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 during four consecutive days and urine samples were collected before intake and 24 h after each citrus juice intake. Urine samples were analyzed by nanoHPLC-q-TOF, followed by Principal Component Analysis (PCA) and Student´s t-test (P<0.05). The PCA analysis showed a separation between two groups (before and after citrus juice consumption). This approach allowed identifying four endocrine compounds as significant metabolites up-regulated which belonged to the steroid biosynthesis pathway by citrus juice intake (tetrahydroaldosterone-3-glucuronide, cortolone-3-glucuronide, testosterone-glucuronide and 17-hydroxyprogesterone). Additionally, these results confirmed the importance of using non-targeted metabolomics technique to obtain new endogenous up- or down-regulated metabolites so as exploring metabolic pathway impacted as consequence of food intake.

Keywords: Citrus juice, endogenous metabolites, metabolomics, steroids, urine.

Highlights:

1. Metabolomics is a powerful tool for identification of metabolites associated with dietary intake
2. Four urinary endocrine metabolites were identified as significant after citrus juice intake
3. The metabolites after 4 days of citrus juice intake belonged to the steroid pathway
4. This metabolomics approach provides mechanistic support to previous targeted studies

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 fiber, 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 field of food and nutrition and others fields like pharmacology, medicine and toxicology (Wishart, 2008). This technique is focused on high-throughput characterization 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 dietary habits, the circadian rhythm causes important effects on the 24 hours kinetic evolution of the urine metabolome (Llorach, Garrido, Monagas, Urpi-Sarda, Tulipani, Bartolome, 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, Abate, Alocci, Rossi, Stinchelli, Molaioni, 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 to physiological pathways. Thus, we could be able to relate them to their beneficial effects on the organism (Llorach, et al., 2010). Therefore, metabolomic technologies allow gaining a further insight on the metabolic pathways linked to food intake and 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

124 endogenous metabolites influenced by citrus intake and others types of the food
125 exposures.
126 The aim of this work was to analyze the influence of citrus juice intake during
127 four consecutive days in the urinary human metabolome and to study changes at
128 metabolic level using nanoHPLC-qTOF-Metabolomics as tool for the identification of
129 the discriminating metabolites responsible for this alteration.
130
131 **2. Materials and methods**
132
133 2.1. *Commercial standards and reagents*
134 The 17-hydroxyprogesterone, theobromine and sodium azide were purchased
135 from Sigma-Aldrich (St. Louis, Missouri, USA), α-cortolone and testosterone-
136 glucuronide were provided by Fountain limited (Malta), all LC-MS grade solvents such
137 as water, acetonitrile and formic acid were obtained from J.T Baker (Phillipsburg, New
138 Jersey, USA).
139
140 2.2. *Citrus juice composition*
141 Juice used for this study was prepared at pilot scale by "Hero España, S.A"
142 (Alcantarilla, Murcia, Spain). It consisted of a mixture of orange juice (*Citrus sinensis*
143 (L.) Osbeck) and lemon juice (*Citrus limon* (L.) Burm). The juices were packaged in
144 individual 200 mL Tetra brik® and maintained at 4ºC until delivery. Juices were totally
145 stable according to previous studies (González-Molina, Moreno, & García-Viguera,
146 2008).
147
148

149 2.3. *Human subjects and study design*

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

164 During the study, the volunteers followed a strict diet absent of fruits and
165 vegetables and any products that might contain direct or indirect plant phenolic. The list
166 of forbidden foods and beverages are shown in Table 2. This strict diet was initiated
167 during the previous week to the beginning date of the assay.

168 On the other hand, the chemical composition and energy value of the dietary
169 intake including citrus juice intake, as well as the percentage contributed by the juice to
170 the daily intake were summarized in Table 3 (data calculated by the software available
171 on the website (http://www.invesalia.es/evaluacion/) with the additional assistance of
172 the Spanish and USDA databases (http://www.bedca.net/ and
173 http://www.nal.usda.gov/fnic/foodcomp/search/). Two servings of juice (400 mL)

