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Identification of phenolic metabolites in human urine after the intake of a functional food made from grape extract by a high resolution LTQ-Orbitrap-MS approach

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ABSTRACT

Grape pomace (GP) is known to be a rich source of polyphenols with biological activity which may be used as functional ingredients for the development of new health-promoting products. Numerous studies have reported that bioactive compounds may act through multiple mechanisms. In order to verify the oral absorption and metabolism of grape polyphenols, we performed a prospective, randomised and cross-over acute study in 12 volunteers with two interventions: 500 mL of a functional beverage enriched with 200 mL of GP and 500 mL of a control beverage without GP. In this work, liquid chromatography coupled with an electrospray ionization hybrid linear ion trap quadrupole-Orbitrap-mass spectrometry (LC/ESI-LTQ-Orbitrap-MS) technique has been used to accurately identify phenolics in GP and human urine. In GP, 41 phenolic compounds were identified mainly procyanidins, phenolic acids and flavonols, and in human urine over 70 metabolites of phenolic compounds including microbiota metabolites, glucuronides and sulfate derivatives were detected. Overall, high resolution mass spectrometry (HR-MS) enhances the identification of a large variety of polyphenols and their metabolites with great mass accuracies for all molecular ions.

1. Introduction

The Mediterranean lifestyle has often been defined as a healthy way of living with the consumption of cereals, fruits, vegetables and red wine playing a central role (O'Keefe et al., 2014). Epidemiological studies have shown such a polyphenol rich diet is associated with a reduced risk of developing cardiovascular disease for which the natural polyphenols have been attributed as one of the major responsible elements (Tresserra-Rimbau et al., 2014; Tresserra-Rimbau et al., 2015).

During winemaking, the phenolic compounds of grapes are transferred to the wine, but a high proportion still remains in the solid winemaking by-products. Grape pomace (GP) is an industrial waste and consists basically of grape seeds, skin and stems. This subproduct has been postulated to be a good source of bioactive compounds, such as polyphenols with a variety of biological activities and numerous protective effects against chronic diseases, mainly cardiovascular pathologies (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2010; Cao, Wang, Gao, Zhang, & Qiu, 2015; Janega et al., 2014; Jara-Palacios et al., 2015; Kar, Laight, Rooprai, Shaw, & Cummings, 2009; Park, Edirisinghe, Choy, Waterhouse, & Burton-Freeman, 2016; Rodriguez-Rodriguez et al., 2012). In recent years, there has been an increased interest in the use of by-products rich in polyphenols to develop a functional food or nutraceutical for the maintenance of human health (Kammerer, Claus, Carle, & Schieber, 2004; Kulkarni, DeSantos, Kattamuri, Rossi, & Brewer, 2011).

The biological activity of grape phenolics depends on their bioavailability, which is defined as the proportion of nutrient that is digested, absorbed and metabolised. Anthocyanins, procyanidins, flavonols, flavan-3-ols, phenolic acids and stilbenes are some of the polyphenols present in grapes, GP and derivatives (Cavaliere et al., 2008; Jara-Palacios et al., 2015; Panighel, De Rosso, Dalla Vedova, & Flamini, 2015; Prasain et al., 2009; Sapozhnikova, 2014; Stalmach, Edwards, Wightman, & Crozier, 2011). Most of

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them are present in food in form of esters, glycosides or polymers that cannot be absorbed. These compounds must be hydrolysed by intestinal enzymes or by the colonic microflora to be absorbed. Later, they are rapidly and extensively metabolised in the intestine and liver, and the major modifications of the parent compounds are methylation, glucuronidation and sulfation (Manach & Donovan, 2004). Due to the complexity of food systems, identifying the number of possible derived metabolites found in biofluids is a challenge, because the exact composition, the chemical structure, metabolic profile and concentration are usually unknown and absorption in humans may be highly variable (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

This complexity may be resolved using novel high resolution mass spectrometry (HR-MS) techniques that are able to report unknown compounds in urinary excretion. In this context, linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) provides single-stage mass analysis delivering molecular mass information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MS^n) allowing structural information. Exact mass measurements and molecular formula assignment are essential for the characterisation of molecules found in low amounts. Accurate mass measurement of the product ions, formed in MS^n experiments, facilitates the elucidation of the unknown compounds present in complex combinations (Ávarez-Fernadez, Cerezo, Cañete-Rodríguez, Troncoso, & García-Parrilla, 2015; Edmands et al., 2015; van der Hooft et al., 2012; López-Gutiérrez, Romero-González, Martínez Vidal, & Frenich, 2016; Vallverdú-Queralt et al., 2014, 2015).

The purpose of the current study was to provide accurate and comprehensive identification of a wide range of phenolic metabolites found in a grape extract beverage made of GP and in urine samples after its consumption using LTQ-Orbitrap analysis.

