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Analytical Methods

Direct analysis of glucuronidated metabolites of main olive oil phenols in human urine after dietary consumption of virgin olive oil

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ABSTRACT

In the present study we report on a UPLC-MRM validated method for the simultaneous direct analysis of main glucuronidated metabolites of olive oil phenols: tyrosol, hydroxytyrosol and its *O*-methyl metabolite homovanillyl alcohol in human urine after dietary olive oil ingestion. The developed method was linear within the concentration range 20–2000 ng/mL with adequate recovery of analytes (>86%). Intra- and inter-day precision and accuracy were according to standard requirements for method validation criteria. Using the developed method, urinary concentrations and excretion rates of glucuronides of olive oil phenols were successfully estimated in an intervention study with 11 healthy volunteers supplemented with a dietary dose of virgin olive oil (VOO) (50 mL). Therefore, about 13% of the consumed olive oil polyphenols were recovered in 24-h urine, where 75% of them were in the form of glucuronides (3′- and 4′-*O*-hydroxytyrosol glucuronides, 4′-*O*-glucuronides of tyrosol and homovanillyl alcohol) and 25% as free compounds.

1. Introduction

A number of epidemiological studies have provided evidence of the health benefits derived from the Mediterranean diet against cancer and cardiovascular diseases (La Vecchia, 2009; Trichopoulou, Costacou, Bamia, & Trichopoulos, 2003). The main characteristics of such a diet are its richness in natural vitamins and antioxidants, from vegetables and fruits, and a high content of monounsaturated fatty acids, olive oil being the main source of fat (Trichopoulou et al., 2003). The biological benefits of olive oil consumption are not only limited to its high content of monounsaturated fat, olive oil minor components also display bioactive properties (Covas et al., 2006; Fitó et al., 2000). Phenolic compounds, the most studied olive oil minor components, belong to the hydrosoluble fraction of olive oil. Some of the most representative phenolic compounds in olive oil are hydroxytyrosol (HOTYR) and tyrosol (TYR) and their respective secoiridoid derivatives, oleuropein and ligstroside (Servili & Montedoro, 2002). They have been shown to exhibit strong antioxidant properties that contribute to the protection of olive oil against lipid rancidity. Recent intervention clinical trials have provided evidence that the phenolic content of an olive oil contributes to the protection in humans against lipid oxidative damage in a dose dependent manner (Covas et al., 2006; Weinbrenner et al., 2004).

One of the first steps in linking the biological activities of phenol compounds of dietary origin to health benefits in humans is to demonstrate their bioavailability from diet. Several intervention studies in human and animal models have reported that phenolic compounds are rapidly absorbed in a dose dependent manner with the phenolic content of the olive oil administered (Visioli et al., 2000; Weinbrenner et al., 2004). Olive oil phenolic compounds are extensively metabolized in the gut and liver and, thus, in biological fluids they are found mainly as phase II metabolites (e.g. glucuronides and sulphates) of HOTYR, TYR and 3-*O*-methylconjugate of HOTYR (homovanillyl alcohol, HVAlc) (Miró-Casas et al., 2001, 2003). In rats administered with HOTYR, both orally or intravenously, both glucuronide and sulphate conjugates of HOTYR and HVAlc were detected in different biological matrices and tissues (D'Angelo et al., 2001; Tuck, Hayball, & Stupans, 2002). Despite inter-species differences (Visioli et al., 2003), the first pass intestinal and hepatic metabolism plays an important role in the

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bioavailability and disposition of olive oil phenolic compounds to such an extent that their free forms are present only at very low concentrations in biological fluids (Miró-Casas, Covas, Farre, et al., 2003; Miró-Casas et al., 2001).

The biotransformation of HOTYR and TYR into their phase II metabolites was predicted to negatively influence their activities as antioxidants (Nenadis, Wang, Tsimidou, & Zhang, 2005). Phase II metabolites are generally considered to be pharmacologically inactive and targets for excretion. However, some phase II metabolites (e.g. glucuronides) of food derived antioxidants, such as catechins, have been reported to be biologically active (Lu et al., 2003). Moreover, in a study in rats (Tuck et al., 2002) it was reported that the urinary excreted 3′-*O*-HOTYR-glucuronide, but not its 3′-*O*-sulphate conjugate, was a more potent antioxidant (by DPPH test) than its parent compound HOTYR.

The prevalent presence of olive oil phenols in the form of phase II metabolites within the organism following olive oil phenolic compounds ingestion (e.g. as olive oil, olive oil phenol extracts and pure olive oil phenols) was acknowledged in many studies, (Miró-Casas, Covas, Farre, et al., 2003; Miró-Casas et al., 2003; Tuck, Freeman, Hayball, Stretch, & Stupans, 2001; Visioli et al., 2003) however no direct methods for their identification and quantification in biological fluids were reported. The main limitation was the unavailability of corresponding standards. Therefore, olive oil phenol metabolites have not been quantified accurately until now as well as their metabolic excretion rates have never been estimated.