174 provided 115.56 mg of flavanones and flavones. Juice intake was conducted during four
175 consecutive days, with consumption 400 mL of juice each day . This volume of intake
176 was selected because is coincident with the daily dietary ingestion of two glasses of
177 juice (at breakfast and dinner). 4 days were enough to investigate if the juice could show
178 a preliminary effect in the human body by the continuous physiological occurrence of
179 bioactive compounds from juice. On day 1, the urine samples were collected twice:
180 before citrus juice intake (control samples) and another when juice was firstly consumed
181 by volunteers. The urine was collected every 24 hours from the first day to a day after
182 the last juice intake. Urine day 0 and day 4 were used for the metabolomic analysis.
183 These urine samples were aliquoted in presence of sodium acetate (10 mM final
184 concentration) and stored at -80 ºC until posterior analysis (Saude & Sykes, 2007).
185
186 2.4. *Sample preparation*
187 Urine samples (500 µL) were softly thawed in a fridge at 4 ºC prior to analysis
188 and then were centrifuged at 11000 *x g* for 5 min. Supernatant (200 µL) was filtered
189 through 0.45 µm Millex®-HV filter units (Millipore, Concord Road, Billerica, MA,
190 USA) and transferred to amber glass vials for injection at the HPLC-q-TOF analysis.
191 Two classes of quality control (QC) were used for metabolomic analysis quality. QCs
192 were MS grade water samples and theobromine solution (20 µM) and were injected in
193 three times in the batch: beginning, middle and end.
194
195 2.5. *HPLC-q-TOF analysis*
196 Chromatographic separation was performed on an 1100 serie HPLC system
197 (Agilent Technologies, Waldbronn, Germany) equipped with on-line degasser, auto-
198 sampler, quaternary pump, and thermostatic column compartment. The column ACE 3

C18: 150 x 0.075 mm, 3 μm from Symta S.A.L (Madrid, Spain) was used. The mobile phase consisted of (A) MilliQ-H_2O 0.1% HCOOH and (B) acetonitrile 0.1% HCOOH. The injection volume was 6.25 nL and the flow rate was 312 nL/min for the urine samples and quality controls (QCs). A gradient with the following rates (v/v) of phase B (t, %B) was used for the determination of metabolites (0, 0); (1, 0); (10, 10); (11, 10); (17.5, 100); (19.5, 100); (19.6, 0); (23, 0).

The MS acquisition was performed by a Bruker MicroTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany). The ESI analyses were carried out with capillary and end plate offset voltages of -4500 and -500 V in positive mode, and 4000 and 500 V in negative mode. Nitrogen was used as both nebulizer and drying gas. The nebulizer gas pressure was 1.6 bar, the drying gas temperature 200 ºC and its flow rate 8.0 L/min. Spectra were acquired at the *m/z* 50-900 range. In order to calibrate the mass axis, a 10 mM sodium formate solution in 1:1 isopropanol-water was introduced into the ESI source at the beginning of each HPLC run using a divert valve.

Bruker Daltonics software packages micrOTOF Control v.2.3, HyStar v.3.2 and Data Analysis v.4.0 were used to control the MS (QTOF) device.

2.6. *Data processing and multivariate statistical analysis*

Each LC-MS data set was processed using the Find Molecular Features (FMF) algorithm in the ProfileAnalysis 2.0 software (Bruker Daltonik, Bremen, Germany) to create a feature list for statistical analysis. Each feature in an LC-MS data set is described by its retention time (RT), m/z value and its intensity. The parameters of the FMF algorithm were set to the following values: S/N (signal to noise) threshold: 5, correlation coefficient threshold: 0.7, minimum compound length: 10 and smoothing width: 1; MS spectra type, line spectra.

To process the batches of LC-MS data a transformation into a tabular format, called bucketing, was required. In our analyses the retention time range was (0.02; 23.04 min) and mass range was (50; 900 Da). The bucket was placed according to its RT and m/z. . The bucket intensity values were normalized to the largest bucket value in each sample. Normalization step is important to ensure the comparative parameters among different samples. Retention time alignment was performed with an algorithm from Podwojski and colleagues taking non-linear retention time shifts into account (Podwojski, Fritsch, Chamrad, Paul, Sitek, Stühler, et al., 2009).

Principal Component Analysis (PCA) was performed using ProfileAnalysis 2.0 after Pareto scaling. PCA-based methods usually constitute the first step in evaluating metabolomic data. PCA is involved in the calculation of linear combinations of the original data (PCs), reducing the dimensionality of a data set and allows identifying the most influential variables (Werth, Halouska, Shortridge, Zhang, & Powers, 2010). Whereby each successive PC explains the maximum amount of variance possible in the data set. In this way, PCA converts data obtained from high-throughput instrumental analysis into a visual presentation: score plot (display the values of LC-MS analysis) and loading plot (represents the values of buckets). Additionally a Student's t-test was carried out in all samples comparing the average intensity values of both sample groups, with a *P*-value <0.05 considered significant.

