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Phenolic metabolites in plasma and tissues of rats fed with a grape pomace extract as assessed by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Grape pomace extract (GPE) is a rich and relatively low-cost source of phenolic compounds. However, little is known about the main GPE metabolites in mammals, which could help explain the observed health-promoting effects. This study investigated the presence of parent compounds from flavanol, flavonol and stilbene families and their metabolites in rat plasma and tissues after an acute intake of GPE in doses of 300 and 600 mg kg/body weight. The measurement of free compounds and their metabolites was performed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Results showed the presence of epicatechin, epicatechin methyl-glucuronide, epicatechin methyl-sulphate, catechin, catechin-glucuronide, quercetin methyl-glucuronide, resveratrol-3-glucuronide, resveratrol-4-glucuronide and resveratrol-3-sulphate in plasma, which was dose dependent. The most abundant measured compound in plasma was epicatechin-glucuronide. The presence of glucuronidated and methyl-glucuronidated forms of catechin were observed in the liver at both doses, while epicatechin-glucuronide and methyl-glucuronide were detected only upon intake of 600 mg GPE/kg body weight. At this dose epicatechin-glucuronide and methyl-glucuronide were also detected in muscle, and catechin methyl-glucuronide in adipose tissue. Results show the main GPE metabolites present in rat tissues after oral consumption, contributing to better understand the health benefits of GPE and its potential utilization as a functional ingredient.

1. Introduction

Phenolic compounds are plant secondary metabolites widely distributed in fruits and vegetables with various characterized functions and several proposed beneficial effects [1,2]. These compounds, particularly flavonoids, are currently receiving significant attention because of their health-promoting effects including those against obesity-associated pathologies, such as type 2 diabetes, metabolic syndrome, cardiovascular diseases, and cancer, among others [3–6]. The positive properties demonstrated for functional foods, and the increase of consumers' awareness for healthy foods, highlights the need to find natural alternatives for the food industry [7]. Grape pomace (GP) is obtained from the winemaking process as the residue remaining after fermentation, mainly constituted by skins and seeds of berries. GP is a potentially abundant and relatively low-cost source of a wide range of phenolic compounds including the most abundant monomeric and oligomeric flavanols (catechin, epicatechin, procyanidins), flavonols (quercetin), anthocyanins and stilbenes (resveratrol) with potential biotechnological utilization in food and pharmaceutical industries as natural or functional ingredients [5,8]. In addition, grape pomace extract (GPE) is a concentrate product obtained from GP which is mainly constituted by phenolic compounds present at higher concentrations than those found in the crude by-product.

Phenolic compounds present in foods are highly metabolized before their absorption [9]. After the ingestion of food and beverages rich in phenolic compounds, the mayor absorption occurs in the small intestine. Typically, phenolic compounds except flavanols, are found in glycosylated forms or polymerized and must be hydrolyzed by intestinal enzymes present in the brush border of the small intestine or by the colonic microflora before they could be absorbed. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver forming sulphate, glucuronidated and/or methylated

Abbreviations: GP, grape pomace; GPE, grape pomace extract; SPE, solid phase extraction; UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry

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metabolites [10,11]. Throughout digestion, hydrolysis and metabolism change the molecular structure of these compounds, leading to a large number of different molecules [12].

Previous reports showed that GPE can exert beneficial health effects, showing an anti-inflammatory effect in diet-induced obese mice [13], an antioxidant activity in rats [14], counteracting the adiposity and hyperglycemia in Type 2 diabetic mice [15], mitigating hepatic steatosis in db/db mice [16] and lowering plasma triacylglycerides and phospholipids in rats fed a high fat diet [17]. Furthermore, GP or GPE supplementation prevents diet-induced metabolic alterations and adiposity in rats with metabolic syndrome [18–20]. However, little is known about the main GPE-derived phenolic metabolites present in plasma and tissues, their biological targets, their distribution and availability that may account for some of the observed health-promoting effects.

The aim of this study was to assess the availability, distribution and levels of metabolites of the most abundant polyphenol families (flavanols, flavonols and stilbenes) in rat plasma, liver, muscle and visceral adipose tissue after oral intake of two doses of malbec GPE. The measurement of free compounds and their metabolites was performed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

2. Material and methods

2.1. Standards and solvents

Standards of catechin (≥99%), epicatechin (≥95%), *trans*-resveratrol (≥99%), quercetin hydrate (95%), procyanidin B1 (≥90%), procyanidin B2 (≥90), the internal standard (IS) catechol and L (+)-ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions (1 mg/ml) of the above compounds were prepared in methanol. Further dilutions were prepared monthly in methanol and stored in dark-glass bottles at −20 °C. Calibration standards were prepared in ultrapure water with 0.1% (v/v) formic acid:acetonitrile 95:5.

HPLC-grade acetonitrile, methanol, acetone, formic acid and glacial acetic acid were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Ethanol was purchased from Merck (Sao Paulo, Brazil). Ortho-phosphoric acid 85% (w/v) was purchased from Sintorgan S.A. (Buenos Aires, Argentina). Ultrapure water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA). OASIS HLB (divinylbenzene-co-*N*-vinylpirrolidone polymer) 60 mg SPE cartridges were purchased from Waters (Milford, MA, USA).