In the majority of the studies, the concentrations of phenolic compounds tested in *in vitro* experiments to achieve biological effects display a large disparity with those observed *in vivo* in the human body after real-life doses of phenolic-rich foods. Concentrations of total phenols in biological fluids (after an enzymatic digestion of samples) have been used as a reference in many experiments without taking into account the contribution of free phenol compounds (presumed biologically active) and their metabolites (presumed biologically inactive) to this overall quantitative estimation. It is, therefore, mandatory to have a proper estimation of the expected concentrations of phenols, and their main metabolites, in humans after phenolic-rich food consumption. These data are required in order to establish the range of concentrations to be tested *in vitro* and *in vivo* in the evaluation of their biological activities.

The availability of HOTYR and TYR metabolites, e.g. their glucuronides (Khymenets et al., 2006), can allow characterising qualitatively their metabolic disposition and estimating quantitatively the contribution of each metabolic pathway. These results should be combined with those obtained in studies designed at the evaluation of their biological activity. Their potential biological activity should allow to review past clinical studies or to design new ones where the contribution of phenol compounds to biological effects should be revised. At this stage it is proposed that this evaluation should be performed applying alternative experimental approaches to those applied until now.

The aim of the present study was to develop a direct and simple method for detection and quantification of the main olive oil phenolic metabolites, glucuronides of TYR, HOTYR and its O-methylated metabolite HVAlc, in human urine in order to estimate the role and the rates of glucuronidation of phenols derived after real-life doses of virgin olive oil.

2. Methods and materials

2.1. Reagents and chemicals

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) (HOTYR) and tyrosol (2-(4-hydroxyphenyl)ethanol) (TYR) were purchased from Extrasynthèse (Extrasynthèse, Lyon, France). Homovanillyl alcohol (HVAlc), 3-(4-hydroxyphenyl)propanol (HOPhPr) (used as internal standard, I.S.2) were supplied by Sigma–Aldrich (Sigma–Aldrich Inc., St. Louis, MO). Synthetic urine UriSub® (CST Technologies Inc., Great Neck, NY) was used for the UPLC-MRM method validation. Methanol (MeOH) and acetonitrile (ACN) were of analytical grade from Scharlau (Scharlau Chemie, Barcelona, Spain). Mobile phase was filtered with 0.22 μm nylon filters (Whatman, Brentford, UK). Sodium bisulphite, acetic acid, ammonium hydroxide, hypochloric and phosphoric acid were supplied by Merck (LiChrosolv®, Barcelona, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France).

Glucuronides: 4′-*O*-hydroxytyrosol (4′-*O*-Gluc-HOTYR) and 3′-*O*-hydroxytyrysol (3′-*O*-Gluc-HOTYR) glucuronides, 4′-*O*-tyrosol glucuronide (4′-*O*-Gluc-TYR), 4′-*O*-homovanillyl alcohol (4′-*O*-Gluc-HVAlc), and 4′-*O*-hydroxyphenylpropanol (4′-*O*-Gluc-HOPhPr) (used as internal standard, I.S.1) (Fig. 1) glucuronides were synthesized according to a method previously described (Khymenets et al., 2006).

2.2. Subjects, diet, study design and sample collection

Six healthy male (aged 22–28) and five female (aged 20–44) volunteers were recruited. The institutional ethics' committee (CEIC-IMAS) approved the protocol and the participants signed an informed consent. All volunteers were healthy on the basis of a physical and medical examination and standard biochemical and haematological tests.

The VOO used in this investigation was of Spanish origin and had been utilised in former studies (Cicero et al., 2008). The amount of total HOTYR, HVAlc, and TYR in VOO was determined as previously described (Miró-Casas, Covas, Fitó, et al., 2003; Miró-Casas et al., 2001).

Prior to the dietary intervention, volunteers followed a one week wash-out period in which sunflower oil was provided as a source of fat for all purposes. During the first four days of the wash-out period, participants were asked to follow an antioxidant-controlled diet consisting of no more than two pieces of fruit, two servings of vegetables or legumes, two cups of tea or coffee per day, and the total avoidance of wine, beer, and olive oil. During the last 3 days of the wash-out period, and on the intervention day (food provided at the clinical trials unit), the volunteers followed a strict low-phenolic compound diet. Phenolic-rich foods (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cocoa, marmalade, olive oil, and olives) were totally excluded from the participants' diet. On the intervention day, at fasting state, 50 mL (44 g) of VOO were administered in a single dose with bread (200 g).

Spot urine was collected at 8 a.m. at fasting state prior to VOO administration and from 0–6 h and 6–24 h after VOO consumption on the intervention day. Urine samples were preserved with sodium bisulphite (1 mM final concentration) at acidic conditions (0.24 M HCl final concentration) and stored at −20 °C prior to use.

Blood samples were collected in EDTA-containing tubes immediately before (0 h) and 1 h, 6 h, and 24 h after VOO intervention. Plasma samples were obtained by centrifugation of whole blood directly after being drawn and were preserved at −20 °C prior to use.

2.3. Analysis of free and glucuronoconjugated HOTYR, TYR, and HVAlc in urine samples by SPE-UPLC-MRM

Prior to analysis a mix of internal standards was added to each tube (final concentration of 500 ng/mL I.S.-1 and 1000 ng/mL I.S.-2.) and dried under nitrogen (25 °C, 10–15 psi, 1 min). After thawing at room temperature, aliquots of 1 mL of urine were distributed to glass tubes. Samples were diluted 1:1 with 4% H_3PO_4 and