2. Material and methods

2.1. Chemicals and samples

All samples and standards were handled without exposure to light. Gallic, hippuric, caffeic, dihydrocaffeic, gentisic, syringic, caftaric, homovanillic, protocatechuic, homoprotocatechuic, hydroxybenzoics, hydroxyphenylacetics, hydroxyphenyl propionics, *p*-coumaric, ferulic and chlorogenic acids, procyanidin dimer type B1 and B2, quercetin, catechin, epicatechin, epicatechin gallate, epigallocatechin, peonidin, rutin, naringenin, resveratrol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and quercetin-*O*-hexoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-hydroxyhippuric acid was purchased from Bachem Americas (Torrance, CA, USA), *trans*-resveratrol-3-*O*-sulfate and glucuronide were purchased from Toronto Research Chemicals (North York, ON, Canada) and *trans*-resveratrol-4-*O*-glucuronide was purchased from Cayman Chemical (Ann Arbor, MI, USA). Hydrochloric acid 35% was obtained from Panreac Química S.A. (Barcelona, Spain). Acetonitrile of HPLC grade was obtained from Sigma-Aldrich (St. Louis, MO, USA) and formic acid (≥98%) from Panreac Química S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by the Millipore System (Bedford, USA).

Miguel Torres S.A. provided the red GP in opaque glass bottles, which were kept refrigerated until analysis.

2.2. Intervention beverages

Polyphenol-rich grape extract beverages were made with GP, diet soda and water to a final volume of 500 mL. The two interventions followed by the volunteers were: (1) control beverage with 100 mL of diet soda and 400 mL of water and (2) a functional beverage with 100 mL of diet soda, 200 mL of GP and 200 mL of water, adjusted to the same volume in both interventions.

2.3. Volunteers

A total of 12 healthy subjects were recruited (6 males and 6 females; mean age: 24 ± 4.47 years; BMI range: 23.87 ± 4.24 kg/m^2; age range: 20–40 years) and admitted to participate after they signed an informed consent form. The subjects were controlled with a medical questionnaire, measures of anthropometric parameters and blood pressures, and blood biochemical analyses (lipidic profile, glucose, glycated haemoglobin). All participants were healthy, non-smokers and reported no previous history of cardiovascular, hepatic or renal disease. Individuals who reported consuming any nutritional supplements, were allergic or intolerant to grapes or wine or addicted to alcohol or drugs were excluded. None of the participants received any pharmacological treatment throughout the process.

2.4. Study design

The study was an open, controlled, randomised and crossover trial. Before each intervention, participants followed a three-day wash-out period in which they were requested to avoid consuming grape, grape products and wine. Also, on the day prior to the intervention they followed a polyphenol-free diet. To ensure adherence to the dietary guidelines, technicians provided a list of permitted and forbidden foods and beverages and a sample menu for assistance. The volunteers were also asked to fill in a food reminder questionnaire during the wash-out period.

For this acute study and after 8 h of fasting, subjects consumed 500 mL of beverage/70 kg body weight. Urine samples were collected prior to the intervention (0 h) and 24 h after the intake of the beverage (24 h). After measuring the volume of urine excreted, samples were stored at −80 °C until the analysis.

The study protocol was approved by the University of Barcelona's Bioethics Commission and registered in the International Standard Randomized Controlled Trial Number as ISRCTN06777936.

2.5. Grape pomace and urine treatment

For the mass spectrometry analyses, the GP and urine samples collected at time 0 and 24 h were centrifuged at 3000*g* for 10 min at 4 °C (discarding sediments). Thereafter, 1 mL of hydrochloric acid 0.1 M was added to 1 mL of GP or 1 mL of each urine sample, which were then, filtered through a 0.22 μM PTFE membrane filter.

2.6. LC-high resolution mass spectrometry and experimental conditions

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a resolution of 30,000 at *m/z* 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 40 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 320 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target $5 \cdot 10^5$ for MS mode and $5 \cdot 10^4$ for MS^n mode). Red GP, urine samples and standards were analysed in the FTMS scan mode at a resolving power of 30,000 at *m/z* 400 and data-dependent MS/MS events acquired at a resolving power of 15,000. The most intense ions detected in full scan MS triggered data-dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions not intense enough for a data-dependent scan were analysed in MS^n mode with the Orbitrap resolution also set at 15.000 at *m/z* 400. An isolation width of 2 amu was used and precursors were fragmented by collision-induced dissociation C-trap (CID) with normalised collision energy of 35 V and an activation time of 10 ms. The maximum injection time was set to 100 ms with two micro scans for MS mode and to 1000 ms with one micro scan for MS^n mode. The mass range in FTMS mode