camino, & García, 2004). These compounds are hydrolysed into the free forms, HT and tyrosol, partially during olive oil storage (Lavelli, Fregapane, & Salvador, 2006) and extensively during gastrointestinal digestion (Pereira-Caro et al., 2012). Thus the biological activity associated with olive oil phenols could be attributed to these free compounds, which are not bitter. Although synergism among different olive phenols is possible, numerous studies have focussed on HT, demonstrating its potential as a nutraceutical or functional ingredient, largely due to its ortho-diphenolic structure, in contrast to tyrosol, with lower biological activity (Hu, He, Jiang, & Xu, 2014). Taking into account that HT is the most representative phenol in olive oil and the positive health claim approved by EFSA, this phenol has attracted the interest of the food industry. HT is easily and cost-effectively recovered from alperujo, a solid by-product obtained in olive oil extraction (Fernández-Bolaños et al., 2005), adding value to this abundant by-product of the olive oil industry. In addition, the lack of bitterness of HT could facilitate its incorporation into foods in amounts adequate to reach the recommended consumption of 5 mg/day

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(EFSA, 2011) without providing a bitter taste to the HT-enriched food. These facts facilitate the potential use of HT as a bioactive food ingredient.

The biological activity of phenolic compounds depends on their bioavailability and metabolic fate, as well as on their digestive accessibility, which is determined by the release from the food matrix and efficiency in transepithelial passage. Several clinical trials have evidenced that olive oil phenolic compounds are absorbed in a dose-dependent manner and extensively recovered in urine as HT-derived metabolites (Rubió et al., 2012; Suarez et al., 2011). Most of these metabolites are common with those obtained in dopamine biotransformation (Gu et al., 2008). However, the food matrix may play a crucial role in phenols' bioavailability, since interaction between polyphenols and other food components, such as proteins, carbohydrates, dietary fibre, fat or alcohol, can occur, affecting HT absorption (revised by D'Archivio, Filesi, Vari, Scazzocchio, & Masella, 2010). Tuck, Freeman, Hayball, Stretch, and Stupans (2001) observed that the bioavailability of HT and tyrosol was higher when administered in an olive oil solution than in an aqueous solution. Similarly, Visioli et al. (2003) showed that the urinary excretion of HT in humans was higher when consumed as a natural olive oil component than when HT was added to refined olive oil, and the latter, in turn, was higher than when HT was added to a non-polar matrix such as yogurt. Recently, an olive leaf extract, administered in capsules, yielded higher oleuropein plasma concentrations than when it was given as a liquid formulation (De Bock et al., 2013). All these studies raise concern about the formulation of nutraceuticals and functional foods based on phenolic olive oil or HT-enriched extracts.

Cereal-based food products are daily consumed, worldwide, in large quantities and, in particular, biscuits are a common food consumed by different population groups (children, adolescents and adults). Enriching bakery products, such as biscuits, with HT can be an interesting strategy to increase the range of dietary products which provide HT, favouring the intake of this bioactive phenol that is almost exclusively present in olive oil and derived products. However, the bioavailability of HT in this food matrix should be clearly established, as well as the effect of consuming HT-enriched baked products on the levels of oxidised low density lipoproteins (oxidised-LDL). Therefore, the aim of the present study was to evaluate the bioavailability of HT incorporated into biscuits in comparison with non-enriched biscuits. Based on HT's well-known antioxidant capacity and a recent health claim approved by EFSA, the effects of consuming the phenol-enriched biscuits on postprandial blood antioxidant status were evaluated by measuring serum antioxidant capacity, using FRAP, ABTS and ORAC techniques, and plasma oxidised-LDL levels, compared to the non-enriched biscuit.

2. Materials and methods

2.1. Chemical reagents and materials

All biscuits used in this study were supplied by Nutrexpa, S.L. (Barcelona, Spain): non-enriched biscuits (control biscuit, C-B) and HT-enriched biscuits (HT-B). Considering that the losses of phenolic compounds in baked products range from 20% to 47% (Reis, Rai, & Abu-Ghannam, 2014), biscuits were enriched at 6.25 mg of HT/30 g (average biscuit portion size), assuming that 20% of HT is lost during baking.