2.7. *Metabolite identification*

Metabolites were identified on the basis of their exact mass and molecular formula, which was compared to those registered in the Human Metabolome Database (www.hmdb.ca) and ChemSpider Database (www.chemspider.com).

Metabolite identification was carried out using SmartFormula (Bruker Daltonik, Bremen, Germany), which assigns the theoretically possible elemental composition for a particular *m/z* value. SmartFormula evaluates the formula suggestion from both the accurate mass and the isotopic pattern information using the Sigma algorithm (a combined value for the standard deviation of masses and intensities for all peaks). The mass tolerance value was set to ≤5 mDa. The charge was 1, and filter H/C element ratio was set from 1 to 3 as complementary information for identification MS/MS spectra are considered by SmartFormula 3D.

3. Results and discussion

Urine samples from eight volunteers were collected at 24 hours after the intake of 400 mL citrus juice (two glasses per day, 1 glass at 8:00 and the other at 18:00) during four consecutive days. The urines were centrifuged and filtrated for the injection into a nanoHPLC-qTOF to generate the molecular ions within a 50-900 mass range (see Experimental section). After alignment of each peak, we obtained a data table (bucket table) with approximately 2500 mass features with its *m/z*, retention time, and intensity of each of them. Urine samples were analyzed by multivariate analysis (MVA) to visualize the most important variation on the direction of the data set and characterize the changes in the urinary metabolic profile of each volunteer.

3.1. *Principal Component Analysis results*

Using LC-MS with MVA approach, fingerprinting of human urine was obtained. PCA plots showed in Figure 1 were used to determine the metabolomics different profiles among the analyzed samples. Each point within the scores plot represented an individual sample, whereas points in the loading plot showed how principal components

are related to the original buckets. To correct for a no constant signal variance, the data set was scaled using the Pareto method (Gaquerel, Heiling, Schoettner, Zurek, & Baldwin, 2010). The two first principal components (PCs), PC 1 (42.2%) and PC 2 (18.4%) express 60.6% of the total LC-MS dataset variance in negative mode and 57.3 % (39.5% PC1 + 17.8% PC2) in positive mode. In this analysis six hundred masses were generated approximately (after applying a bucket filter of 50%), four of which were identified and selected as the most significant masses (two in negative mode and two in positive mode) (P<0.05) (Table 4). In addition, other metabolites that appeared as significant in Student´s t-test (P<0.05) and that remained unidentified have also been included in Table 4. The score plot has showed that the analyses are distributed in the sub-space determined by the selected PCs. In this study, we observed that there are clear clusters of samples from treated and control volunteers appearing separated from one another. The loading plots showed which buckets were responsible of this behaviour (Figure 1). Therefore, buckets 539.2502 *m/z*; 10.87 min, 541.2682 *m/z*; 11.00 min (negative mode), 465.2443 *m/z;* 13.16 min and 331.2214 *m/z*, 12.62 min (positive mode) were responsible of the groups' segregation.

Likewise, the bucket statistical plots help to visualize the findings from the PCA results related to one specific bucket. The horizontal and vertical axis recorded the analyses and the intensity corresponding to the bucket value (relative values in the y-axe to indicate the tendency of the individual metabolites, 0 is not an absolute data), respectively. The Figure 2 showed four buckets, which corresponded to the previously described statistically significant metabolites (P<0.05) linked to citrus juice intake. In these graphs, the separation between treatment and control groups displayed an interesting dispersion, where the selected metabolites were only present in the citrus juice consumption group, whereas in the control group their intensity was much lower.

Concerning the diet and their variables (as it has been mentioned in the Experimental Section), volunteers only consumed the citrus juice (2 glasses) as the only plant-based origin food of a normal, constant and balanced diet for four consecutive days. In fact, previous several studies have supported that metabolites identified in our study did not result from ingestion of a diet rich in protein or fiber (Rasmussen LG, 2012; Rasmussen, Winning, Savorani, Ritz, Engelsen, Astrup, et al., 2011). Therefore, these results have evidenced that non-targeted approach provided new useful significant metabolites linked to citrus juice intake, which contribute to the development of the food metabolome as an important part of the human urinary metabolome (Mazzarino, et al., 2011).

