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Metabolite profiling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance markers

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ABSTRACT

An understanding of causal relations between phenol intake and its beneficial effects on health is hindered by the lack of robust biological markers of its exposure. This is particularly relevant in mid/long-term nutritional intervention studies. An analytical methodology based on UPLC–MS/MS has been developed to determine the metabolites of the phenolic compounds from olive oil and thyme in biological fluids after a sustained intake of two phenol-enriched olive oils for their further use as compliance biomarkers. In a randomized, double-blind, controlled, cross-over trial, 33 hypercholesterolemic volunteers received during 3 weeks 25 mL/day of (1) raw Virgin Olive Oil with a low phenolic content as a control (80 mg total phenols / kg oil; VOO), (2) Functional Virgin Olive Oil enriched with its own phenolics (500 mg total phenols / kg oil; FVOO), and (3) Functional Virgin Olive Oil enriched with its own phenolics plus complementary phenolics from Thyme (500 mg total phenols / kg oil, 50% from olive oil and 50% from thyme respectively; FVOOT). Plasma and 24 h-urine samples were collected. The results showed that some hydroxytyrosol (HT) metabolites presented low specificity as biomarkers of intake. However, hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate appeared to be suitable biomarkers for monitoring compliance with olive oil intake as their values in plasma or/and 24-h urine were significantly higher after FVOO compared to baseline pre-intervention concentrations. They were also significantly correlated with the monitored level of compliance. On the other hand, metabolites derived from thyme were more specific, thymol sulfate and hydroxyphenylpropionic acid sulfate being the metabolites with the largest increase in both plasma and 24-h urine, whereas urinary *p*-cymene-diol glucuronide presented the greatest increase post-treatment. Their urinary excretion values also displayed significant correlations with the level of compliance and they were defined as FVOOT compliance biomarkers. This study enabled robust quantitative and qualitative compliance biomarkers after the ingestion of two phenol-enriched olive oils to be determined and provided a thorough analysis of the true phenolic exposure after a sustained consumption that could be further related to expected biological effects.

1. Introduction

The European Food Safety Authority Scientific and Technical Guidance has recently provided the criteria for authorizing health claims for foods. This clearly establishes that relevant human interventional studies have to be presented to substantiate any claim. While studies in animal or in vitro models may provide supporting evidence (e.g. in support of a mechanism), human data are essential to substantiate the health claim (EFSA Panel on Dietetic Products, 2011). Over the past decade, a significant number of human nutrition intervention studies have been conducted with the goal of establishing the exact bioefficacy of various subclasses of polyphenols as protection against chronic degenerative diseases (Del Rio et al., 2013; Kay, Hooper, Kroon, Rimm, & Cassidy, 2012). Nevertheless, few validated biomarkers of polyphenol exposure are available, which hinders establishing any relationship between exposure and effects (Kay, 2010). This link is essential to decide whether the negative outcome of a controlled trial (i.e., a lack of functional change in response to supplementation) can be related to the

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basic hypothesis as a clinical effect or as a lack of compliance among the participants in the trial (Puiggròs, Solà, Bladé, Salvadó, & Arola, 2011).

The dietary intake of nutrients and non-nutrient components in human intervention studies is usually determined using dietary assessment methods, such as diet diaries. There are considerable difficulties in assessing polyphenol intakes using this traditional approach, thus highlighting the need for validated biomarkers of their intake. Biological markers have been used as an alternative over recent years (Kay, 2010). However, the relationship between dietary intake and the resulting concentrations of biomarkers in body fluids is highly complex, and, thus, very few validated biomarkers of dietary exposure are available. Various studies have measured total urinary polyphenols as biomarkers of fruit and vegetable intake in order to characterize and quantify habitual food intakes in a diet pattern (Krogholm, Haraldsdóttir, Knuthsen, & Rasmussen, 2004; Krogholm et al., 2012; Mennen et al., 2006; Nielsen, Freese, Kleemola, & Mutanen, 2002). Alternatively, more specific biomarkers can be monitored when we are interested in a given food or food ingredient.

Consistent clinical intervention trials have supplied evidence that the phenolic compounds (PC) of virgin olive oil contribute to protecting humans against lipid oxidation in a dose-dependent way (; Covas, Nyyssönen, et al., 2006; Weinbrenner et al., 2004). The absorption and excretion of olive oil PC following an acute intake in humans have been studied previously (García-Villalba et al., 2010; Miro-Casas et al., 2001, 2003; Visioli et al., 2000; Vissers, Zock, & Katan, 2004) and attempts to monitor olive oil phenolic consumption as a biomarker for intervention compliance have focused in the analysis of total HT (Covas, de la Torre, et al., 2006;; Marrugat et al., 2004). However, no studies have been performed to determine individual polyphenol metabolites in biological fluids after a sustained intake of olive oil. Additionally, there is a modern trend towards flavoring olive oils with herbs and spices to improve their sensorial profile. This could be turned to advantage to look at the combined or synergic beneficial health effects of polyphenols and a novel approach would consist of developing functional olive oils further enriched with their own PC combined with PC from other sources.

Our aim was to identify biomarkers for olive and thyme PC after a 3-week dietary intervention with phenol-enriched olive oils within the frame of a randomized, double-blind, crossover, and controlled nutrition intervention trial. Two functional phenol-enriched olive oils were evaluated, one with its own PC (FVOO) and a second one further enriched with thyme PC (FVOOT). A sensitive and reliable analytical method was developed to detect phenol metabolites in plasma and urine samples to identify the most appropriate compliance markers and eventually relate them to the expected biological effects.