All solvents and reagents were of analytical grade unless otherwise stated. Trichloroacetic acid, iron (III) chloride hexahydrate, potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), catechol, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), fluorescein, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azino bis-(3-ethylbenzo thiazoline-6-sulphonic acid) diammonium salt (98%) (ABTS), and 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) were from Sigma–Aldrich (Madrid, Spain). Methanol, sodium hydrogen phosphate, potassium dihydrogen phosphate, acetonitrile, hexane, hydrochloric acid (37%), formic acid, and acetonitrile (HPLC grade) were acquired from Panreac (Madrid, Spain).

HT was recovered with 95% purity from olive oil wastewaters following a patented industrial system (Fernández-Bolaños et al., 2005).

2.2. Subjects and study design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Ethics Committee of Hospital Universitario Puerta del Hierro in Majadahonda (Madrid, Spain). Volunteers' recruitment was carried out by giving short talks and placing advertisements at Complutense University. Written informed consent was obtained from all volunteers.

A randomized, controlled, crossover, double-blind study was carried out in 13 subjects (3 men and 10 women), aged 22–37, with body mass index between 20 and 30 kg/m^2. They were non-smoker, non-vegetarian, non-pregnant women, not suffering from any chronic pathology or gastrointestinal disorder. The volunteers should not have taken any medication or nutritional supplement during the last six months prior to the study. The sample size was estimated by attending to previous bioavailability studies (De Bock et al., 2013; Garcia-Villalba, Larrosa, Possemiers, Tomas-Barberan, & Espin, 2014; Martinez-Lopez et al., 2014; Rubió et al., 2012; Suarez et al., 2011).

The study was carried out at the Human Nutrition Unit in ICTAN, on two different days, separated by two weeks. On each intervention, after an overnight fast, volunteers consumed 30 g of biscuits (4 units), with a glass of water. HT-B and C-B presented identical composition regarding macronutrients (g/30 g of biscuit): proteins: 2.1, carbohydrates: 19.0, dietary fibre: 1.8 (soluble: 0.3 and insoluble: 1.5), sugars: 6.3, other carbohydrates: 11.9, total fat: 4.8 (saturated: 0.9, monounsaturated: 3.2, and polyunsaturated: 0.7), water: 0.6, calories: 131 kcal/30 g. A polyphenol-free breakfast (consisting of bread, cheese and milk) and lunch (consisting of a hot dog and yogurt) were provided to the volunteers 2 and 4 h, respectively, after consuming either C-B or HT-B. Water and isotonic beverages were available *ad libitum*. For the two days previous to each intervention, participants were also instructed not to consume olive products and alcohol, to avoid interferences, as well as chocolate, fruit juices, coffee, tea, wine, oranges, tangerines, apples, grapes, strawberries and other berries, beet, onion, aubergine and celery. Volunteers were asked to complete a 24 h food intake recall the day before each intervention in order to control any possible food restriction incompliance. Blood samples were collected in BD Vacutainer® tubes (Becton, Dickinson and Company, New Jersey, USA), with or without EDTA, to separate plasma and serum, respectively, at baseline ($t = 0$) and 0.5, 1, 2, 3, 4, 5 and 6 h after consuming the biscuits. Plasma and serum were separated by centrifugation (10 min, 1500 *g*, 4 °C) and aliquots stored at −80 °C prior to analysis. Urine samples were collected in 24 h urine plastic collection containers containing 0.5 g of ascorbic acid to preserve the polyphenols in urine during storage. Intervals of urine collection were −2–0, 0–3, 3–6, 6–12 and 12–24 h; total volume was recorded, and aliquots were maintained frozen at −20 °C.

2.3. Phenolic composition of biscuits

To determine the HT content of biscuits, fat and phenolic fractions, finely crushed biscuits (1 g) were extracted, using