3.2. *Identification of the discriminant metabolites*

The discriminant *m/z* values described by MVA were determined qualitatively by comparison with three commercially available steroids (17-hydroxyprogesterone, α-cortolone, and testosterone-glucuronide). The comparison proved that the accurate mass and retention time of the tentatively identified metabolites by molecular formula and database search matched those of the standards. In positive electrospray ionization mode, testosterone-glucuronide standard showed a retention time of 13.1 min and 465.2443 *m/z* with a daughter ion of 289.2089 *m/z* (-176 amu, corresponding to the loss of glucuronic acid) as previously published by Antignac and colleagues (- , & Bizec, 2005). 17-hydroxyprogesterone standard exhibited m/z 331.2214 with a retention time of 12.7 min. These *m/z* values and retention times matched with those described in the urine samples (Table 4) (H.-J. Cho, Kim, Lee, Chung, & Choi, 2009). However in negative mode, α-cortolone was injected (cortolone-3-glucuronide not commercially available) and showed *m/z* 365.2406 that

corresponded to the base peak after cortolone-3-glucuronide fragmentation (Sumner, Amberg, Barrett, Beale, Beger, Daykin, et al., 2007). In addition, the characteristical fragments at *m/z* 271 and 253 were also detected after the fragmentation event coincident with those found for the MS/MS event of the cortolone standard (H.-J. Cho, Kim, Lee, Chung, & Choi, 2009). The other metabolite, tetrahydroaldosterone glucuronide (m/z 539.2502), was tentatively identified with a mass range tolerance of <5 mDa by comparison with the theoretical mass at m/z 539.2497 attributed by Human Metabolome Database.

In the past decade, significant advances in LC-MS technology that facilitated routine analyses of steroid hormones (Stanczyk & Clarke, 2010). Using this technique, the specific glucuronide fragment *m/z* 113 has been well-suited for monitoring steroid glucuronides, although the loss of glucuronic acid (-176 amu) has been also observed (Lutz, Lutz, & Lutz, 2006). In a previous work, by Gadzala-Kopciuch and colleagues have reported that testosterone-glucuronide is the major form in urine (Gadzała-Kopciuch, Ričanyová, & Buszewski, 2009).

These low-molecular weight metabolites identified represent the intermediate and final products within the steroid biosynthesis pathways (map00140) (www.genome.jp/kegg/) (Figure 3). Steroid hormones constitute an important class of metabolites with different biochemical and physiological functions. All steroid hormones are synthesized from cholesterol through a series of reactions belonging to the pregnenolone pathway (Sirén, Seppänen-Laakso, & Orešič, 2008). The core structure of steroid is the cyclopentanoperhydrophenanthrene four–ring hydrocarbon nucleus (You, 2004). During the first metabolic phase, steroids are biochemically converted to other steroids by the oxidation, hydroxylation or reduction of functional groups and, in their phase II, metabolism includes the formation of glucuronide and

348 sulfate conjugative forms, which promotes their excretion and may produce biologically
349 active metabolites (Jäntti, Tammimäki, Raattamaa, Piepponen, Kostiainen, & Ketola,
350 2010). Those steroids possessing a 3β-hydroxyl structure are mostly excreted as
351 sulfates, whereas steroids with 3α- or 17β-hydroxyl group appear as glucuronide
352 conjugation in urine (Hintikka, Kuuranne, Leinonen, Thevis, Schänzer, Halket, et al.,
353 2008). Less than 3% of the steroids are excreted unconjugated by urine (Gomes,
354 Meredith, Snape, & Sephton, 2009). In our current study, three metabolites were
355 glucuronidated while the intermediate metabolite remained unconjugated like. This fact
356 has been previously stated in the steroid pathway (Figure 3).
357
358 3.3. *Steroidogenesis and citrus juice intake*
359 The effect of diurnal variation in the urinary metabolome constitutes an
360 important issue that has been demonstrated in previous metabolomic studies in humans
361 (Lenz, Bright, Wilson, Morgan, & Nash, 2003). In this way, the endogenous urinary
362 metabolite profiles could have a considerable inter-subject variability and could be
363 influenced by the physiological state. Regarding diet, it may modulate the intestinal
364 microbiota and, therefore by doing so can modify the bioavailability and metabolism of
365 nutrients. This fact is reflected in the biochemical urinary profile. Indeed, the microbial
366 metabolome has been defined as of great relevance in relation with the urinary
367 metabolome (Rezzi, Ramadan, Fay, & Kochhar, 2007).
368 Steroidogenesis has been firstly defined though the urinary steroid excretion by
369 valid and sensitive devices like GC-MS, but in the past years has been investigated by
370 liquid chromatography tandem mass spectrometry that provide an integrated picture of
371 an individual urinary metabolome (Krone, Hughes, Lavery, Stewart, Arlt, &
372 Shackleton, 2010). At our current study, steroid conjugates (tetrahydroaldosterone-3-

glucuronide, cortolone-3-glucuronide, and testosterone-glucuronide) and 17-hydroxyprogesterone have been identified as metabolites significantly contributing to changes in the urine metabolome after the citrus juice intake (Table 4) and no statistical differences in the concentration of them were found between men and women (Figure 2). There are few studies about the isolation and characterization of cortisol metabolites (cortolone-3-glucuronide) and tetrahydroaldosterone glucuronide metabolites in human urine (Grose, Nowaczynski, Kuchel, & Genest, 1973; Kornel & Saito, 1975). On the contrary, there are many reports about 17-hydroxyprogesterone and testosterone-glucuronide (Cho, Lee, Choi, Lee, & Chung, 2009; Lutz, Lutz, & Lutz, 2006; Storbeck, Swart, Africander, Conradie, Louw, & Swart, 2011). Some studies reported that exposure to different factors, such as diet, physical exercise, and distinct environment factors could cause alterations in the biosynthesis or inactivation of endogenous steroids, that may affect the bioavailability of steroid hormones. However, and to the best of our knowledge, there are no studies linking directly the consumption of citrus juices at diet dose to the steroid metabolites. We can hypothesise the mechanisms through which juice components influence the excretion of these specific metabolites. Being the conjugative activity of glucuronyl tranferases not incredibly specific, a phase II metabolism competition with other metabolites could take place. Another possibility could be related to the short term upregulation of phase II enzymes due to the increased presence of substrates (polyphenols) which ends up in increased glucuronidation of steroidal metabolites, which in turn are increasingly excreted. In fact, there are previous studies where polyphenol colonic metabolites are excreted for more than 48 h after ingestion and may significantly interfere with phase II metabolism (Del Rio, Rodríguez-Mateos, Spencer, Tognolini, Borges & Crozier, 2012). This urine steroid profiling may

enrich the nutritional utility of citrus juice in the investigation of defects in the metabolic pathways (Chan, Taylor, Tiu, & Shek, 2008).

3.4. *Influence of citrus bioactive components on the human physiological pathways.*

Citrus fruits have been valued as a fundamental part for a healthy diet, it is well established that citrus juice constitutes an interesting source of phenolic compounds (mainly flavanones) and other nutrients and non-nutrients compounds (vitamins, minerals, dietary fibre, essential oils, organic acids, and carotenoids) (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010). These bioactive components may regulate gene expression at the transcriptome and may exhibit a potential for modulating human metabolism (Giménez-Bastida, Martínez-Florensa, Espín, Tomás-Barberán, & García-Conesa, 2009; Morin, Nichols, Zalasky, Davis, Manthey, & Holland, 2008). Studies in humans on bioavailability of these compounds allowed developing and observing that the peak plasma concentration of hesperetin was 1.28 ± 0.13 μmol, after ingestion of 1 litre of orange juice. Flavanones metabolites appeared in plasma 3 h after juice ingestion and reached a peak between 5 and 7 h (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003). Then returned to baseline level at 24 h and their relative urinary excretion was 7.9 ± 1.7% after of juice intake (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003). Besides, there are other citrus juice ingredients with reported biological activities in the human body like vitamins, limonoids, carotenoids or alkaloids, among others. We should not forget that it is a multicomponent food matrix where the bioactive components can act in a synergistic, antagonistic or isolated manner at physiological level, equally the bioavailability of these compounds can be affected by the food matrix (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001; Gil-Izquierdo, Gil, Tomás-

