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Orange juice affects acylcarnitine metabolism in healthy volunteers as revealed by a mass-spectrometry based metabolomics approach

Vanessa Moreira[a,1], Elisa Brasili[b,c,*,1], Jarlei Fiamoncini[d], Federico Marini[e,f], Alfredo Miccheli[e], Hannelore Daniel[d], Jennifer Ji Hye Lee[b], Neuza Mariko Aymoto Hassimotto[b,c], Franco Maria Lajolo[b,c]

[a] Department of Pharmacology, Paulista School of Medicine, Federal University of São Paulo, São Paulo, Brazil
[b] Department of Food Science and Experimental Nutrition, School of Pharmaceutical Science, University of São Paulo, São Paulo, Brazil
[c] Food Research Center (FoRC), CEPID-FAPESP (Research Innovation and Dissemination Centers São Paulo Research Foundation), São Paulo, Brazil
[d] ZIEL Institute for Food and Health, Technische Universiät München, Munich, Germany
[e] Department of Chemistry, University of Rome "La Sapienza", Rome, Italy
[f] Department of Food Science, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

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ABSTRACT

Citrus juices, especially orange juice, constitute rich sources of bioactive compounds with a wide range of health-promoting activities. Data from epidemiological and *in vitro* studies suggest that orange juice (OJ) may have a positive impact on lipid metabolism. However, the effect of orange juice intake on blood lipid profile is still poorly understood.

We have used two different blood samples, Dried Blood Spots (DBS) and plasma, to assess the effect of two-week orange juice consumption in healthy volunteers by a mass-spectrometry based metabolomics approach. DBS were analysed by liquid chromatography mass spectrometry (LC-MS) and plasma samples were analysed by the gas chromatography mass spectrometry (GC–MS).

One hundred sixty-nine lipids including acylcarnitines (AC), lysophosphatidylcholines (LysoPC), (diacyl- and acyl-alkyl-) phosphatidylcholines (PC aa and PC ae) and sphingomyelins (SM) were identified and quantified in DBS. Eighteen fatty acids were identified and quantified in plasma. Multivariate analysis allowed to identify an increase in C3:1, C5-DC(C6-OH), C5-M-DC, C5:1-DC, C8, C12-DC, lysoPC18:3, myristic acid, pentadecanoic acid, palmitoleic and palmitic acid and a decrease in nervonic acid, C0, C2, C10, C10:1, C16:1, C16-OH, C16:1-OH, C18-OH, PC aa C40:4, PC ae C38:4, PC ae C42:3, PC ae C42:4 and cholesterol levels after orange juice intake.

A two-week period of orange juice intake could affect fatty acids β-oxidation through mitochondrial and peroxisomal pathways, leading to an increase of short-chain acylcarnitines and a decrease of medium and long-chain acylcarnitines. This is the first report analyzing the effect of orange juice intake in healthy volunteers using a dried blood spot-based metabolomics approach.

1. Introduction

Citrus juices, especially orange juice (OJ), constitute rich sources of vitamin C and bioactive compounds such as flavanones hesperidin and naringenin with a wide range of health-promoting biological activities (Li & Schluesener, 2017).

Several studies have shown that consumption of orange juice reduces oxidative stress and inflammation helping in preventing atherosclerosis, heart failure, Alzheimer's disease and other immunological disorders (Azzini et al., 2017; Rampersaud & Valim, 2017; Rangel-Huerta et al., 2017). These health effects were mainly associated to a lowering of blood lipids and lipid peroxidation, decreased oxidized LDL and maintenance of low levels of IL-6 and C-reactive protein (Escudero-López et al., 2015; Zheng et al., 2017). On the other hand, new food biomarkers are needed to evaluate the effect of diet on health and to check adherence to dietary recommendations and healthy eating patterns. In recent years, metabolomics has emerged as a key tool in search for novel biomarkers of citrus consumption providing new insight in nutritional science. In particular, metabolic profiling strategies allowed the identification of proline betaine as urinary biomarker of citrus fruit

* Corresponding author.
E-mail address: elisa.brasili@uniroma1.it (E. Brasili).
[1] These authors contributed equally to this work.