2.2. GP sampling

This study was conducted using GP of *Vitis vinifera* L. cv. Malbec, harvested in 2017. The material was provided by a local winery from the vineyards of the Mendoza's region in Argentina. The winemaking was conducted with daily pumping and contact of the skins and seeds with the juice for 11 days. After this, must was pressed, and the fresh GP was obtained, placed in ice-cooled boxes for transportation to the laboratory, and stored at −20 °C until processing. The recovery of the phenolic compounds from the GP was performed via solid–liquid extraction as previously described [8]. Herein, 80 g of fresh GP was ground with a laboratory mixer, and the powder extracted at a 5:1 solvent-to-sample ratio using ethanol:water, 50:50 v/v) as the solvent. The extraction was carried out for 2 h under continuous stirring at 60 °C. The preparation was filtered through a filter paper and concentrated at low pressure using a rotary evaporator at 40 °C. The concentrated extracts were freeze-dried for 96 h at 0.12 bar and −45 °C in a Free Zone 2.5 equipment (Labconco, Missouri, USA). Freeze dried extracts were placed in sealed tubes and kept in the dark at −20 °C in a dry atmosphere until analysis or preparation of diets.

2.3. Animal studies

All animal studies were conducted in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Cuyo (CICUAL, Protocol approval no. 36/2014). Ten-weeks-old male Wistar rats were housed under controlled conditions of temperature (23 ± 1 °C) and light (12 h light/dark cycle), and were fed a standard rat chow (Gepsa-Feeds, Buenos Aires, Argentina) and water ad libitum. Animals were fasted for 16 h with only access to tap water. Rats weighing 320 ± 23 g at dosing were randomly divided into three groups (n = 6 each) receiving: 300 mg GPE/kg of body weight or 600 mg GPE/kg of body weight, or the vehicle (ethanol:water, 50:50, v/v) (control group). The average intake of phenolic compounds in rats that received 300 and 600 mg GPE/kg body weight was 752 and 1503 μg, respectively. Both doses of GPE, dissolved in ethanol:water 50:50 (v/v), and the vehicle were administered by intraesophageal gavage, at a volume of 0.6 ml/rat. Two hours later, animals were anesthetized with ketamine (50 mg/kg body weight) and acepromazine (1 mg/kg body weight), and blood was collected from the abdominal aorta into EDTA-containing tubes. Plasma was obtained after centrifugation at 1000 × g for 15 min at 4 °C. In addition, liver, epididymal adipose tissue and soleus muscle were weighed, flash-frozen in liquid nitrogen and then stored at −80 °C until assayed. Two hours after the acute ingestion of GPE was chosen to sample the plasma and tissues because this is the time that corresponds with previously reported maximum plasma concentration of metabolites [9,21,22].

2.4. Extraction of phenolic compounds and their metabolites from GPE, plasma and tissues

The method used to extract free phenolic compounds and their metabolites from GPE, plasma and tissues was based on Serra et al. [23]. Briefly, 25 mg of GPE were resuspended in 2 ml of ultrapure water with 1% (v/v) of formic acid and extracted by solid-phase extraction (SPE) as described below.

An aliquot of 0.75 ml of plasma was diluted 1:1 (v:v) with ultrapure water, then 20 μl phosphoric acid 85% (v/v) and 50 μl catechol (IS) (20 mg/l) were subsequently added prior to the SPE. Freeze-dried liver, muscle and adipose tissue (200 mg) were added with 50 μl of ascorbic acid 1% (w/v), 50 μl catechol (IS) (20 mg/l) and 100 μl phosphoric acid 4% (w/v). Samples were mixed and subsequently extracted with 400 μl of water:methanol: 4% (w/v) phosphoric acid (94:4:1, v:v:v), sonicated during 30 s in a freeze water bath to avoid heating, and centrifuged for 15 min at 15,800 g at 20 °C. This procedure was repeated four times. The supernatants obtained in each extraction were collected, and centrifuged for 3 min, at 13,500 × g at 20 °C. Then, the extracts were treated with SPE to concentrate the compounds of interest before UHPLC-MS/MS analysis. The SPE of GPE, plasma and tissue extracts were performed by using OASIS HLB cartridges (60 mg, Waters, Milford, MA, USA). For this purpose, samples were passed through HLB SPE cartridges previously conditioned with methanol and a 0.2% (v/v) acetic acid solution (5 ml each). The loaded cartridges were washed with 3 ml ultrapure water and 5 ml of 0.2% (v/v) acetic acid solution. The retained analytes were eluted with 2 ml of acetone:Milli-Q water: 0.2% (v/v) acetic acid (70:29.5:0.5, v:v:v) and collected in glass tubes. Afterwards, the extract was evaporated to dryness (SpeedVac concentrator), and the residue re-suspended in 0.2 ml of initial mobile phase and injected into the chromatographic system.

2.5. Phenolic compounds quantification by UHPLC-MS/MS

The analysis of phenolic compounds and their biological metabolites were performed by UHPLC-MS/MS. The UPLC analysis of extracts was performed using a Waters Acquity Ultra-Performance TM liquid

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