Barberán, & Ferreres, 2003; Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002; Mullen, Archeveque, Edwards, Matsumoto, & Crozier, 2008). The results of the current study provide information of the up-regulation of four steroidal metabolites (upon 2 glasses of citrus juice for 4 days) giving mechanistic support to other widely targeted assays describing the effects of the citrus juice intake on the cholesterol and bone metabolisms. The four steroid metabolites (testosterone-glucuronide, cortolone-3-glucuronide, 17-hydroxyprogesterone and tetrahydroaldosterone-glucuronide) over synthesized in the human body after ingestion of the juice are included in a pathway where the cholesterol is the precursor compound (Table 4 and Figure 3). This overactivation of this pathway to these endogenous metabolites could lead to a higher demand of cholesterol as primary substrate of the enzymes of steroid metabolism (primarily governed by the P450 enzyme, CYP11A1), supporting the hypocholesterolemic effect of chronic citrus juice intake (Benavente-García & Castillo, 2008; Cesar, Aptekmann, Araujo, Vinagre, & Maranhão, 2010; González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010; Krum, 2011; Kumar, Cantor, Allen, Riccardi, & Cox, 2002; Kurowska, Spence, Jordan, Wetmore, Freeman, Piche, et al., 2000; Pang, Wang, Mok, Lai, Chow, Leung, et al., 2010; Pikuleva, 2006). Besides, among the 3 end-point significant metabolites, testosterone-glucuronide plays an important role in the maintenance of health bone by prevention of osteoblast apoptosis and stimulation of osteoclast apoptosis (Clarke & Khosla, 2009). Like in cardiovascular and cholesterol metabolism concerns, this action has been related to citrus flavonoids, particularly, flavanones which provide potentially improvement of bone health by inhibition and/or activation of the different enzymes and stimulation of the osteoblast differentiation by BMP (Bone Morphogenetic Proteins) or MAPKs signalling pathways (Habauzit, Nielsen, Gil-Izquierdo, Trzeciakiewicz, Morand, Chee, et al., 2009; Habauzit, Sacco, Gil-Izquierdo, Trzeciakiewicz, Morand,

Barron, et al., 2011; Horcajada, Habauzit, Trzeciakiewicz, Morand, Gil-Izquierdo, Mardon, et al., 2008; Trzeciakiewicz, Habauzit, Mercier, Barron, Urpi-Sarda, Manach, et al., 2010; Trzeciakiewicz, Habauzit, Mercier, Lebecque, Davicco, Coxam, et al., 2010; Wong & Rabie, 2006; Yu, Wang, Walzem, Miller, Pike, & Patil, 2005). In the same way, other citrus components like β-crytoxanthin inhibited bone resorption in bone tissue cultures and may reduce the risk of osteoporosis (Yamaguchi, 2008).

4. Conclusions

In this study, 4 days of 2 glasses (400 mL) of citrus juice consumption by healthy volunteers were enough to induce changes in the human metabolome linked to the steroid biosynthesis pathway by nanoHPLC-q-TOF-metabolomics. Therefore, a dietary dose of citrus juice was able to produce short-term effects on endogenous metabolites without requiring a chronic intake of it. This approach allowed identifying four endocrine compounds of the urine metabolome as significant metabolites up-regulated by citrus juice intake (tetrahydroaldosterone-3-glucuronide, cortolone-3-glucuronide, testosterone-glucuronide and 17-hydroxyprogesterone). These results confirmed the importance of using non-targeted metabolomics technique to obtain new endogenous up- or down-regulated metabolites so as exploring metabolic pathway impacted as consequence of food intake demonstrating that not only the exogenous metabolites are relevant. These discriminant metabolites provided mechanistic support to other targeted effects of citrus juice intake by clinical trials like cholesterol and bone metabolisms. Regarding cholesterol metabolism, the overactivation of the steroid biosynthesis pathway of the cited endogenous metabolites could lead to a higher demand of cholesterol as primary substrate of the enzymes of steroid metabolism. Concerning to the bone metabolism, the increase of the four metabolites upon citrus

juice intake, three of them, corresponding to end-point of steroid biosynthesis pathway may contribute in the remodelling bone processes of the human body. Particularly, testosterone plays an important role in the maintenance of health bone by prevention of osteoblast apoptosis and stimulation of osteoclast apoptosis. Therefore, these results have evidenced that non-targeted approach provided new useful significant metabolites linked to citrus juice intake, which contribute to the development of the food intake-related metabolome as an important part of the global human urinary metabolome. This is the first metabolomic study linking directly the consumption of citrus juices at dietary dose to the steroid metabolites. Future studies with higher number of volunteers and longer period of juice intake will be useful to confirm this preliminary data.

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FIGURE CAPTIONS

Figure. 1. PCA. Scores (A) and Loading (B) plot PC1 *vs* PC2. Score plot assigned to the urine samples collected at baseline (circle) and after citrus juice intake (triangle). Lower half of the loading plot shows the buckets discriminative (*m/z* and RT) after citrus juice consumption.

Figure. 2. Bucket statistic plots corresponding to four identified metabolites, showing behaviour and intensity at baseline and after citrus juice intake (y-axis values correspond to the relative intensity of the hormone metabolite).

Figure. 3. Steroid biosynthesis pathway showing the structure of the four metabolites identified and its precursor, cholesterol.

1

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

2 Data are represented by mean ± SD.

3

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)

4
5

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 5)
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 %)
B-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.

Table 4. Putatively identified metabolites positively correlated to the citrus juice intake by human volunteers (the metabolites are indicated according to the hierarchical order provided by *t*-test at $P<0.05$)

Retention Time (min)	Detected Mass (*m/z*)	Metabolite putative identification	Putative Neutral Molecular formula	Ionization mode	Theoretical mass (*m/z*)
9.90	462.9711	Unidentified metabolite	-	$[M-H]^-$	-
9.97	424.0162	Unidentified metabolite	-	$[M-H]^-$	-
10.00	689.1288	Unidentified metabolite	-	$[M-H]^-$	-
10.70	537.2361	Unidentified metabolite	-	$[M-H]^-$	-
10.87	539.2502	Tetrahydroaldosterone-3-glucuronide (tentative)	$C_{27}H_{40}O_{11}$	$[M-H]^-$	539.2497
11.00	541.2682	Cortolone-3-glucuronide	$C_{27}H_{42}O_{11}$	$[M-H]^-$	541.2654
11.03	413.2009	Unidentified metabolite	-	$[M-H]^-$	-
11.42	567.1747	Unidentified metabolite	-	$[M+H]^+$	-
12.24	509.1639	Unidentified metabolite	-	$[M+H]^+$	-
12.62	331.2214	17-Hydroxyprogesterone	$C_{21}H_{30}O_3$	$[M+H]^+$	331.2267
13.16	465.2443	Testosterone-glucuronide	$C_{25}H_{36}O_8$	$[M+H]^+$	465.2483
13.40	561.3377	Unidentified metabolite	-	$[M+H]^+$	-

Figure(s)
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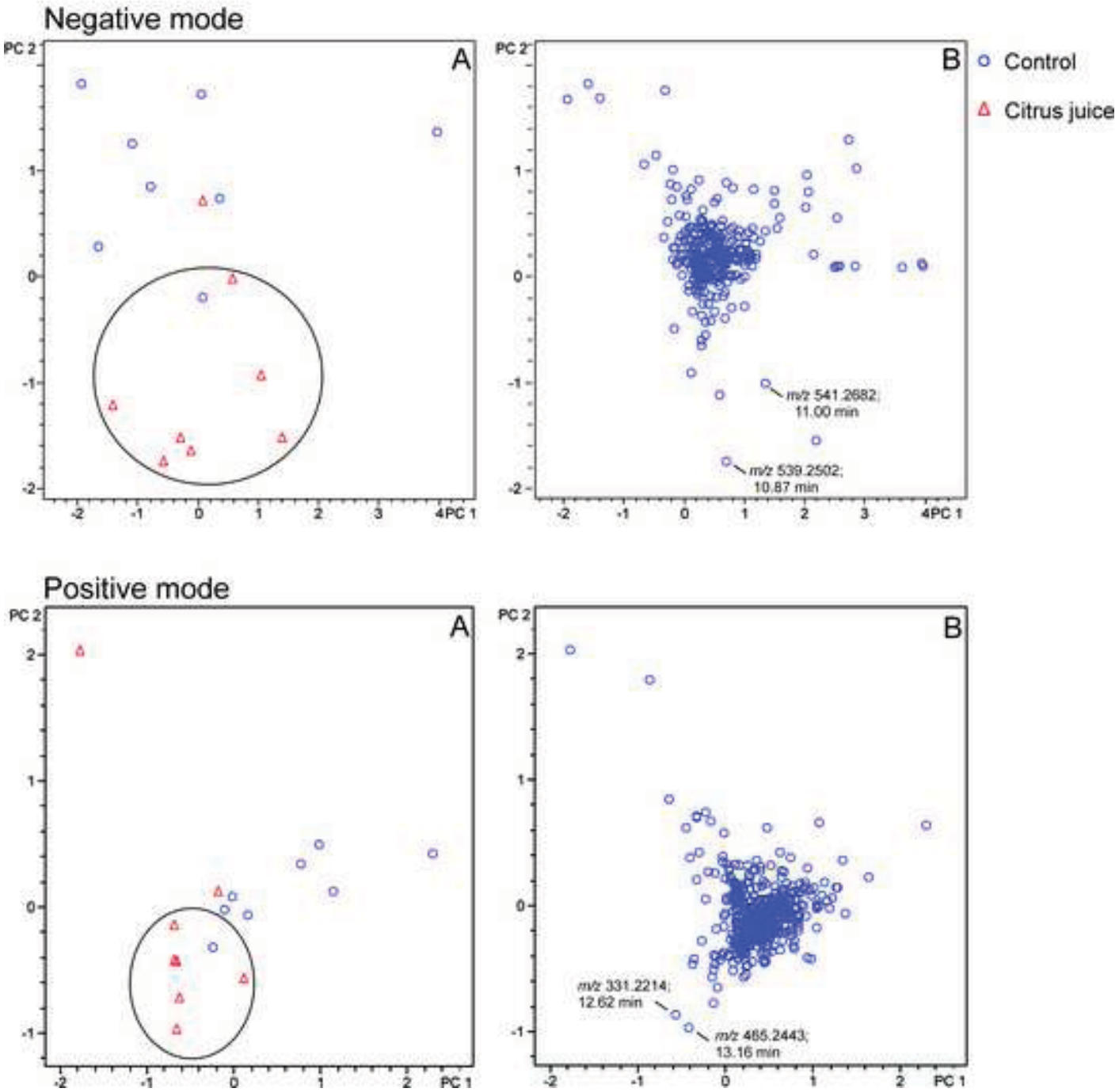
Figure 1. PCA. Scores (A) and loadings (B) plots PC 1 vs PC 2



Figure(s)

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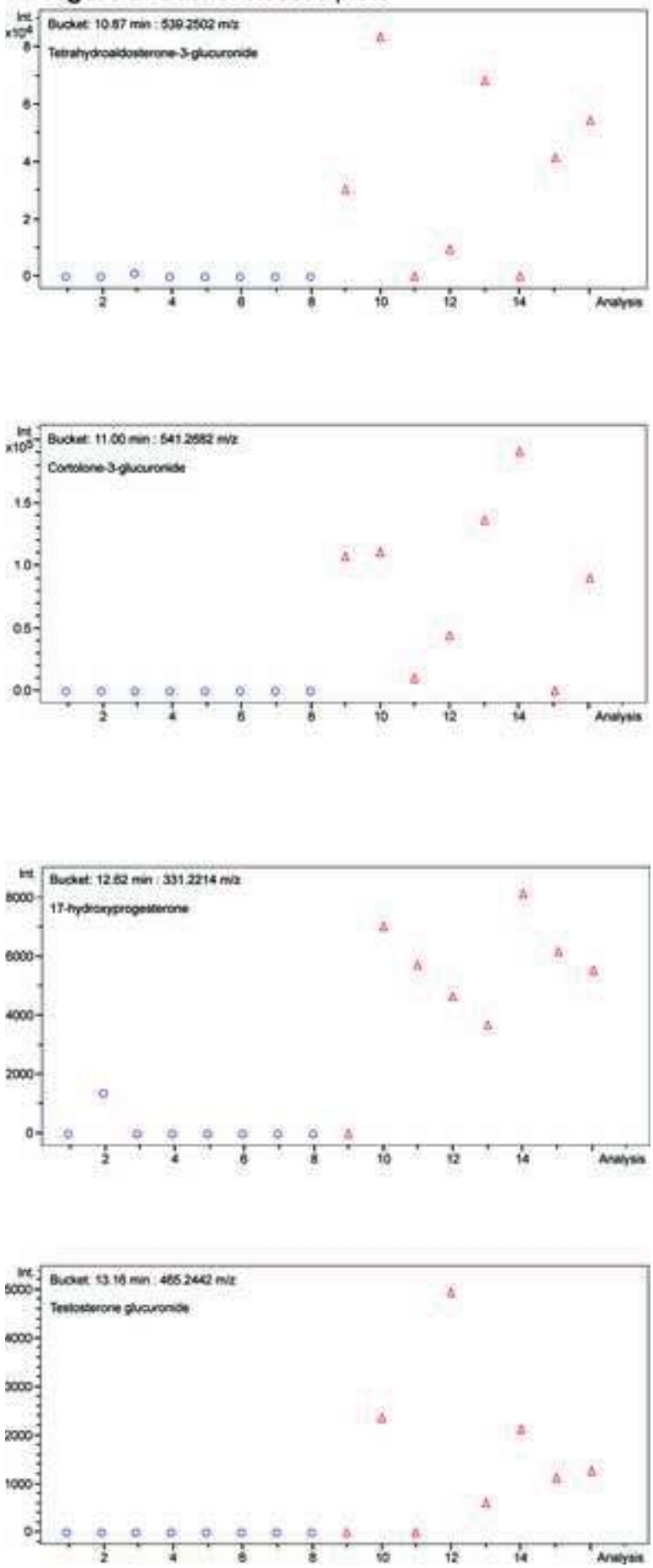
Figure 2. Bucket statistic plots



Figure(s)

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