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intake (Heinzmann et al., 2010; Lang et al., 2017; Lloyd, Beckmann, Favé, Mathers, & Draper, 2011). Yet, an untargeted serum metabolomics-driven approach was also used to predict orange juice consumption. Rangel-Huerta et al. (2017) observed a decrease in serum levels of hydroxyoctadecadienoic acid (9-HODE + 13-HODE) and dihydroxyoctadecanoic acid (12,13-DiHOME and 9,10-DiHOME) and an increase in 12-hydroxyeicosatetraenoic acid (12-HETE) levels after high-polyphenol orange juice supplementation.

Recently, the analysis of dried blood spot (DBS) matrix, commonly used in newborn screening for the diagnosis of inborn errors of metabolism, has been extended and became a praticable tool for many different applications ranging from therapeutic drug monitoring (Li & Tse, 2010), genomics (Tarini & Goldenberg, 2012), proteomics (Chambers, Percy, Yang, & Borchers, 2015) and metabolomics (Michopoulos, Lai, Gika, Theodoridis, & Wilson, 2009; Wilson, 2011). Using this methodology blood samples are typically obtained from heel or finger pricks and spotted onto filter paper for analysis. DBS offers some essential advantages over whole blood, plasma or serum samples, including a minimally invasive and easy to perform sampling, as well as a significantly lower volume of required sample compared to the venous collection and a low cost of sample transport (Gao et al., 2017; Zukunft, Sorgenfrei, Prehn, & Adamski, 2013). In addition, DBS analysis could lead to the detection of whole blood markers that might not be present in plasma or serum samples.

In the present study we used two different blood samples, DBS and plasma, to assess the effect of two-week orange juice consumption on lipid metabolism in healthy volunteers by a mass-spectrometry based metabolomics approach. DBS were analysed by liquid chromatography mass spectrometry (LC-MS) and plasma samples were analysed by the gas chromatography mass spectrometry (GC–MS). Multivariate statistical models were constructed to explore the relationship between the lipids in dried blood spot and plasma and the orange juice intake.

2. Materials and methods

2.1. Chemical composition of orange juice

Orange juice obtained from local producers was used. Flavonoid identification and quantification were performed as described in Brasili et al. (2017). Briefly, a total of 5 mL of orange juice was centrifuged for 10 min at 4 °C and 7000 g. An aliquot of the supernatant (1 mL) was filtered and injected (10 μL) into a 1260 Infinity Quaternary LC System (Agilent Technologies, USA) with an autosampler and a quaternary pump, coupled to a DAD. Elution of the analytes was achieved on a column Prodigy 5 μm ODS3 reversed-phase silica (250 mm × 4.60161 mm) (Phenomenex Ltd., UK) with a flow rate of 1 mL/min at 25 °C. The mobile phase consisted of 0.5% formic acid in water (solvent A) and 0.5% formic acid in acetonitrile (solvent B). The eluates were monitored at 270 and 525 nm. Peak identification was carried out by the combined information provided by comparison of retention times, diode array spectral characteristics and mass spectra, measured by LC- ESI-MS/MS, with the internal standards and the data available in the literature. The equipment of LC-ESI-MS/MS was a Prominence liquid chromatograph (Shimadzu, Japan) linked to an ion trap mass analyzer (Esquire, Bruker Daltonics, Billerica, MA, USA) with an electrospray ionization (ESI) interface in negative mode for flavonoids. The mass spectrometer operating conditions were as follows: collision energy was 4000 V and capillary temperature was 275 °C. The analysis was carried out using a full scan from m/z 100 to 1500.

Quantification was done by calibration curves of internal standards (Extrasynthese, Genay, France).

The content of vitamin C was evaluated through the reduction of ascorbic acid. This compound was extracted with metaphosphoric acid (0.3% vv^{-1}) and quantified by reversed-phase HPLC, in a Hewlett-Packard 1100 system, with an autosampler and a quaternary pump, coupled to a diode array detector (DAD). The column used was μ-Bondapack (300 mm × 3.9 mm i.d., Waters, Milford, MA, USA) column and elution (flow rate of 1.5 mL/min) was carried out in isocratic conditions with 0.2 M sodium acetate/acetic acid buffer (pH 4.2), monitored at 280 nm. Total ascorbic acid was estimated after reduction of dehydroascorbic acid (DHA) with 10 mM dithiothreitol, with a calibration curve of ascorbic acid (0–600 mg/L).

2.2. Antioxidant activity

The antioxidant capacity of orange juice was assessed by free radical-scavenging activity according to Brand-Williams, Cuvelier, and Berset (1995) method and by an oxygen radical absorbance capacity assay according to Prior et al. (2003) method. To determine the radical scavenging activity, a 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol was prepared. An aliquot of 50 μL of deproteinated sample was added to 150 μL of this solution. The decrease in absorbance was determined at 517 nm using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Hercules, CA, USA) when the reaction reached a plateau (after 20 min). The area under the curve was calculated. The results were expressed as millimole Trolox equivalents. To evaluate the oxygen radical absorbance capacity, 25 μL of diluted sample (1:300) was added to 150 μL of sodium fluorescein (40 nM) and incubated for 15 min at 37 °C. Subsequently, 0.6 mL of AAPH (4 mM) as a peroxyl radical generator and Trolox as a control standard were added, and the fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength of 520 nm, every 5 min for 120 min. Final results were calculated from a standard curve using different concentrations of Trolox (12.5–100 μM) and expressed in millimole Trolox equivalents. Measurements were recorded on a Synergy TM HT-multimode microplate reader (Biotek Instruments, Winooski, VT, USA).

2.3. Subjects and study design

Fifteen healthy volunteers between 20 and 45 years old (7 males and 8 females) with a body mass index (BMI) of 22.5 ± 3.5 kg/m^2 (mean ± SD) participated in a longitudinal study. Informed consent was obtained from all individual participants included in the study. Exclusion criteria included diabetes, gastrointestinal and cardiovascular diseases, liver and kidney dysfunctions, intake of supplements or antibiotics, current smokers, suspected or definite history of alcohol or drug abuse history, being pregnant. The study protocol was approved by the Ethics Committee of the Faculty of Pharmacy at University of São Paulo (ref: 10207012.6.0000.0067). During the study period, participants were forbidden to consume citrus fruits other than test orange juice. After a 7 day washout period (citrus-free diet), subjects were asked to consume a 250 mL of orange juice, twice daily for 15 consecutive days. A total of 8 mL of blood by venous puncture was collected in heparinized tubes at the start, corresponding to 7 day after washout period (T0) and at the end of the study (T1), period related to 15 days after orange juice intake. Aliquots of whole blood were spotted onto Guthrie card filter papers (Whatman no. 903 Protein Saver TM cards, formerly Schleicher & Schuell, Keene, USA) for the DBS analysis. The Guthrie card filter papers were left to dry for at least 4 h at room temperature and were stored at −20 °C in a foil bag with a desiccant package pending further analysis. The blood tubes were then centrifuged and the resulting plasma was stored at −20 °C until further analysis.

2.4. Biochemical parameters

Concentrations of glucose, triglycerides, total cholesterol (TC), HDL and LDL levels in plasma were determined by spectrophotometry using commercial kits (Labtest, Brazil) adapted to a biochemical analyzer equipment (LabMax 240, Labtest, Santa Clara, Brazil). The determination of LDL-cholesterol was calculated using the Friedwald formula.