2. Material and methods

2.1. Olive oil preparation and characterization

VOO with a low phenolic content (80 mg total phenols / kg oil) was used as a control condition in the intervention and as an enrichment matrix for the preparation of two phenol-enriched olive oils with a total phenolic content of 500 mg total phenols / kg oil but with different phenolic composition. FVOO was enriched with its own PC by adding a phenol extract obtained from freeze-dried olive cake. FVOOT was enriched with its own PC and complemented with thyme PC using a phenol extract made up of a mixture of olive cake and dried thyme. Hence, FVOOT contained 50% of olive PC (hydroxytyrosol derivates) and 50% of thyme PC (flavonoids, phenolic acids and monoterpenes) (Table 1). The procedure for obtaining the phenolic extracts and enriched oils was previously developed in our laboratory (Rubió, Motilva, et al. 2012). For the wash-out period, a commercial common olive oil (blend of refined and a small percentage of virgin olive oil) kindly provided by Borges Mediterranean Group was used. The total phenolic content of the olive oils was measured with the Folin–Ciocalteu method (Vázquez Roncero, Janer Del Valle, & Janer Del Valle, 1973). The phenolic profile of the olive oils was analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) using the method previously described (Rubió, Motilva, et al., 2012). Representative chromatograms of the three studied olive oils are shown in Fig. 1 of Additional Information.

A consumer acceptance test was performed to assess the overall opinion of the three olive oils at the time of the intervention. A specific profile sheet was set up where volunteers had to assign a score on a 7-point category scale (dislike very much, dislike moderately, dislike slightly, neither like nor dislike, like slightly, like moderately and like very much). The scores were converted to whole numbers between 0 and 6 respectively to calculate the means and the standard deviation.

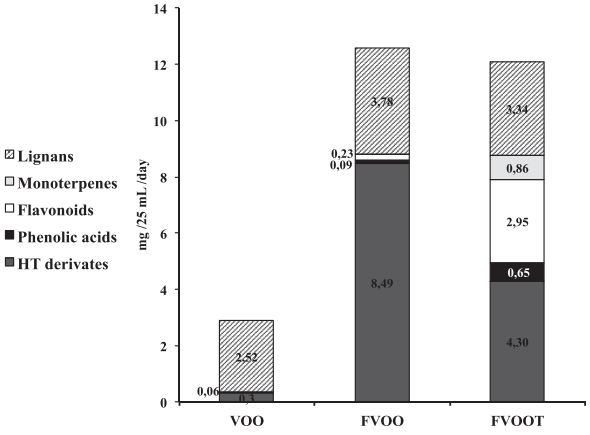
2.2. Study design

The study was a randomized, double-blind, crossover, controlled trial with 33 hypercholesterolemic volunteers (total cholesterol > 200 mg/dL)

Table 1
Phenolic daily intake through 25 mL of VOO (Virgin Olive Oil; 80 mg total phenols/kg oil), FVOO (Functional Virgin Olive Oil enriched with its own phenolics; 500 mg total phenols/kg oil) and FVOOT (Functional Virgin Olive Oil enriched with both its own phenolics (50%) and phenolics from Thyme (50%); 500 mg total phenols/kg oil).

Phenol (mg phenol/25 mL/day)	VOO	FVOO	FVOOT
Hydroxytyrosol derivates			
Hydroxytyrosol	0.01 ± 0.00	0.21 ± 0.02	0.12 ± 0.00
3,4-DHPEA-AC	n.d.	0.84 ± 0.06	0.39 ± 0.04
3,4-DHPEA-EDA	0.04 ± 0.00	6.73 ± 0.37	3.43 ± 0.29
3,4-DHPEA-EA	0.26 ± 0.04	0.71 ± 0.06	0.36 ± 0.03
Phenolic acids			
p-Hydroxybenzoic acid	n.d.	0.02 ± 0.00	0.06 ± 0.00
Vanillic acid	n.d.	0.07 ± 0.00	0.13 ± 0.01
Caffeic acid	n.d.	0.00 ± 0.00	0.06 ± 0.00
Rosmarinic acid	n.d.	n.d.	0.41 ± 0.03
Monoterpenes			
Thymol	n.d.	n.d.	0.64 ± 0.05
Carvacrol	n.d.	n.d.	0.23 ± 0.02
Flavonoids			
Luteolin	0.04 ± 0.00	0.18 ± 0.02	0.21 ± 0.02
Apigenin	0.02 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
Naringenin	n.d.	n.d.	0.20 ± 0.02
Eriodictyol	n.d.	n.d.	0.17 ± 0.01
Thymusin	n.d.	n.d.	1.22 ± 0.09
Xanthomicrol	n.d.	n.d.	0.53 ± 0.06
7-Methylsudachitin	n.d.	n.d.	0.53 ± 0.09
Lignans			
Pinoresinol	0.05 ± 0.00	0.12 ± 0.00	0.10 ± 0.05
Acetoxipinoresinol	2.47 ± 0.19	3.66 ± 0.31	3.24 ± 0.28

Values in the table provide the individual phenolic characterization of the olive oils expressed as means ± SD of mg phenols/25 mL oil/day. In the graph below the distribution by phenolic groups is represented with total values for each group.



3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone.