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Non-targeted metabolomic approach reveals urinary metabolites linked to steroid biosynthesis pathway after ingestion of citrus juice

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ABSTRACT

Citrus juice intake has been highlighted because of its health-promoting effects. LC–MS based metabolomics approaches are applied to obtain a better knowledge on changes in the concentration of metabolites due to its dietary intake and allow a better understanding of involved metabolic pathways. Eight volunteers daily consumed 400 mL of juice for four consecutive days and urine samples were collected before intake and 24 h after each citrus juice intake. Urine samples were analysed by nanoHPLC-q-TOF, followed by principal component analysis (PCA) and Student's *t*-test ($p < 0.05$). PCA showed a separation between two groups (before and after citrus juice consumption). This approach allowed the identification of four endocrine compounds (tetrahydroaldosterone-3-glucuronide, cortolone-3-glucuronide, testosterone-glucuronide and 17-hydroxyprogesterone), which belonged to the steroid biosynthesis pathway as significant metabolites upregulated by citrus juice intake. Additionally, these results confirmed the importance of using the non-targeted metabolomics technique to identify new endogenous metabolites, up- or down-regulated as a consequence of food intake.

1. Introduction

The benefits of plant foods in general and citrus fruits and juices in particular, are potentially due to their high content in phytochemical compounds. These bioactive molecules can be defined as compounds present in fruit and vegetables that may exhibit a potential for modulating human metabolism (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003). The beneficial effects of citrus fruits intake are attributed to vitamin C, citric acid, folate, limonoids, essential oils, dietary fibre, carotenoids such as lutein, zeaxanthin, β-carotene and β-cryptoxanthin and especially phenolic compounds (mainly flavonoids) (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010; Mangels, Holden, Beecher, Forman, & Lanza, 1993). Flavanones, hesperetin, and naringenin are the most abundant phenolic compounds in citrus fruits and constitute the major part of the total flavonoids intake in many European countries (Gil-Izquierdo, Gil, Tomás-Barberán, & Ferreres, 2003). Citrus flavonoids, alone or in synergy with other compounds have a wide range of biological activities, such as antioxidant, vascular, estrogenic, anti-inflammatory, tumour cytotoxicity, antimicrobial effects, and protection against cardiovascular diseases (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001).

Over the past few years, metabolomics has emerged as a new approach in the field of food and nutrition and others fields like pharmacology, medicine and toxicology (Wishart, 2008). This technique is focused on high-throughput characterisation of small molecule metabolites in biological samples (Krastanov, 2010). The metabolome can be detected by non-invasive surgical samples including saliva, plasma, serum or urine (Sugimoto, Wong, Hirayama, Soga, & Tomita, 2010). Among them, urine is one of the most used biofluids for metabolomic trials. The metabolome can be modulated by internal or external factors such as diet, which affects the urinary metabolome, producing significant changes in its qualitative and quantitative profile. The identification of the food intake-related metabolome is highly relevant to correlate the dietary habits with the expected healthy activity of the bioactive compounds and to identify new metabolites of their consumption (Wishart, 2008). In the same way, and besides the

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dietary habits, the circadian rhythm causes important effects on the 24 h kinetic evolution of the urine metabolome (Llorach et al., 2010). Urine composition (influenced by intrinsic and extrinsic factors) affects its pH and therefore conditions the rate of microorganism growth and the kinetics rate of metabolites in urine (Mazzarino et al., 2011). These sensitive multifactorial responses of the human body can be developed by metabolomics.

Metabolomics analyses have been traditionally classified as targeted and non-targeted. Targeted analyses are focused on a specific group of metabolites, whereas untargeted metabolomics are focused on the detection of many separate groups of metabolites, to achieve specific fingerprints or metabolite patterns (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). Both approaches have provided highly valuable information in a wide variety of studies. Using metabolomic tools, we could find new metabolites of food intake and also over- or down-regulated endogenous metabolites associated with physiological pathways. Thus, we could relate them to their beneficial effects on the organism (Llorach et al., 2010). Therefore, metabolomic technologies allow a further insight on the metabolic pathways linked to food intake and those implicated in the origin of pathological conditions, and the starting-point to their prevention by adequate food intake. However, there is a lack of experimental data on metabolomics, giving as significant compounds endogenous metabolites influenced by citrus intake and others types of food exposure.

The aim of this work was to analyse the influence of citrus juice intake over four consecutive days on the urinary human metabolome and to study changes at metabolic level using nanoHPLC-qTOF-Metabolomics as a tool for the identification of the discriminating metabolites responsible for these changes.

2. Materials and methods

2.1. Commercial standards and reagents

The 17-hydroxyprogesterone, theobromine and sodium azide were purchased from Sigma–Aldrich (St. Louis, MO); α-cortolone and testosterone-glucuronide were provided by Fountain limited (Malta). All LC-MS grade solvents such as water, acetonitrile and formic acid were obtained from J.T Baker (Phillipsburg, NJ).

2.2. Citrus juice composition

Juice used for this study was prepared at pilot scale by "Hero España, S.A" (Alcantarilla, Murcia, Spain). It consisted of a mixture of orange juice (*Citrus sinensis* (L.) Osbeck) and lemon juice (*Citrus limon* (L.) Burm). The juices were packaged in individual 200 mL Tetra brik® containers and maintained at 4 °C until delivery. Juices were totally stable according to previous studies (González-Molina, Moreno, & García-Viguera, 2008).

2.3. Human subjects and study design

Eight Caucasian volunteers participated in the study (4 male and 4 female), their physical characteristics are represented in Table 1. For the selection of volunteers, it was taken into account that they were healthy according to medical parameters, blood and urine samples were collected to assess biological variables and haematocrit, thus confirming the eight volunteers' health participating in the study. Volunteers were not smokers, not pregnant, have had stable feeding habits, were not vegetarians, and none reported a history of heart disease or received any medication. This study followed the guidelines set by the Helsinki Declaration (http://www.fda.gov/ohrms/dockets/dockets/06d0331/06D-0331-EC20-Attach-1.pdf). We must be aware of the limitations of the subjects in clinical trials when designing the study: volunteers were informed of the right to participate or not in research and to withdraw their approval at any time. Informed consent of each individual involved in the trial was signed (Speid, 2010). The study was approved by the Bioethics Committee.

During the study, the volunteers followed a strict diet absent of fruits and vegetables and any products that might contain direct or

Table 1
Characteristics of volunteers involved in the study.

All subject	Women (*n* = 4)	Men (*n* = 4)
Height (m)	1.60 ± 0.06	1.84 ± 0.04
Weight (kg)	53.75 ± 6.50	84.83 ± 10.51
BMI (kg m^{-2})	16.73 ± 1.60	23.05 ± 3.35
Age (y)	38 ± 7	36 ± 5

Data are represented by mean ± SD.

Table 2
List of food and beverages prohibited during the assay.

Forbidden beverages	Forbidden foods
Fruit juices, nectars	Fruits, except watermelon, melon and pineapple
Coffee	Vegetables, garlic, parsley, potatoes, mushrooms, soybeans
Tea	Yogurt with fruit pieces
Beer, wine, champagne, cider, whiskey, rum and cognac	Cereal bars, nuts
Shakes, cocoa	Cocoa and derivates
Soft drinks and beverages	Chocolate
	Jams, ice cream
	Brown sugar, brown rice, brown bread
	Sausages, pickles
	Honey
	Olives, olive oil (restricted use 1 spoonful/day)

Table 3
Nutritional composition and energy value of the dietary intake and citrus juice intake during the study.

	Daily intake	Citrus juice (400 mL)[a]
Carbohydrates (g)	214.02	36 (16.82%)
Total sugar (g)	38.1	13.28 (34.85%)
Glucose (mg)	10290	6240 (60.64%)
Sacarose (mg)	9750	2560 (26.26%)
Fructose (mg)	8000	4480 (56%)
Lactose (mg)	9400	–
Maltose (mg)	660	–
Proteins (g)	129.83	1.76 (1.35%)
Fat (g)	55.7	0.12 (0.21%)
Calcium (mg)	929.79	83.84 (9.02%)
Iron (mg)	12.53	0.52 (4.15%)
Magnesium (mg)	371.25	78.72 (21.20%)
Potassium (mg)	4762.2	1394.5 (29.28%)
Sodium (mg)	2074.5	14.64 (0.70%)
Zinc (mg)	16.33	0.584 (3.57%)
Copper (mg)	1.43	0.128 (8.95%)
Vitamin C (mg)	162.4	154.8 (95.32%)
Vitamin E (mg)	5.04	0.15 (2.97%)
Vitamin B_1 (mg)	1.22	0.024 (1.96%)
Vitamin B_2 (mg)	1.22	0.015 (1.23%)
Vitamin B_3 (mg)	27.81	0.091 (0.32%)
Vitamin B_6 (mg)	2.14	0.08 (3.73%)
Vitamin A (μg)	220.1	3.00 (1.36%)
β-carotene (μg)	100.55	1.00 (0.99%)
Total polyphenols (mg)	115.56	115.56 (100%)
Flavanones (mg)	86.12	86.12 (100%)
Flavones (mg)	29.44	29.44 (100%)
Energy value (kcal)	1857.93	152 (8.18%)

[a] The rate per cent between brackets indicates the nutritional percentage of contribution of the juice to the total daily diet.